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Isolation and Characterization of Probiotic Bacteria from Laphet (Myanmar's Traditional Fermented Tea Leaves)

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Abstract:
Background*: Probiotics are live microbial food constituents that affect the microflora and provide health benefits to the host when consumed in sufficient quantities. Laphet (Myanmar's traditional fermented tea leaves) is one of the most consumed daily snack throughtout Myanmar and contains Latic Acid Bacteria (LAB) which play an important role in probiotics. The aim of this study was to use the catalase assay and the type of fermentation to assess the features and biochemical properties of LAB.* ***Materials and Methods****: Lactic acid bacteria (LAB) were identified using particular media, especially MRS Agar, and the antimicrobial activity was tested using test bacteria Escherichia coli O157, Staphylococcus aureus.* ***Results****: The study's findings were as follows: The result of the catalase assay and the type of fermentation were negative catalase and homofermentative. Antibacterial activity indicates that the lactic acid bacteria obtained could inhibit pathogenic bacteria E. coli, S. aureus. We detected the LAB using normal laboratory assays and 16S rRNA sequencing techniques. Blast analysis resulted in Laphet sample which confirmed that the bacterial strains are Lactiplantibacillus plantarum, Lactiplantibacillus sp. and Lactiplantibacillus argentoratensis.*
Key Words*: isolation, characterization, probiotics, bacteria, Laphet*

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I. Introduction

Laphet (Myanmar's traditional fermented tea leaves) is one of the most popular consumption snacks in Myanmar and is formed by the fermenting tea leaves (*Camellia sinensis*). Laphet is produced through tea leaves (*Camellia sinensis*) by fermentation with limited air passages. In the begining, the young leaves are harvested from the plantation. The tea leaves are selected through a fermentation process, which consists of steaming for approximately 5 minutes, removing excess water, re-selecting tea leaves, packing them into clay pots, and pressing the leaves with heavy weights. The fermentation process should be checked periodically. The naturally forming microbes ferment the tea leaves thoroughly.

The bitter taste of tea leaves is reduced during the fermentation process, although the actual taste of tea leaf is bitter. Laphet is an ancient and national specialty that is consumed by almost everyone in the country, regardless of race or religion, during family gatherings, monasteries, and traditional activities. As a fermentation product made from green tea, Laphet has an abundance of bacterial activity and antioxidant properties [1].

Latic Acid Bacteria (LAB) and yeast are the two most common groups of microbial clusters discovered in Laphet samples [2]. Gram-positive bacteria in the cocci or bacilli-shape, facultative that are catalase-negative, motile, and produce lactic acid as a the end product metabolism during carbohydrate fermentation called Latic Acid Bacteria [3]. LAB probiotics are labeled as homofermentative or heterofermentative based on their metabolic processes. Under anaerobic conditions homofermentative LAB ferments carbohydrates primarily to produce lactic acid. In heterofermentative LAB, sugars are fermented to produce ethanol, CO_2, and less lactic acid [4]. LAB is a member of the Eubacteriales order, as well as the Streptococcaceae and *Lactobacillaceae* families, and do not generate cytochrome or spores. Lactobacillus, Lactococcus, Bifidobacterium, Tetragenococcus, Vagococcus, Weissella, Streptococcus, Leuconostoc, Aerococcus, Oenococcus, Carnobacterium, Enterococcus, Sporolactobacillus, and Pediococcus are the representative genera of LAB [5]. Nonpathogenic, viable in negligible pH medium, LAB is represented as probiotic has the ability to grow on media with significant amounts of bile salts, adherent and invading epithelial cells, antibacterial properties, and health benefits [6].

Because of their beneficial effects on health and well-being, probiotics have gained widespread acceptance as human and animal supplements [7,8]. Probiotics impact the microbial composition associated with allergy, cancer, diabetes, inflammatory disorders, heart disease, and dyslipidemia [9]. In the last decade, the antioxidant activity of LAB and LAB-related compounds has been extensively described [10]. Due to the

abundance of LAB and yeast, Laphet can be considered as a rich source of probiotic microorganisms [2]. In this study, we aimed to isolate, identified the probiotic-potential bacteria especially LAB from Laphet.

