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Ameliorated Viability of Lactic Acid Bacteria in Fruit Juice Isolated from Indigenous Dahi with Prebiotics (*Asparagus falcatus* and *Zingiber officinale*)

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ABSTRACT

Dahi is a fermented milk product containing probiotic lactic acid bacteria. This study aimed to isolate, identify, and characterize lactic acid-producing bacteria from native Dahi and evaluate their viability in orange juice using natural prebiotics. Dahi samples were obtained from local shops in Chattogram and Bogura, Bangladesh. Lactic acid-producing bacteria were isolated using MRS (de Mann Rogosa and Sharpe) medium. The isolated bacteria were identified through colony morphology, biochemical tests, and probiotic characteristics. Molecular identification was performed using polymerase chain reaction (PCR) targeting conserved 16S rDNA regions. Isolates of the genus *Lactobacillus* and *Lactococcus lactis sp. Lactis* were confirmed and used to develop probiotic orange juice. Prebiotics (*Asparagus falcatus* and *Zingiber officinale*) were added to the juice to support probiotic growth. The inoculated cell's viability and the juice's physicochemical parameters were evaluated during fermentation (48 hours) and storage (28 days). All fruit juice samples showed a mean number of viable cells of at least 1×10^5 CFU/mL during the 48-hour fermentation and 28-day storage in the refrigerator. Using natural prebiotics positively affected the survival of lactic acid bacteria, as demonstrated by bacterial colony growth on Petri dishes. Developing probiotic fruit juice enriched with prebiotics could be an effective alternative for individuals allergic or intolerant to milk-based products. Incorporating lactic acid bacteria from native Dahi into orange juice, combined with natural prebiotics, resulted in viable probiotic cells throughout fermentation and storage.

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Introduction

Dahi, a naturally fermented yogurt, is significant in Indian culinary culture and has been consumed for centuries. Commonly consumed in India, Pakistan, Bangladesh, and neighboring countries, dahi is considered one of the oldest and most widely consumed fermented milk products (Mudgal & Prajapati, 2017). Fermented dairy products provide sufficient nutritious meals with a wide range of flavors, aromas, and textures, which are important in enhancing the human diet (Sarkar, 2008).

Probiotics and lactic acid bacteria are essential in maintaining gut health and promoting overall well-being. Lactic acid bacteria (LAB) are a class of gram-positive bacteria that produce lactic acid as their primary product of fermented sugar (Deng et al., 2022). Probiotics are beneficial bacteria that are alive, nonpathogenic, and play a vital function in the host's microflora (De Vrese & Schrezenmeir, 2008). Lactic acid bacteria (LAB), which include *Lactobacillus* sp., *Bifidobacterium* sp., and *Enterococcus* sp., are the most common probiotic microorganisms (Klein et al., 1998). Lactic acid-forming bacteria are Gram-positive, non-spore-forming, cocci or rod-shaped, catalase-negative, and fastidious bacteria frequently isolated from milk and dairy products. They are nonpathogenic to humans and animals and are Generally Recognized as Safe (GRAS) organisms (Fuchs et al., 2008). Probiotics are "live microorganisms that provide a health benefit to the host when administered in sufficient amounts." Probiotics in food have several health benefits,

including lowering blood cholesterol, improving gastrointestinal function, boosting the immune system, and lowering the risk of colon cancer (Berner & O'Donnell, 1998).

Prebiotics are a dietary component crucial in promoting the growth and activity of beneficial microorganisms in the gut. They are non-digestible fibers that serve as a source of nutrition for specific strains of bacteria, primarily bifidobacteria and lactobacilli, which are known to affect human health positively. Prebiotics are often composed of dietary fibers and oligosaccharides. *Asparagus falcatus* is a popular prebiotic source that includes inulin-like compounds. *Asparagus falcatus* can be employed in the food industry as an inulin source (Mudannayake et al., 2015). Several studies have found that adding inulin or fructooligosaccharides to the skim milk medium increased the development of several *Lactobacillus* and *Bifidobacterium* strains (Kaewarser et al., 2023). Ginger has antimicrobial action in vitro against inflammatory gut bacteria like *E. coli* and *Klebsiella pneumonia* (Salehi et al., 2018). Both ginger and turmeric promote the growth of beneficial *Bifidobacterium* and *Lactobacillus* species while inhibiting the growth of numerous *Ruminococcus* species isolated from clinical samples (Lu et al., 2017). Fruits and vegetables have long been considered ideal for probiotic production because they contain the necessary nutrients (Sheehan et al., 2007). Because they have taste characteristics that appeal to people of all ages and are viewed as healthful and refreshing foods, there is a nascent interest in developing fruit juice with probiotics by integrating prebiotics (Sheehan et al., 2007; Nagpal et al., 2012). Many probiotic foods currently accessible are milk-based, such as fermented milk and yogurt. However, they come with a few drawbacks, including lactose intolerance and a rise in the consumer's cholesterol level. So, it was investigated whether fruit juice could be used to grow probiotic bacteria. As a result, the current study sought to evaluate the cell development of indigenous probiotic bacteria in the presence of natural prebiotics and to generate probiotic fruit juice.

