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Isolation and Molecular Characterization of Probiotic Bacteria and Cell Free Supernatant Analysis from Borassus flabellifer Fruit Pulp

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Abstract

Probiotic microorganisms have gained increasing attention for their potential role in promoting human health by enhancing gut microbiota balance, modulating immunity, and preventing gastrointestinal infections . Among the diverse microbial sources, fruit-based ecosystems are emerging as promising reservoirs of lactic acid bacteria (LAB) [3,4]. This study was designed to isolate and characterize LAB from the ripened fruit pulp of Borassus flabellifer (palmyra palm), a tropical fruit known for its nutritional and medicinal values [5,6]. The isolated strains were subjected to comprehensive morphological, physiological, biochemical, and molecular analyses, including 16S rRNA gene sequencing [7,8]. The dominant isolate was identified as Lactobacillus plantarum, a well-known probiotic species [9,10]. Additionally, a sterile cell-free supernatant (CFS) was prepared from the identified strain and characterized to confirm the presence of functional bioactive components [11,12]. These findings support the probiotic potential of B. flabellifer and highlight its applicability as a natural, functional source for probiotic and bioactive compound development [13,14].

Keywords: Probiotics, Lactobacillus plantarum, Cell-free supernatant, Borassus flabellifer, Lactic acid bacteria (LAB), 16S rRNA, HPLC analysis, Functional food, Plant-based probiotics, Antioxidant metabolites.

Introduction

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [1]. Over the past decades, probiotics have emerged as a significant area of interest in food science, human health, and biotechnology due to their role in maintaining intestinal microbial balance, stimulating immune responses, and preventing gastrointestinal diseases [2,3]. Among the wide variety of probiotics, lactic acid bacteria (LAB), especially those belonging to the genus Lactobacillus, are the most extensively studied and widely used because of their Generally Recognized As Safe (GRAS) status, fermentative metabolism, and robust resilience in gut environments [4,5]. Traditionally, LAB have been isolated from dairy-based fermented products. However, the increasing prevalence of lactose intolerance, dairy allergies, and vegan preferences has accelerated interest in plant-based sources for probiotic exploration [6,7]. Fruits and vegetables, particularly those undergoing natural fermentation, have become a focal point for isolating potential probiotic strains [8]. These matrices not only provide a non-dairy alternative but are also rich in prebiotics and other nutrients that support the viability and growth of beneficial microbes [9,10]. Borassus flabellifer, commonly known as the palmyra palm, is a tropical fruit bearing tree native to South and Southeast Asia. Its ripe fruit pulp is consumed widely in southern India and is cherished not only for its unique flavor and nutritional benefits but also for its traditional medicinal value [11]. The fruit is rich in sugars, vitamins, polyphenols, and antioxidants, making it a favorable niche for microbial colonization [12,13]. Despite its traditional use and biochemical richness, limited scientific research has been conducted to explore the microbiological properties of B. flabellifer. This study aims to isolate and identify potential probiotic LAB from the fruit pulp of B. flabellifer. The isolated strains were characterized using morphological, biochemical, and molecular methods, including 16S rRNA sequencing. Furthermore, the cell-free supernatant (CFS) of the identified LAB was extracted and analyzed for the presence of bioactive metabolites using High-Performance Liquid Chromatography (HPLC). The xedfr ework intends to bridge the gap between traditional knowledge and modern scientific validation, offering insights into the probiotic potential and biofunctional properties of B. flabellifer-associated LAB. 2. Materials and Methods 2.1 Sample Collection and Preparation.

Materials and Method

Sample collection

The ripened fruits of *Borassus flabellifer* was collected from the local formers of srivilliputhur, Virudhunagar district.

Isolation of probiotics

Freshly collected samples were taken to the laboratory and surface sterilized. The hard skin was carefully removed to collect the fruit pulp. Then crushed with morter and pestle. The juices were serially diluted and plated on De Man- Rogosa -Sharpe agar (MRS agar) a selective medium for isolation of lactic acid producing microorganisms.[3,12,13]

Identification of lactic acid producing microorganisms
The lactic acid producing microorganism were identified based on morphological, physiological and biochemical characterization method.

Morphological and physiological characterization of lactic acid producing microorganisms
Colony Morphology
The morphological characteristic feature of bacterial colony such as shape, colour, size, opacity, elevation, edge and smell were used to identify and classify bacteria. [14]To determine colony morphology, the distinct colonies were selected from the plates and then pure culture was made. [16]The pure culture of the selected strain was streaked on MRS agar plates for 48 hrs at 37°c to produce single colonies. The morphology was then observed and studied.[25]

Gram Staining
Gram staining has been used for classifying bacteria on the basis of their forms and cellular morphologies. Gram stain has segregated all bacteria into two large groups: those organisms that retain the purple colour of the primary stain (crystal violet) on the cell well are described as gram-positive and those cells that retain the pink colour of the counterstain (safranin) are gram-negative[14]. To perform gram staining, a small amount pf culture was added to a drop of water slide and spreaded to an even thin film with an inoculation loop, and then heat fixed on the slide by passing over a gentleflame[16].
The slide was flooded with crystal violet for 30 seconds, rinsed with water and flooded with Gram's iodine (a mordant, that binds the crystal violet to the peptidoglycan layer of gram-positive cell wall) and rinsed again with water after 1 min. The slide was then flooded with alcohol (a decolorizing agent, that removes stain from the gram-negative cells) and rinsed with water. Then, the slide was counterstained with safranin and rinsed with water after 1 min. The slide was air dried and viewed under bright field compound microscope[25].

