Integrative Bioscience Nexus

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Fermented Rice as a Reservoir of Potential Probiotic Bacteria: Isolation, Identification, and Functional Characterization

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Abstract

Fermented rice, known as 'Panta Bhat,' in South Asian countries, is a common dietary staple rich in beneficial bacteria that may have probiotic properties. This research aimed to isolate, identify, and analyze the probiotic characteristics of two bacterial strains from fermented rice. Through 16S rRNA gene sequencing, sequence analysis, and a homology search in the NCBI database, these isolates were identified as *Enterococcus durans* JCM 8725 and *Enterococcus faecium* ATCC 19434, showing 98.86% and 98.83% similarity, respectively. The probiotic potential, functional characteristics, safety, and stress tolerance of these strains were assessed *in vitro*. Both strains demonstrated promising probiotic attributes, including resistance to 0.3% bile salts, robust growth across various salt concentrations (with optimal growth at 1% NaCl, and resilience in acidic pH ranges (with optimum growth at pH 4). In the pepsin tolerance assay, *E. durans* and *E. faecium* showed viable counts of 4.544 and 4.158 log CFU/mL, respectively, indicating good survivability under gastrointestinal stress. Both isolates were non-amylolytic, non-hemolytic, and lacked gelatinase activity, indicating a positive safety profile. Antibiotic susceptibility tests revealed that *E. durans* was responsive to five antibiotics, while *E. faecium* was sensitive to three. The isolates demonstrate strong antibacterial activity against *Bacillus subtilis* and *Pseudomonas* sp., and inhibit biofilm formation in *S. aureus*, *S. flexneri*, *B. subtilis*, and *Salmonella* sp. Given their favorable functional properties and safety profile, these isolates show promise as probiotics. However, further in vivo and mechanistic studies are required to validate their use in food and pharmaceutical applications.

Keywords: Fermented rice, panta bhat, probiotics, *E. durans*, *E. faecium*, probiotic potential

Academic Editor: Dr. Ming Lu

Received: 15 May 2026
Revised: 25 June 2026
Accepted: 29 June 2026
Published: 05 July2026

1. Introduction

Fermentation is one of the oldest and most sustainable biotechnological processes employed for food preservation and enhancement. Through the metabolic activities of microorganisms, fermentation transforms raw food substrates into products with improved shelf life, safety, sensory attributes, and nutritional value [1]. In recent decades, fermented foods have attracted considerable scientific and industrial attention owing to their association with human health benefits and their role as natural reservoirs of beneficial microorganisms [2]. Among the diverse microbial populations involved in food fermentation, lactic acid bacteria (LAB) represent the dominant and most functionally significant group due to their long

history of safe use and their capacity to produce a variety of bioactive metabolites [3][4]. Probiotics are defined as live microorganisms that confer health benefits on the host when administered in adequate amounts [5]. Increasing evidence suggests that probiotic bacteria can modulate gut microbiota composition, improve intestinal barrier integrity, enhance nutrient absorption, regulate immune responses, and inhibit the colonization of enteric pathogens through competitive exclusion and the secretion of antimicrobial compounds [6]. Effective probiotic strains must be able to resist acidic pH, bile salts, and digestive enzymes, have antibacterial action against pathogens, survive gastrointestinal conditions, and retain a high safety profile [3]. Consequently, the identification of novel probiotic strains from traditional fermented foods has become an active area of research [7].

Fermented rice, commonly known as Panta Bhat in Bangladesh and neighboring countries, is a spontaneously fermented food prepared by soaking cooked rice in water overnight. It has long been associated with improved digestion, cooling effects, and enhanced nutritional benefits. During fermentation, naturally occurring microorganisms metabolize available carbohydrates and produce organic acids, primarily lactic acid, resulting in a gradual decline in pH that suppresses spoilage and pathogenic microorganisms while favoring acid-tolerant LAB. Concurrent oxygen depletion creates anaerobic niches that further promote fermentative LAB, while ambient temperature additionally shapes microbial succession [7]. Using culture-dependent and molecular (16S rDNA) approaches, Jeyagowri et al. identified several LAB species from naturally fermented rice in Sri Lanka, including *Limosilactobacillus fermentum*, *Latilactobacillus curvatus*, *Latilactobacillus graminis*, *Weissella confusa*, and *Pediococcus pentosaceus*. Similar findings have been reported in other traditional fermented rice and cereal-based products of South and Southeast Asia, where members of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Weissella*, and *Enterococcus* contribute to acidification, flavor development, food preservation, and the production of bioactive compounds [8] [9].

Among LAB, the genus *Enterococcus* is considered the third largest within the group and has received increasing scientific interest for its dual significance in food fermentation and probiotic applications [10]. Enterococcus species are facultative anaerobic, Gram-positive, catalase-negative bacteria commonly isolated from fermented foods and the gastrointestinal tracts of humans and animals, where they contribute to flavor development and produce enterocins. Enterocins are antimicrobial peptides with inhibitory activity against important foodborne pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Clostridium* spp.*, and Bacillus* spp. [10][11]. In particular, *E. faecium*, *E. durans*, and *E. faecalis* have demonstrated promising probiotic properties, including acid and bile tolerance, adhesion to intestinal epithelial cells, and immunomodulatory potential [10][11].

