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Identification and Probiotic Evaluation of Lactic Acid Bacteria Isolated from Coconut Inflorescence Sap (Neera)

Shaima[1], Hiba Mariyam[2], M.K. Fasla Nasri[1], B. Asha[2], S. Shabanamol[2], M. Shonima Govindan[3]

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ABSTRACT

Background: Recent years have seen an increase in study interest in LAB's separation and description from a range of conventional fermented foods and items. Several traditional fermented foods were used to isolate probiotic bacteria because their probiotic properties have not been thoroughly investigated.

Methods: In the current study, the lactic acid bacteria (LAB) that were isolated from Neera, the palm of coconut flower sap extract, were tested for their potential as probiotics. Many parts of India enjoy this naturally fermented beverage. Following sample collection, Neera is carried in an ice box and maintained at 4°C for the duration of the experiment. After analysis, the pH was determined to be 6.5. The LAB strain from Neera was isolated and identified by examining its colony form, physiological characteristics and biochemical analysis. It was given the name SHC1. It is discovered that the isolated strain is catalase negative, gram positive and nonmotile. Identifying Lactobacillus species was further supported by the isolates' effective anaerobic growth at pH 6.5 on MRS agar plates.

Result: As per the findings of the acid tolerance test, the isolated LAB strain is able to withstand a broad pH range and flourishes in acidic conditions. This study's Lactobacillus species was resistant to bile salt and they were able to grow and survive at varying concentrations of bile salt. The LAB isolate had antioxidant activity in the current investigation, as demonstrated by the results of the DPPH scavenging assays, which indicated 38% inhibition at 10-1 ppm concentration of bacterial isolate. The isolate of SHC1 was identified as *Leuconostoc mesenteroides* with NCBI Accession No: NR113912.1 based on BLAST analysis of the sequencing data and 16s rDNA sequencing, which revealed the possible isolate exhibiting eminent probiotic activity. A BLAST comparison with *Leuconostoc mesenteroids* (Gen Bank accession number NR) showed 100% similarity.

Key words: Biochemical characterization, Molecular identification, Phytochemical analysis.

INTRODUCTION

Since the probiotic properties of numerous traditional fermented foods have not been well investigated, probiotic bacteria were extracted from these meals. Lactic acid bacteria (LAB) were investigated for their potential as probiotics because Neera is a naturally fermented beverage that is consumed all over India. The coconut palm flower's sap extract was used to isolate LAB. Probiotics are made from the more competitive and high-performing isolates of lactic acid bacteria (LAB), which are isolated using different food matrices (Bromley and Uchman, 2003).

Probiotics are live organisms that, when consumed in sufficient quantities, enhance the host's health by restoring the microbial balance in the system. Probiotic use has been the focus of many studies on animal diseases, particularly those affecting the gastrointestinal and vaginal systems (Pineiro and Stanton, 2007). Through a variety of methods, such as attachment to epithelial cells, immune system regulation and antimicrobial chemical production, they are skilled at stopping the spread of pathogenic organisms. Their ability to bio-preserve food and their usefulness as a fermentation starter culture under closely watched conditions are demonstrated by this. Accordingly, there has been an increase in interest in recent years in the isolation and characterization of LAB from a range of traditional fermented foods and products (Alonso *et al.*, 2018).

In several parts of India, people drink this naturally fermented beverage. Probiotic-containing dietary products are becoming more and more popular. Fermented milks,

[1]Department of Biotechnology, SAFI Institute of Advanced Study (Autonomous), Vazhayur-673 640, Malappuram, Kerala, India.
[2]Department of Microbiology, SAFI Institute of Advanced Study (Autonomous), Vazhayur-673 640, Malappuram, Kerala, India.
[3]Department of Biochemistry, SAFI Institute of Advanced Study (Autonomous), Vazhayur-673 640, Malappuram, Kerala, India.

