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Isolation and Identification of probiotic bacteria from natural Neera to extend the shelf life of fresh fruits and vegetables

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Abstract

Packaging and processing techniques in the food sector can adversely impact both consumer health and the environment. Consequently, there's a growing demand for minimally processed foods that retain their nutritional and sensory qualities while ensuring extended shelf life. Edible coatings have emerged as a promising solution, offering improved quality, safety, and functionality for perishable items like fruits and vegetables. These coatings regulate water diffusion, gas permeability, and oxidation, and can be applied through dipping, spraying, or coating methods. A recent study focused on isolating probiotic bacteria from Neera samples collected near Choutuppal in Nalgonda, Telangana. Ten bacterial strains were cultivated from these samples on MRS agar and subsequently sub-cultured to obtain pure cultures. Morphological analysis confirmed the purity of each culture. The isolates were then assessed for antimicrobial activity against spoilage-causing microorganisms in fruits and vegetables. Biochemical tests, including catalase, methyl red, oxidase, starch hydrolysis, citrate utilization, and Voges-Proskauer tests, were conducted to characterize the isolates. Among the ten strains, isolate 3 demonstrated the most promising characteristics, including strong antibacterial activity. Molecular identification using universal 16S rRNA primers identified this isolate as Levilactobacillus brevis. Phylogenetic analysis using Mega-4 bioinformatics software further confirmed its identity. This strain exhibited excellent performance in bile salt tolerance tests and demonstrated other probiotic activities, highlighting its potential as a functional food ingredient. The findings underscore the significance of probiotics in enhancing food quality and safety, offering a natural solution to meet consumer preferences for healthier and longer-lasting food options.

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Introduction

Fruits and vegetables are an important part of a healthy diet due to their low-calorie content (Charlton *et al.*, 2014) and several health benefits (Berger *et al.*, 2010). Consuming a diet rich in fruits and vegetables has been shown to lower the risk of several diseases, including cardiovascular disease, colon cancer, obesity, and diabetes (More *et al.*, 2020). They have become increasingly popular in recent years, and their richness of nutrients makes them necessary for everyday use (Leneveu-Jenvrin *et al.*, 2020). Producing minimally processed fruits and vegetables has helped meet the rising demand for fresh produce (both whole and cut) in many developed countries over the past decade. This is because these foods are both nutritious and easy to prepare. Fruits and vegetables that can be stored for longer without losing their quality are one way in which minimal processing techniques are replacing more conventional ones (De Corato *et al.*, 2020; Hasan *et al.*, 2020). Food quality declines during preservation, a major issue for food manufacturers and a significant contributor to food waste. Recently, novel and effective food handling methods have been invented to lead to the extension of food safeguarding, shelf-life extension, and, thus, food waste decrease (Stan *et al.*, 2019; Stan, 2020; Verma *et al.*, 2021; Chitrakar *et al.*, 2021; Nezami, 2020). But not all of these cutting-edge technologies are viable commercial solutions because they influence customer behavior (Rabadán *et al.*, 2021; Stefanoiu *et al.*, 2018). Edible packaging, coatings, and films are novel solutions to this issue because they protect perishable goods, delaying spoilage from microorganisms and preventing loss of moisture and gas (Dehghani *et al.*, 2018).

Research into edible packaging systems is increasing annually as more people seek less conventional and more nutritious foods. Senses of smell, taste, and sight can be preserved using edible films and coatings as the principal packaging material for goods with edible ingredients. The ripening of produce coated with edible films is slowed, and its shelf life is extended (Hassan *et al.*, 2018; Ulusoy *et al.*, 2018). Wax was applied to oranges in China in the 12th and 13th centuries to create an edible coating. Edible coatings produced from boiling soybeans were developed in Japan in the 15th century (Tural *et al.*, 2017) and were used to enhance the visual appeal of various foods. The edible packaging industry has proliferated in the past few years, with a projected valuation of $1097 million by 2023 (Mamtani *et al.*, 2021). Edible packaging has two separate applications in the food sector. Edible coatings can be sprayed directly onto the food item or onto a prefabricated film that is then coiled around the food item (Suhag *et al.*, 2020).