Materials and Methods

Sample Collection

Thirty dahi samples were obtained from local markets in Chattogram and Bogura, Bangladesh. The collected samples were immediately transported to the laboratory and stored aseptically at 4°C to avoid contamination and degradation.

Isolation of Lactic Acid Bacteria

MRS (De Man, Rogosa and Sharpe) media (Himedia, India) and Nutrient Agar (Oxoid, England) were used to isolate and cultivate lactic acid bacteria. The growth media were made according to the manufacturer's directions. One gm of samples was homogenized after being diluted with 10 mL of distilled water. The serially diluted (10^{-5}) sample was spread on an MRS agar plate and incubated anaerobically (BL GasPak 100 Anaerobic system, BD Biosciences, Sparks, MD, USA) for 24 hours at 37°C (De et al., 2010). Subcultures yielded pure bacterial colonies.

Identification of Isolated Cultures

Bacteria were isolated from dahi samples using MRS (de Mann, Rogosa, and Sharpe) broth and agar. The physiological, biochemical, and morphological characteristics of individual whitish-colored colonies were examined. Various biochemical assays, including Gram staining, motility, indole, catalase, and oxidase tests (Cheesbrough, 1987), and morphological evaluations (De Man et al., 1960), were conducted to assess the isolates. Additionally, the isolates were subjected to sugar fermentation tests using sucrose, galactose, lactose, maltose, and mannitol (Krishnaveni et al., 2020).

Antimicrobial Activity

To test the antibacterial activity of isolated lactic acid-producing bacteria, three pathogenic bacteria were chosen (*Escherichia coli* ATCC-8739, *Staphylococcus aureus* ATCC-6538, and *Pseudomonas aeruginosa* ATCC-9027). The disk diffusion method was used with Mueller Hinton agar plates. Test pathogens were seeded on Mueller Hinton agar plates. A pure cultivated isolate in MRS broth was centrifuged at 6000 Xg for 15 minutes to extract the supernatant. A sterile membrane filter with a pore diameter of 0.22 µm was used to filter the supernatants. Blank discs were deposited with 15 µl supernatant of each isolate. The cultivated plates with discs were then incubated for 24 hours at 37°C. After incubation, the growth inhibition zones were measured (Campos et al., 2006).

Antibiotic Sensitivity Test

The antibiotic sensitivity test of isolates was evaluated using the disk diffusion method. The isolates were disseminated on Mueller Hinton agar media using sterile cotton buds. Antibiotic disks (azithromycin, ceftriaxone, amoxicillin, cefixime, streptomycin, and chloramphenicol) were evenly spaced around the plate's surface. The plates were kept at 4°C for 2 hours for optimal antibiotic diffusion. The plates were then incubated for 24 hours at 37°C. The zone of inhibition was observed, and the diameter of the zone was measured for antibiotic sensitivity or resistance (Abbasiliasi et al., 2017).

Temperature Tolerance Test

The chosen isolates were inoculated into several MRS broth tubes and incubated for 24 hours at various temperatures, including 25°C, 37°C, and 45°C. Isolates were then cultivated on an agar medium using the pour plate method. The plates were then incubated at 37°C for 24 hours and compared for growth (Mourad and Bettache, 2018). The growth of the isolates was measured at different time intervals, including 12, 18, and 24 hours.

Assessment of probiotic properties

Effect of pH

Isolated cultures were seeded into MRS broth tubes at different pH levels, including pH 3, 5, and 7, and incubated for 48 hours at 37°C. Using the pour plate method, 0.1 mL of inoculated culture was poured onto MRS agar medium from each tube and incubated at 37°C for 48 hours. The pH tolerance of isolated cultures was determined by observing their growth on MRS agar media (Jideani et al., 2021).

Effect of bile salt

Various bile salt concentrations were used to make MRS broth (1.0%, 2.0%, and 3.0 %). The medium was then given to each tube at a rate of 10 mL. Inoculation was done using the isolates chosen. After being inoculated with an equal number of inoculums, the tubes were incubated for 48 hours at 37°C. Following the incubation period, 0.1 mL of the culture from each concentration was developed in the agar medium using pour plate technique. The plates were then incubated at 37°C for 24 hours before being compared for growth (Mohanty and Ray, 2016).

NaCl tolerance

The selected isolate was inoculated with varied NaCl concentrations (1%, 5%, and 10%) in sterilized MRS broth tubes and incubated at 37°C for 24 hours. Following incubation, 0.1 mL of each tube's culture was used to grow in agar media using the pour plate method. After a 24-hour incubation at 37°C, the plates were examined for comparative growth (Jideani et al., 2021).

Molecular Identification of Bacteria by Polymerase Chain Reaction (PCR)

DNA extraction

DNA was extracted from the acquired isolates using the traditional boiling procedure with some modifications (Dashti et al., 2009). This was accomplished using the two-fold boiling procedure. To begin, 200 mL deionized water was poured into a 2 mL Eppendorf tube, and a loop of fresh colonies (about 5-6) was removed from the agar plate and transferred to the Eppendorf tube. The tubes were then vortexed for a few seconds to create a homogeneous cell solution before being boiled for 15 minutes at 99°C. This process was carried out once more. The suspensions were immediately cooled at -20°C for 5 minutes after they had been boiled. Finally, the Eppendorf tubes with cell suspension were centrifuged at 10000 rpm for 5 minutes and around 100 µl of supernatant containing bacterial DNA was collected.