Corresponding Author: M. Shonima Govindan, Department of Biochemistry, SAFI Institute of Advanced Study (Autonomous), Vazhayur-673 640, Malappuram, Kerala, India.
Email: shonima.g@siasindia.org
ORCID: https://orcid.org/0009-0001-6615-0998

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ice cream, buttermilk, cheese, milk powder and yogurts are among the primary dairy-based products (Stanton *et al.*, 2001). Among the non-dairy food items include cereals, nutrition bars, soy-based products and various juices (Ewe, 2010; Sheehan *et al.*, 2007). In recent years, there has been a lot of interest in studying the symbiotic relationship between the bacterial occupants and their human host. The gut microbiome refers to the group of bacteria and their collective genomes that are found in the gastrointestinal tract (Marchesi, 2015). The human gut microbiome is crucial for homeostasis, health and illness. The beneficial benefits of gut bacteria are primarily linked to immunological response, hunger and gastrointestinal function (Thaiss *et al.*, 2016; Azcarate-Peril *et al.*, 2017; Fetissov, 2017; Van de Wouw *et al.*, 2017). Numerous adverse effects have also been documented in a variety of illnesses and ailments, with many of these linked to changes in gut bacterial composition (Jiang *et al.*, 2015; Miele *et al.*, 2015; Aron-Wisnewsky *et al.*, 2016; Leung *et al.*, 2016; Budden *et al.*, 2017; Wilson Tang and Hazen, 2017; Yu and Schwabe, 2017; Schirmer *et al.*, 2018; Van de Wouw *et al.*, 2017). With the right marketing, Neera might develop into a wholesome beverage that offers a healthy substitute for aerated drinks. The goal of the current project is to separate and describe the powerful probiotic bacteria found in coconut neera in order to create a probiotic beverage.

MATERIALS AND METHODS

Sample collection

Fresh Neera, the sample, was taken off the coconut palm. Neera Technicians from Vadakara Coconut Farmers Producer Company Limited assisted in the collection of the samples. The experiment was conducted in Department of Biochemisrty, SAFI Institute of Advanced Study (Autonomous), Vazhayur, Malappuram, Kerala during the year 2023-2024.

Chemicals

All of the analytical-grade chemicals and lab supplies used in the investigation were supplied by HIMEDIA, Sigma and Merck.

Phytochemical analysis

The Neera was initially screened for qualitative phytochemicals. The phytochemical methods described Molisch's tests for carbohydrates, Mayer's and Wagner's tests for alkaloids, frothing test for saponins, Biuret test for protein, Ninhydrin test for amino acids, Lead acetate test for phenols, flavonoids, carotenoids and terpenoids.

Isolation of lactic acid bacteria from neera

Strains were isolated using samples of Neera (naturally fermented nectar of coconut palm) in line with the procedure outlined by Poornachandra Rao *et al.* (2015). After being enriched with a de Man, Rogosa and Sharpe (MRS) broth, approximately 1 milliliter of the material was grown for 24 hours at 37°C in an anaerobic environment. The enhanced broth samples were plated on MRS agar, diluted with buffer saline and then grown for 24 hours at 37°C in an anaerobic environment. After that, MRS agar plates were used to subculture the colonies with distinctive morphologies. The viable cultures were kept in MRS slants, while the stock cultures were kept at 4°C for additional examination.

Biochemical characterization of the selected strain

Biochemical characterisation and morphological analysis were used to identify the isolates. Bergey's handbook was used to identify the isolates by rummage-sale. All colonies' microscopic characteristics, including color, size and form, were noted before the staining process was carried out. The hanging drop method, oxidase, catalase, indole, MR-VP, citrate utilization, urease, triple sugar iron agar, starch hydrolysis, gelatin and indole generation tests were used to examine the isolates for motility.

Acid tolerance test

A modified version of Erdorul Ozlem and Erblr Feryal's, 2006 approach was used to conduct the test. The isolate's optimum development at different pHs was checked by subculturing a single isolated colony from a 1% (v/v) fresh overnight culture of the isolate in MRS broth with pH values of 2,4,5 and 6.5 adjusted using NaOH (1.0 M) or HCl (1.0 M). The colony was then incubated at 37°C for 24 hours. Using a spectrophotometer that measures optical density at 600 nm versus uninoculated broth, the growth of bacteria was then evaluated to see how well the isolates could flourish at different pH levels.