Biochemical Characterization

Indole Production Test
The objective of the indole synthesis test was to find out if a bacterium could break down the amino acid tryptophan into indole. Test bacteria were added to peptone broth, which was then cultured for 24 hours at 37 °C to measure the synthesis of indole. After the incubation period, the tube was immediately filled with 5 drops of Kovac's reagent (P-dimethyl amino benzaldehyde: 5 gm, hydrochloric acid: 25 ml, amyl alcohol: 75 ml). The development of a cherry red color in the reagent layer on the medium's surface after the addition of Kovac's reagent signified a positive indole test. The reagent layer showed a negative indole response and stayed yellow to hazy[14].

Methyl Red Test

The ability of microorganisms to oxidize glucose and produce a high concentration of acid end products was determined using the methyl red test. Test tubes filled with sterile MR-VP broth were infected with the isolated organisms, and the tubes were then cultured for 24 to 48 hours at 37 degrees Celsius. 7 to 8 drops of methyl red indicator were applied after incubation, and the development of red colour indicated that the experiment was effective[16].

Voges Proskauer Test

The Voges-Proskauer test identified the synthesis of non-acidic or neutral end products including butanediol and acetyl methyl carbinol (acetoin). The Voges-Proskauer test identified the synthesis of non-acidic or neutral end products including butanediol and acetyl methyl carbinol (acetoin). Inoculated into sterile MR-VP broth tubes, the organisms were cultured at 37°C for 24 hours. Barritt's reagents A and B were added after incubation. A positive result was indicated by the colour changing to pink.

Citrate Utilization Test

Certain organisms possessed the ability to use monoammonium phosphate as their only supply of nitrogen and citrate as their only source of carbon. Consequently, alterations in the medium's indicator were observed, indicating a change in the pH of the medium. Simmon's citrate medium was prepared, sterilized, and stored slantwise in tubes, which were then left to solidify in order to conduct the test. After being streaked on the slant, the test organism was incubated for 24 hours at 37 °C. The colour of slant's changed from green to Prussian blue, suggesting an effective result.

Catalase Test (Blazevic and Ederer, 1975)

Test organisms were injected into nutrient slants, which were then incubated for 24 hours at 30 degrees Celsius. After the incubation period, one ml of 3% hydrogen peroxide was added to each tube and observed for gas bubbles. The occurrence of gas bubbles was indicated positive for catalase[4][16][25].

Screening for Probiotic Bacteria

Culture Medium for Bacterial Growth

The isolated colonies were inoculated in 100ml of MRS Medium containing (g/1), The flasks were inoculated with 5 % inoculum and kept in shaker for 48 hours at 200rpm and 37o c. After 48 hours of incubation, this culture broth was used for further screening assays.

Genomic DNA extraction

The cultures grown on MRS broth for 48 hours at 37o c was centrifuged at 4600rpm for 3min. Bacterial genomic DNA was isolated. The pellet was resuspended in 400 ml of sucrose TE buffer. Lysozyme was added and make upto final concentration at 8mg/ml-1 which incubated for 1 h at 37o c. In the tube, 100 ml of 0.5M EDTA (pH 8.0), 60 ml of 10% SDS and 3 ml of proteinase K and make upto 20mgmL1 and then

incubated at 55o c for overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamyl alcohol (24:1) and then added ethanol, collected the precipitated. The DNA pellet was resuspended in a sterile distilled water.

16s rRNA Polymerase Chain Reaction] analysis (Rawlings, 1995)

Isolation of the cultures were used for amplification of the 16S ribosomal RNA (rRNA) conserved region was performed. The universal primers were for reverse and forward (3'CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG5')and(5'CCAAGCTTCT AGACGGITACCTTGTTACGACTT3') The reaction mixture contained 2 mM Mgcl2, 0.1 µM deoxynucleoside triphosphate (dNTP) mixture, 0.5 µM of each primer, 1.5/u, DNA polymerase and 2 µl of template DNA and was then made up to a final volume of 50 µl using sterile nuclease-free water. Amplification was performed using the thermal cycler and the PCR cycling parameters consisted of initial denaturation at 94°c for 4 min followed by 30 cycles of 94°c for 30 s, 53°c for 30 s, and 72°c for 90 s, and then a single final extension step of 72°c for 5 min. The 1600-bp PCR product was visualised on a 1.0% agarose gel, stained with ethidium bromide (0.5 µg/ml). The amplified PCR products were purified using the DNA as per manufacturer's instructions and were sequenced in accordance with the Big Dye Terminator Version 3.1 Sequencing Kit[17][21][26].

Molecular characterization

The most conserved 16srRNA gene sequences from prokaryotic cells were considered as most powerful tool to identify the unknown bacterial species. The 16s rRNA genes from four samples were amplified by using the polymerase chain reaction and sequenced. The obtained forward and reverse sequences were aligned and the low-quality regions of the sequences were trimmed. The obtained final sequences were subjected to BLAST (Basic local alignment search tool) analysis[20].

Species Identification

16s rRNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between isolated bacteria from marine water samples. The 16s rRNA gene sequences of the selected bacterial isolates were sequenced. The BLAST search was performed for l6s rRNA gene sequences of the selected bacterial isolates at NCBI database using nucleotide BLAST. Subsequently, the l6s rRNA gene sequences for different bacterial strains which showed maximum similarity from BLAST result were taken for phylogenetic analysis using Mega 6.0 version and nucleotide frequency count analysis using main work benchsoftware[27].