The food sector needs help with customer acceptability regarding novel manufacturing methods, such as edible coatings and films (Vital *et al.*, 2018), even though these can assist in extending the shelf life of numerous food items. Consumer acceptability is vital to the production of effective food products. Hence insight into how consumers create and interpret opinions about novel technology and goods is essential for food chain invention (Stan *et al.*, 2019; Siegrist *et al.*, 2021). Several studies have been conducted to determine whether or not consumers will embrace novel processing technologies and techniques, such as nanotechnology (Peters *et al.*, 2016), radio frequency (Stefanoiu *et al.*, 2018), food irradiation (MacRitchie *et al.*, 2014), and edible coatings and films (Wan *et al.*, 2007). This investigation examines the current state of knowledge regarding the use of edible formulations on a variety of less processed fruits and vegetables, with a particular emphasis on the scientific aspects of this practice, involving coating ingredients and composition, implementation techniques, and the impact on food shelf life and quality, which involves nutritional quality.

Probiotics are live bacteria that help humans stay healthy. Maintaining viability and metabolic activity is essential from when food is harvested until the consumer consumes it. Although protecting against these microbes is crucial, the question of when and where to release them still needs to be answered. There is no issue with release when employing edible

films or food coatings because they are both ingested with the food. However, these coatings help extend the storage life of perishable commodities like fresh produce. Beneficial microbes, or probiotics, aid humans when consumed in adequate amounts. Protecting against harmful germs, boosting mucus formation, and improving gastrointestinal mucosa proliferation are all functions these microorganisms perform well. Additionally, they contain immunogenic qualities that lessen the side consequences of diarrhea, avoid intestinal inflammation, lower blood cholesterol levels, prevent allergies, and regulate genital and urinary tract diseases (E Silva *et al.*, 2014). One of the most efficient ways to obtain probiotics is by incorporating them into your food, including cornflakes (Dadgar *et al.*, 2014), pomegranate juice (Khanbagi Dogahe *et al.*, 2015), dough (Javanmard *et al.*, 2013), cheese (Tavakoli *et al.*, 2016), yogurt (Massoud *et al.*, 2015; Beheshtipour H *et al.*, 2012), processed milk (Beheshtipour *et al.*, 2013), and grape drink (Malganji *et al.*, 2016). Helpful substances and bioactive substances are produced by probiotic varieties during their residency, including peptides with opiate and antithrombotic effects, attached linolenic acid, and propionic acid (Massoud *et al.*, 2015; Gholami *et al.*, 2014; Farhadi *et al.*, 2013).

Using these beneficial microbes has been reported to decrease oxidative stresses and inflammatory mediators (Mohammadi *et al.*, 2015; Mohammadi *et al.*, 2015), as well as remove poisons and heavy metals (Massoud *et al.*, 2020; Siahmoshteh *et al.*, 2016). As stated by Hosseini *et al.* (2013) and Soheili *et al.* (2011), prebiotics are used to promote the development of probiotics. More research into the human gut microbiome can lead to identifying hitherto unrecognized prebiotics and probiotics (Gómez *et al.*, 2016). Probiotics' survival and metabolic activity must be preserved through food manufacturing, after ingestion, and within the gastrointestinal system (Nguyen *et al.*, 2016). For example, lactic acid bacteria have been shown to increase the nutritional value of foods by contending with pathogens for nutrients (such as vitamins, minerals, trace elements, and peptides) and by creating organic acids and bacteriophages (antimicrobial peptides) to combat spoilage during storage. Thus, using probiotics might lengthen the period that vegetables and fruits can be stored, avoiding being linked to their antagonistic effects (Alegre *et al.*, 2011). Protecting fruits and vegetables with edible coverings has become a common practice recently. By limiting postharvest moisture loss, gas exchange, respiration, and oxidative processes, edible coating with semi-permeable films might extend the storage life of fruit (Khodaei *et al.*, 2019; Petriccione *et al.*, 2015). Films and coatings for edibles can be fabricated from a wide range of biocompatible materials, including lipids, polysaccharides, proteins, and their respective combinations (Pereira *et al.*, 2016). The food packaging business and the network of edible polymer films can both benefit from the incorporation of probiotics. An alternate strategy for managing dangerous microbes and bolstering food safety is provided by incorporating probiotics and other active chemicals into the structure of biopolymers. Research on both probiotics and food packaging has increased over the past two decades (Espitia *et al.*, 2016), yet there has been relatively little research on the use of probiotics in food packaging. It was first suggested in 2007 (Tapia *et al.*, 2007) that probiotics may be used in consumable films. Therefore, there is continuing investment in the study and production of probiotics films and coatings for proactive packaging. These coatings and films could serve as viable replacements for transporting probiotics. Active or bioactive packing, like probiotic material for packaging, can improve food stability and even positively affect the health of the customer. This research aimed to determine which strains of lactic acid bacteria could be isolated from Neera to improve food safety and shelf life without sacrificing nutritional value.