Test for bile salt tolerance test

To examine the strains' bile salt resistance, a modified Gilliland and Colleagues, (1984) approach was used. This test examines the greatest growth after isolates have been inoculated into MRS broth tubes containing 0.5, 1, 1.5, 2 and 3% bile salts. After 18 to 24 hours of incubation at 37°C, bacterial growth was measured using absorbance at 600 nm. MRS broth devoid of bile salt served as the experiment's control.

Antioxidant assay

Blois (1958) approach was used to analyze the DPPH Scavenging activity. Before being used, a newly made methanolic solution (0.1 M) of DPPH was stored at 4°C in the dark. One milliliter of the material was mixed with one milliliter of methanolic DPPH solution. The sample was swapped out for a 100% methanol 28 solution to create the control reaction. The combination was allowed to sit at room temperature in the dark for half an hour. Determine the absorbance using a UV visible spectrophotometer set to 517 nm.

% DPPH Scavenging Activity=

$$100\times \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}}$$

Antibiotic susceptibility test

The isolate's antibiotic susceptibility was evaluated on MRS agar plates using the antibiotic disc diffusion method and the antibiotic range recommended by EFSA standards.

Hemolytic activity

After streaking fresh bacterial cultures onto blood agar media, they were cultured for 24 hours at 37°C. Next, the isolates were checked to see if there was a clean zone encircling the colonies. Greenish zones indicate alpha hemolysis, clear zones indicate beta hemolysis and the lack of zones showing no hemolysis is referred to as gamma hemolysis. According to Halder *et al.* (2017), only colonies with gamma hemolysis were chosen; colonies exhibiting beta or alpha hemolysis were not included.

16S rRNA gene amplification and phylogeny analysis

Global bacterial primers were used to molecularly characterize isolated bacterial strains based on 16S rRNA conserved gene sequences. Bacterial Genomic DNA Purification Kit HiPurATM (MB505, Himedia) Using the conformist PCR method, the targeted gene sequence was expanded. The size of the amplified fragments was then investigated by running the final result to 1.5% gel electrophoresis. MEGA software was used to phylogenetically analyze the nucleotide sequences that were recovered after the amplified samples and relevant sequencing segments were sent for sequencing. Using the BLAST tool, bacterial isolates were further verified at the species level using a BLAST search of the GenBank/EMBL database. In order to grant these strains unrestricted access, 16S rRNA gene sequences were submitted to the GenBank database and assigned accession numbers.

RESULTS AND DISCUSSION

Collection of coconut nectar neera

The sample, fresh neera, was removed off the coconut palm. The samples were promptly put in an icebox and brought to the laboratory for further analysis. They are kept at 4°C for the length of the experiment. The pH was found to be 6.5 after analysis.

Isolation of lactic acid bacteria from neera

The LAB strain was separated and the result is displayed in Fig 1. The isolated strain was given the designation SHC1.

Phytochemical analysis for neera

The results of the Phytochemical analysis of Neera are presented in Table 1.

Biochemical characterization of LAB isolate

Characteristic the results of the biochemical analysis of Lab isolate SHCI are displayed in Table 2.

Probiotic potential of LAB isolate SHC1 tested *in vitro*

Acid tolerance test

Acid tolerance facilitates the study of strain survival in low pH gastric juice conditions. The findings of an examination performed at 37°C to ascertain the SHC1 strain's resistance to acidic pH conditions are displayed in Fig 2.

Bile tolerance test

The isolate's capacity to withstand bile salts facilitates the evaluation of its metabolic activity and small intestine colonization *in vitro*. Fig 3 displays the results of an examination of the bile tolerance of the SHC1 strain.

Antioxidant assay of LAB isolate SHC1

Table 3 displays the findings of an analysis of the SHC1 strain's capacity to scavenge DPPH.

Hemolytic activity

Fig 4 displays the lab isolate's hemolytic activity, which demonstrates gamma hemolysis and the lack of a zone surrounding colonies.

