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Full Length Article

In Vitro Safety Evaluation of Novel Phyto Lactic Acid Bacteria Isolated from Fruits as Potential Probiotics

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Abstract

This study focused on the isolation of lactic acid bacteria (Probiotics) from non-dairy sources like fresh fruits including olive (*Olea europea*- Gamlik), mango (*Magnifera indica* L. cv. Chaunsa) and banana (*Musa cavendish* Basrai), which are available in local markets of Pakistan. The three novel strains of the enterococcus genus were isolated from these fruits. These species of enterococcus were molecularly identified with 16S rRNA gene sequencing. *Enterococcus durans* JCM8725 from olive, *Enterococcus faecium* MW 585366 from mango and *Enterococcus faecium* NBRC 100486 from banana were identified. These strains were positive for lipolytic and lactic acid production and negative for amylolytic, proteolytic, cellulolytic activities, D*Nase* and hemolytic. *E. durans* JCM8725 demonstrated the highest survival rate viable cells count 7.58 Log CFU/mL at pH 2.0. Similarly, after four hours in 0.3% bile salt. *E. durans* JCM8725 had shown higher stability (7.88 Log CFU/mL). *E. durans* JCM8725 had shown elevated Cell Surface Hydrophobicity (CSH) at 51% and auto-aggregation at 44%. Due to these properties, *E. durans* JCM8725 had an inhibitory effect on pathogenic strains *Escherichia coli* ATCC25922, *Pseudomonas. aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC25923. Antibiotic susceptibility test (AST) against imipenem, vancomycin, penicillin, chloramphenicol and streptomycin revealed that *E. durans* JCM8725 exhibited antibiotic susceptibility over all antibiotics while other two strains were resistant to streptomycin. The *E. durans* JCM8725 had the highest DPPH scavenging activity, 42.5%. The strains were found safe. The hemolytic activity and the D*Nase* test were negative in the *in-vitro* safety analysis.

Keywords: Nondairy LAB; Phytoprobiotics; Molecular identification; 16S rRNA; *In vitro* safety

Introduction

The modern food industry has witnessed an increase in attention and demand due to the perceived health benefits and improved taste of its products. There have been constant rises in the use of functional foods that have nutritional value, particularly fortified foods, including probiotics. Probiotics, when incorporated into functional foods, offer various health advantages. This rise in the market is due to the probiotic's association with health benefits and innovative technological intervention (Sehrawat *et al.* 2022). Probiotics are live microorganisms and when they are dispensed in controlled amounts of about 10^6-10^9 CFU/g, they furnish the host with health benefits (FAO/WHO 2006). Due to increasing concerns related to lactose intolerance and fermented dairy products, which are rich in cholesterol and fats portions, there is an urge to explore more sources, particularly a vegetarian lifestyle-based has increased (Aspri *et al.* 2020). To address customers' growing concerns about food origin, the extraction of probiotics from non-dairy sources, both fermented and non-fermented, is gaining attention. This includes sources such as vegetables, sauerkraut, kefir, bakery products, beverages, fish-based sausages and soy-based such

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as soy curd, soy yogurt and soy milk (Kumar *et al.* 2022). Non-dairy sources encompass both fermented and unfermented products, including cereal, bread bakery products, puddings, fruits, vegetables, juices, purees, pulp, beverages, meat, fish and sausages. Fruits and vegetables are used for the isolation of lactic acid bacteria. *Lactobacillus pentosus* are isolated by chilly and Nagi Naspati (*Pyrus communis*) and *Lactobacillus fermentum* is isolated by PRSI Peru (Pyrus pyraste) using 16S rDNA (Khan *et al.* 2023a).

The 16S rRNA sequencing technique allows for microbial composition analysis by examining the genetic material within the ribosomal RNA of bacteria (Sengun *et al.* 2024). The fruit-based lactic acid bacteria *Enterococcus faceium* PP 627037 was isolated from *Musa acuminate* and identified by using 16S rRNA sequencing. then it was followed by the analysis of fragment sequences gene base sequence of 16S rRNA and finally, it was compared with DNA reported in the BLAST program about the gene bank (Pava *et al.* 2024). Bananas, cherries, oranges, plums and sapota fresh fruits are the vectors for the isolation of Weissella paramesenteroides, which are valued by having bacteriocin and exopolysaccharide production ability. Five Weissella paramesenteroides strains are isolated from fruits. These strains are screened and selected based on tolerance at highly acidic conditions, high cell surface hydrophobicity, bile-induced biofilm formation and antimicrobial activity (AMA). Furthermore, these strains do not possess virulent traits, such as hemolysis, D*Nase* production and biogenic amine production (Khan *et al.* 2023b).

Keeping in view the need and demand of non-dairy lactic acid bacteria, the study was designed to evaluate the probiotic potential of probiotic strains isolated from the local fruits of Pakistan. Mango fruits (*Mangifera indica*) Chaunsa variety was procured from Mango Research Institute Multan, Pakistan. Meanwhile, the banana peels (*Musa cavendish* 'Dwarf Cavendish' Basrai) and olive pomace (*Olea europea*) were obtained from Rashdi Agriculture Farm Khesana (Mori, Tando Allahyar, Sindh, Pakistan) and Barani Agriculture Research Institute (Chakwal, Punjab, Pakistan). The probiotics strains, *Enterococcus faecium* MW 585366, *Enterococcus durans* JCM 8725 and *Enterococcus faecium* NBRC 100486, were isolated at the National Institute for Genomics and Advanced Biotechnology (NIGAB) at the National Agriculture Research Centre, Islamabad, Pakistan.

