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Isolation and Functional Characterization of Probiotic Bacteria from *Borassus flabellifer* for Therapeutic Applications

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Abstract:

- ***Introduction:***

Traditional fermented foods serve as vast, yet often underexplored, reservoirs of probiotic microorganisms. This study investigates the screening, isolation, and characterization of potential probiotic bacteria from naturally fermented Palmyra palm (*Borassus flabellifer*) fruit extract.A primary focus was placed on identifying isolates with high β-galactosidase activity to address lactose intolerance.

- ***Methods:***

Five bacterial isolates were initially collected under aseptic conditions. After preliminary screening via cultural, microscopic, and biochemical assays, five Gram-positive, catalase-negative isolates were shortlisted for comprehensive probiotic evaluation. These candidates were subjected to Survival assays under simulated gastrointestinal conditions (acid and bile tolerance). Cell surface characterization, including auto-aggregation, co-aggregation with pathogens, hydrophobicity, and biofilm formation. Functional quantification of β-galactosidase, safety and antioxidant profiling, including antibiotic susceptibility, and hydroxyl radical scavenging.

- ***Results:***

Of the initial pool, 5 isolates displayed strong adhesion-related traits, with one strains emerging as superior probiotic candidates. These five strains demonstrated a high tolerance to gastric acidity and bile salts. Significant co-aggregation with Salmonella Typhi (up to 78.87%) Klebsiella pneumoniae (up to 74.85%) and (up to 68.85%) and Escherichia coli. Notable hydroxyl radical scavenging (57.68–70.66%) and a clean safety profile (antibiotic-susceptible).

- ***Discussion:***

These discoveries emphasize the potent probiotic and functional attributes of bacteria derived from Palmyra nectar. Their ability to survive the gastrointestinal transit, lactose digestion, and abide by the intestinal mucosa suggests high therapeutic value. These strains hold significant promise for the development of functional foods and nutraceuticals tailored to enhance microbiome balance and alleviate lactose absorption.

Keywords: *Borassus flabellifer, Probiotic Isolates, Characterization, Antibiotic Sensitivity, Antimicrobial Activity and DPPH Radical Scavenging Activity.*

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I. INTRODUCTION

When administered in a sufficient quantities, probiotics a live microorganisms benefit the host's health. They are essential for maintaining the equilibrium of the gut microbiota, boosting immunological responses, and averting a number of gastrointestinal illnesses. *Lactobacillus, Bifidobacterium*, and *Saccharomyces* are common probiotic genera that are frequently utilized in nutraceuticals and functional foods. Because they synthesis biologically active

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substances that promote health and prevent infective diseases, Probiotics are widely Recognized as Safe (GRAS) to take [1]. Several probiotic strains act as secretome rich of secondary metabolites that are important for intercellular signaling and genomics integrity, highlighting their suitability for use in food matrices and nutritional supplements [2].

The concept of probiotics most likely originated in 1908 when Eli Metchnikoff, the winner of the Nobel Prize, proposed that Bulgarian peasants' long lifespans were caused by their consumption of fermented milk products. Lilly and Stillwell coined the term "probiotic" in 1965 to refer to chemicals secreted by one organism that promote the growth of another. In 2021, Marteau [3] and colleagues details the "microbial preparations or components of microbial cells that have a beneficial effect on health and well-being." The gastrointestinal (GI) tract has a surface area of more than 400 m^2 and contains the highest concentration of commensal microorganisms [4].

Investigating new and natural sources of probiotics, especially from plant-based substrates, has drawn more attention in recent years. Rich in sugars, fibers, vitamins, and bioactive compounds, palm fruits from species like *Elaeis guineensis* (oil palm) and *Borassus flabellifer* (palmyra palm) foster the development of isolates [5,6]. Diverse microbial communities are frequently involved in the traditional fermentation of palm fruit products, indicating their potential as a source of advantageous probiotic strains.

The *Borassus flabellifer* is a tall, upright palm with large, fan-shaped leaves. The Greek word *Borassus* refers to the fruit's leathery covering, while flabellifer means "fan-bearer." Jaggery palm, Palmyra palm, toddy palm, and wine palm are some synonyms for the plant. This species is found all over the world, from Australia to Africa. It is found throughout India's tropical regions, particularly in West Bengal, Bihar, and the coast of the peninsula. It is frequently grown. The plant's various parts are used to treat a variety of conditions, including liver and spleen enlargement, antiperiodic, heart burns, and secondary syphilis. In addition to these pharmacological applications, the plant's juice is used to make jellies, health drinks, and other products.

Probiotic microorganisms from palm fruit are isolated using selective culturing methods to produce pure strains, which are then identified and confirmed to be probiotic through morphological, biochemical, and molecular characterization [2] Adhesion ability, antimicrobial activity against pathogens, acid and bile tolerance, and safety evaluation are important features [3] Investigating palm fruit as a source of probiotics aids in the creation of affordable, plant-based probiotic products as well as the identification of new strains with distinctive functional characteristics. In order to assess the probiotic microorganisms' potential uses in both industry and health, this study focuses on their systematic isolation, characterization, and optimization from palm fruit.

II. MATERIALS AND METHODS

In the Tirupati region, farmers in Chittoor district sold us ice apples that were harvested directly from their trees. Prior to extraction, the fruits were peeled and cleaned with tap water. Palmyra palm fruit juice was prepared by manually extracting the pulp in aqueous phase at a pulp to water ratio of 1:2 until the homogenous suspension was formed. The palmyra palm extract was pasteurized for five minutes at 65 °C [7]. After being heated and cooled, the palmyra palm fruit juice aqueous extract was placed in a screw cap bottles and refrigerated at -20°C until needed.