Materials and methods

Study Location and Sample Collection

A total of 3 samples were collected from the local areas of Choutuppal, Nalgonda, and Telangana, and processed for isolating new bacterial strains.

Media

HiMedia Laboratories Pvt. Ltd., Mumbai, India, was contacted for the acquisition of *Lactobacillus* (MRS) agar, broth, and an antibiotic susceptibility disc. The pathogens were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India, and their accession numbers are as follows: *Bacillus subtilis MTCC 10403, Escherichia coli MTCC 4430, Pseudomonas aeruginosa MTCC 424, Micrococcus luteus MTCC 1809, and Salmonella typhimurium MTCC 98*.

Bacterial Isolation from Neera

The microbial population in the obtained neera samples was enriched by inoculating them into enrichment media (MRS broth media). After letting the batters ferment overnight, they were diluted serially (phosphate saline 0.1 M, pH 7.2) and poured onto a plate of MRS agar that had hardened. 24-48 hours of anaerobic incubation were conducted at 37 degrees Celsius. Pure cultures were streaked onto an MRS agar plate after being selected from colonies with distinct morphological features. Isolated settlements were sub-cultured in MRS broth and kept at four °C until storage. The isolated strains were processed into an extract (CE) and supernatant (CS) according to the protocol described by Jo *et al.* (2021). Remote pure cultures were then subcultured onto slants for additional study using the spread plate method. The morphological, biochemical, and molecular analyses relied on these pure cultures.

Antibacterial activity

Preparation of nutrient agar plates followed by the spreading of lab cultures of pathogenic bacteria, including *Pseudomonas fluorescens* (MTCC 9768), *Escherichia coli* (MTCC 424), *Staphylococcus aureus* (MTCC 96), *Klebsiella pneumoniae* (MTCC 272), and *Bacillus subtillis* (MTCC 3053). The paper-dip approach is then used to place the triggered samples and wait 24 hours. After 24 hours of incubation, a distinct zone of bacterial inhibition was visible surrounding the selection and quantified. Anti-bacterial activity-displaying models are used in further research (Mohamed *et al.*, 2020).

Biochemical identification of the bacterial isolate PKN 3

Gram staining, indole test, methyl red test, Voges-Proskauer test, citrate utilization, catalase test, and glucose fermentation test were used to identify bacterial isolate PKN 3 morphologically. Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986) was used for all tests.

Evaluation of probiotic attributes in vitro

Acid and bile salt

With a few tweaks, the acid and bile salt tolerance experiments were conducted using the same methods as Pan *et al.* (2009) stated. The MRS broth (pH two and (0.3 and 1%) ox gall salt) was infected with 100 L of the isolates and incubated at 37°C. The samples were counted after 0, 2, and 4 hours of incubation.