However, the use of enterococci as probiotics remains contentious since certain strains include virulence characteristics and transferable antibiotic resistance genes [10]. Therefore, comprehensive strain-specific safety evaluation, including assessment of hemolytic activity, gelatinase production, antibiotic susceptibility, and antagonistic activity against pathogens, is essential before their application in food or therapeutic products [10].

Despite Panta Bhat's cultural significance and widespread usage in Bangladesh, the variety and probiotic functioning of the LAB community have received scant attention. Panta Bhat therefore provides a promising but underexplored source of potentially beneficial LAB, necessitating more research to discover and characterize novel probiotic candidates with potential uses in food, nutrition, and health.

2. Materials and Methods

2.1. Preparation of rice samples

White rice (100 g) was cooked with water (1:3 ratio) for 30 minutes to achieve a soft texture. For fermentation, 70 g of the cooked rice was soaked in sterile distilled water (1:3 ratio) for 12-16 hours in 250 mL clay pots at ambient temperature (27 °C).

2.2. Collection and isolation of bacterial samples

Homemade fermented rice samples were collected aseptically in sterile containers. The fermented rice suspension was filtered using Whatman filter paper to obtain a consistent filtrate. To enrich the bacterial population, 100 µL of the filtrate was added to De Man, Rogosa, and Sharpe (MRS) broth and incubated at 37 °C for 24 hours. Serial dilutions of the enriched culture were spread on MRS agar and incubated at 37 °C for 24 hours. Morphologically distinct colonies were chosen and purified through repeated streaking on new MRS agar plates until pure cultures were obtained.

2.3. Molecular identification

2.3.1. Genomic DNA extraction and quantifications

Bacterial isolates stored at -80 °C for molecular identification were revived in MRS broth and cultured overnight at 37 °C. The cultures were streaked onto MRS agar plates to ensure purity, and then a single colony from each isolate was grown in MRS broth for genomic DNA extraction. The EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech Co., Ltd., Beijing, China) was used to extract DNA from selected bacterial strains, following the manufacturer's instructions. The extracted DNA PCR product was tested for purity using a Nano drop spectrophotometer and gel electrophoresis. DNA extract was stored at -20 °C for future use.

2.3.2. PCR Amplification of 16S rRNA gene

To prepare the PCR reaction, add 25 µL of the Tag 2× Master Mix (buffer, polymerase, and dNTPs), 1 µL of forward primer, 1 µL of reverse primer, and 2 µL of UPH_2O. After adding 49 µL of the solutions to a sterile PCR tube, 1 µL of gDNA was used as a template. The amplification reactions were performed using a thermal cycler as per Mulaw et al. instructions. To amplify the 16S rDNA gene, the universal primers 27F 5´AGAGTTTGATCCTGGCTCG´ and 1492R 5´TACGGCTACCTTGTTAGGACTT´3' were employed. The purified DNA extract was utilized as a template to amplify the 16S rRNA gene using PCR. To initiate PCR, heat the mixture to 95 °C for 12 minutes, denature the DNA at 95 °C for 30 seconds before annealing at 55 °C for 30 seconds. After annealing, single-stranded DNA was extended at 72 °C for 2 minutes using Taq polymerase enzyme. After the final PCR cycle, elongation was performed at 72 °C for 4 minutes. A final hold was employed at 4 °C for 50 min.

2.3.3. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate the PCR products. TAE buffer (40 mM Tris acetate, 1 mM EDTA) was used to prepare a 1.5% (w/v) agarose gel, which was then heated to melt it. The melted gel was mixed with the fluorescent tag (ethidium bromide, 0.5% µg/mL). In a mold with a comb inserted at one end for wells, the molten gel was allowed to solidify. The solidified gel was put in an electrophoresis apparatus with TAE buffer. PCR product and a DNA molecular-weight size marker (DNA ladder, 100 pb−1.5 kb) were applied in the wells before an electrophoresis run. After the power was switched off, the gel was gently removed from the gel tank. The gel was analyzed after being exposed to UV light.

2.3.4. Sequence analysis and identification

BioEdit Sequence Alignment Editor was used to assemble and modify the acquired 16S rRNA gene sequences. To ascertain the isolates' closest taxonomic affinities, the modified sequences were compared with reference sequences found in the GenBank database using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST).