- *Isolation and Purification of Probiotic Bacteria from the Palm Fruit Juice Samples*

In a separate test tubes, the fermented ice apple sample was dissolved in MRS broth, and then incubated for 24 hours at 37°C in an aerobic environment. A serial dilution technique was employed to reduce the quantity of microorganisms in the sample. To accomplish this, a microbial suspension was made by suspending and stirring 10 milliliters of the fermented ice apple sample in 90 milliliters of distilled water. For instance, a blank would be created by pipetting 10 milliliters into 90 milliliters of water to create a $10^{-2,}$ 10^{-3}, $10^{-4,}$ 10^{-5}, 10^{-6}, and 10^{-7} dilution.

- *Selection and Purification of Microbial Isolates*

Nutrient agar and De-Man Rogosa & Sharpe agar (MRS) plates were inoculated using a dilution factor of 10^{-7} from the serial inoculation. A clean petri dish was filled with a volume of 0.5 ml 10^{-7} dilution sample. To check for bacterial growth, the sample was spread out and covered with nutrient agar using the spread plate technique. It was then incubated for 24 hours at 37°C. The total number of colonies from each dilution was calculated by counting the colonies [8]. Using the streak plate method, bacteria were isolated on MRS agar, cultured for 24 hours at 37°C, and then refrigerated at 4°C on MRS agar slants until further analysis [9]. Colonies displaying diverse morphological traits were carefully picked and subjected to repeated sub culturing on MRS agar across three successive transfers to ensure purity and stability of the isolates.

- *Primary Identification and Preliminary Screening of Isolates*

Potential isolates were underwent for the the Gram staining test, and the stained cells were observed under a light microscope to determine their morphological characteristics. A small part of the colony was added with a drop of 3% aqueous KOH on a glass slide to find out is the isolate cells are negative for a viscosity test. Catalase negative isolates were determined by using Three percent (3%) hydrogen peroxide. The identified rod-shaped, gram-positive cells which shows negative for both catalase and stringing were re cultured and kept in sterile 20% glycerol stocks, and stored at 20°C [10]. All selected isolates were grown 3 times in MRS medium prior to the further experiment.

- *Biochemical Characterization*

Biochemical tests, including starch hydrolysis, indole test, methyl red test, Voges-Proskauer test, citrate utilization

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test were performed on bacterial isolates cultured on MRS agar for 48 hours. All analyses were carried out by the procedures outlined in Bergey's Manual of Systematic Bacteriology [11].

➢ *Bacterial Strains and Culture Conditions*

Five pathogenic indicator strains were selected for the assessment of antimicrobial assay. *Salmonella Typhi*, *Klebsiella pneumoniae*, *Escherichia coli*. All bacterial strains were cultivated in Luria-Bertani (LB) broth (Hi-Media Laboratories Pvt. Ltd.) at 37°C for 24 h.

➢ *Functional Categorization of Probiotic Traits*

- *Resistance to Acidic and Bile Conditions*

The isolates' resistance to bile and acid was assessed using the method outlined by [12] Bhushan et al. (2020). The overnight culture was harvested and made a pellet by centrifugation at 8000xg for 10 minutes and then pellet was suspended in PBS (pH 7.2). Approximately 109 CFU/mL cell suspensions were employed to various acidic conditions (pH 1, 2 and 3) and MRS broth with cell suspension had been supplemented with different amounts of ox gall (0.25%, 0.5%, and 0.75%) and incubated at 37 °C for 0, 3, and 6 hours. The control was MRS broth without ox gall (pH 6.5). A measure of cell viability, was then calculated by gradually diluting samples and spreading them onto MRS agar plates.

➢ *Resistance to Simulated Gastrointestinal Juices*

The procedure described by Bao et al. was used in the formulation of artificial GI fluids [13]. For gastric juice, 100 mL of 0.2 % sterile saline was mixed with 0.35 g of pepsin and pH was brought to 2.5 by using 1 mol/L HCl. A membrane filter with a pore size of 0.22 µm was used to filter the fluids. In order to prepare the artificial intestinal fluid, 0.9 g of trypsin and 1.8 g of bovine bile salts were liquified in 100 mL of distilled water which contained 0.2 g of sodium chloride and 1.1 g of sodium bicarbonate and the pH was adjusted to 7.0 with 1 M sodium hydroxide. After that, the fluid was filtered by a 0.22 µm membrane filter. This artificial intestinal fluid was inoculated with bacterial cultures. After inoculation, the viable CFU was performed using the plate count method at 0, 3, and 6 hours.

➢ *Assay of Temperature and NaCl Stress Tolerance*

The method of [14] Abushelaibi et al. (2017) was used to assess the salt and temperature with slight modifications. The MRS broth was cultured with probiotic isolate and with stand at different temperatures like 25 °C, 37 °C, and 55 °C in a incubation time period of 24hrs. At the end of incubation time, the broth was harvested and plated on MRS agar and leave it for 48 hrs at 37°C. In the same way, the 24 hrs inoculum was added into MRS liquid broth containing sodium chloride at, 2%, 4% and 6% concentrations. After 24 hrs of seeding, the growth rate of the probiotic isolation was evaluated using count assay.

➢ *Assay of β-Galactosidase Production*

With a minor modifications, the method described by [15] Rai and Tamang (2022) was used to determine β-galactosidase synthesis. After the incubation, the bacterial culture was precipitated by centrifugation at 12,000×g for 4 minutes at 4°C. The precipitate was washed with PBS rinses and resuspended in a MRS broth. An ultrasonic processor (frequency >20 kHz) was used to sonicate one milliliter of the suspended cells for five minutes in order to lyse the cells. After lysis, the cell suspension was centrifuged and the supernatant was collected and reacted with 4 ml of 4 mg/ml o-nitrophenyl-b-D-galactopyranoside (ONPG, 4 mg/mL). After adding 2% lactose to the reaction mixtures, they were incubated for 24 hours at 37°C. Following incubation, the aliquots underwent another centrifugation, two PBS rinses, and reconstitution in the identical buffer. After that, the suspensions were placed in a water bath at 37 °C for 15 minutes. Finally, the reaction was stopped by adding 0.5 mL of 1 M sodium carbonate, and the absorbance was measured at OD 560 nm.b-Miller Units were used to calculate the B-galactosidase activity Miller Units(MU) = (OD420nm/OD560nm X volume X time) X1000.