Molecular identification of PKN 3 based on 16S rRNA gene sequence

The PKN 3 isolate was sent to MACROGEN (Seoul, Korea) to be sequenced with universal 16S rRNA primers to determine its molecular identity.

Mega-4 was used for the phylogenetic analysis in this study. The sequence of 16S rRNA has been uploaded to NCBI.

Results and discussion

Morphological studies

Gramme staining was used to determine the morphological identity of the isolates. Isolates were discovered to be a creamy white tint and to have an uneven, spherical shape. The sizes of the isolates varied widely. Flat to undulating in height, completely opaque around the edges.

Antibacterial activity

MTCC 3053 *Bacillus subtilis*, MTCC 96 *Staphylococcus aureus*, MTCC 274 *Klebsiella pneumoniae*, MTCC 424 *Escherichia coli*, and MTCC 9768 *Pseudomonas fluorescens* were used to assess the antibacterial properties of neera isolates. Plant extracts made with methanol performed significantly better than those from other solvents (Figs 2–6).

Table 1. Morphological studies of the pure isolates.

Isolate no	Color	Shape	Size	Elevation	Margin	Opaque
Isolate 1	Cream white	Irregular	large	Raised	Filiform	Non-Transparent
Isolate 2	Cream white	Circular	Large	Raised	Entire	Non-Transparent
Isolate 3	Cream white	Irregular	Medium	Raised	Undulate	Non-Transparent
Isolate 4	Cream white	Circular	Large	Raised	Entire	Non-Transparent
Isolate 5	Cream white	Irregular	Medium	Flat	Undulate	Transparent
Isolate 6	Cream white	Irregular	Medium	Flat	Undulate	Non-Transparent
Isolate 7	Cream white	Circular	Small	Slightly raised	Entire	Non-Transparent
Isolate 8	Cream white	circular	Medium	Flat	Entire	Transparent
Isolate 9	Cream white	irregular	Large	Flat	Undulate	Non-Transparent
Isolate 10	Cream white	irregular	small	flat	entire	Non-Transparent

Table 2. Zone of inhibition of isolates against pathogens.

Isolate no	*Bacillus*	*staphylococcus*	*Klebsiella*	*E. coli*	*Pseudomonas*
Isolate 1	0	0.7cm	0.8cm	0	1.2cm
Isolate 2	0	0	0.5cm	1.0cm	1.1cm
Isolate 3	1.0cm	0.7cm	0.6cm	0.6cm	1.7cm
Isolate 4	0	0	0	0.4cm	1.1cm
Isolate 5	0	0	0.7cm	0	1.0cm
Isolate 6	1.0cm	0.4cm	0	0	1.5cm
Isolate 7	0.7cm	0	0	0	1.5cm
Isolate 8	1.2cm	0	0	0.6cm	1.0cm
Isolate 9	1.0cm	0	0	0.6cm	1.0cm
Isolate 10	0.8cm	0	0	0	1.3cm
Amp antibiotic	1.9cm	1.0cm	1.0cm	1.3cm	2.0cm

Ten bacterial isolates were used to assess the antibacterial activity, and the results are summarised in Table 2 Isolate 3 (1.7cm) had the best antibacterial activity versus Pseudomonas, followed by isolate 6 (1.5cm) and isolate 10 (no action). Isolate 1 had the highest antibacterial activity against *Klebsiella pneumonia* MTCC 272 at 0.8cm, while isolates 6–10 had none. Antibacterial activity against *Bacillus subtilis* MTCC 3053 was harmful to Isolate 1, 2, 4, and 5, positive for Isolate 8, and highest for Ampicillin (1.9cm). Ampicillin showed similar significant action (0.80 cm) against *Staphylococcus aureus* MTCC 96 (1.0 cm), isolate 3 (0.7 cm), and no activity against isolates 2, 4, 5, 7, 8, 9, and 10.

Table 3. Morphological characteristics of isolates.