Material and Method

Sample collection

The fruits used for isolation of lactic acid bacteria were olive, *Olea europea* var. Gamlik, besides their pomace from the Barani Institute of Agriculture, BARI, Chakwal; mangoes, *Mangifera indica* var. Chaunsa, from the Mango Research Institute, MRI, Multan and bananas, *Musa cavendish* var. Dwarf-Basrai, from Tando Allah Yar, Sindh. The fruits were well cleaned with running tap water to eradicate dirt, dust and microbes before placing in a refrigerator.

The isolation of lactic acid bacteria

The isolation of lactic acid bacteria from fruit was based on a protocol developed by Kamaliya *et al*. (2023). The fruits were sterilized with 70% ethanol and the surfaces were rinsed using sterilized distilled water. Fruits were chopped and crushed under aseptic conditions. Fifteen grams (15 g) of fresh fruit pulp were added to 150 mL of peptone water and homogenized for 5 min at room temperature. From the first peptone water dilution, secondary dilutions 10–1 to 10–5 were performed and 100 μL aliquot of each dilution was spread over De Man Rogosa and Sharpe agar (MRSA) (Merk, USA) and incubated for 24 h at 37°C (Fig. 1). Pure single colonies were obtained by sub-culturing distinct colonies in terms of morphology. Morphology, Gram-staining and Catalase-oxidase tests characterized the isolated colonies.

Molecular characterization and identification

The genome DNA contents of purified single colonies were extracted through an Invitrogen Mini DNA extraction kit (Thermo Fisher Scientific, USA). The extracted DNA was sent to a private laboratory (Macrogen, Korea) for molecular identification. Universal primers *i.e*., 9F (5'-GAGTTGATCCTGGCTCAG-3') and universal reverse primers 1510R (5'- GGCTACCTTGTTACGA-3') were used for the amplification of the 16S rRNA gene (Nawaz *et al.* 2019). The PCR reaction mixture included 50 μL of total volume by using 25 μL Master Mix and Taq DNA Polymerase Platinum® (Invitrogen, Waltham, USA) beside 2 μL primers, 1 μL DNA sample and 20 μL Milli-Q water. The 1.5 kb DNA ladder was used as the standard. The gel was placed under the Gel Documentation System to visualize and capture the fluorescent images of DNA bands. PCR products were purified using AMPureXP® commercial DNA purification kit (Indianapolis, USA). The sequences were obtained from the Illumina-based system in San Diego, USA; the MiSeq Sequencing System. These were then matched to those in the GenBank database, developed by the National Center for Biotechnology Information from Maryland, USA, through its BLAST Algorithm (Christoff *et al.* 2017). The maximum likelihood method through the MEGA 11 software was thereafter adopted to produce the phylogenetic tree (Tamura *et al.* 2021).

Enzymatic and metabolic characterization

The isolates of lactic acid bacteria were tested for enzymatic activities such as amylolytic, proteolytic, lipolytic and cellulolytic activities. To assess amylolytic activity, 10 μL of fresh test culture was spotted on starch plates. A mixture of gram starch and gram nutrient agar in a one-to-one

Fig. 1: Isolating Phyto-lactic acid bacteria from (**A**) Banana (*Musa cavendish* var. Basrai), (**B**) Olive (*Olea europea* var. Gamlik) and (**C**) Mango (*Magnifera indica* var. Chaunsa) on (**D**) MRS media to isolate pure colonies

proportion was dissolved into 100 mL distilled water and plates were incubated at 37°C for 48 h with streaked starch agar. The incubation period at which the plates received 1% Gram's iodine solution was left for further 10 min. Clear zones around the culture spot indicated a positive result for amylolytic activity (Yahya *et al.* 2021). To analyze the lipolytic activities, the 1.0% tween 80 with phenol red as indicator was used in TSA media (Sigma-Aldrich, 22091). Fresh cultures were streaked onto TSA plates supplemented with Tween 80. The plates were incubated for 48 h and a color change from red to orange was considered positive (Techo *et al.* 2022). Li *et al.* (2018) evaluated cellulolytic activity by using Luria Bertani agar (Sigma-Aldrich, 41106200), which was made by incorporating 1% w/v carboxymethyl cellulose. The agar plate was streaked with the colonies and incubated at 39°C for 48 h. After incubation, petri plates were stained with 0.1% w/v Congo red; therefore, there would be a clear appearance of clear halos, thus indicating both positive and negative results. LAB was plated on brain heart infusion agar (BHI Sigma-Aldrich, 70138) plates that had been amended with 0.25% w/v calcium carbonate ($CaCO_3$). Incubation took place at 30°C for 5 days. The LAB strains were streaked on BHI-$CaCO_3$ agar plates. Lactic acid production was observed since clear zones started appearing around the streaks of bacteria (Aboseidah *et al.* 2017).