II. Material and Methods

Material

The laphet sample namely Zayan Laphet was collected from the municipal market of Tharkayta, Yangon, Myanmar.

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1 gram Laphet was used and dissolved in peptone water (10^{-1}) and vortexed until homogeneous. After that, took 1000µl of 10^{-1} and moved it to 10^{-2} peptone water and vortexed until homogeneous. This process continued until 10^{-5}, 10^{-4}, and 10^{-5} were moved to Petri dishes and MRS agar medium was added using the pour method and incubated at room temperature for 48 hours. Observation of colony growth and counting 10^{-4} and 10^{-5}.

Characterization of isolate bacteria

In this test, prepared a bacterial culture from liquid media, dropped it on a glass object which was then also dripped with 3% of H_2O_2 (hydrogen peroxide), and let stand for 1 minute. Observe the presence or absence of bubbles. The formation of bubbles indicates that the bacteria are aerobic (catalase positive), but if no bubbles formed, it indicates that the bacteria are anaerobic (catalase negative). To determine the fermentation type A Durham tube and MRS broth were used to investigate gas formation from glucose.

Acid Resistance Test

1mL bacterial culture was inoculated on 9ml of MRS Broth media and incubated at 37°C for 24 hours. Furthermore, 1 mL of MRS Broth bacterial suspension was put into a test tube which contained 9 mL of MRS Broth without pH control (control) and MRS Broth pH 3 (pH adjusted in the presence of 5N HCl) and incubated for 90 minutes. The pH 3 cultures and control were then diluted to 10^{-6} before being grown on MRS medium and incubated at room temperature for 48 hours. To identify the viable microorganisms, the Colony Forming Unit (CFU) was determined. The viability (%) will be calculated by comparing the number of cells previous and post incubation. The greater the viability ratio, the more tolerant the bacteria are to low pH, the more tolerant the bacteria are to lower pH.

Bile Salts Resistance Test

1 mL bacterial culture was inoculated on 9 mL of MRS Broth media and incubated at 37^{o}C for 24 hours. After that, 1 mL of MRS Broth bacterial culture was put into a test tube containing 9 mL of MRS Broth in absence of oxgall control (control) and MRS Broth with oxgall 0.3% then incubated for 1day. Subsequently, oxgall 0.3% and standard culture were mixed to 10^{-6} and spread into MRS medium for 48 hours at 37^{o}C using the spread technique. To identify viable microorganisms, the Colony Forming Unit (CFU) was determined. The viability (%) will be calculated by comparing the number of cells before and after incubation The greater the viability ratio, the more tolerant the microorganisms were to bile salts.

Antimicrobial Activity

Using the disk diffusion method on two microorganisms, namely *Escherichia coli* O157 and *Staphylococcus aureus* ATCC 25923, an antimicrobial resistance test was carried out. 1 mL lactic acid bacteria culture was centrifuged at 10.000 rpm for 5 minutes at 27 °C temperature, the supernatant was used for microbial resistance. In a petri dish, mix 20 mL of Natrium Agar (NA) medium with 0.2% regenerated testing microbes and leave to unwarm. Then, in NA medium, holes were made with a radius of ±6.5 mm. Afterward, injected 50 µL bacterial supernation latic acid bacteria (LAB). Finally, it was incubated at 37^{o}C. After 24 hours, the focus shifted to a translucent zone with a spherical form.

Isolation of the DNA genome of LAB using 16SrNA

Identification of LAB genomic DNA was carried out by following to the method of Amelia R. *et al.* , 2021 [3]. Before working with PCR primers, the purification column was discarded, the microtube containing the DNA solution was stored at -20 °C.

Preparation of PCR primer (16SrNA)

The isolation of genomic DNA from pure bacterial colonies was amplified by PCR. The DNA amplification reactions were carried out in a *Mupid-Exu Thermocycler* using a forward primer F 16S-27F (5'AGA GTT TGA TCC TGG CTC AG-3') and a reverse primer R 16S-1492R (5'GTT 'TAC CTT GTT ACG AACTT-3'). The PCR mixture uses materials such as Forward 16S-27F and Reveres 16S-1492R primers, Master Mix, Template, and

dH20.