S. No.	Morphological characteristics	Observations
1	Surface	Smooth, Creamy
2	Opacity	Translucent
3	Color	Off white
4	Motility	Non motile
5	Gram staining	Positive
6	Cell shape	Rods

Table 4. Biochemical tests against bacterial isolates.

S. No.	Tests performed	Observations
1	Indole	Negative
2	Methyl red	Positive
3	Vogues Proskauer	Negative
4	Citrate	Negative
5	Catalase	Negative
6	Oxidase	Negative
7	Starch hydrolysis	Positive

All of the isolates tested positive for antibacterial activity against *Pseudomonas fluorescens* MTCC 9768, with Ampicillin producing the best results (2.0cm), Isolate three coming in second (1.7cm), Isolates 7 and 8 tied for third (1.5cm), and so on.

Fig. 1. Neera sample collected from the local area.

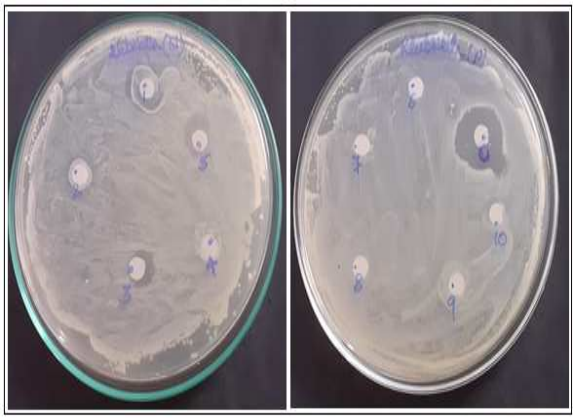

Fig. 2. Neera sample antibacterial activity against *Klebsiella*.

Gram staining techniques were used to conduct morphological analyses of the isolates (Table 3).

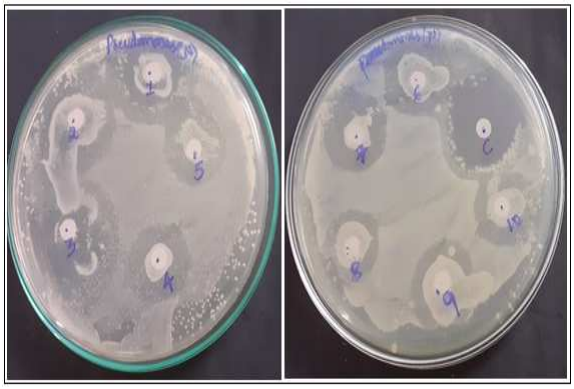

Fig. 3. Neera sample antibacterial activity against *pseudomonas*.

The research discovered that the isolates' surfaces were creamy and smooth, with a translucent opacity. The isolates were non-motile and appeared to be an off-white tint. Isolates were gram-positive and rod-shaped when tested.

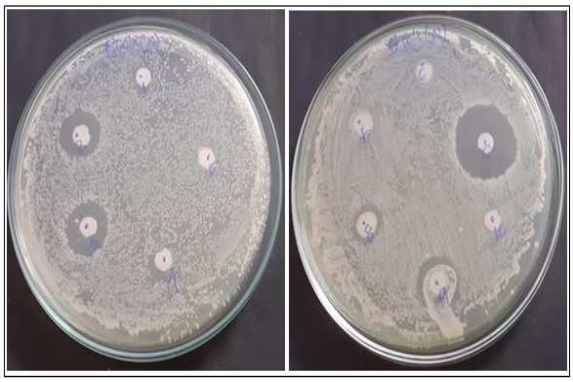

Fig. 4. Neera sample antibacterial activity against *E coli*.

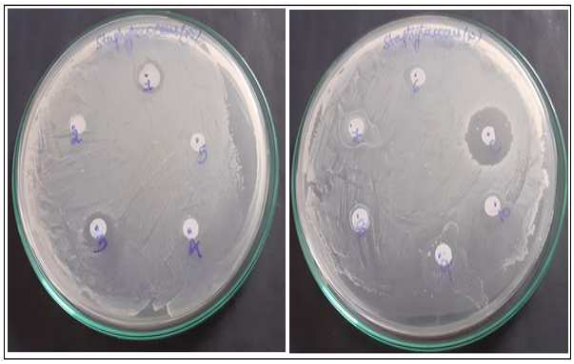

Fig. 5. Neera sample antibacterial activity against *staphylococcus*.