➢ *Evaluation of Antioxidant Potential*

- *Preparation of Test Sample*

✓ *Extracellular Supernatant*

The bacterial strains were grown for 24 hours at 37°C in MRS medium until they reached the exponential phase. Following incubation, cultures were centrifuged at 4,000×g for 10 minutes at 4°C. The cell free supernatant (CFS) was then carefully collected for DPPH activity analysis.

➢ *Intracellular Extracts*

Following three complete PBS rinses, the bacterial cells were extracted and subjected to low-temperature ultrasonication (400 W, 5-second pulses interspersed with 5-second pauses, repeated 80 cycles). The cell lysate was disrupted, centrifuged at 8,000×g for 20 minutes, and the resultant supernatant was collected as the Intracellular extract (ICE) for use in the assessment of antioxidant activity.

➢ *DPPH Radical Scavenging Assay*

The modified method of [16] Fan et al 2017 was used to estimate the DPPH radical scavenging activity [16]. A 1ml of ECS or ICE was treated with 0.4 mM DPPH in ethanol. The reaction mixer was kept in a dark at 37°C for 30 min. After incubation, the % scavenging activity of ECS or ICE was observed at OD_{517nm} and calculated with the following formula.

Scavenging Activity (%) =1 − (A1 − A2) =Ao 100

Ao = Absorbance of the control group (distilled water alone).

A1 = Absorbance of the test sample reacting with DPPH.

A2 = Absorbance of the blank group (absolute ethanol replacing

The DPPH solution).

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➢ *Antibacterial Susceptibility Test*

Antibiotic resistance was estimated by using the method Bauer et al [17] Kirby-Bauer disk diffusion method. Probiotic isolates were inoculated in MRS broth and incubated at 37 °C for 24 h. After incubation, bacterial suspension was swabbed with sterile swabs across the surface of Mueller-Hinton agar (MHA) plates. Five antibiotic discs (6 mm; Hi-Media, Mumbai, India). Ciproflaxin, Ampicillin, Erythromycin and Azythromycin each 10 micro grams were selected and placed on the media and the plates wer incubated at37 °C for 24 h.

➢ *Molecular Characterization*

- *Amplification of 16S rRNA Gene*

DNA was isolated from the probiotic isolate using the traditional phenol:chloroform:isoamyl alcohol (25:24:1) method proposed by Sambrook et al (Sambrook et al., 1989). Primers 2F (5′-AGAGTTTGATCCTGGCTCA-3′) and 1390R (5′-TACG GTTACCTTGTTACGACTT-3′) were used to amplify the 16s rRNA using the purified DNA as a template. The PCR reaction mixture was prepared in a 50ml vials, it contains 1× Taq buffer with MgCl2, 50 mM of each dNTP, 0.2 mM of each primer, 1 unit of Taq DNA polymerase, and approximately 100 ng of template DNA. The PCR procedure began with a denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 45 sec, primer annealing at 55°C for 1 min, and an extension phase at 72°C for 90sec. For ten minutes, a last elongation step was carried out at 72°C. A QIAamp 96 thermal cycles was used to accomplish amplification. To verify effective amplification, the PCR product was examined by electrophoresis on a 1.5% agarose gel. Sequencing was done using an Applied Bio systems TM 3730xl DNA Analyzer after amplification. Low-confidence areas were removed after the original sequence data's quality was assessed. The CAP3 sequence assembly algorithm was used to put together high-quality reads into contigs. Following assembly, the sequences were aligned and contrasted with reference 16S. To ascertain taxonomic affinities, rRNA gene sequences were obtained from the NCBI GenBank database and examined using the BLAST sequence alignment program.

➢ *Statistical Analysis*

The results were displayed as the mean ± standard deviation (SD) after each experiment was independently repeated three times. GraphPad Prism (version 5.0; GraphPad Software) was used for statistical analysis. To compare group differences, one-factor and two-factor analyses of variance (ANOVA) were used. The significance level was set at $p < 0.05$, $p < 0.01$, $p < 0.0001$, and non-significant (ns).

III. RESULTS

➢ *Screening and Purification of Microbial Isolates*

MRS agar medium was used to isolate probiotic bacteria from Ice apple fermented samples, where 10 samples were identified. Based on the morphological, biochemical and potential characteristics, five isolates (PB1, PB2,PB2, PB3, PB4 and PB5) were selected for phenotype screening and exhibited a circular and irregular shape, small and medium size, transparency, undulant margin, slightly elevated clear white, white color and gram positive (Table-1).

Table 1 Colony Morphology of Isolated Strains from Ice Apple.

Isolate	colour	shape	Size	Elevation	Margin	Opacity
1	Cream white	Irregular	Large	Slightly raised	Undultant	Not transparent
2	Cream white	Circular	Small	Raised	Entire	Not transparent
3	white	Circular	Large	Slightly raised	Entire	Not transparent
4	Cream white	Irregular	Medium	flat	Entire	Not transparent
5	white	Circular	Large	Flat	Entire	Not transparent

➢ *Biochemical Profiling of Isolate*

In the present study, all the tested bacterial isolates were exhibited as Gram-positive and rod-shaped. All strains are also non-motile, non-spore-forming, and catalase negative. The carbohydrate fermentation process and carbohydrate utilization capacity was evaluated by using Hicarbo kit. The results were shown in (Table-2).

IMVIC and biochemical tests were performed to all the five isolates. The results were showed as, all the strains were shown as negative for indole test. After the incubation period of 24hrs at 35 degrees Celsius, the formation of mixed acid fermentation in glucose media from each isolates were not observed and confirms the negative for methyl red test. For VP test (Voges Proskauer), the isolates which were grown on VP medium, those were negative to turn into red. All isolated strains performed negative on a citrate utilization, nitrate reduction, urease test and positive for starch hydrolysis (Table.3).