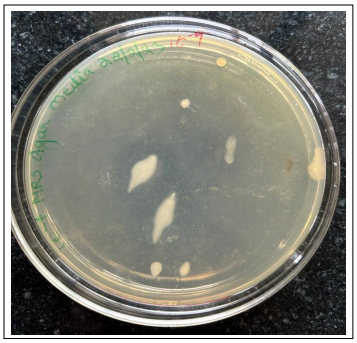


Fig 1: LAB isolate SHC1.

Table 1: Phytochemical analysis.

Constituents	Result
Alkaloid	+ve
Carbohydrates	+ve
Saponin	-ve
Protein	+ve
Amino acid	+ve
Phenols	+ve
Flavanoids	+ve
Carotenoids	-ve
Terpenoids	+ve

Table 2: Phenotypic and biochemical characterization of LAB isolate.

Test	Isolate (SHC1)
Gram staining	+ve
cell shape	Cocci shaped
motility	Non-motile
Indole	-ve
Methyl red	-ve
Voges-proskauer	-ve
Citrate utilization	-ve
Urease	-ve
Catalase	-ve
Oxidase	-ve

Molecular identification of bacterial isolates

The isolate of SHC1 identified *via* BLAST analysis of the sequencing data is Leuconostoc mesenteroides, NCBI Accession No. NR113912.1. This was based on the BLAST analysis of the sequencing data following 16SrRNA gene sequence analysis and phylogenetic tree (Fig 5 and 6) construction by neighbor joining. Saitou and Nei, 1987 novel approach to reconstructing phylogenetic trees: the neighbor-joining method. 10.1093/oxford journals. molbev.a040454. SHC1 between Leuconostoc mesen teroids NBRC 100496 reveals 99% similarity.

The purpose of this study was to isolate Neera bacteria and evaluate their probiotic capabilities. Among the nations that use a lot of neera are India, Sri Lanka, Africa, Malaysia, Indonesia, Thailand and Myanmar. It is a medicinal and healthful beverage. Neera is typically extracted twice a day, in the morning and the evening, using the tapping process that is collected from the coconut's inflorescence. Neera is maintained at 4°C for the duration of the experiment since they are extremely vulnerable to spontaneous fermentation at room temperature within a few hours of extraction. After analysis, the pH was determined to be 6.5. Colony morphology, physiological and biochemical characterization were used to identify the LAB strain that was isolated from Neera. This strain was given the designation SHC1. It is discovered that the isolated strain is non-motile, gram positive and catalase negative. The isolates' successful growth at pH 6.5 on MRS-agar plates under anaerobic circumstances provided additional evidence that they were *Lactobacillus* species. The growth of bacteria can be significantly impacted by pH. It is necessary for probiotic microbes to be able to tolerate the low pH of the human gut. Depending on the types of food eaten, the length of time it takes for food to digest (up to 3-4 hours) and the frequency of feedings, the pH in the human stomach can vary between 1.5 to 4.5. According to the results of the acid tolerance test, the isolated LAB strain can tolerate a wide range of pH values and thrives in acidic environments. Another crucial element in determining the survival of *Lactobacillus* species is bile salts. The growth medium in this investigation were supplemented with 0.5-3% bile salts, which matched the amount seen in the

Table 3: DPPH Scavenging activity of LAB isolate SHC1.

Concentration (ppm)	DPPH Scavenging activity (percentage inhibition)
Control	-
10^{-1}	38.4±0.3
10^{-2}	1.30±0.2
10^{-3}	1.12±0.05
10^{-4}	0.42±0.1
10^{-5}	0

Data shown are Mean±SD of triplicates.

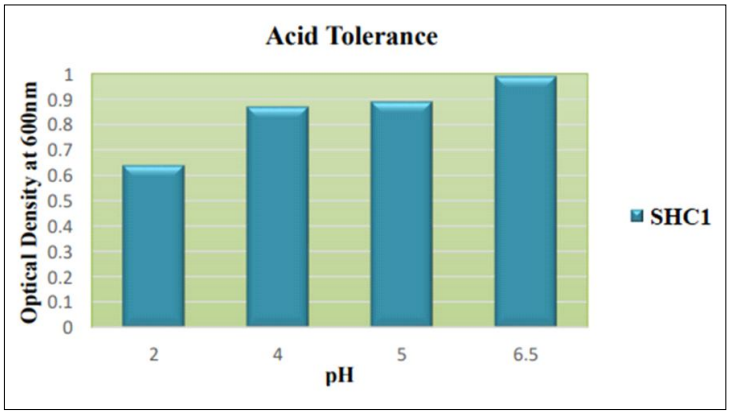


Fig 2: Acid tolerance analysis of LAB isolate SHC1 isolated from Neera.