Amplification of PCR product

Lactobacilli and *Lactococci* were finally identified using PCR (Polymerase Chain Reaction) and PCR product amplification. Table 1 lists the primers that detect and confirm isolated LAB. The PCR mixtures for detecting *Lactobacillus* were made with 11.75 µl of master mix, 5µl of each primer, 2.5 µl of DNA template, and 0.75 µl of nuclease-free water for a total of 25 µl. The PCR mixture of *Lactococcus lactis* was prepared with 12.5 µl of master mix, 2µl of each primer, 4µl of DNA template, and 4.5µl of nuclease-free water for a total of 25 µl. A thermal cycler was used for amplification. All reactions were carried out in a 25 µl final volume. The thermocycler conditions for *Lactobacillus* (29 cycles) were as follows: initial denaturation 94℃ for 3 minutes, final denaturation 94℃ for 3 seconds, annealing 55℃ for 30 seconds, extension 72℃ for 3 minutes, final elongation 72℃ for 10 minutes, and final holding 4℃ (Gebreselassie et al., 2016). Thermocycler conditions of Lactococcus lactis (35 cycles) were followed as initial denaturation 94℃ for 5 minutes, final denaturation 94℃ for 40 seconds, annealing 58℃ for 40 seconds, extension 72℃ for 1 minute, final elongation 72℃ for 10 minutes, and final holding 4℃ (Pu et al., 2002).

Agarose gel electrophoresis

Agarose gel electrophoresis was employed to visualize the PCR products. In 50 mL of TAE buffer, 0.75 g of agarose was dissolved. The solution was boiled for two minutes and then cooled to approximately 50 °C, after which 5 µL of ethidium bromide was added. The agarose gel was prepared, placed in the gel casting platform, and the combs were inserted. The gel was left to solidify at room temperature for 20 minutes. The gel was then transferred into an electrophoresis apparatus filled with 1X TAE buffer. Wells were created using a hard gel comb for sample loading. A gel gap was used to load 5.5 µL of PCR products and 3 µL of loading dye per sample, along with a 1 kb plus DNA marker to determine the amplicon size. Electrophoresis was performed at 90 volts and 120 amps for 35 minutes. The PCR products were visualized under UV light using a BDA digital system from biometra GmbH, Germany.

Preparation of Orange Juice

The orange fruit was purchased at a nearby Chattogram market and brought to the laboratory for juice extraction. The orange juice was made manually according to the instructions in Figure 1.

Preparation of Natural Prebiotics

Commercial asparagus (*Asparagus falcatus*) powder, ginger (*Zingiber officinale*) powder, and starch were purchased from the super shop of Mart Promoters Limited (Khulshi Mart), Chattogram, Bangladesh. Asparagus and ginger powders were employed as prebiotics in this investigation. The paste was also thickened with starch and prepared by following the formula in Table 2. All the ingredients were weighed and combined roughly in a cup. After that, the cup was filled with enough water to produce a paste.

Preparation of Isolated LAB Cultures

LAB (*Lactobacillus* sp. and *Lactococcus lactis* subs *Lactis*) were isolated from a 1×10^5 CFU/mL plate and cultivated with MRS (De Mann, Rogosa, and Sharpe) broth media for 24 hours at 37°C. Microbial growth increased the turbidity of the broth after the incubation period.

Preparation of Probiotic Orange Juice

Orange juice was prepared manually to incorporate the prebiotic, which boosts the development of probiotic bacteria. Probiotic orange juice was prepared as described in Figure 1.

Preparation of Experimental Treatments

The effect of probiotic inclusion was examined using four different treatments (Figure 2). Airtight-capped sterile glass bottles were used to make the experimental treatments. The treatments were as follows: Treatment 1 (T1) = (100 mL orange juice + 5% *Lactobacillus* inoculum + 0.5% prebiotics); Treatment 2 (T2) = (100 mL orange juice + 5% *Lactobacillus* inoculum); Treatment 3 (T3) = (100 mL orange juice + 5% *Lactococcus* inoculum + 0.5% prebiotics); Treatment 4 (T4) = (100 mL orange juice + 5% *Lactococcus* inoculum). For each treatment, each criterion was assessed in triplicate.

Table 1. Primers used to detect and confirm lactic acid bacteria.

Target organism	Primer	Primer sequence (5'-3')	Fragment size (bp)	Reference
Lactobacillus	LAC1F LAC2R	AGCAGTAGGGAATCTTCCA ATTTCACCGCTACACATG	340	Gebreselassie et al., 2016
L. lactis sp. lactis	LacF LacreR	GTACTTGTACCGACTGGAT GGGATCATCTTTGAGTGAT	161	Pu et al., 2002

Table 2. Formula of prebiotics composition 0.5% (w/v) for juice.