Nucleotide Sequence analysis

The full-length sequences obtained were matched with NCBI using BLAST (Altschul et al., 1997). Multiple sequence analysis was carried out using CLUSTALX (Thompson et al., 1997) and neighbor-joining plot using PHYLODRAW (Perriere

were used to construct the phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

Phylogenetic Tree

In general, the portion of 16s rRNA sequence from distantly related organisms were remarkably similar, which means that sequences from distantly related organisms can be precisely aligned, marking the true differences were easy to measure. In this regard, the sequences of 16s rRNA were being extensively used to determine the taxonomy, phylogeny (evolutionary relationships) and to estimate the rate of species divergence among bacteria. [8]Thus, the 16s rRNA sequences of the isolated four bacterial species from marine water samples were multiply aligned with their respective BLAST hits using Clustal W and their evolutionary relationship among another similar bacterial genus were also plotted by phylogenetic tree as NJ (Neighbour joining) plot [19].

***Lactobacillus plantarum* cultivation**

Lactobacillus plantarum was cultured using de Man, Rogosa and Sharpe (MRS) broth, a nutrient-rich medium specifically formulated to support the growth of lactic acid bacteria. A loopful of the bacterial isolate was inoculated into sterile MRS broth and incubated at 37□°C for 24–48 hours under anaerobic conditions to promote optimal cell growth and metabolic activity. The culture was monitored for turbidity as an indicator of bacterial proliferation. The harvested culture was then used for subsequent experimental procedures[2][16][22][18]**.**

Preparation of Cell Free Supernatant

Lactobacillus plantarum A7 and the commercial probiotic strain were cultured in MRS broth and incubated anaerobically at 37°C for 24 hours. Following incubation, the cultures were centrifuged at 4000 rpm for 15 minutes at 4°C to remove the bacterial cells. The supernatant was then filtered using a 0.22 µm membrane filter to obtain a sterile cell-free supernatant (CFS), which was stored at 4°C for subsequent assay.

Cell Free Supernatant Confirmatory test

The bacterial culture was centrifuged at 6000 rpm for 15 minutes at 4□°C to separate the cells from the medium. The supernatant was collected and passed through a 0.22□µm syringe filter to obtain a sterile, cell-free extract. To confirm the absence of live bacterial cells, a small aliquot of the filtered supernatant was streaked on MRS agar plates and incubated at 37□°C for 24 hours. No visible bacterial growth confirmed the sterility of the supernatant, validating its use in downstream applications[23][24].

FTIR Spectroscopy

TThe spectra were collected in the range of 4000–400 cm^{-1} using an attenuated total reflection (ATR+7]=p7[‘ accessory. The FTIR analysis helped confirm the functional groups on the surface of SeNPs that might be responsible for stabilizing the

nanoparticles.For metabolite analysis, FTIR spectroscopy was employed to monitor both intracellular and extracellular metabolites, such as glucose and citric acid, throughout the synthesis process. High-throughput screening (HTS) FTIR was used in combination with micro-bioreactors, with spectra collected from 4000–400 cm^{-1} in ATR mode. Partial least squares regression (PLSR) was applied to correlate the FTIR spectral data with metabolite concentrations[29].

High Performance Liquid Chromatography

High-Performance Liquid Chromatography was employed to analyze the bioactive components present in the sample. The filtered extract was injected into an HPLC system equipped with a reverse-phase C18 column. The mobile phase consisted of a gradient mixture of solvents, typically water with 0.1% formic acid (solvent A) and acetonitrile or methanol (solvent B), delivered at a constant flow rate of 1.0 mL/min. The detection was carried out at a specific wavelength using a UV-Vis or photodiode array detector, depending on the target analytes. Retention times and peak areas were compared against standard compounds to identify and quantify the constituents.[6][9][10].

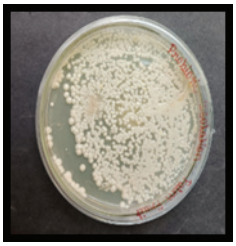
Results and Discussion

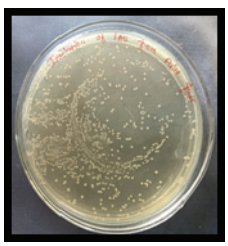
Sample collection

Fig 1.1 whole palm fruit Fig 1.2 Pulp of Palm Fruit

The sap samples were aseptically collected from naturally ripening *Borassus flabellifer* fruits located in and around Srivilliputhur, Virudhunagar district, Tamil Nadu, India—an area known for its traditional palmyra cultivation. Samples were placed in sterile containers, chilled during transport to preserve microbial integrity, and immediately processed upon arrival in the laboratory for subsequent enrichment and isolation studies

Isolation of LAB





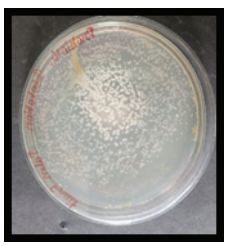


Fig – 2.1 LAB 1

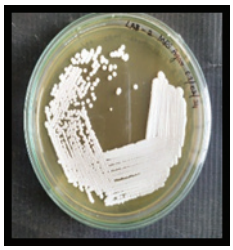


Fig -2.2 – LAB 2

Fig – 2.3 LAB 3

Out of approximately 120 morphologically distinct isolates obtained from *Borassus flabellifer* sap, three LAB strains that produced clear halos on MRS–$CaCO_3$ agar were chosen, repeatedly sub-cultured to purity, and maintained as standalone cultures.
Ripe *Borassus flabellifer* sap was aseptically collected and serially diluted before being plated on De Man, Rogosa & Sharpe (MRS) agar supplemented with 0.5% (w/v) calcium carbonate ($CaCO_3$), which facilitates the identification of acid-producing colonies by clear halos. Plates were incubated at 30–37□°C for 24–48□h, allowing acidogenic lactic acid bacteria (LAB) to develop visible zones. Distinct colonies exhibiting clear zones were selected and repeatedly streaked on fresh MRS agar to obtain pure cultures. Purified isolates were preserved in glycerol stocks at –80□°C. This method yielded approximately five exopolysaccharide-producing LAB strains—belonging to *Fructobacillus fructosus* and *Leuconostoc mesenteroides*—demonstrating the effectiveness of enriched MRS–$CaCO_3$ media for isolating functional LAB from palmyra pulp sap