Probiotic properties of phyto probiotic strains

Acid bile tolerance test

The ability of LAB isolates to survive in acidic conditions was measured through the experimental methods described by Sohail *et al.* (2011). The MRS broth was amended with 1.0 *N* HCl to attain the 2.0 pH, the incubation time intervals were 5 min, 30 min, 60 min, 90 min and 120 min. The viable cells were counted by using MRS agar plates for the cell count method and an incubation time of 24 h to determine Log CFU/mL. The oxgall bile salt (Oxoid) 0.3% was used. The MRS broth was supplemented with 0.3% oxgall bile salt and incubated for 4 h. The total plate count was done and viable cell count Log CFU/mL was used to express the result. Zero-hour incubation time was also done to compare the change in viability by the plate count method.

Cell surface hydrophobicity (CSH) and auto-aggregation (AGG)

Adhering LAB to hydrocarbons was assessed through the cell surface hydrophobicity (CSH) test and clump-forming ability was assessed through the auto-aggregation test as described by Khan *et al.* (2023b). The results were calculated by using the following equation:

$$\text{Activity } \% = A^{o} - A/A \times 100$$

Where, A^{o} and A present initial and final absorbance, respectively.

Antimicrobial activities of the identified strains

The methodology of Khan *et al.* (2023b) was followed in establishing the antimicrobial activity of LAB isolates. The strains were grown on MRS broth. The freshly grown broth was centrifuged at 4000 × g for 10 min at 4ºC. The supernatant was collected and pellets were discarded. The supernatant was filtered, followed by the passage through a 0.22 μm sterile filter syringe of the supernatant. The 100 μL of *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC25923 pathogenic cultures were poured onto MHA plates before forming wells in the agar surface. 100 μL portions of filtered LAB supernatants to individual wells, which went through incubation at 37°C for 24 h. Observers checked the surrounding areas of the wells for any positive outcomes. The zones were formed. The formation of zones shows the presence of bacteriocins in LAB strains.

Antibiotic susceptibilities of the phyto-lab strains

A disk diffusion method served as the technique for a susceptibility test of antibiotics in LAB isolates. Muller-Hinton agar (Oxoid, CM0 337) was evenly poured on plates then freshly grown inoculum was poured on MHA plates. A minimum of five antibiotic disks became positioned on top of the agar surface. The inoculated plates required an incubation at 37°C for 18 to 24 h period. Every screened isolate fall under one of three outcome categories described by CLSI (Golnari *et al.* 2024) based on imipenem IPM (10 μg), vancomycin VA (30 μg), penicillin P (10 μg), streptomycin S (10 μg) and chloramphenicol C (30 μg) response. The zones inhibited by each antibiotic disk were measured using the scale of millimeter. The diameter of each zone formed by antibiotics was measured. The results were interpreted as Resistant (R), intermediately resistant (I) or Sensitive (S).

***In-Vitro* and *In-Vivo* safety evaluation of phyto-lactic acid bacteria strains**

Hemolytic activity

To test the hemolysin-producing ability of the lactic acid bacteria isolates, we utilized a 5% defibrinated sheep blood source obtained from Oxoid, UK, based on the procedure of Golnari *et al.* (2024). The presence of a clear halo around the colonies was an indication of positive hemolysis, where the actual color was β-hemolysis and no zones around the colonies $^{\gamma}$- hemolysis.

D*Nase* test

D*Nase* agar (Oxoid-CMO 0321) was used to determine D*Nase* activity. The DNA broke down by the microbes and its hydrolysis occurred. To check the safety of isolated strains, the *DNase* activity was evaluated. The freshly grown LABs colonies were streaked on D*Nase* agar plates. After overnight incubation at 37ºC, the isolates were considered D*Nase* positive with the formation of clear colored areas around the colonies (Meena *et al.* 2022).

***In vitro* antioxidant activity**

The DPPH free radical scavenging activity assay followed the method described by Khan *et al.* (2023b). The experiment started with making a 400 μM DPPH solution in methanol. A mixture containing each suspended LAB isolate along with ascorbic acid received 2 mL of the DPPH solution that had been prepared previously. During incubation at room temperature, the mixture received 30 min of exposure before measuring the absorbance at 517 nm. A control system consisted of mixing 2 mL of methanol with 2 mL of the DPPH solution. The 1.0 mg/mL concentration of ascorbic acid as their positive control solution. The % inhibition of DPPH in LAB strains was determined through the use of the following formula:

$$\text{Antioxidant } \% = \frac{(1 - \text{A sample})}{\text{A control}} \times 100$$

Results

Initial description of phyto probiotics strains

The lactic acid bacteria were isolated from the fruits, shows that among the 50 bacterial isolates from fruits 15 olives, 31 bananas and 4 mangoes 42 of them were phenotypically and genotypically characterized and identified as gram-positive, oxidase-catalase-negative bacteria (Fig. 2). From these isolates, 35 belonged to lactic acid bacteria. Three isolates with the best performance were selected during further probiotic evaluation from each type of fruit. Their morphological characteristics shown in Table 1.