Ingredients	Amount (Gram)
Asparagus (*Asparagus falcatus*)	0.4 g
Ginger (*Zingiber officinale*)	0.08 g
Starch	0.02 g
Total	0.5 g

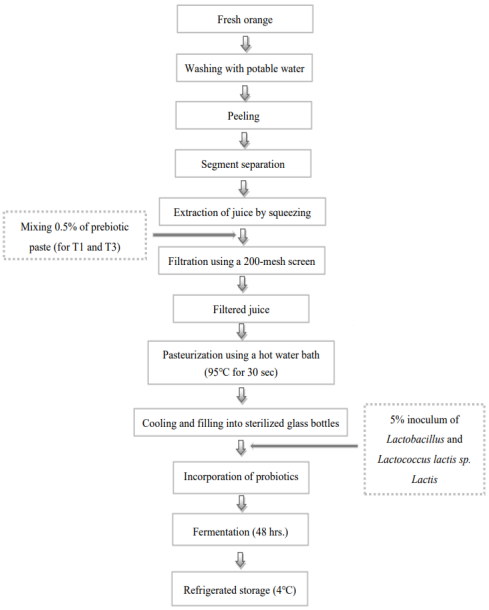

Figure 1. Preparation procedure of probiotic orange juice with prebiotics

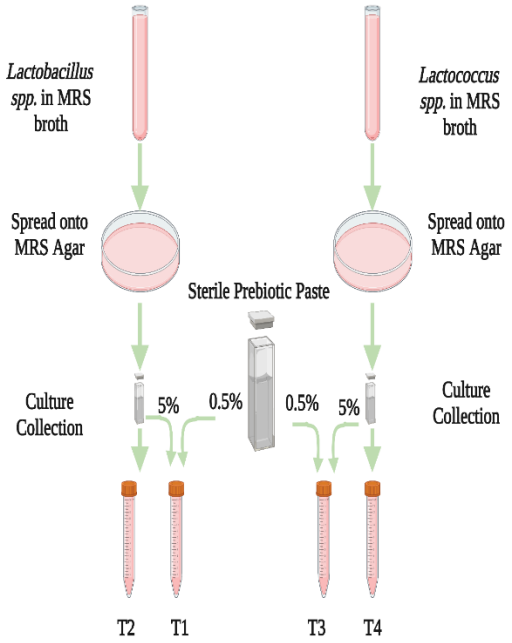

Figure 2. Technological flowsheet to produce the treatments of probiotic juice

Prebiotics and Cell Viability

The experimental treatment cultures were incubated at 37°C for 48 hours. The growth measurements were assessed at 12 hours, 24 hours, and 48 hours. Plate count using MRS agar media was used to determine cell viability (CFU/mL) in the treatments with and without prebiotics at various intervals during the incubation period (12 hours, 24 hours, and 48 hours).

Storage Temperature (4°C) and Cell Viability

The four experimental treatments were kept in the refrigerator (4°C) for four weeks after a 48-hour fermentation. The viability of the isolates was determined using the plate count method every seven days.

Physicochemical Properties of Orange Juice During Fermentation

Physicochemical parameters of treatments (T1, T2, T3, and T4) were investigated at different time intervals (0, 12, 18, and 24 hours) during the fermentation period, including total soluble solids (TSS in ºBrix), pH, and titratable acidity (TA in %). The Brix refractometer (DANOPLUS Brix Refractometer) was used to calculate total soluble solids (TSS). The pH of the treatments was determined using a Ruolan Lab pH meter. A simple acid/base titration method was used to calculate the percent titratable acidity (Lee et al., 2005).

Statistical Data Analysis

MS Excel 2010 and GraphPad Prism 9.0 were used to analyze variance (ANOVA) and student's t-test on the quantitative data of cell viability. A 5% significance level ($p<0.05$) was used to measure the result's significance.

Results

Isolation and Identification of LAB

During the microscopic examination of isolates, bacilli and cocci were discovered. Lactic acid bacteria isolated from different sources of Dahi were identified as *Lactobacillus* and *Lactococcus* sp. based on their morphological, cultural, physiological, and biochemical characteristics described by Bergey's Manual (Garrity and Holt, 2001) (Figure 3 & Table 3).

Antibiotic Susceptibility Assay

A total of six different antibiotic discs (azithromycin, ceftriaxone, amoxicillin, cefixime, streptomycin, and

chloramphenicol) were used to evaluate the sensitivity of the isolates. The zone of inhibition (mm) was assessed to examine the sensitivity of isolates. Azithromycin sensitivity was higher in both bacilli (*Lactobacillus* sp.) and cocci (*Lactococcus lactis*) (Figure 4). Most antibiotics had a zone of inhibition ranging from 4 to 15 mm, indicating that isolates were less sensitive (Figure 3). Bacilli (*Lactobacillus* sp.) were shown to be more sensitive to chloramphenicol, while cocci (*Lactococcus lactis*) were found to be resistant (Figure 4). This findings demonstrated most of the starins exhibited resistance to the that antibiotics used in the study.