Biochemical Characterization

The indole assay evaluates the microbe's ability to metabolize the amino acid tryptophan into indole (Table 4). The appearance of a red hue in the media indicates the existence of indole.

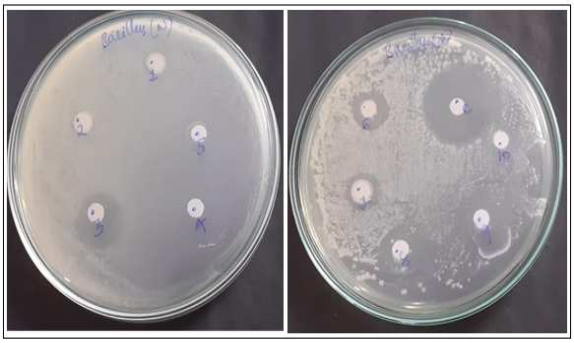

Fig. 6. Neera sample antibacterial activity against *Bacillus*.

The sample fails because the top layer of isolates does not become the expected cherry red in the Indole test. We added a few drops of a methyl red solution to MR media after incubating bacterial isolates in the medium at 35 degrees Celsius for 48 hours to show the presence of mixed acid fermentation metabolites in glucose media. The isolates above tested positive for the MR test because they turned red when exposed to methyl red. The VP test involved inoculating the VP medium with bacterial isolates, incubating the mixture at 35 degrees Celsius for two days, and adding alpha-naphthol and potassium hydroxide.

Fig. 7. Molecular identification of isolates 3.

The conversion of glucose to acetone produces a pleasant red color change. When V-P reagents I; and II were added to the test tubes, the isolates did not change color. Isolates failed the VP test because they did not become red when subjected to the V-P test.

Bacteria can convert citrate to oxaloacetate, which is then converted to pyruvate and carbon dioxide. The change in color from green to bright blue after 48 hours of incubation indicates that the media's pH has increased (to greater than 7.6).

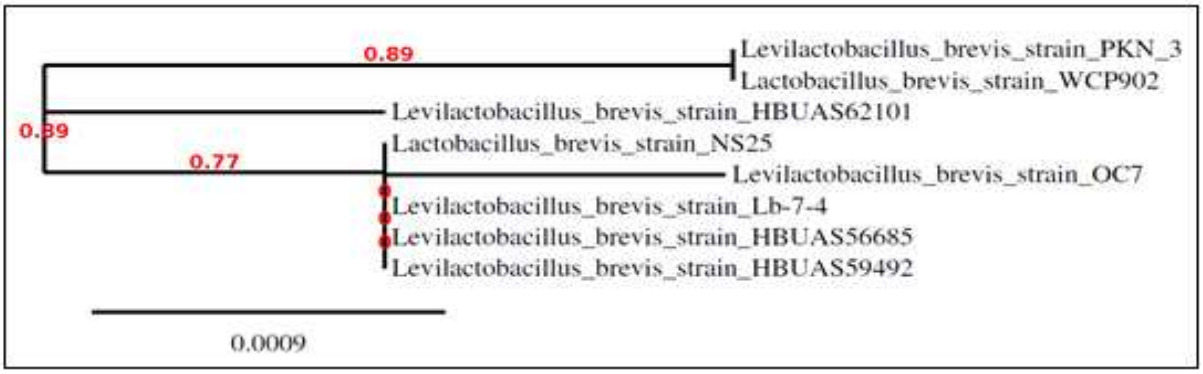

Fig. 8. Phylogenetic tree of isolates 3.

All of the isolates have been observed to have the same medium color. All ten isolated specimens tested negative.