Colony Morphology

Table 1.1 Colony Morphology of LAB Isolates

	LAB 1	LAB 2	LAB 3
Color	White	White	White
Form	Round	Punctiform	Round
Elevation	Raised	Raised	Raised
Margin	Entire	Entire	Entire

On MRS–$CaCO_3$ agar, all three isolates (LAB□1–3) displayed white, opaque colonies, in line with typical lactic acid bacteria profiles. Each colony was raised, projecting slightly above the agar surface and presenting entire (smooth) margins, indicating uniform growth without edge irregular While these shared characteristics confirm their LAB nature, the isolates showed notable differences in shape and size: LAB□1 and LAB□3 formed well-defined round colonies, whereas LAB□2 produced much smaller, punctiform (pinpoint) colonies—a sign of more compact growth Such variations, including colony color, form, elevation, and margin, are essential first-level phenotypic markers used to distinguish bacterial strains before proceeding to microscopic and molecular identification These observed traits not only align with standard microbiological descriptors but also lay a solid foundation for further physiological and taxonomic assessment of the isolates.

3.3 Gram Staining

LAB1-gram positive, rod shape	LAB2-gram positive, rod shape	LAB3-gram positive, rod shape

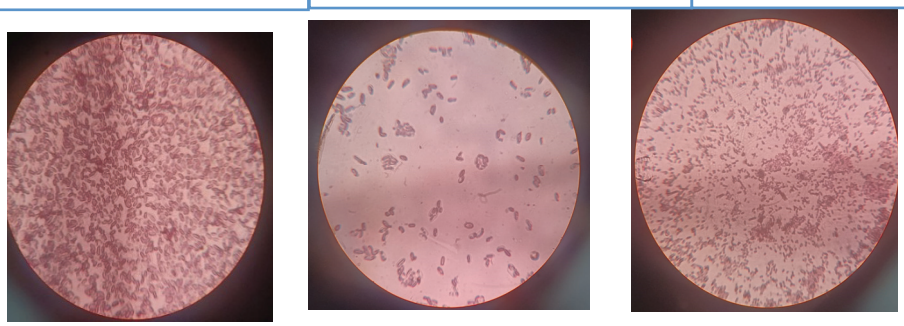


Fig 3.1 The Gram staining of LAB Isolates

Table 2.2 Gram Staining of LAB Isolates

	LAB 1	LAB 2	LAB 3
Gram Staining	Positive, Rod	Positive, Rod	Positive, Rod
Motility	Non-Motile	Non-Motile	Non-Motile

All three isolates were confirmed to be Gram-positive, rod-shaped bacteria and showed no motility, meaning they lacked structures like flagella and did not move on semi-solid media. This combination of traits—rod morphology, Gram-positive cell

walls, and absence of motility—is typical of lactic acid bacteria. These characteristics support their preliminary identification as members of the LAB group, which are known for fermenting sugars into lactic acid under low-oxygen conditions and are commonly found in fermentation-based ecosystems.

3.4 Biochemical Characterisation

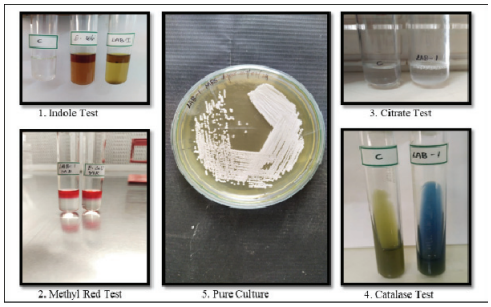


Fig 3.2 LAB 1

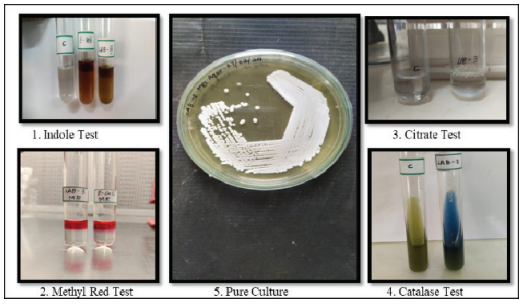


Fig 3.3. LAB 2

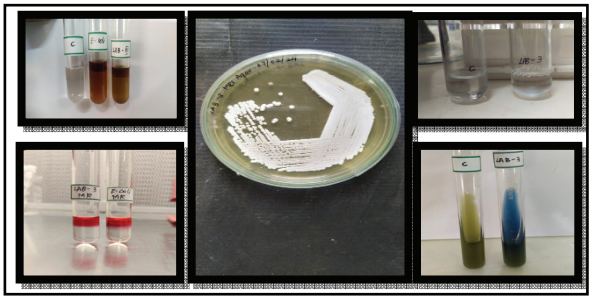


Table 3 Biochemical Characterisation of LAB Isolate

Test	LAB 1	LAB 2	LAB 3
Indole Test	Negative	Negative	Negative
Methyl Red	Positive	Positive	Positive
Citrate Test	Positive	Positive	Positive
Catalase Test	Positive	Positive	Positive

The biochemical characterization of bacterial isolates from Lab 1, Lab 2, and Lab 3 revealed consistent results across all tested parameters. All three isolates tested

negative for the Indole test, indicating the absence of tryptophanase enzyme and the inability to convert tryptophan into indole. In contrast, the Methyl Red test was positive in all cases, demonstrating that the isolates perform mixed acid fermentation and produce stable acidic end products. Similarly, the Citrate test showed positive results for all isolates, suggesting that the bacteria can utilize citrate as their sole carbon source, facilitated by the enzyme citrate-permease. The Catalase test was also uniformly positive, confirming the production of the catalase enzyme which decomposes hydrogen peroxide into water and oxygen, indicating that the bacteria are likely aerobic or facultatively anaerobic. These consistent biochemical responses suggest that the isolates share similar metabolic capabilities, supporting their potential identification as closely related bacterial strains.