Antimicrobial Activity

Probiotics also can prevent harmful bacteria from multiplying and colonizing the gut. Three pathogenic bacteria (*E. coli* ATCC-8739, *S. aureus* ATCC-6538, and *P. aeruginosa* ATCC-9027) were used to investigate the antibacterial activity of the isolated probiotic bacilli and cocci. The disc diffusion method was used to evaluate antimicrobial activity. Antimicrobial activity was assessed by measuring the zone of inhibition (mm). Cocci was less resistant to gram-negative organisms (*E. coli* and *P. aeruginosa)* than bacilli but more resistant to gram-positive bacteria (*S. aureus*) (Figure 5). In addition, both isolates demonstrated a protective effect against pathogens in the test (Figure 5).

Growth at Different Temperature Ranges

At varying temperatures (25°C, 37°C, and 45°C) and time intervals (12, 18, and 24 hours), the growth of isolated bacilli (*Lactobacillus* sp.) and cocci (*Lactococcus lactis*) was investigated. With increasing temperature ranges up to 45°C, cell proliferation of both isolates (*Lactobacillus* and *Lactococcus lactis*) considerably increased ($p<0.05$). However, at the same temperature, the length of incubation had no effect ($p>0.05$). According to the data, *Lactococcus lactis* showed a higher temperature tolerance than *Lactobacillus* sp. (Table 4).

Probiotic Properties of Isolates

In this experiment, isolates of bacilli (*Lactobacillus* sp.) and cocci (*Lactococcus lactis*) were able to grow in a variety of pH ranges (pH 3, pH 5, and pH 7). However, *Lactococcus lactis* showed better growth at lower pH (Table 5). High bile salt concentrations inhibit bacterial growth. With a varied bile salt concentration (0.3%, 1.0%, and 1.5%), *Lactococcus lactis* grew faster than *Lactobacillus* sp. (Table 5). *Lactococcus lactis* had lower growth at bile salt concentrations of up to 1.5%, while *Lactobacillus* had no growth. The presence of NaCl hinders the growth of certain microorganisms. The isolates (bacilli and cocci) could not survive at a concentration of 10% NaCl, although they grew more rapidly at 1% and 5% NaCl concentrations (Table 5).

Table 3. Microscopic and biochemical assessment of the isolates.

Isolates	Biochemical Tests									
	G. Stain	Motility	Indole	Catalase	Oxidase	Sucrose	Galactose	Lactose	Mannose	Mannitol
Lactobacillus	+	–	–	–	–	+	+	+	+	–
L. lactis	+	–	–	–	–	+	+	+	+	+

Note: + = Positive; – = Negative

Table 4. Cell growth at different temperature ranges.

Temperature ranges	Growth of microorganisms ($\times 10^5$ CFU/mL) (Mean±SD)					
	Lactobacillus sp.			*Lactococcus lactis* sub. *lactis*		
	12 hrs	18 hrs	24 hrs	12 hrs	18 hrs	24 hrs
25°C	165.4 ± 26.71^{a}	176.2 ± 24.61^{a}	183.8 ± 23.96^{a}	238.8 ± 32.05^{x}	248.8 ± 32.81^{x}	257.6 ± 33.61^{x}
37°C	198.2 ± 13.14^{b}	207 ± 11.64^{b}	216.4 ± 17.62^{b}	268.2 ± 25.17^{y}	275.6 ± 27.67^{y}	284.6 ± 28.97^{y}
45°C	150.4 ± 29.52^{c}	154.6 ± 29.24^{c}	157.8 ± 29.83^{c}	284.8 ± 28.42^{z}	293.6 ± 35.87^{z}	300.6 ± 38.37^{z}

Mean values with different superscripts between the rows are significantly different by ANOVA ($p<0.05$).

Table 5. The isolates' acid tolerance, bile tolerance, and salt tolerance tests.

LAB Isolates	pH ranges			Bile salt (%)			NaCl (%)		
	pH 3	pH 5	pH 7	1%	2%	3%	1%	5%	10%
Lactobacillus sp.	+	++	+++	++	+	–	+++	++	–
Lactococcus lactis	++	+++	+++	+++	++	+	+++	+++	–

Note: – = Negative/No growth; + = Small growth; ++ = Moderate growth; +++ = Excellent growth

Figure 3. LAB on MRS agar media

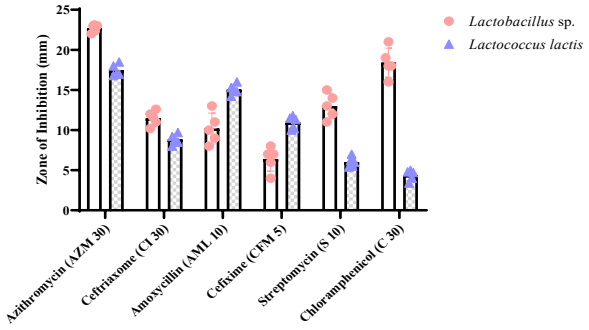

Figure 4. Antibiotic susceptibility test of isolated lactic acid bacteria (LAB)