Molecular identification of LAB

The 16S rRNA amplification gave 5 isolates from olive fruit, 25 isolates from banana fruit and 5 from mango fruit. The product size of these isolates was 1500 base pairs (Fig. 3). The PCR products were purified and partial Sanger sequencing identified all LAB isolates as the three lactic acid bacteria *i.e*., *Enterococcus durans* JCM 8725 (from Olive), *Enterococcus faceium* MW 585366 (from Mango) and *Enterococcus faecium* NBRC 100486 (from Banana). The strains were identified as being from the *Enterococcus* genus. The sequencing of the 16S rRNA gene is the best molecular approach for distinguishing closely related species within a genus (Fig. 3). An evolutionary relation of the isolates and related species in the *Enterococcus* genus is indicated by a 16S rRNA sequence-based phylogenetic tree (Fig. 3). The strains were identified as being from the *Enterococcus* genus. The sequencing of the 16S rRNA gene is the best molecular approach for distinguishing closely related species within a genus. The 16S rRNA sequence (Fig. 4) comparisons of existing databases to identify and evaluate the evolutionary relationship of three lactic acid bacteria isolates. The 16S rRNA sequence of *Enterococcus durans* JCM 8725 (OK137545) isolate was deposited and its homology analysis indicated a 99.86% similarity with *Enterococcus durans* (NR_113257). This confirms *E. durans* JCM 8725 identity and suggests a unique adaptation to the olive environment (Table 2). In addition, the sequence of *Enterococcus faecium* NBRC 100486 (OK178272) showed a 99.54% homology with *Enterococcus faecium* (NR_115764). Although there is a slightly lower degree of similarity compared to the *E. durans* JCM 8725 isolate, the homology remains within a range that unequivocally identifies the isolate as *E. faecium*. The *E. faecium* NBRC 100486 showed 99.69% similarity with *E. faecium* MGFR1. *E. faecium* MW 585366

Table 1: Morphological characteristics LAB isolates

Characteristics	*E. durans* JCM 8725	*E. faceium* NBRC 100486	*E. faecium* MW585366.1
Colonies Color on MRS agar	Small greyish-white colonies	Small greyish-white colonies greyish-white	Small greyish-white colonies
Cell shape	Cocci	Cocci	Cocci
Surface	Smooth	Smooth	Smooth
Entire edge	Smooth	Smooth	Smooth
Cellular arrangement	Chains	Chains	Chains
Gram's staining	+	+	+
Oxidase activity	-	-	-
Catalase activity	-	-	-

Table 2: Percentage homology of LAB strains

Bacterial strains	Accession numbers	% homology	Similarity
Enterococcus durans JCM 8725	OK137545	99.86%	*Enterococcus durans* (NR_113257)
Enterococcus faecium NBRC 100486	OK178272	99.54%	*Enterococcus faecium* (NR_115764)
Enterococcus faecium MW58366	MW585366.1	98.26%	*Enterococcus faecium* (NR_113904)

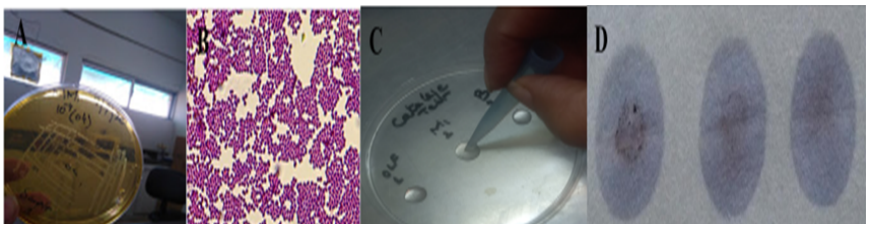

Fig. 2: (**A**) Showing color and shape of phyto-LAB colonies on MRS agar; (**B**) Cocci and gram-positive; (**C**) Catalase negative; (**D**) Oxidase negative

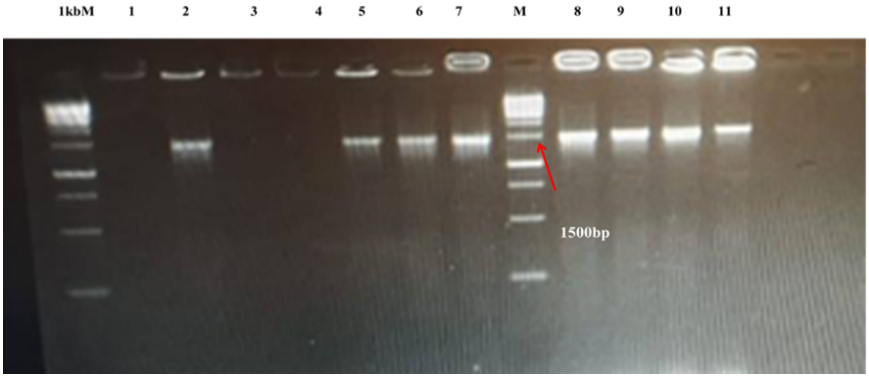

Fig. 3: PCR amplified 16S rRNA gene of phyto-LAB isolates

(MW 585366.1) was positioned within a clade of *E. faecium*, demonstrating its genetic similarity to other LAB strains and supporting its classification. *E. faecium* NBRC 100486 was also grouped with *E. faecium* strains, showing a clear relationship with other LAB and highlighting its phylogenetic distinction. *Enterococcus faecium* MW 585366 (MW585366.1) exhibited 98.26% similarity with *E. faecium* (NR_113904). While this level of sequence similarity is slightly lower compared to the other two isolates, it is still sufficiently high to classify the isolate within the *E. faecium* species. The minor divergence observed could be indicative of strain-level variations or slight evolutionary adaptations specific to the environmental conditions from which the isolate was obtained (*i.e.*, mangoes).