3.5 Acid tolerance test

Table 4 - Acid Tolerance Test

pH	LAB 1	LAB 2	LAB 3
pH 1 0 hr **1 hr** **2 hr** **3 hr**	+ + + +	+ + - -	- - - +
pH 2 0 hr **1 hr** **2 hr** **3 hr**	+ + + +	+ + + -	+ + + +
pH 3 0 hr **1 hr** **2 hr** **3 hr**	+ + + +	+ + + -	+ + + -
pH 4 0 hr **1 hr** **2 hr** **3 hr**	+ + + +	+ + + -	+ + + -

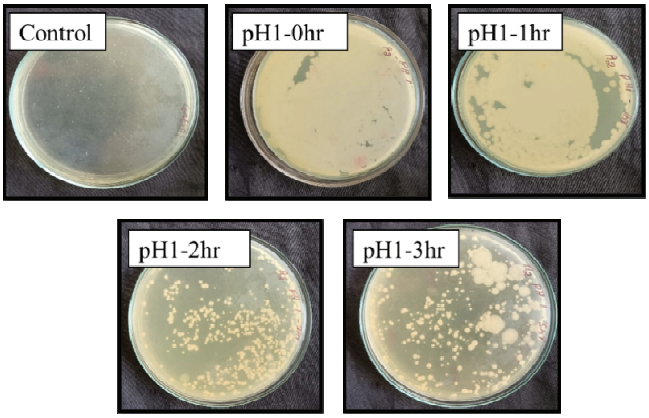
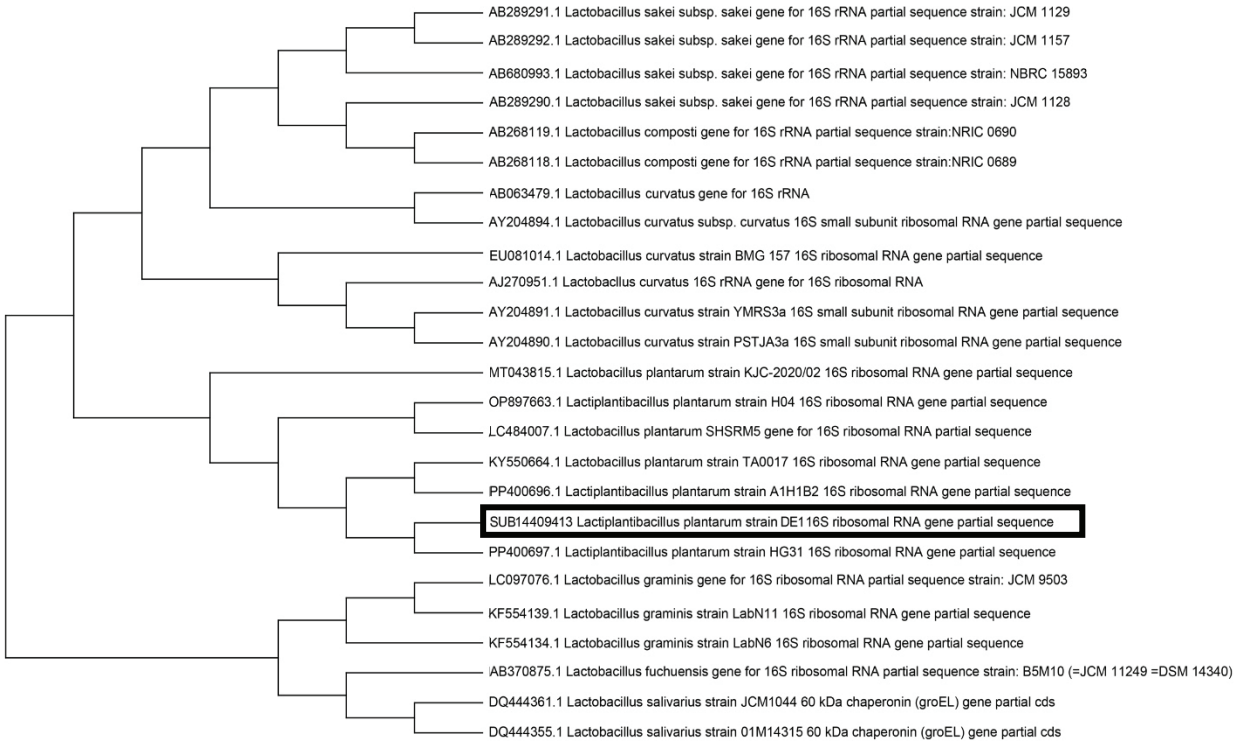


Fig 4 – Acid Tolerance test for LAB Isolates

3.6 Molecular Identification by 16srna and Identification

Plasmid DNA from the probiotic isolates was extracted using the alkaline lysis method as described by Brimboin and Doly (1978). The quality and presence of plasmid DNA were initially confirmed by agarose gel electrophoresis, followed by amplification through Polymerase Chain Reaction (PCR) analysis. Standard molecular weight markers were employed to estimate the size of the amplified DNA fragments, as represented in Fig. 1.10. For molecular identification, primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') targeting the 16S rRNA gene were used. PCR amplification of the probiotic DNA yielded an amplicon size between 1–1069 bp, confirming the presence of bacterial genetic material consistent with probiotic strains Furthermore, phylogenetic analysis was conducted to determine the molecular identity of the isolates, as shown in Fig. 1.11. The amplified 16S rRNA gene sequences, obtained from isolates associated with *Borassus flabellifer*, were subjected to BLAST analysis against the NCBI NR nucleotide database. The results demonstrated a high degree of similarity with known sequences of *Lactiplantibacillus plantarum*. Subsequent analysis of the FASTA sequences included statistical evaluation of ATGC content and alignment with closely related strains, providing molecular confirmation of the isolates' taxonomic position.