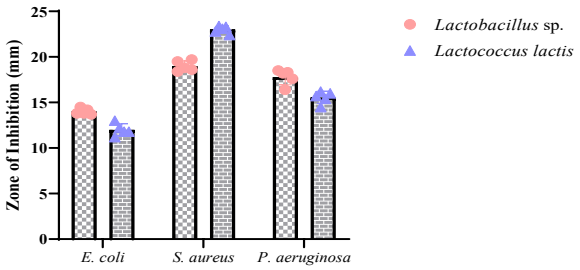

Figure 5. Antimicrobial activity of the isolated LAB against pathogenic bacteria

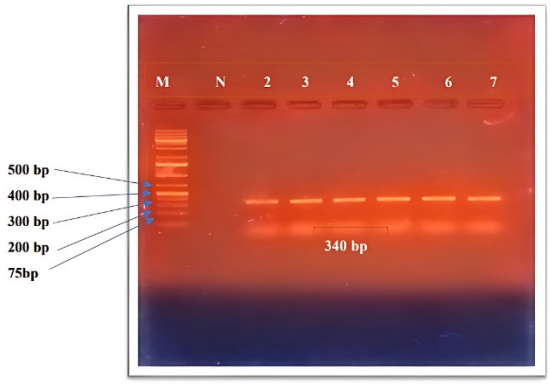

Figure 6. *Lactobacillus* genus-specific PCR assay.
This figure illustrates fragments amplified explicitly by PCR employing the primer *LAC1F* and *LAC2R*. Lane M: 1 kb plus DNA marker, Lane N: negative control. Lanes 2-7: PCR products showing the *LAC* gene-sized amplicon (340bp)

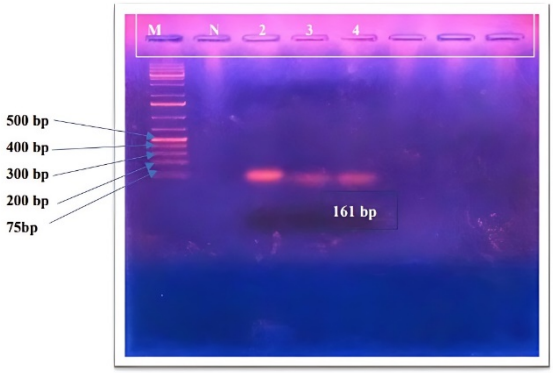

Figure 7. *Lactococcus lactis sp. lactis-specific* PCR assays.
This figure illustrates fragments amplified explicitly by PCR using the primer *LacF* and *LacreR*. Lane M: 1 kb plus DNA marker, Lane N: negative control. Lanes 2-4: PCR products; only Lane 2: PCR product showing the *Lac* gene-sized amplicon (161bp). Lanes 3-4: samples giving negative reaction in PCR

Molecular Identification

Polymerase chain reactions (PCR) were carried out on the genomic DNA of collected isolates (*Lactobacillus* sp. and *Lactococcus lactis*) by using forward primers (LAC1 and Lac respectively) and reverse primers (LAC2 and Lacre respectively) (Figure 6 and 7). For the amplification of 340 bp of the 16S rRNA gene (16S rDNA) of *Lactobacillus*, PCR primers LAC1F and LAC2R were developed (Gebreselassie et al., 2016). Furthermore, utilizing lactic-specific primers, the isolate identified as *Lactococcus* was subjected to molecular identification at the subspecies level.

Probiotic Juice Preparation Using Prebiotics

We produced a functional food as probiotic fruit juice by combining isolated lactic acid bacteria with prebiotics. According to our findings, prebiotic paste aided probiotic development, and the addition of prebiotic paste also increased cell viability (Figure 8). Prebiotics (asparagus and ginger powders) were added to the probiotic isolate inoculated fruit juice to increase the organism's growth. The isolated bacteria grew far better in fruit juice added with prebiotics than in juice not supplemented with prebiotics in the experiment.

Prebiotic Effect on Cell Viability

Isolated lactic acid bacteria were examined for viability with and without prebiotic paste. The studies demonstrated that prebiotic paste improved both organism's (*Lactobacillus* sp. and *Lactococcus lactis*) cell viability (Figure 8). Isolates with prebiotics grew faster at storage temperature than isolates without (Figure 9). However, growth slowed as the storage period lengthened (Figure 8). *Lactobacillus sp.* (T1) grew faster with prebiotics than *Lactococcus lactis* (T3). Cell viability was substantially lower in isolates without prebiotics (T2 and T4).

Fermentation Effects on Juice Properties

During the fermentation phase, various characteristics (Total Soluble Solids, pH, and Titratable acidity) of treatments (T1, T2, T3, and T4) were measured. Each parameter was found to be decreased during fermentation. With the extended fermentation duration, TSS (Total Soluble Solids) and TA (Titratable acidity) were reduced (Figures 10a and 10c). All the treatments had an acidic pH level (Figure 10b).

Discussion

The collected LAB isolates in our study produced white-colored, round, convex, and smooth colonies. Both *Lactobacillus* and *Lactococcus lactis* isolates were confirmed as positive sugar fermenters for sucrose, galactose, lactose, and mannose. These isolates were characterized as Gram-positive, catalase-negative, and non-motile, consistent with previous research findings (Amelia et al., 2020; Roza et al., 2022).

Both lactic acid bacteria isolates (*Lactobacillus* and *Lactococcus*) showed varying levels of antibiotic sensitivity. This result demonstrated that the tested lactic acid bacteria had the highest sensitivity to azithromycin. *Lactobacillus* sp. had more sensitivity to chloramphenicol than *Lactococcus lactis*. The observed results were consistent with the lactic acid bacteria data that had previously been published (Ali et al., 2018; Georgieva et al., 2015; Sharma et al., 2016).