Enzymatic and metabolic activity of lab strains

The isolates of lactic acid bacteria were tested for enzymatic activities such as amylolytic, proteolytic, lipolytic and cellulolytic activities (Fig. 5). The strains were observed positive for proteolytic and lipolytic activities while negative for amylolytic and cellulolytic activities (Table 3).

Probiotic characterization of phyto lactic acid bacteria

Acid and bile salt tolerance

LAB isolates appeared to survive under the acidic pH condition of *in vitro* testing. The survival incubation times under pH 2.0 were 5, 30, 60, 90 and 120 min.

Table 3: Enzymatic and metabolic characteristics of lactic acid bacterial strains

Characteristics	*E. durans* JCM8725	*E. faceium* NBRC100486	*E. faecium* MW 585366
Amylolytic	-	-	-
Proteolytic	-	-	-
Lipolytic	+	+	+
Cellulolytic	-	-	-
Lactic Acid Production	+	+	+

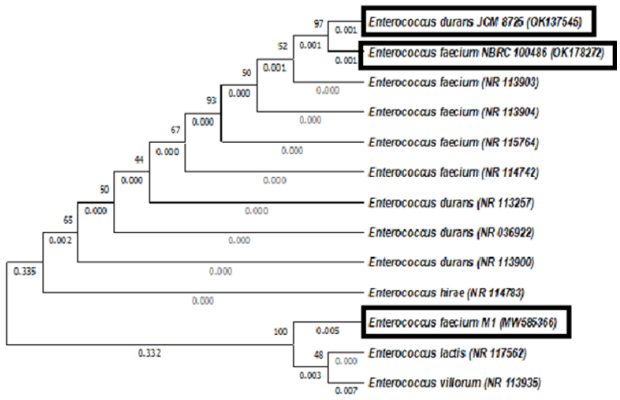

Fig. 4: Phylogenetic tree of the Phyto-LAB strains isolated from fruits (olive, Banana, Mango)

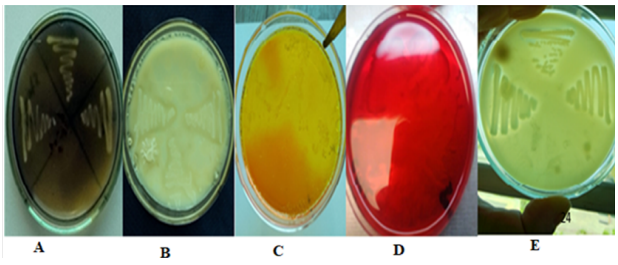

Fig. 5: (**A**) Amylolytic- negative; (**B**) Proteolytic- positive; (**C**) Lipolytic-positive; (**D**) Cellulytic-negative; (**E**) Lactic acid production-positive

The results indicated that no growth occurred in the strains after five minutes of survival at pH 2.0 conditions. In summary, the viability of *E. durans* JCM 8725 was highest at 2.0 pH after 5 min with a Log CFU/mL value of 7.58. Both *E. faecium* MW58366 and *E. faecium* NBRC 100486 possessed 7.25 Log CFU/mL viable cells counts and 7 Log CFU/mL survival after the pH level reached 2.0 after being incubated for 5 min (Fig. 6A). The isolates were able to endure for four hours when exposed to 0.3% bile salts. *E. faecium* MW 585366 exhibited the greatest survival rate of 7.88 Log CFU/mL, followed by *E. faecium* NBRC 100486 at 7.7 Log CFU/mL and *E. durans* JCM 8725 at 7.88 Log CFU/mL, as seen in Fig. 6B. Nevertheless, no significant changes were discovered since the p-value was more than 0.05, which means that the findings were statistically not significant.

Cell surface hydrophobicity (CSH) and auto-aggregation (AAG) assay

Adhering of LAB to hydrocarbons was assessed through cells surface hydrophobicity (CSH) test and clump forming ability was assessed through auto-aggregation (AAG). In this study, xylene hydrocarbon was used. The strain *E. durans* JCM 8725 showed 51%, *E. faecium* MW 585366 showed 40% and *E. faecium* NBRC 100486 had a cell surface hydrophobicity (CSH) of 36.4% (Fig. 7). An auto aggregation (AAG) 44% *E. durans* JCM 8725, 34% *E. faecium* MW 585366 and 33.7% *E. faecium* NBRC 100486 (Fig. 8).