There would be no growth of bacteria if they could not use citrate. The oxidase assay showed similar outcomes. All of these isolates also passed the starch hydrolysis and Casein hydrolysis tests.

Molecular identification of PKN 3 based on 16S rRNA gene sequence

The amplified 16S rRNA sequences of isolate 3 Neera were subjected to evolutionary analyses using the MACROGEN (Seoul, Korea) for sequencing using universal 16S rRNA primers that were > 95% like *Levilactobacillus brevis* strain, thus validating the homology sequences of the isolates (Fig. 7).

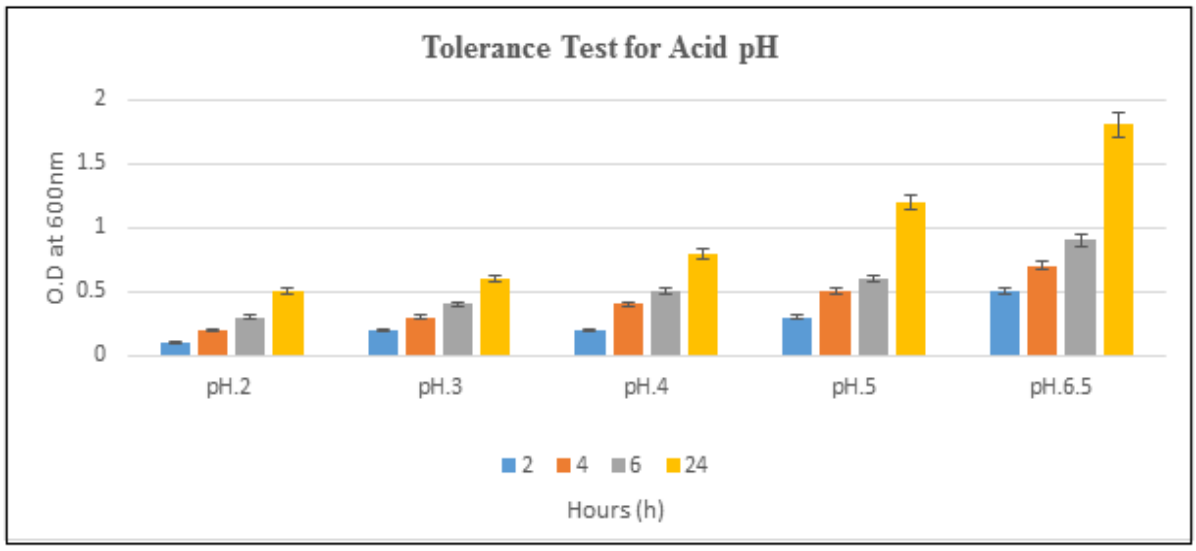

Fig. 9. Tolerance test for acid pH against the isolates of the Neera sample.

Phylogenetic tree construction

Mega-4 was used for the phylogenetic analysis in this study. The sequence of 16S rRNA has been uploaded to NCBI. Three phylogenetic groups representing the genus *Levilactobacillus brevis* were found among the lactic acid bacteria isolated from Neera sample leaves. Strains PKN_3, WCP902, HBUAS62101, NS25, OC7, LB-7-4, HBUAS56685, and HBUAS59492 were shown to belong to the *Levilactobacillus brevis* phylogenetic cluster (Fig. 8).

Tolerance Test for Acid pH

In this study, the selected isolate PKN 3 was used for acid tolerance tests with various pH conditions including pH 2, pH 3, pH 4, pH 5, and pH 6.5. optimum growth was observed at pH 6.5 at 24 hours incubation period (Fig. 9).

Test of Bile Salt Tolerance

For gauging acid and bile tolerance, the survival rate of all isolates examined from pH 2 to 6.5 was

determined under bile circumstances (0.05, 0.1, and 0.3%). Survival of the isolate from pH 2 to 6.5 and tolerance of 0.05 and 0.3% acid bile are shown in Figure 10. Isolate strains had the most effective 24-hour survival rate of 1.8 in 0.05 and 0.3% acid bile, respectively. Specifically, PKN 3 was employed for acid tolerance testing with varying bile salt levels (0.05%, 0.1%, and 0.3%) in this investigation (Fig. 10).