In this investigation, the tested LAB isolates showed inhibitory effects against the pathogens (*E. coli*, *S. aureus*, and *P. aeruginosa*) used. Following the earlier study, more inhibitory effects of isolated LAB against gram-positive pathogens than gram-negative pathogens were found in this study. The lactic acid bacteria inhibit the gram-positive pathogens more due to the production of bacteriocins with a bactericidal effect. However, generating organic acids and hydrogen peroxide may cause adverse effects on gram-negative bacteria.

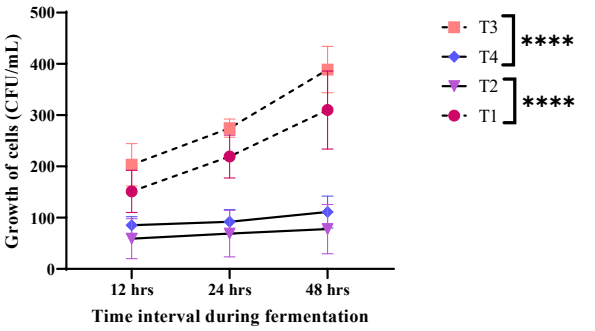

Figure 8. Bacterial cell viability of treatments during fermentation at 37°C.
****Indicates the significant differences between treatments (T1 and T2; T3 and T4), where p≤0.0001 by student's t-test.

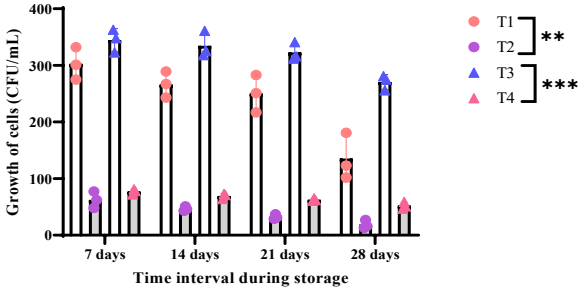

Figure 9. Microbial cell growth and stability at storage temperature.
** Shows the significant differences between T1 and T2 (p≤0.005) and *** reflects the statistical differences between T3 and T4 (p≤0.0002). Student's t-test was carried out to analyze the statistical significance.