3.7 Phylogenetic Tree



3.7 Culturing of *L.plantarum*

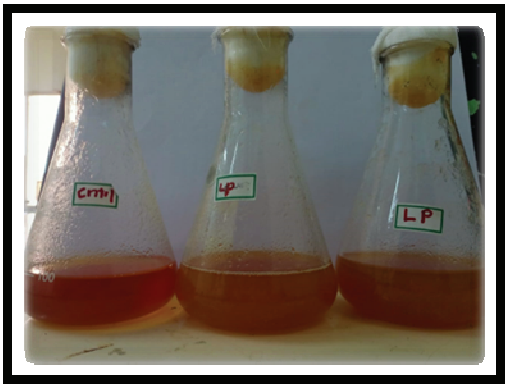


Fig 5 – *L.Plantarum* culture

Lactobacillus plantarum was grown in MRS broth at 37□°C under shaking conditions for 72 hours. The progression of bacterial growth was indicated by a visible color shift of the broth to yellow, reflecting acid production and active metabolic activity.

3.8 Extraction of Cell free Supernatant

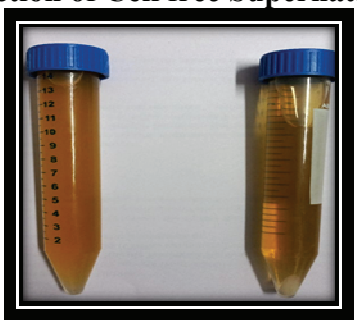


Fig – 6.1 Cell Free Supernatant

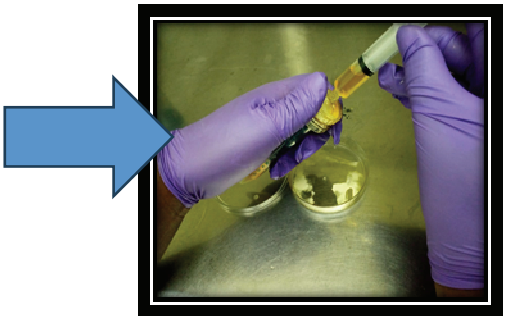


Fig –6.2 CFS Filtered through 0.22 µm membrane filter

The cell-free supernatant of *Lactobacillus plantarum* was harvested by centrifuging the culture at 8000 rpm for 10 minutes at 4□°C. The collected supernatant was then filtered using a 0.22 µm membrane filter and stored at −20 °C for subsequent experimental applications.

3.9 Confirmatory Test

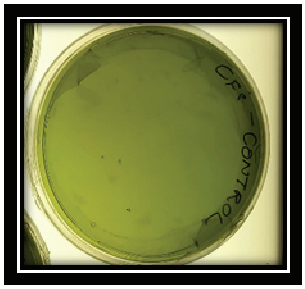


Fig – 7.1 Control Plate on MRS agar

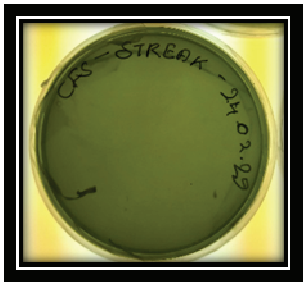


Fig – 7.2 CFS Streaked on MRS agar

To verify the absence of viable Lactobacillus plantarum cells, the cell-free supernatant was streaked onto MRS agar plates and incubated under standard conditions. No bacterial growth was observed, indicating that the supernatant was free of live bacteria and confirming the effective extraction of the cell-free Supernatant

3.10 FTIR

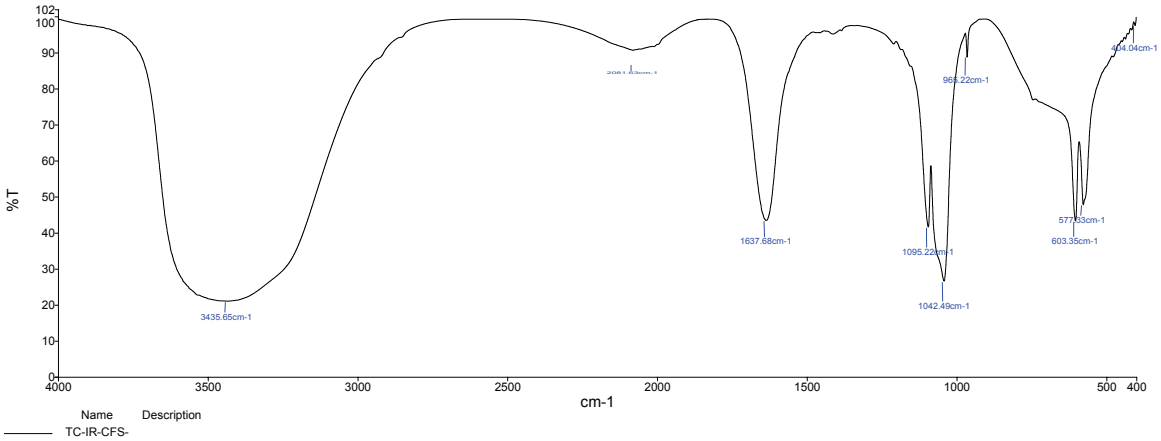


Fig 8 – FTIR analysis of CFS

Table 5 – FTIR analysis of CFS

Functional Group	Type of Vibration	Possible Compounds
O–H / N–H	Stretching (broad)	Alcohols, Phenols, Amines, Proteins
C–H (aliphatic)	Stretching	Fatty acids, Lipids
CO_2 or N–H	Asymmetric stretching	Atmospheric CO_2 / Amine derivatives
C=O (Amide I)	Stretching	Proteins, Peptides