Table 2 Carbohydrate Fermentation Profiles of Probiotic Strains Isolated from Palm Nectar.

Carbohydrates	PB 1	PB 2	PB 3	PB 4	PB 5
Lactose	+	+	+	+	+
Xylose	+	+	+	+	+
Maltose	+	+	+	+	+
Fructose	+	+	+	+	+
Dextrose	+	+	+	+	+
Trehalose	+	+	+	+	+
Raffinose	+	+	+	+	+

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Melibiose	+	+	+	+	+
Galactose	+	+	+	+	+
Sucrose	+	+	+	+	+
L-arabinose	+	+	+	+	+
Mannose	+	+	+	+	+

Table 3 IMViC Biochemical Tests

Isolates	Indole	Methyl red	Voges Proskauer	Citrate Utilization	Nitrate reduction test	Urease test	Starch hydrolysis
PB1	-	-	-	-	-	-	+
PB2	-	-	-	-	-	-	+
PB3	-	-	-	-	-	-	+
PB4	-	-	-	-	-	-	+
PB5	-	-	-	-	-	-	+

➢ *Antimicrobial Assay Activity*

The cell free extract of probiotic isolates showed significant antagonist activity against the pathogenic bacteria such as Salmonella Typhi, Klebsiella pneumoniae, Escherichia coli (Figure-1). Preparation of LB media plates spreading with pathogenic bacteria and then the samples were activated by paper dip technique and incubated for about 24hrs. After incubation, the zone of inhibition was measured (Table.4).

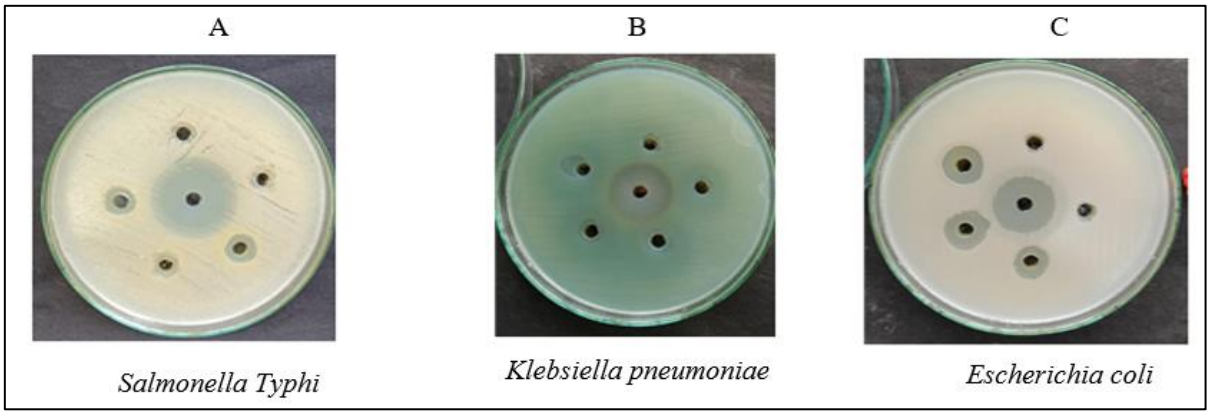

Fig 1 Antimicrobial Activity of Probiotic Isolates

Table 4 Antagonistic Activity of Probiotic Isolates

Test oranisms	PB1	PB2	PB3	PB4	PB5
A. *Salmonella Typhi*,	++	++	++++	++	++
B. *Klebsiella pneumoniae*	+++	+++	+++++	+++	+++
C. *Escherichia coli*	+	+	+	+	+

+ : zone diameter 4-5mm

++ : zone diameter 6-7mm

+++ : zone Diameter more than 7mm

++++: zone Diameter more than 8 mm

One important feature supporting the preservation of gut health is the probiotic isolates' ability to suppress clinically significant human infections. Table 4 summarizes the evaluation of the isolates' inhibitory potential against three harmful bacteria. The studied isolates had notable antimicrobial activity, with various degrees of inhibition against the target pathogens, according to the data. The isolates with the biggest zones of inhibition (ZOI) and inhibition against all tested pathogens were deemed to be the most effective (Figure 1). Different levels of antibiotic activity against the indicator microbes were shown by the probiotic strains' supernatant, indicating variations in the synthesis of antimicrobial chemicals.

➢ *Functional Categorization of Probiotic Traits*

- *Resistance of Probiotics to Stimulated GI Conditions*

✓ *Tolerance to Acidic and Bile Secretions*

The acidic and bile tolerance of five probiotic strains were evaluated for the periods of 1.0, 2.0, and 3.0 at pH levels of 0, 3, and 6 h. The results were shown in Figure 2 A-C. Viability of isolates under acid condition was counted as CFU and expressed the results as Log CFU/ml. All strains exhibited significantly viability and resistance under severe acidic conditions, even at pH 1.0 after 3 hours of exposure. Based on results at all tested pH levels, we observed a

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progressive decline in viability among the isolates after 6hrs incubation. A moderate reduction was observed, yet all strains sustained survival. Ox gall was used to assess the bile salt tolerance of five isolates at concentrations ranging from 0.3% to 0.75% w/v over 0, 3 and 6h as shown in Figure 2D to F. All strains demonstrated a considerable viability throughout the incubation periods. After 6hs of time, at highest concentration (0.75%), the isolates showed strong resistance towards bile secretions.

✓ *Resistance to Gastrointestinal Juice*

All five isolates were exposed to gastric and intestinal environments and exhibited a normal growth. However the number of viable cells were significantly reduced in the gastric juice at a low pH. As shown in Figure. 3A, the isolates grown with a moderate survival rate at the end of 6 hr. The impact of intestinal juice on growth of these isolates were illustrated in Figure 3 B. The results demonstrated that all the strains were able to survive in intestinal fluid, with strain PB 3 exhibiting highest survival rate among five.