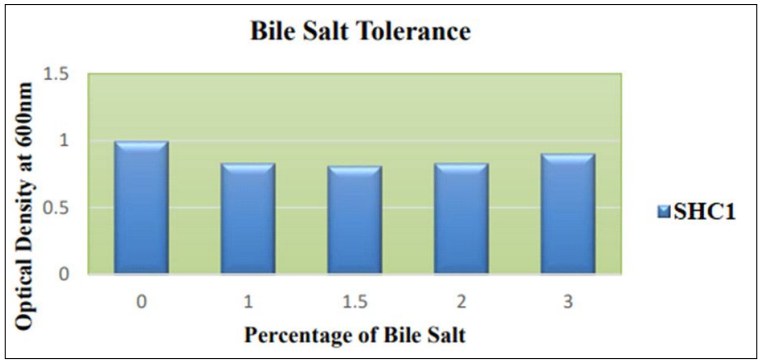


Fig 3: Bile tolerance analysis of LAB isolate SHC1 isolated from Neera.

human digestive tract. Therefore, a 0.3% bile content must be tolerated before probiotic bacteria are chosen for human ingestion. This study's *Lactobacillus* species was resistant to bile salt and they were able to grow and survive at varying concentrations of bile salt. According to the outcomes of the DPPH scavenging tests, the LAB isolate demonstrated antioxidant capacity with a 38% inhibition at a bacterial isolate concentration of 10-1 ppm. The isolate of SHC1 was identified *as Leuconostoc mesenteroides* with NCBI Accession No: NR113912.1 based on BLAST analysis of the sequencing data and 16s rDNA sequencing, which revealed the possible isolate exhibiting eminent probiotic activity. *Leuconostoc mesenteroides*, which was isolated from Coconut Neera, is the subject of this report's probiotic qualities.

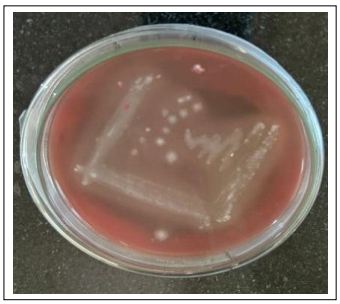


Fig 4: Hemolytic activity.

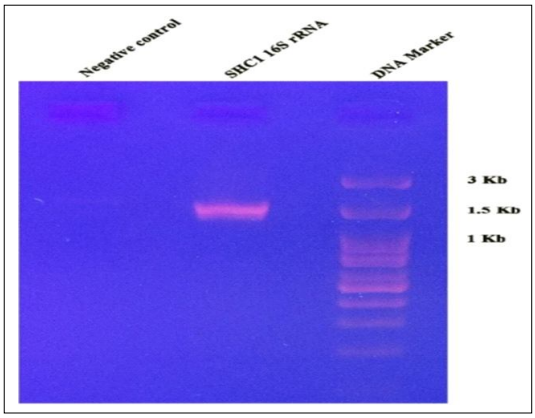


Fig 5: PCR amplification of 16S rRNA of SHC1.