Preparation of 16sRNA gene amplification

The gene amplification was prepared for 16SrNA base on the primers for 16SrNA (Table 1) and the PCR program (Table 2).

Tabel 1. PCR of 16SrNA Primer

Composition	Volume (µl)
Master Mix	12.5
Primer F	1
Primer R	1
DNA (Template)	1
dH_2O	9.5
Total	**25**

Table 2. PCR Program Reaction

Process	**Temperature (°C)**	**Time**
Pre denaturation	95	2 minutes
Denaturation	95	45 seconds
Annealing	56	45 seconds
Extension	72	1 minutes
Final Extension	72	10 minutes
Cooling	**d.**	

Construction for visualizing PCR product

The agar was put into the electrophoresis operating system, then TBE solution was added until the agar was completely soaked. In the agar wells, 5µL of the sample and 5µL of the DNA ladder were inserted Agarose was carried out at 100V for 40 minutes, and the outcomes were examined underneath the Ultraviolet light. After reading the PCR data, the cleaned and generated DNA was delivered to the Genetics Lab for sequencing.

Construction of Phylogenetic tree based on sequencing results

Bidirectional electropherogram sequenced for each sample were edited and contiguous using the SeqManTM application. The sequence of bases that make up the 16S rRNA gene for each bacterial sample were then BLAST on the NCBI website [11]. From the BLAST results, 20 bacterial sample sequence data were selected in the gene bank which were then used for alignment, phylogenetic tree construction, and determining genetic distance using the MEGA 11 program. Alignment was performed using the Clustal W algorithm. The phylogenetic tree was constructed using the Neighbor-Joining method [12] and evolutionary distances were analyzed using the 2-parameter Kimura method [13]. The bootstrap value used was 1,000 [14]. Genetic distances were analyzed using the Pairwise Distances method.

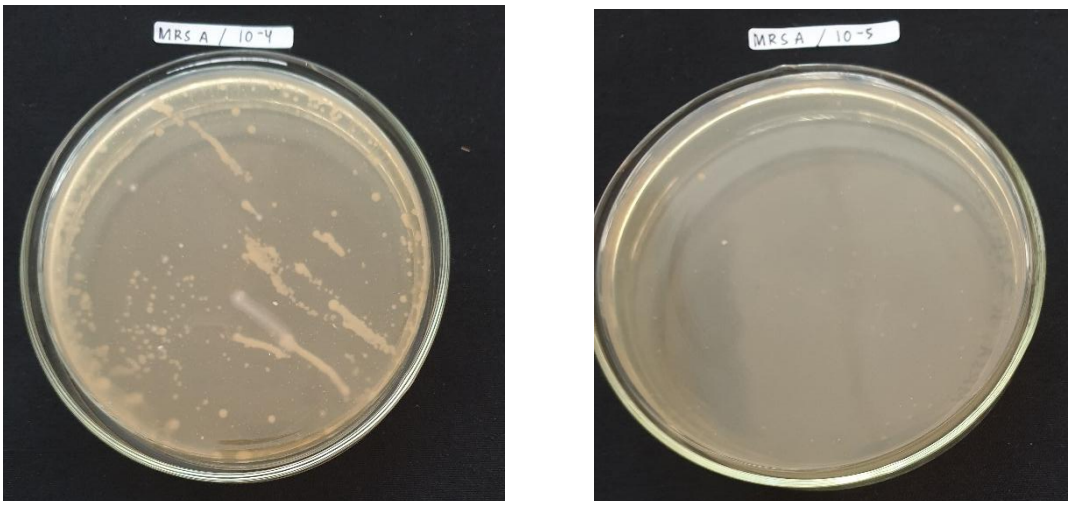
III. Results

Isolation of Lactic Acid Bacteria from Laphet

The total lactic acid bacteria that isolated from Laphet can be seen in (Table 3).

Table 3. Total Colonies of Latic Acid Bacteria from Laphet

Sample Code	Dilution	Total Colony of LAB
Zayan Laphet	10^{-4}	200
Zayan Laphet	10^{-5}	27



A B

Fig. 1. Dilution of 10^{-4} conlonies of Latic Acid Bacteria from Laphet (A) and dilution of 10^{-5} colonies of Latic Acid Bacteria isolated from Laphet (B)

Characteristic of isolate LAB

The results of the catalase test showed negative catalase results (Table 4). Testing of LAB isolates with catalase test was carried out to determine the ability of bacteria to produce catalase enzymes and the tolerance of LAB isolates to oxygen. The catalase test was carried out using 3% H_2O_2 which was dropped on a pure culture of LAB isolates shows the catalase test carried out with H_2O_2 to determine the ability of bacteria.