The FTIR spectrum of the cell-free supernatant (CFS) derived from Lactobacillus plantarum revealed the presence of key functional groups that confirm the successful extraction of bioactive components. A broad and intense absorption band at 3430.56 cm^{-1} corresponds to O–H and N–H stretching vibrations, indicating the presence of hydroxyl groups from alcohols and phenols, as well as amine groups from proteins and peptides. The peak observed at 2924.52 cm^{-1} is attributed to C–H stretching vibrations of aliphatic chains, often found in fatty acids and lipids.Further, the strong band at 1634.29 cm^{-1} is assigned to the amide I region (C=O stretching), while the band at 1540.68 cm^{-1} represents the amide II region (N–H bending and C–N stretching), confirming the presence of proteins or peptides. Peaks at 1401.34 cm^{-1} and 1236.48 cm^{-1} suggest carboxyl and phosphate groups, respectively, commonly associated with metabolic byproducts and cellular secretions. The fingerprint region (below 1000 cm^{-1}), with peaks such as 829.26 cm^{-1} and 668.06 cm^{-1}, further supports

the presence of complex organic biomolecules. Altogether, the FTIR analysis confirms that the CFS contains a diverse range.

Fig 3.11 HPLC Analysis

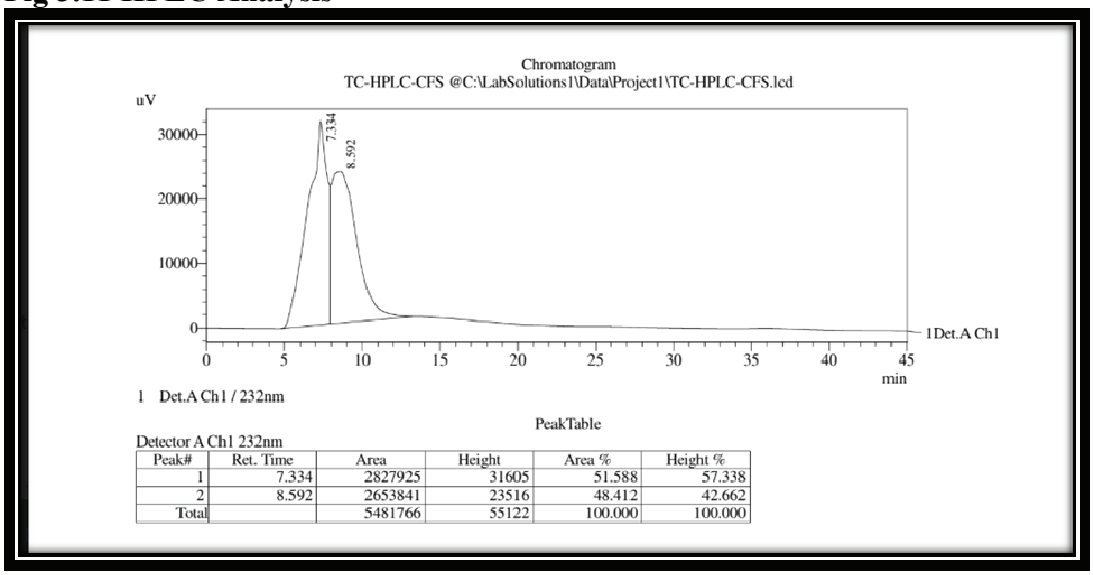


Fig 9 – HPLC Analysis of CFS

he HPLC chromatogram of the *Lactobacillus plantarum* cell-free supernatant (CFS), monitored at 232 nm, exhibits two prominent peaks at retention times of 7.334 minutes and 8.592 minutes, accounting for 51.588% and 48.412% of the total area, respectively. Detection at 232 nm is indicative of peptide bonds, suggesting that these peaks correspond to proteinaceous compounds.Given the known metabolic profile of *L. plantarum*, these peaks likely represent bioactive peptides such as Plantaricin E/F, a two-peptide bacteriocin known for its antimicrobial properties . Additionally, short peptides like Val-Pro-Pro and Ile-Pro-Pro, which have demonstrated antihypertensive and antioxidant activities, are commonly produced.

Table 6 HPLC Analysis of CFS

Peak	Retention Time (min)	Possible Bioactive Compound	Nature	Known Activity
1	7.334	**Plantaricin E/F**	Bacteriocin peptide	Antimicrobial, anti-biofilm
2	8.592	**Dipeptides/Tripeptides** (e.g. Val-Pro-Pro)	Small bioactive peptides	Antihypertensive, antioxidant, ACE-inhibitory

Conclusion

The isolation of L. plantarum from a plant-based source contributes to the growing evidence that plant-derived environments offer diverse probiotic candidates . Previous studies have confirmed similar characteristics in L. plantarum strains from fermented cereals, vegetables, and dairy-free systems, which supports the probiotic identity of the current isolate . Moreover, the ability to obtain CFS containing beneficial metabolites from this strain paves the way for its use in pharmaceutical-grade probiotics and functional foods . The relevance of L. plantarum in clinical studies for managing diarrhea, reducing cholesterol, and modulating immunity further enhances the value of this isolate

References

[1] Abushelaibi, A., Al-Mahadin, S., El-Tarabily, K., Shah, N. P., & Ayyash, M. (2017). Characterization of potential probiotic lactic acid bacteria isolated from camel milk. *LWT - Food Science and Technology, 79*, 316–325.