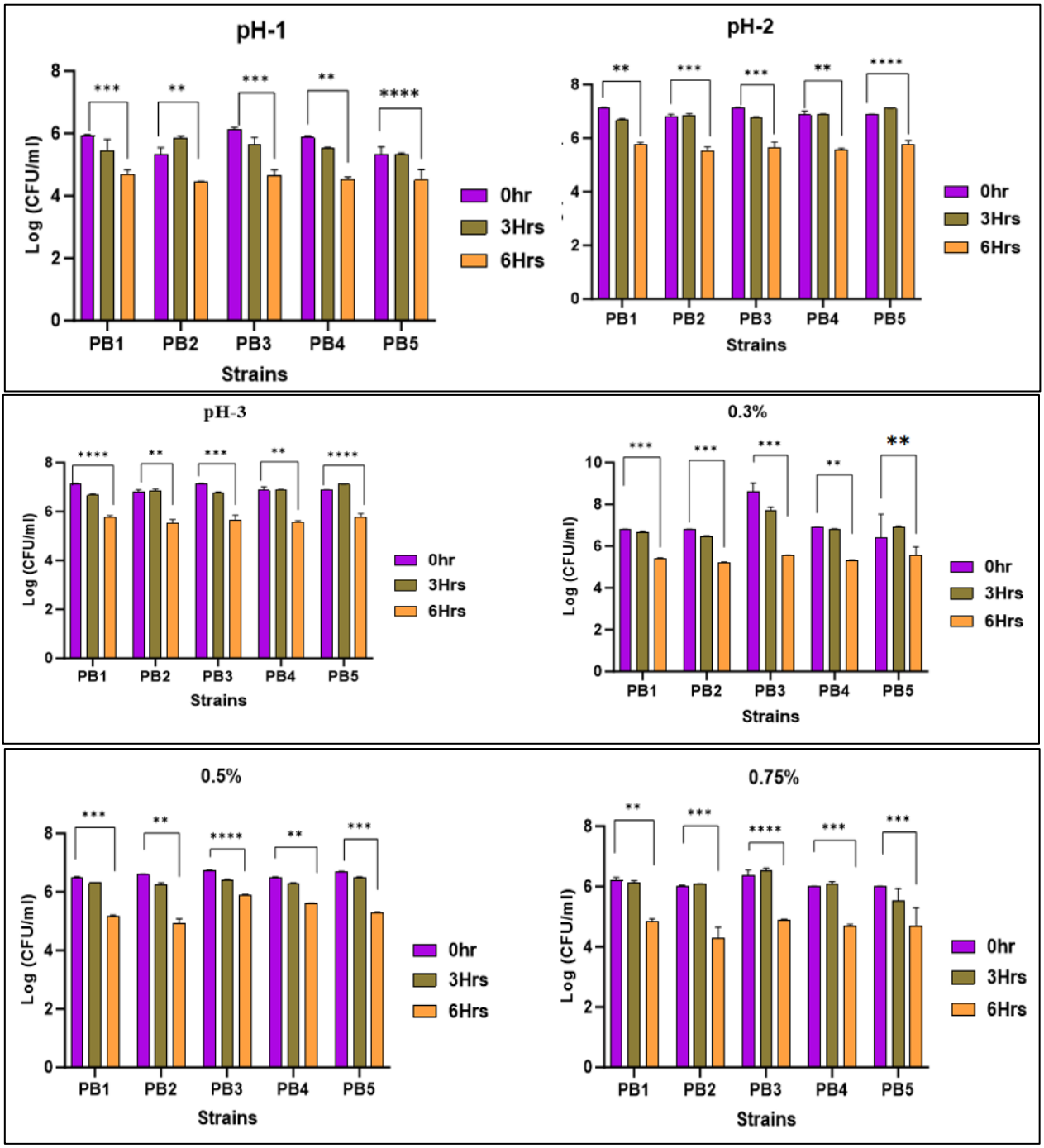

Fig 2 Acid and bile tolerance of five probiotic isolates expressed as Log CFU/mL. Acid tolerance was evaluated at pH 1.0 (A), pH 2.0 (B), and pH 3.0 (C), while bile salt tolerance was assessed at concentrations of 0.3% (D), 0.5% (E), and 0.75% (F). Bacterial survival was measured at 0, 3, and 6 h of incubation at 37 °C. Results are presented as mean values ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using two-way ANOVA; significant differences in survival rates ($p < 0.05$, $p < 0.01$, $p < 0.0001$ and ns) are denoted by an asterisk (*), (**), (****).

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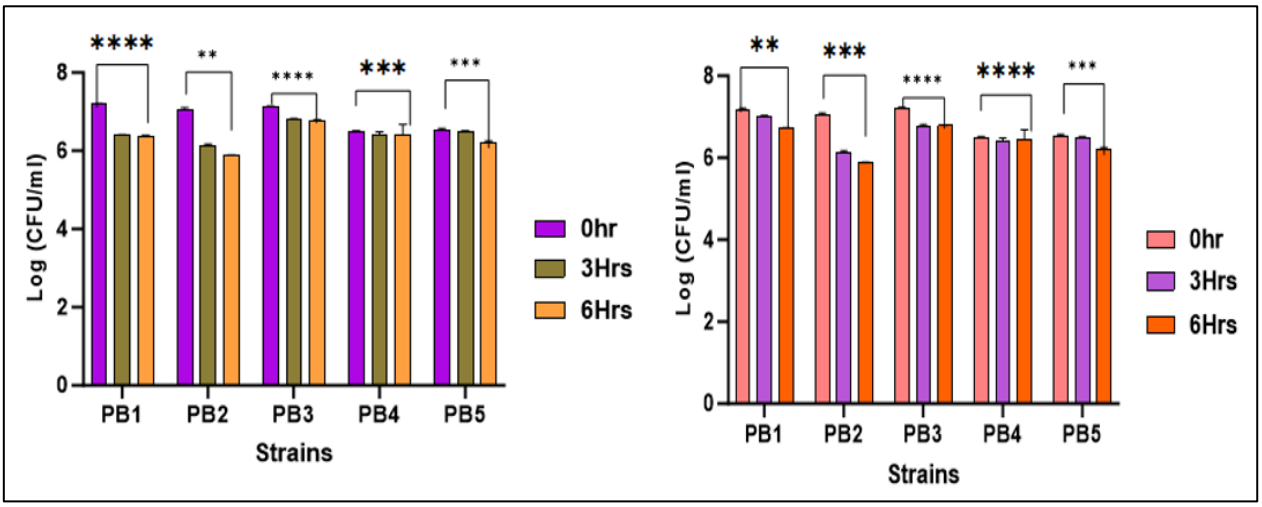