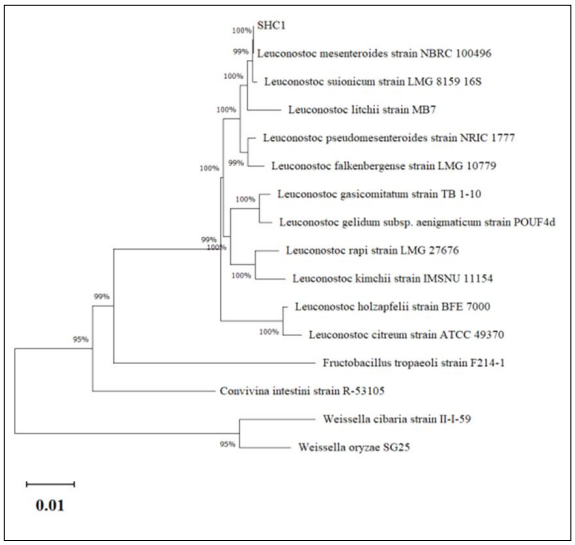


Fig 6: Phylogenetic tree showing the evolutionary relationship of the *Leuconostoc mesenteroides*.

Neera, also known as palm nectar, is a drink made from the sap of the flower of several toddy palm species. Neera extraction usually takes place prior to dawn. It has a transparent, pleasant color. It is also referred to as palm wine and can ferment naturally at room temperature within a few hours of extraction. Neera turns toddy after fermenting. India, Sri Lanka, Africa, Malaysia, Indonesia, Thailand and Myanmar are among the countries that consume a lot of neera. Neera, which has a pH of 6.5 to 7.0, aids in digestion and is heavy in carbs and nutrients. Analysis is done using the fresh Neera that was gathered hygienically prior to daybreak and transported at low temperatures. The location, timing and length of tapping all affect Neera's composition. Ethyl alcohol is produced when neera, which is extracted from the spadix of the coconut palm, naturally ferments because of the inherent presence of bacteria and yeasts (Xia, 2011). Different fermenting food matrices are utilized to separate lactic acid bacteria (LAB) and probiotics are made from the isolates that perform better and are more competitive (Bromley and Uchman, 2003). By restoring the microbial equilibrium in the body, probiotics are live organisms that, when taken in sufficient quantities, help the host's health. An important component of our intestinal microbiota, lactobacilli are the subject of much research into their impact on human health in general. The genus Lactobacillus is of significant economic relevance since it is one of the main families of lactic acid bacteria utilized in food fermentation. Thus, the goal of the current work is to separate and describe powerful probiotic lactic acid bacteria from neera.

CONCLUSION

Since neera is extremely prone to spontaneous fermentation at room temperature within a few hours of extraction, it is transported in an ice box and maintained at 4°C for the duration of the experiment. An analysis of the pH revealed that it was 6.5. Colony morphology, physiological and biochemical characterization were used to identify the LAB strain that was isolated from Neera. This strain was given the designation SHC1.

It is discovered that the isolated strain is catalase negative, gram positive and non-motile. Probiotics can establish themselves in the intestinal system because they can tolerate the bactericidal qualities of bile salts, the typical acidic conditions of gastric fluids and the creation of lactic acid, which stops the growth of other microbes. The isolated LAB strain is tolerant of a wide range of pHs and thrives in acidic environments, according to the results of the acid tolerance test. The isolates of *Lactobacillus*

species used in this investigation grew and survived at varying concentrations of bile salt and demonstrated resistance to it. Numerous discoveries in the last few decades have added to our knowledge of probiotics' antioxidant potential. When humans are under a lot of physical stress, Lactobacillus Spp has been shown to exhibit high antioxidant activity. According to the results of the DPPH scavenging tests conducted in this investigation, the LAB isolate demonstrated antioxidant capacity with a 38% blockage at a concentration of 10-1 ppm of bacterial isolate. 16srDNA sequencing was used to identify the putative isolate exhibiting notable probiotic activity and BLAST analysis of the sequencing data revealed that the isolate of SHC1 was Leuconostoc mesenteroides, NCBI Accession No: NR113 912.1. Gram-positive, facultatively anaerobic, spherical, non-motile, non-sporogenous lactic acid bacteria with an inherent high level of vancomycin resistance. Leuconostoc mesenteroides, which were isolated from Coconut Neera, showed probiotic qualities and encouraging antioxidant activities in the initial investigation of their probiotic potential. in order for Neera to be recommended as a probiotic beverage. The safety assessment of Leuconostoc mesenteroides as probiotics, however, requires more research.

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Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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