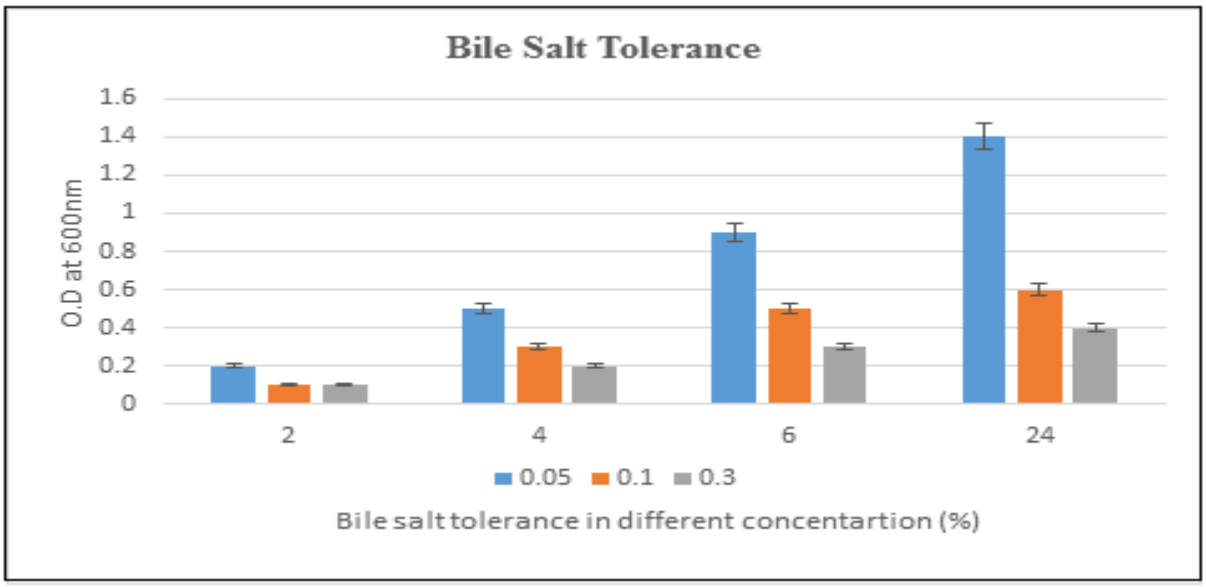

Fig. 10. Bile salt tolerance test for the isolates of Neera sample.

Conclusion

Microorganisms known as foodborne pathogens are naturally present in fresh fruits and vegetables and are typically linked to foodborne disease outbreaks. Despite the widespread use of chemical methods for controlling these infections, the food industry has turned to biological management to maintain safety and reduce pollution in response to consumer desire for natural, "chemical-free," and "more mature" fresh products. There is substantial evidence that Neera isolates protective cultures that can suppress the growth of diseases without altering the flavor of foods, making them a valuable tool for biocontrol. This research aims to identify and characterize the potential probiotics isolated from the Neera sample and tested in a spray formulation designed to extend the freshness of produce. Isolate 3 was determined to be a strain of *Levilactobacillus brevis* depending on its morphological, biochemical, and molecular characteristics. The strain performed admirably in tests measuring acid-bile, pH tolerance, and antibacterial activity—all required for classification as probiotics. These bacterial strains may find applications in many commercial and industrial contexts. Therefore, the isolates should be evaluated as possible probiotics to maintain the nutritional composition and extend the shelf life of the fruits and vegetables. However, more research is needed to do so.

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Declaration

The authors declare no conflict of interest to report regarding this research work.

Ethical approval

The authors confirm that there are no ethical issues in the publication of the manuscript.

Human and animal rights

No animals/humans were used for studies that are the basis of this research

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