Table 4. Biochemical properties of Laphet LAB isolates

Sample	Catalase Test	Type of Fermentation
Test 1	Negative	Homofermentative
Test 2	Negative	Homofermentative

LAB isolates showing negative catalase means no air bubbles are formed due to O_2 gas. This happens because LAB does not produce the catalase enzyme which can convert H_2O_2 into water and oxygen. All isolates showed no formation of O_2 gas bubbles after dropping H_2O_2 indicating negative catalase. LAB includes catalase-negative bacteria so it does not produce air bubbles.

The results of this study were also the same as those conducted and stated that gram-positive LAB was catalase-negative and did not form spores and LAB isolates from Tempoyak from Pariaman produced catalase negative [15].

The lack of bubbles inside the Durham tubes immersed in broth infected with the LAB further demonstrated its homofermentative ability *Lactobacillus* genus of 70 species is divided into three classes, with the majority of homofermentative or heterofermentative Lactobacillus is slightly acid-tolerant than most LAB overall.

Acid Resistance Test

Bron P.A. *et al.* , 2004 reported that when probiotic bacteria is consumed, it is exposed to gastric acidity [16]. As a result, a Laphet LAB acid tolerance test was performed at pH control and pH 3. The number of colonies developing under control was greater (83×10^5 CFU/mL) than the pH3 (39×10^5 CFU/mL) with exit rate of 46.99% in isolate 1 and isolate 2 colonies grew was greater (60×10^5 CFU/mL) than pH3 (31×10^5 CFU/mL) with a viability exit rate of 51.67%. The types of the microbial species that exit at low pH and their strains can reflect the different viability values.

Table 5. Acid Resistance Test

Isolate	Total Colony (x 10^5 CFU/mL)		Viability
	pH Control	pH3	
ISOLATE 1	83	39	46.99%
ISOLATE 2	60	31	51.67%

Bile Salt Resistance Test

Bile salt resistance test should be carried out to prove probiotic in addition to the acid resistance test. Table 6 the isolate showed show that the control was 86 x 10^6 CFU/mL and the cell number decreased to 44 x 10^6 CFU/mL in isolate 1 after a combination of 0.3% bile salt with 51.16% viabilities and isolate 2 showed that 81 x 10^6 CFU/mL was controlled and the decrease in cell number was reduced to 31 x 10^6 CFU/mL after a combination of 0.5% bile salt with 38.27% viabilities .

The capacity of isolated lactic acid bacteria to produce bile salt hydrolase (BHS) is related to their ability to live in bile salts because the isolates contain the BSH enzymes, they are able to convert the harmful to non-toxic physicochemical features of bile salts. The same authors claim that greater concentrations of bile salts can convert the harmful to non-toxic physicochemical features of bile salts. Cell viability is indicated by the ability of some LAB to produce exopolisaccharides (EPS), thus providing defense against bile salt stress (0.15-0.3 %t) and acidic pH (2.0-3.0) [17, 18].

Table 6. Bile Salt Resistance Test

Isolate	Total Colony (x 10^6 CFU/mL)		Viability
	Oxgall Control	Oxgall 0.3%	
ISOLATE 1	86	44	51.16%
ISOLATE 2	81	31	38.27%

Antimicrobial behavior

The result showed that the most broad inhibition zone was detected with *S. aureus* (23.4mm) for isolate 1 and (22.1mm) for isolate 2, followed by *E. coli* (19.8mm) and (18.3mm) respectively (Table 7).