Antimicrobial activities of Lab

The probiotic strains showed antagonistic activity against the tested pathogens. The three probiotic strains suppressed the growth of *E. coli* ATCC 25922, with the strain *E. durans* JCM8725 showing substantial action against *E. coli* ATCC 2922 in contrast to the other two probiotic strains (Fig. 9). It was observed that *S. aureus* ATCC 2593 was inhibited by *E. durans* JCM8725 and *E. faecium* MW 585366 and no significant difference was observed between their activities. No inhabit activity was observed by the strain *E. faecium* NBRC100486 (Fig. 9). The strains *E. durans* JCM8725 and *E. faecium* NBRC100486 equally inhibited *S. aureus* ATCC 25923, while *E. faecium* MW 585366 did not affect the *P. aeruginosa* ATCC 27853.

Antibiotics susceptibility test (AST) of LAB

It was discovered through inhibition zone measurements that all antibiotics displayed sensitivity toward *E. durans* JCM8725, but *E. faecium* MW 585366 and *E. faecium* NBRC100486 showed no response to streptomycin testing (Fig. 10).

***In vitro* and *in vivo* safety evaluation of lab**
Hemolytic activity

The LAB strains were found to be a hemolytic negative on 5% sheep blood agar. All LAB strains were non-hemolytic in nature (Fig. 11A).

D*Nase* activity

The strains were found D*Nase* negative as no pink zones were formed around the streaked colonies (Fig. 11B).

Antioxidant activity DPPH (Diphenyl-2- Picrylhydrazyl) assay

The DPPH scavenging method was utilized to measure antioxidant activity. All the LAB strains exhibited antioxidant activity. The highest percentage was recorded for *E. durans* JCM 8725 at 42.5 %, whereas *E. faecium* NBRC 100486 was recorded at 32.5% (Fig. 12).

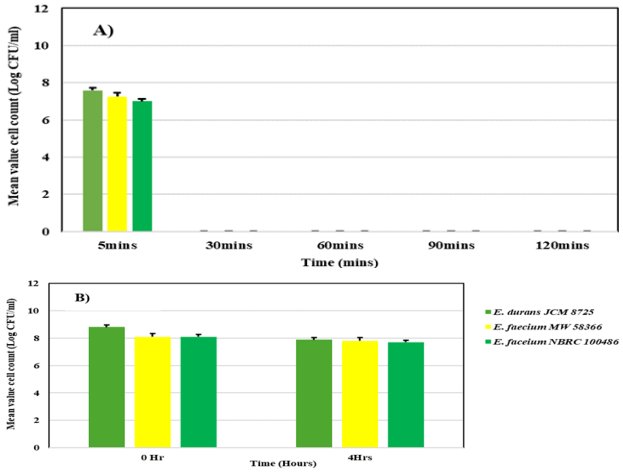

Fig. 6: (**A**) Survival of LAB isolates in pH 2.0 after 5 min of exposure; (**B**) survival of Phyto-LAB isolates in 0.3% bile salts after exposure of 4 h

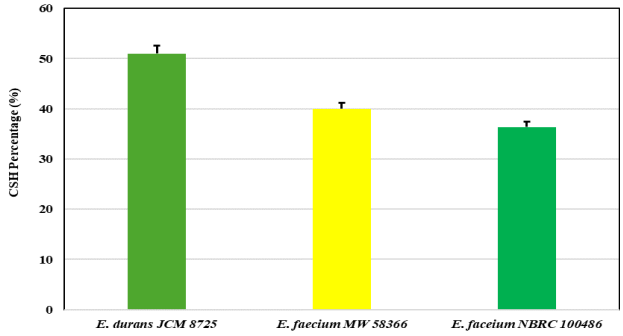

Fig. 7: Cell surface hydrophobicity (CSH) of Phyto LAB strains

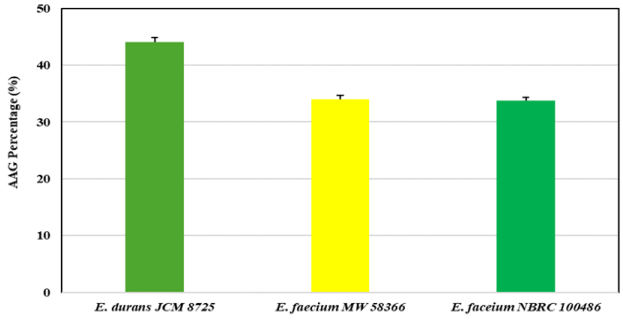

Fig. 8: Auto-aggregation (AAG) ability of Phyto LAB strains

Discussion

An evolutionary relation of the isolates and related species in the *Enterococcus* genus is indicated by a 16S rRNA sequence. The clustering of the isolates with reference strains, particularly *E. faecium* and *E durans*, further supports the accuracy of the species-level identification. The close genetic relationship of *E. durans* JCM 8725 and *E. faecium* NBRC 100486 through the conserved nature of

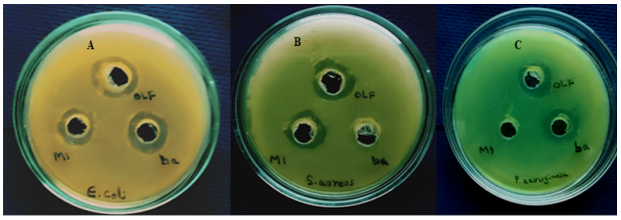

Fig. 9: Antimicrobial activity of phyto-lactic acid bacteria strains against. (**A**) *E. coli* ATCC 25922; (**B**) *S. aureus* ATCC 2593; (**C**) *P. aeruginosa* ATCC 27853