Fig 3 In vitro tolerance of five probiotic isolates to artificial gastrointestinal conditions represented as Log CFU/mL. (A) Survival in gastric fluid and (B) artificial intestinal fluid was monitored at 0, 3, and 6 h of incubation at 37 °C. Data represent the mean ± standard deviation (SD) of three independent replicates. Statistical analysis was performed using two-way ANOVA; significant levels in survival rates ($p < 0.05$, $p < 0.01$, $p < 0.0001$) are denoted by an asterisk (*), (**) (****), respectively.

✓ *Salt and Temperature Tolerance Assessment*

The salt and thermal resistance of five probiotic isolates were evaluated over 24 hr incubation time at 2%, 4% and 6% NaCl concentration (Figure 4 A-C). The results demonstrated a inverse relationship between salt levels and microbial survival rate, as salt concentrations rose over time, the growth viability decreases. Based on the observation, at 6% NaCl levels for 24 hour incubation time resulted in a significant reduction in CFU across all isolates. In studies of temperature sensitivity at 25 °C, 37 °C. and 55 °C (Figure 4 D), the isolates exhibited optimal growth in 24hrs incubation time on the test range of temperature. The Optimum growth peak was observed at 25 °C and 37 °C and the growth was dramatically declined at 55 °C, these results indicated that the isolates are sensitive to high temperature.

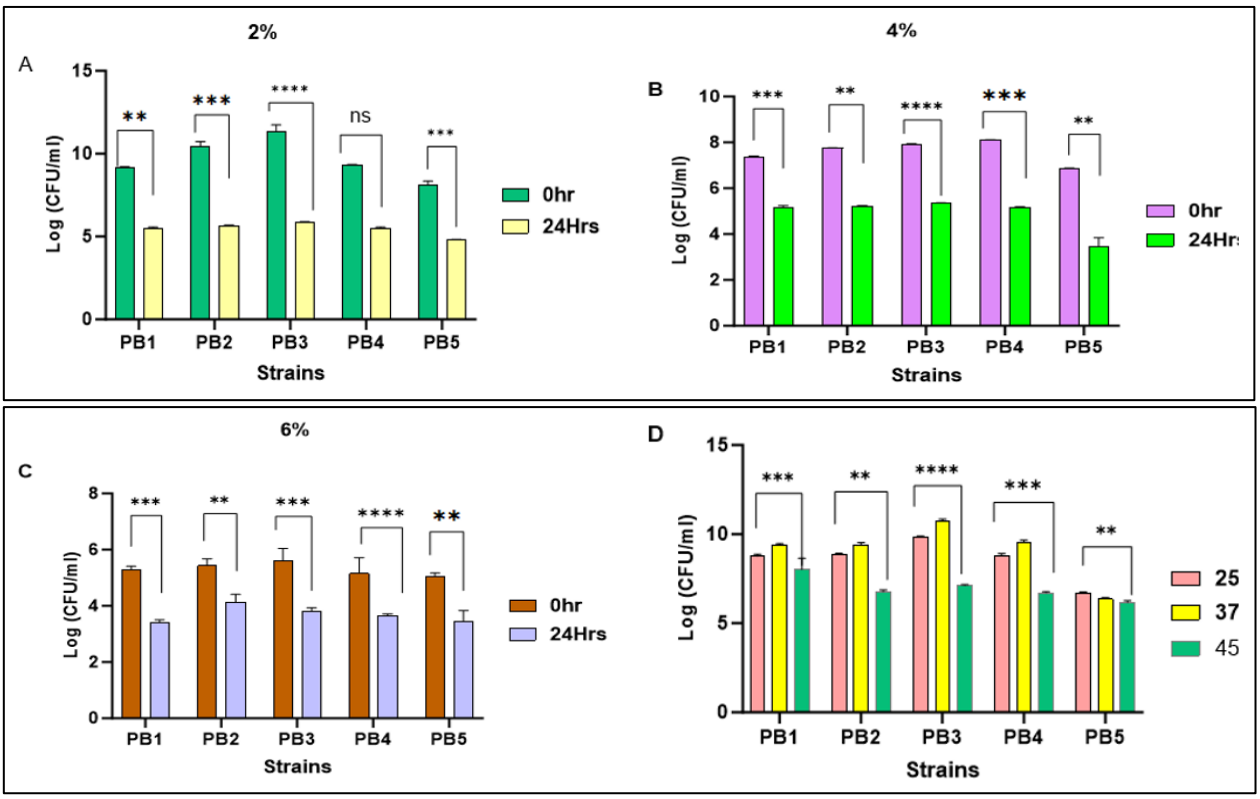

Fig 4 Effect of varying NaCl concentrations and incubation temperatures on the viability of five probiotic isolates, expressed as Log CFU/mL. (A-C) Survival in 2%, 4%, and 6% NaCl concentrations, respectively, measured at 0 and 24 h. (D) Temperature tolerance assessed after 24 h of incubation at 25 °C, 37 °C, and 55 °C. Data are presented as mean ± standard deviation (SD) from three independent replicates. Statistical analysis was performed using two-way ANOVA; significant levels ($p < 0.05$, $p < 0.0001$) are indicated by asterisks (*), (****) and ns, ***$p < 0.001$.