Table 7. Antimicrobial of Laphet LAB

Source of resistance	Bacteria test (mm)	
	SA	*EC*
ISOLATE 1	23.4	19.8
ISOLATE 2	22.1	18.3

The zones of inhibition can be classified as four schemes, weak (<5 mm), medium (5-10 mm), strong (>10-20 mm), and very strong (>20-30 mm) according to Morales G. *et al*., 2003 [19]. Thus, the inhibition activity of Laphet LAB against *S. aureus* is categorized as very strong. Following the study of Pratama Y.E. *et al.*, 2021, when testing *S. aureus*, the largest clear zone was around 18-31.36 mm which was significantly higher than this study [17]. Compared to Amelia R. *et al*., 2021, the inhibition zones were *E. coli* (23.28 mm) and *S. aureus* (7.63 mm), In this study, the inhibition zone was larger in *S. aureus* and slightly lower in *E. coli* [3]. We can conclude that antimicrobial effect of the LAB from Laphet against bacterial pathogens was significance.

Molecular identification by 16sRNA method

The PCR sequencing results are shown in (Figure 2) below. The DNA Amplification of one isolate was 1395 pb, and the primers used in this exploration were efficient in finding bacteria. The 16S rRNA gene is reported globally and has a logical sequence measure [20].

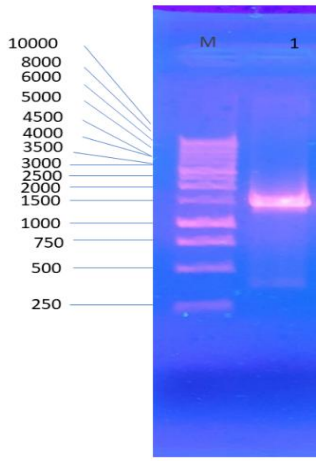


Fig. 2. The results of PCR sequencing the isolate bacteria from Laphet

Based on the PCR and BLAST results, the isolate bacteria from Laphet had 99.71% similarity with *Lactiplantibacillus plantarum* and *Lactiplantibacillus argentoratensis*, *Lactobacillus plantarum subsp. argentoratensis* strain 99.78% parallel and *Lactiplantibacillus plantarum strain LP26* had parallel with 99.64% (Fig. 3).

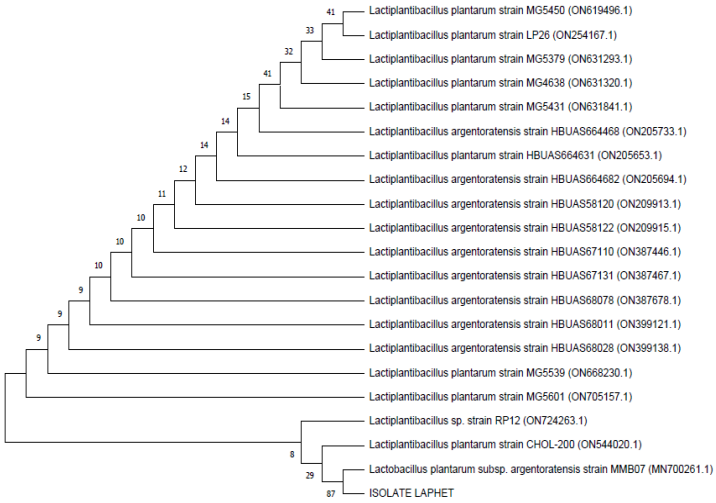


Fig. 3. The phylogenetic tree of Laphet sample

L. plantarum strain was isolated from *dadiah* from *Sijunjung* along with DNA sequence with 1525 bp has been documented by Syukur S. et al. , 2014 [21]. Isolates with a 97 percent similarity can be regarded the comparability group according to Dighe A.S. *et al*., 2004 [22]. Nurhikayani R. et al. ,2019 reported has similar result with this study in that two samples of four were *Lactobacillus plantarum* with 100 % similarity using BLAST analysis [23].

IV. Conclusion

Laphet is the one the daily consuming traditional food in Myanmar that is made up by fermented tea leaves (Camellia *sinensis*) that has a lot of LAB. The isolated LAB from Laphet has the potential as a probiotic, viable in medium with low pH and high concentration with bile salt and has antimicrobial activity. Molecular and bioinformatic analysis revealed that the LAB in Laphet are *Lactiplantibacillus plantarum, Lactiplantibacillus argentoratensis, Lactiplantibacillus sp.*strain and *Lactobacillus plantarum subsp. Argentoratensis* strains

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