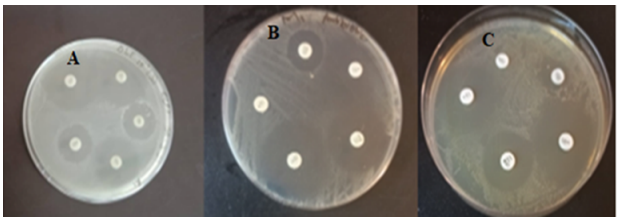

Fig. 10: Antibiotic susceptibility test (AST) of the Phyto-lactic acid bacteria strains. (**A**) *E. durans* JCM 8725; (**B**) *E. faecium* MW 585366; (**C**) *E. faecium* NBRC 100486

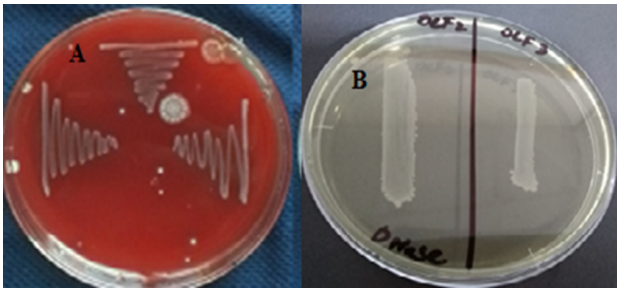

Fig. 11: (**A**) Hemolytic activity; (**B**) D*Nase* activity of Phyto Lactic acid bacteria

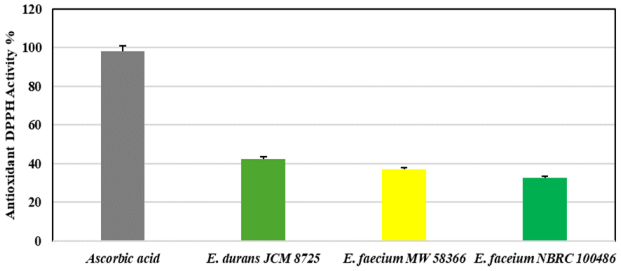

Fig. 12: Percentage of antioxidant DPPH assay of Phyto LAB strains

the 16S rRNA gene in these species can be demonstrated by even the minor differences among the sequences that are highly preserved. The identification of these lactic acid bacteria (LAB) as species within the *Enterococcus* genus has significant implications. LABs, particularly those within the *Enterococcus* group, are known for their beneficial roles in fermentation and as probiotics. Understanding their genetic identity and phylogenetic relationships helps

establish their potential utility in various applications, such as food fermentation, bio preservation and potentially in therapeutic contexts. The study revealed that 31 enterococci are isolated from watermelons, tomatoes, pomegranates, mango, papaya, bananas and dates (Al-Kharousi *et al.* 2022). The bacteria *E. casseliflavus* which was obtained from fresh guava produces bacteriocin according to Todorov *et al.* (2023). A probiotic strain was isolated from blue cherry fruit and identified as *Enterococcus faecium* MYSBC14 by 16S rRNA (Naik *et al.* 2023). Fermented leaves of vegetables and vegetables were the noble source of lactic acid bacteria. *E. faecalis* was isolated from fermented vegetables and molecularly identified by 16S r RNA (Ajibola *et al.* 2023). Another study by Arellano *et al.* (2024) revealed that enterococcus sp was isolated from the chilies (doenjang). The *E. faecium* was isolated from doenjang and molecularly identified by using 16S rRNA a universal primer. The plant source *Musa acuminata* was the source for the isolation of *E. faecium* PP 627037. The *E. faecium* PP 627037 was molecularly identified by using 16S rRNA sequence (Pava *et al.* 2024). Another strain of lactic acid bacteria *Enterococcus faecalis* was molecularly identified by 16S rRNA. This was isolated from fermented turnip juice (Sengun *et al.* 2024). The recent studies supported our work that fruits and vegetables are the potential and novel source of lactic acid bacteria and molecularly identified using 16S rRNA sequence. The isolation from olives, showing high genetic similarity to known *E. durans* strains, might be a candidate for further probiotic research or industrial applications. *E. faecium*, on the other hand, is significant for its strength and widespread presence in both environmental and host-associated niches. The isolates from bananas, olive and mangoes, with slight genetic variation, might represent strains with unique properties relevant to their fruit-based origins (Table 2). These isolates could offer insights into how LAB adapts to different environmental niches and their potential application in fruit fermentation processes.