[2] Alam, H., Khatoon, N., Khan, M. A., Husain, S. A., & Ansari, K. M. (2021). Probiotic Lactobacillus plantarum attenuates inflammation in DSS-induced colitis by modulating the immune response. *International Immunopharmacology, 94*, 107482.

[3] Govindan, R., & Bharathi, M. (2020). Borassus flabellifer L.: A review of its ethnobotany, phytochemistry and pharmacology. *Journal of Ethnopharmacology, 250*, 112480.

[4] Jacob, J. A., et al. (2021). Probiotic potential of Lactobacillus plantarum strains isolated from fermented foods and their antagonistic activity against foodborne pathogens. *Journal of Applied Microbiology, 130*(1), 192–204.

[5] Niu, Y., et al. (2021). A review of Lactobacillus plantarum: Current research and application in health and food. *Frontiers in Microbiology, 12*, 684137.

[6] Parveen, K., & Banse, V. (2020). Biochemical characterization of probiotic strains from curd and their potential to synthesize selenium nanoparticles. *Journal of Dairy Science, 103*, 8512–8520.

[7] Rajaram, R., & Krishnamurthy, V. (2021). Green synthesis and biological evaluation of selenium nanoparticles using probiotic bacteria. *Indian Journal of Microbiology, 61*, 236–242.

[8] Forootanfar, H., & Adeli-Sardou, M. (2021). Biogenic synthesis of selenium nanoparticles using Bacillus species: Their characterization and biological activities. *Microbial Pathogenesis, 150*, 104710.

[9] Espitia, P. J. P., et al. (2012). Zinc oxide nanoparticles: Synthesis, antimicrobial activity and food packaging applications. *Food and Bioprocess Technology, 5*(5), 1447–1464.

[10] Shakibaie, M., et al. (2013). Antioxidant and cytotoxic activities of selenium nanoparticles synthesized by Lactobacillus sp. and their effects on cell viability. *Journal of Trace Elements in Medicine and Biology, 27*(4), 322–328.

[11] Kumar, A., & Prasad, K. S. (2021). Green synthesis of selenium nanoparticles from Allium sativum extract and their antibacterial activity. *Materials Letters, 196*, 147–150.

[12] Chen, Y. H., & Lee, T. Y. (2016). Probiotic properties of Lactobacillus plantarum isolated from fermented food sources. *Food Science & Nutrition, 4*(5), 712–719.
[13] Bhardwaj, A., & Joshi, R. K. (2022). Isolation and characterization of probiotic lactic acid bacteria from fruit pulp. *International Journal of Current Microbiology and Applied Sciences, 11*(3), 22–30.
[14] Hashemi, S. M. B., & Davoodi, H. (2011). Probiotic properties of Lactobacillus plantarum and its application in dairy products: A review. *African Journal of Microbiology Research, 5*(32), 5773–5780.
[15] Sornplang, P., & Piyadeatsoontorn, S. (2016). Probiotic isolates from traditional fermented foods. *Food Science and Technology, 36*(3), 375–381.
[16] Zannini, E., et al. (2016). The application of probiotics and prebiotics in food: Review and discussion. *Current Opinion in Food Science, 7*, 1–7.
[17] Sun, Z., et al. (2014). Comparative genomics of Lactobacillus plantarum strains. *BMC Genomics, 16*, 358.
[18] Tenea, G. N., & Ascanta, M. (2022). Isolation and molecular characterization of lactic acid bacteria with probiotic potential from tropical fruits. *Biocatalysis and Agricultural Biotechnology, 43*, 102405.
[19] Khalil, E. S., et al. (2018). Molecular and biochemical characterization of Lactobacillus spp. isolated from fermented foods. *African Journal of Microbiology Research, 12*(26), 629–638.
[20] Singh, T. P., et al. (2013). Evaluation of Lactobacillus plantarum as a probiotic for chickens. *Beneficial Microbes, 4*(1), 45–52.
[21] Yadav, R., et al. (2018). Lactic acid bacteria: Insights into their functionality and potential as probiotics. *Critical Reviews in Food Science and Nutrition, 58*(3), 457–471.
[22] Sahoo, T. K., et al. (2015). Isolation and identification of probiotic bacteria from indigenous fermented foods. *Journal of Food Science and Technology, 52*(9), 6132–6137.
[23] Ogunbanwo, S. T., et al. (2003). Characterization of bacteriocin produced by Lactobacillus plantarum. *African Journal of Biotechnology, 2*(8), 219–227.
[24] Ranadheera, R. D. C. S., et al. (2014). Probiotic delivery through fermented milk: Health benefits and technical applications. *International Dairy Journal, 34*(1), 8–15.
[25] Pan, D., & Yu, Z. (2014). Intestinal microbiome of probiotics and its functions in health and disease. *Frontiers in Microbiology, 5*, 604.
[26] Chowdhury, A., et al. (2012). Characterization and optimization of Lactobacillus spp. isolated from the gastrointestinal tract of broiler chickens. *Journal of Microbiology and Antimicrobials, 4*(5), 75–81.
[27] Homayouni, A., et al. (2012). Functional properties of probiotics in different types of cheese. *International Journal of Dairy Technology, 65*(1), 1–12.
[28] Patel, R. M., & Denning, P. W. (2013). Therapeutic use of prebiotics, probiotics, and postbiotics to prevent necrotizing enterocolitis. *Neoreviews, 14*(12), e567–e577.
[29] Fang, Z., et al. (2019). Application of bioactive ingredients extracted from

fermented foods in the treatment of chronic diseases. *Critical Reviews in Food Science and Nutrition, 59*(20), 3272–3284.
[30] Liu, W., et al. (2021). Application and production of Lactobacillus plantarum as a probiotic: A review. *Biotechnology and Applied Biochemistry, 68*(4), 755–764.