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✓ *β-Galactosidase Production*

The assessment of β-galactosidase activity in probiotic isolates was conducted to evaluate their efficiency in lactose hydrolysis. According to the results (Figure. 5A), Among the isolates, the maximum β-galactosidase activity was exhibited by PB3 (230 ± 5.1IU/mL), followed by PB5 (191 ± 7.63 IU/mL) and PB2 (179 ± 4.59 IU/mL). In contrast, the minimal activity was observed in PB1 (124± 4.59 IU/mL). Overall, enzyme production varied considerable among the five isolates. Nevertheless, all isolates demonstrated the appreciable level of enzyme activity, indicating their potential role in facilitating the lactose digestion in the host.

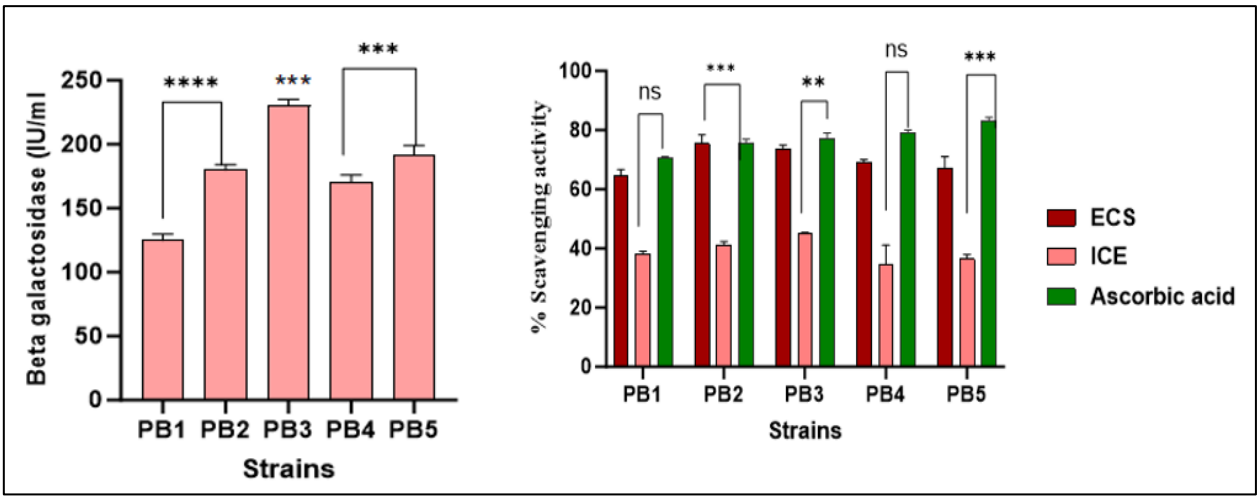

Fig 5 Functional Properties of Five Probiotic Isolates. (A) β-Galactosidase Activity of Five Probiotic Isolates Estimated After 24 h of Incubation. (B)

✓ *Evaluation of Antioxidant Potential*

The antioxidant activity of both extra cellular supernatant (ECS) and Intracellular extract was evaluated by DPPH radical scavenging activity (Figure. 5B). Among the tested samples, the ECS sample exhibited superior antioxidant activity, specially, PB3 showed the potential scavenging activity at approximately 82.5%, all other isolates maintained scavenging potential with more than 50%. In contract, lowest DPPH scavenging activity was displayed by ICE samples. Within this group, PB3 recorded with 45.29% activity. overall results, ICE sample displayed lower scavenging activity compared with ECS samples. The standard reference, ascorbic acid, yield a scavenging activity 84.21%.

✓ *Antibiotic Susceptibility Test*

Antibiotic susceptibility of probiotic isolates were performed using different drug classes. Thezone of inhibition was calculated. In the current study, the isolates showed resistance to antibiotic such as Ciproflaxin, Ampicillin, Erythromycin and Azythromycin (Figure.6).

Fig 6 Antibiotic Susceptibility of Representative Probiotic Isolates PB3 and PB5 Determined Using the Disk Diffusion Method Against Various Antibiotics.

The results indicates that the given antibiotic resistance patterns of all isolates could be considered safe, reinforcing their applicability for potential probiotic applications (Table-5)

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Table 5 Antibiotic Susceptibility Profiles of the Two Probiotic Isolates.

Strains	***Zone of Inhibition (mm)***			
	Ciproflaxin	***Ampicillin***	***Erythromycin***	***Azythromycin***
PB3	4	7	6	5
PB5	1.5	1	3	2

➢ *Molecular Characterization*

- *Amplification of 16S rRNA Sequences*

16S rRNA gene sequencing was used to molecularly identify the most promising probiotic isolates PB3. Based on the functional properties we selected PB3 for molecular characterization. Agarose gel electrophoresis verified PB3 LAB bacteria produced PCR amplification of roughly 1500 bp. The NCBI's BLAST tool was used to retrieve the 16S rRNA sequences and then uploaded to the Genbank repository (https://www.ncbi.nlm.nih.gov/genbank/). A high degree of similarity with previously published 16S rRNA genetic sequences found in the GenBank database repository was found by sequence alignment. The isolate PB3 was identified as *Lactobacillus plantarum.*

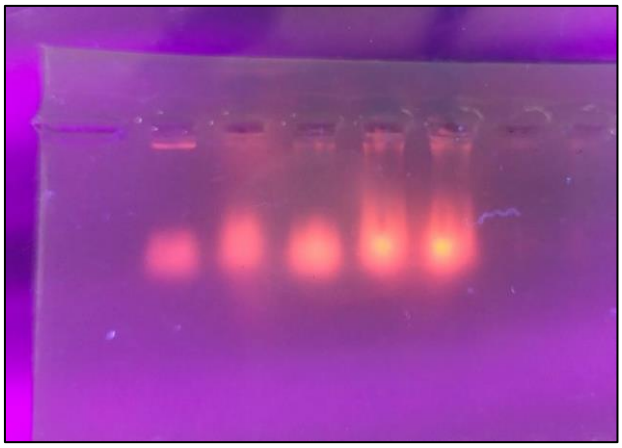

Fig 7 PCR Amplification Products of the Isolated Probiotic Strains (PB1, PB 2, PB 3, PB 4, PB5) Visualized on a 2% Agarose Gel. Lane 1: DNA Ladder (100 bp–3000 bp), Lanes 2–5: PCR Amplicons of the Respective Isolates.