The strains were observed positive for proteolytic and lipolytic activities while negative for amylolytic and cellulolytic activities (Table 3). The observed results indicated that the strains exhibited proteolytic and lipolytic activities but were deficient in amylolytic and cellulolytic activities. This outcome aligns with earlier findings where lactic acid bacteria isolated from fruits displayed diverse enzymatic profiles. The presence of proteolytic and lipolytic activities in the LAB isolates suggests their potential to metabolize complex macromolecules, thereby enhancing nutrient availability in the gut (Wang and Ji 2019). Strains with lipolytic activities may find applications in weight management and cholesterol assimilation (Huang *et al.* 2019). However, the absence of amylolytic and cellulolytic activities can be considered advantageous, as it reduces the risk of unwanted fermentation and gas production in the gut (Garber *et al.* 2020). The enterococcus species can produce lactic acid by hydrolysis of sugars. *E. faecium* WH51-1 shows the ability to produce lactic acid using corn-s steep water (Selim *et al.* 2021). *Enterococcus hirae* ds 10 produced lactic acid during the fermentation of beet molasses (Abdel-Rahman *et al.* 2021). The strains determine their potential to colonize the human gastrointestinal tract, employing cell surface hydrophobicity testing (Farid *et al.* 2021). Additionally, Farid *et al.* (2021) also tested its ability to auto-aggregate using lactic acid bacteria, which is an essential characteristic in determining its prolonged persistence in the gut. The three probiotic strains suppressed the growth of *E. coli* ATCC 25922, with the strain *E. durans* JCM8725 showing substantial action against *E. coli* ATCC 2922 in contrast to the other two probiotic strains (Fig. 8). It was observed that *S. aureus* ATCC 2593 was inhibited by *E. durans* JCM8725 and *E. faecium* MW 585366 and no significant difference was observed between their activities. No inhabit activity was observed by the strain *E. faecium* NBRC100486 (Fig. 8). The strains *E. durans* JCM8725 and *E. faecium* NBRC100486 equally inhibited *S. aureus* ATCC 25923, while *E. faecium* MW 585366 did not affect the *P. aeruginosa* ATCC 27853. Sakandar *et al.* (2019) revealed that the LAB strains had inhibitory activity against ATCC strains with different grades of antagonism. The latest research studies reported the inhibition of ATCC pathogens by lactic acid bacteria that can be isolated from fruits. These lactic acid bacteria can produce the bacteriocins. It confirmed the safety level of strains and also stated its ability to enhance the positive influences on the gut microbiota (Raheem *et al.* 2021). *Enterococcus hirae* R44 was found in freshwater fish and having antimoicrobial compounds class II and enerolysin (Li *et al.* 2024).

The Clinical and Laboratory Standard Institute used the classification scheme of resistant (R) and intermediate resistant (I) and sensitive (S) to evaluate resistance levels (Golnari *et al.* 2024). The LAB strains demonstrate general antibiotic sensitivity but their streptomycin resistance in these strains indicates concern regarding antibiotic resistance within specific LAB populations. Interestingly, while evaluating the antibiotic susceptibility profile of the lactic acid bacteria strains, the *E. faecium* NBRC 100486 and *E. faecium* MW 585366 strains exhibited resistance to streptomycin but demonstrated sensitivity to the other tested antibiotics. Intrinsic resistance was found in many probiotics (LAB), a natural resistance against the antibiotics found in the lactic acid bacteria (Li 2020). This susceptibility pattern aligns with the general trend observed in probiotic strains (Aghamohammad and Rohani 2023). The blue cherry fruit was a source of probiotic *Enterococcus faecium* MYSBC14. The probiotic was resistant to streptomycin (Naik *et al.* 2023). The lactic acid bacteria *Enterococcus* sp. isolated from broccoli was found resistant to streptomycin (Ragab *et al.* 2024). These findings aligned with our results all three strains are resistant to streptomycin.

LAB strains cannot lysis the red blood cells. The finding was supported by the study of *Enterococcus faecium*

isolated from Turkish pastrima (Dinçer and Kıvanç 2021). Another study has shown the parallel results that enterococcus strains obtained from different sources are non-hemolytic (Faris and Atya 2023). The non-hemolytic property made the LAB strains safe for consumption. As all LAB strains did not degrade the DNA molecule. *Enterococcus faecalis was* found D*Nase* negative (Hussain *et al.* 2025).

The three strains of *Enterococcus* sp were evaluated for antioxidant potential. *Enterococcus faecium* ST7119ea; 7119: *Enterococcus faecium* ST651ea; *Enterococcus faecium* ST7319ea were analyzed for antioxidant potential. *Enterococcus faecium* ST7319ea has the highest antioxidant potential at 79%, whereas *Enterococcus faecium* ST651ea has 68% antioxidant activity (Rwubuzizi *et al.* 2023). Another strain *Enterococcus faecalis* 84B has 81.1% antioxidant activity (Ali *et al.* 2023). These findings all supported that *Enterococcus* spp., which are probiotic, have antioxidant potential. Thus in view, the novel phyto lactic acid bacteria possess the safe characteristics and could be a novel non-dairy source of the isolation of probiotics.

Conclusion

Fresh fruits are a good source of lactic acid bacteria. These might be a future candidate as phyto-probiotics. The enterococcus strains isolated from local fruits are non-hemolytic and D*Nase* negative. These strains also have antioxidant properties and antibiotic susceptibility. These strains require further whole genome sequence studies for the identification of virulence genes.

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Author Contribution

FF and AS planned the research project, SG provided the Lab work and chemical assistance, AA facilitated the statistical data analysis, AR, BLAST the sequencing data at NCBI and identified the strains and MU and SK assisted in lab work and formatted the final draft of the write-up.

Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

The corresponding author will be eligible to provide data on request.

Ethics Approval

Not applicable to this research study.

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