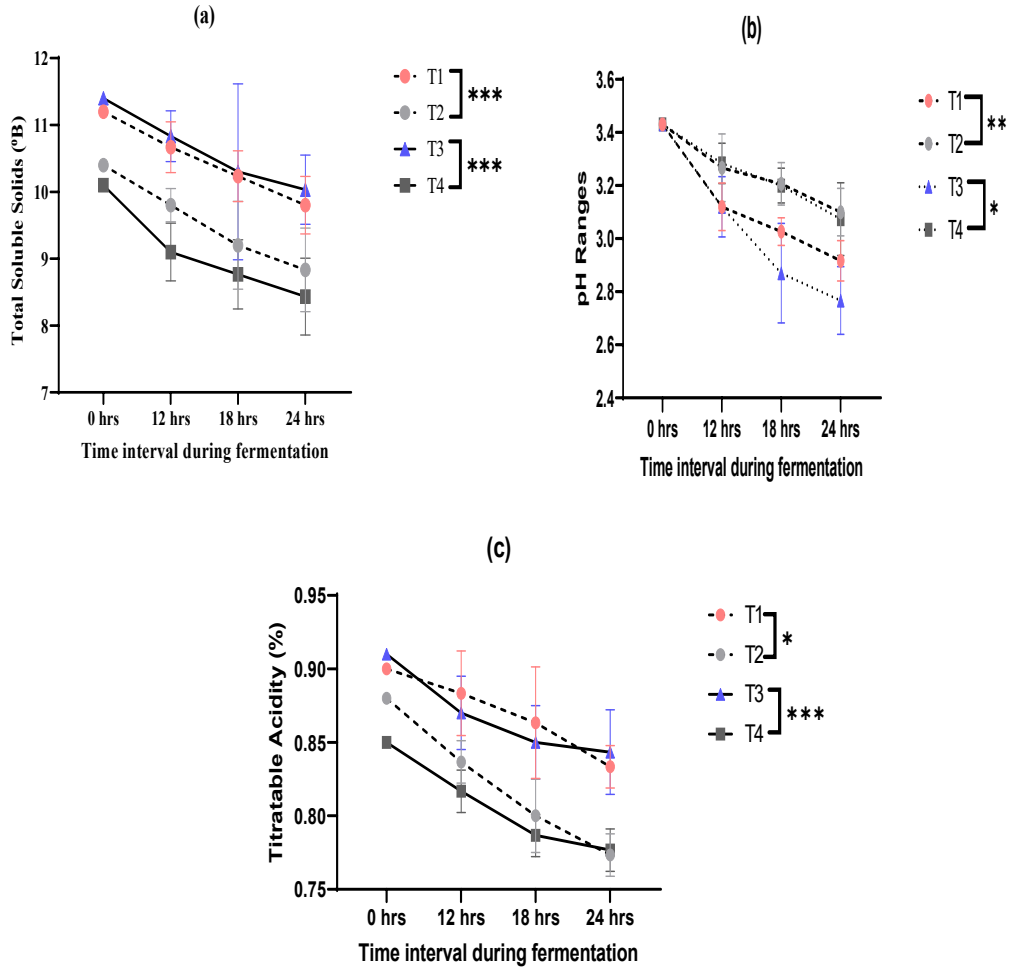

Figure 10. Changes in physicochemical parameters of treatment groups, i.e., *Lactobacillus* pair (T1 and T2) and *Lactococcus lactis* pair (T3 and T4) during fermentation (a) TSS (^{0}B) were highly significant within the pairs (***p≤0.0004), (b) pH ranges of first pair (T1 and T2) and second pair (T3 and T4) were also significant (**p≤0.005; *p≤0.01) and (c) Titratable acidity (%) was also statistically significant in case of both pairs (*p≤0.017; p≤0.0002).

The previous study finding also supports this investigation report (Ibrahim et al., 2021). Additionally, the isolates (*Lactobacillus* and *Lactococcus lactis*) demonstrated improved growth with typical cell proliferation up to 45°C, as described in earlier studies. But compared to *Lactobacillus* sp., *Lactococcus lactis* grew more rapidly at 45 °C (Akabanda et al., 2014; Angmo et al., 2016; Maqsood et al., 2013).

The pH has a significant impact on bacterial development. The ability to withstand bile salts and an acidic pH is necessary for lactic acid bacteria to have probiotic potential. These findings revealed that the isolates can grow at lower pH (3 and 5). Earlier investigations reported similar findings (Haddadin et al., 2004; Harun-ur-Rashid et al., 2007; Hoque et al., 2010). The isolated bacterial strains in this investigation

demonstrated resistance to bile salts at a concentration of 1.5% and acid at pH 3. The highest bile concentration in a healthy human intestine is 0.3%; hence probiotic bacteria must be able to withstand this concentration before being chosen for human ingestion. Different species of bacteria may not thrive as well due to the suppressive nature of NaCl. In our investigation, LAB isolates grew at 1% and 5% concentrations of NaCl but not at 10% concentration, which prevented growth. Hoque et al. (2010) reported a similar finding of salt-tolerant (1-9% of NaCl) LAB isolated from Bangladeshi yogurt. Our study finding also agrees with the report suggested by (Ibourahema et al., 2008).

Initial association based on biochemical results aided in choosing appropriate molecular methods for further gene identification. The isolates were examined using PCR genes, which are used to identify *Lactobacillus* spp. and *Lactococcus lactis* sp. *lactis*. The findings were consistent with prior studies in which isolates were identified as *Lactobacillus* sp. and *Lactococcus lactis* sp. *lactis* by sequencing their 16S rRNA genes with an analogous PCR primer (Gebreselassie et al., 2016; Pu et al. 2002). LacF and LacreR PCR primers were used to amplify 161bp of the 16S rRNA gene (16S rDNA) of *Lactococcus lactis* sp. *lactis* (Buyukyoruk et al., 2010).

Lactic acid bacteria were used to produce probiotic orange juice, while a prebiotic mixture of asparagus and ginger powders was added to orange juice, creating a symbiotic combination. This approach suggests that prebiotics have a considerable effect on the survival of lactic acid bacteria in fruit juice beverages; compared to juice without prebiotics, fruit juice containing prebiotics had a greater cell viability of inoculation lactic acid bacteria.

During the fermenting stage, different physicochemical parameters (Total soluble solids, titratable acidity, and pH) of the juice were changed. Similar findings were previously observed for several fruits and vegetable juices. TSS, pH, and titratable acidity in our product (probiotic juice) decreased during fermentation due to bacterial (probiotic) growth using nutrients (Nguyen et al., 2019).

Conclusion

The capacity of two isolated indigenous lactic acid bacteria (*Lactobacillus* sp. and *Lactococcus lactis* subsp. *Lactis*) to survive with prebiotics was investigated in this experiment. During the fermentation and storage of orange juice, the cell viability of these lactic acid bacteria was discovered to be relatively high. The prebiotics mixture (*Asparagus falcatus* and *Zingiber officinale)* enhanced the growth of the probiotics in the juice, according to this study. Lactic acid bacteria belong to a group of microorganisms that are extremely strong. To optimize the probiotic natural product supplementation with prebiotics (*Asparagus falcatus* and *Zingiber officinale)*, isolated lactic acid bacteria were added to the fruit beverage. The vulnerability scope of lactic acid bacteria in natural fruit juice with prebiotics was determined during this experiment, and it was discovered that prebiotics substantially affect the growth of isolated strains in fruit juice. This finding suggests that fruit juice could be a viable choice for persons who cannot ingest probiotic dairy products due to hypersensitivity or lactose intolerance. Fruit drinks that contain probiotics are healthier because fruits are inherently high in crucial macro and micronutrients.

Declarations

Conflicts of Interest

All authors declare that there are no conflicts of interest regarding the research.

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Availability of Data and Materials

The manuscript provides all the data (tables and graphs) generated during the study.

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