IV. DISCUSSION

This study emphasized to identifying and evaluating bacterial isolates for their potential as active probiotics. This study adds value to traditional fermented foods rich an unexplored quantity of probiotic microorganisms. Especially the study concentrated on the isolation and characterization of probiotics from an Ice apple fermented sample. A natural fruit widely consumed with nutritive values [18].

In this study, five bacterial isolates were screened for probiotic properties, among these, one specific strain (PB3) demonstrated probiotic efficiency, including robust GI adaptability, salt and temperature tolerance, enzyme production, antibacterial, antibiotic resistance and antioxidant activities. Furthermore, In addition to assessing these probiotic qualities, our study evaluated the strains on safety for both therapeutic and preventive action against pathogenic infections at intestine.

The stomach low pH and bile salt concentration in the small intestine are obstacles to the survival of probiotic bacteria [19]. These acidic pH is essential to improve the digestion but at the same time, it create a harsh environment for probiotic bacteria. High bile alt levels can cause bacterial cell membrane rupture and decrease bacterial survival in the gut [20] The survival ability of probiotic bacteria under these conditions is evaluated by studying the acid tolerance and bile salt resistance [21, 22]. In this study five strains were recovered from ice apple fruit extract were employed for acid and bile tolerance. In this study five probiotic strains recovered from ice apple fruit extract were employed for acid and bile tolerance testing. Probiotic strains isolated from ice apple have been shown to withstand bile concentrations up to 0.5%, with viable cells range between 7.01± 0.01 and 9.21 ± 0.12 log CFU/mL after 3 and 6 hours of exposure [23]. Likewise, at a 0.25% bile salt concentration, viable counts remained between 8.59 ± 0.11 and 5.53 ± 0.05 log CFU/mL after 3 h, and 6.42 ± 0.07 to 4.82 ± 0.08 log CFU/mL after 6 h of incubation.

Another significant characteristic of probiotics is survival under gastric conditions [24]. Many ingested microbes are eliminated by the at pH 2.0 & pH 8.0 environment of gastric juices [25]. The Probiotic isolates showed a notable resistance to GI conditions and artificial intestinal fluids. These results are consistent with previous reports, where viable counts increased from 7.18 ± 0.06 to 7.93 log CFU/mL after 3 h and 6 h of incubation, respectively [23]. Under the same conditions, in the current findings, our isolates demonstrated significant average viable counts in both gastric (5.98 ± 0.01 – 6.78 ± 0.07 log CFU/mL) and intestinal (5.71 ± 0.02 – 7.57 ± 0.06 log CFU/mL) conditions after 3 h and 6 h of incubation.

During 24hrs of incubation all isolated strains showed optimal growth at 25°C and 37°C. The maximum cell growth was observed at 37°C, ranging from 8.75 ± 0.02 to 9.8 ± 0.04 log CFU/mL. At the higher temperature of 45°C, the growth markedly decreased to 6.17 ± 0.01 log CFU/mL. This growth pattern regarding thermal sensitivity is correlated with the results of Zang et al [26]. In the similar way, the isolates also exhibited NaCl tolerance at levels of 2% and 4% , demonstrate good tolerance. At the 6% Na Cl level, declined growth was observed, indicating that higher salt levels adversely affect the viability of the cells.

Lactose predigestion during food fermentation and β-galactosidase activity in the intestinal tract are the two primary pathways by which probiotic bacteria alleviate the lactose intolerance [15]. In the present research, all isolated strains exhibited significant β-galactosidase activity, with fluctuating enzyme levels between individual strains. These data confirms that while the enzyme is universally present across the isolates, certain strains possess superior catalytic

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potential. Such variability highlights the promise of these probiotics for use in targeted applications, particularly those requiring robust lactose hydrolysis and improved digestive health.

To evaluate free radical scavenging activity of probiotic isolates, bacterial cell-surface components were utilized. The present analysis showed that probiotic cell components, specifically extracellular supernatant (ECS) and Intracellular cell extract (ICE), demonstrated significant antioxidant activity particularly regarding DPPH radical scavenging. When comparing the scavenging activity of the two components, the ECS exhibited the highest DPPH scavenging potential, indicating that primary metabolites play a key role in neutralizing the free radicals. This results were partially correlate with the findings of Wang et al [27], who reported that Intracellular cell extracts of *Bifidobacterium Strains* possess higher DPPH and superoxide scavenging activity. Furthermore, a strain dependent nature of antioxidant activity was observed across all probiotic isolates.

V. CONCLUSION

This study isolated five probiotics strains from naturally fermented ice apple fruit extract and fermented Ice apple fruit extracts and characterized them on their morphology, tolerance to salt, temperature, pH, gastrointestinal tolerance, and antimicrobial, antibiotic and molecular identification. Among the isolate PB3 emerged as the most promising strain, demonstrating high survival rate under high GI conditions and adopted to intestinal environments. These investigations highlights their potential application as a functional probiotics in food or pharmaceutical formulations. The future study focusing on in vivo evaluation, safety assessment and mechanism involved in host-microbe interactions will further develop their activity and create a way for developing novel probiotic products.

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- *Conflict of Interest*

The authors declare no conflict of interest.

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