2.4. Probiotic potential tests

2.4.1. Bile salt tolerance test

The bile salt tolerance of the isolates was performed following the method described by Liu et al. with minor adjustments [13]. The isolated bacteria were grown in MRS broth containing 0.3% oxgall for six hours, and their absorbance at 600 nm was measured after 0, 2, 4, and 6 hours to assess their growth. The experiment was carried out in triplicates

2.4.2. Salt (NaCl) tolerance test

The isolates' ability to withstand NaCl was evaluated in MRS broth supplemented with different NaCl concentrations (1–5%, w/v). After inoculating 100 µL aliquots of overnight cultures into the specified media, the cultures were anaerobically incubated for 48 hours at 37 °C. Bacterial growth was subsequently quantified using a spectrophotometer by measuring the optical density at 600 nm (OD_{600}). Every assay was carried out in triplicate [14].

2.4.3. Acid tolerance test

The isolated bacterial strains were cultured in MRS broth, and acid resistance was examined in MRS broth adjusted with1M hydrochloric acid (HCl) to obtain final pH values of 2, 3, and 4. The inoculated cultures were incubated at 37 °C, and at 0,1,2,and 3 hours of incubation, the viability of the bacterial strains was determined by measuring the absorbance of the inoculated cultures at 600 nm [15].

2.4.4. Pepsin tolerance test

Pepsin tolerance was assessed using a slightly modified version of the Pinto et al. procedure [16]. The isolates were inoculated into MRS broth containing 3 mg/mL pepsin, and the pH was lowered to 2.5 using 1M HCl. The cultures were cultured for 2 hours at 37 °C before being serially diluted and spread on MRS agar. The plates were incubated at 37 °C for 24 hours, and viable bacterial counts were calculated and reported as logarithmic colony-forming units (CFU/mL).

2.5. Phenotypic characterization

2.5.1. Amylolytic (starch hydrolysis) test

Starch agar plates were used to assess the isolates' amylolytic activity. After streaking the isolates onto the medium's surface, they were incubated for 24 to 48 hours at 37 °C. After incubation, Gram's iodine solution (0.5% iodine crystals and 1.5% potassium iodide) was poured over the plates. Iodine reacts with starch and produces blue-black coloration. A clear halo surrounding the bacterial growth showed starch hydrolysis due to extracellular amylase activity, whereas the absence of a clear zone was considered a negative result for starch breakdown [17].

2.5.2. Hemolytic activity test

The method described by Shivani & Sathiavelu (2024) [18] was used to evaluate the hemolytic activity of LAB isolates. The isolates grown in MRS broth were streaked on blood agar plates containing 5% (v/v) sheep blood, and the plates were then incubated for 24 hours at 37 °C. The strains' hemolytic activities were demonstrated as follows: Alpha (α) hemolysis, indicated by a tiny area of the media discolored from green to brown, results from the decrease of hemoglobin to diffuse methemoglobin. Gamma (γ) hemolysis is defined by no noticeable changes in the media, while beta (β) hemolysis is characterized by a translucent, pale-yellow zone surrounding the colonies, indicating complete RBC hemolysis.

2.5.3. Gelatinase activity test

The isolates' gelatinase activity was assessed using MRS agar supplemented with 3% (w/v) gelatin. The isolates were streaked onto the medium's surface and incubated at 37 °C for 24 to 48 hours. After incubation, the plates were inspected for gelatin hydrolysis by looking for clear zones around the bacterial growth. The formation of a distinct halo surrounding the colonies indicates gelatinase synthesis, while the absence of a clear zone suggests a negative result [19].

2.6. Antibiotic sensitivity test

All isolates were tested for antibiotic susceptibility using the disk diffusion method on Mueller-Hinton agar medium in compliance with Clinical and Laboratory Standards Institute standards [20]. Initially, isolated strains were seeded on MRS plates at 37 °C for 18 to 24 hours. Sterile swabs were used to inoculate Müeller–Hinton agar plates with bacterial solutions that had been adjusted to an inoculum of approximately 10^8 CFU/mL (0.5 McFarland standard). Following a 24-hour incubation period at 37 °C, the diameters of the inhibition zones were determined, and the isolates' susceptibility was assessed in accordance with CLSI guidelines [21].

Table 1. List of antibiotics used to assess antibiotic sensitivity of the bacterial isolates.

Serial No.	Name of antibiotics
1	Chloramphenicol (30 µg/Disc)
2	Gentamicin (10 µg/Disc)
3	Ampicillin (10 µg/Disc)
4	Kanamycin (30 µg/Disc)
5	Doxycycline (30 µg/Disc)
6	Cefixime (5 µg/Disc)
7	Cefuroxime (30 µg/Disc),
8	Aztreonam (30 µg/Disc)
9	Ciprofloxacin (5 µg/Disc)
10	Ceftazidime (30 µg/Disc)
11	Vancomycin (30 µg/Disc)
12	Erythromycin (15 µg/Disc)
13	Penicillin (10 µg/Disc)
14	Azithromycin (15 µg/Disc)
15	Cefotaxime (30 µg/Disc)

2.7. Antibacterial activity

The antagonistic activity of the isolates was assessed against two pathogenic bacteria, *namely Bacillus subtilis* and *Pseudomonas* sp., using the disc diffusion assay with minor modifications. To acquire cell-free supernatants (CFS), the isolates were cultivated in MRS broth and centrifuged at 4,000 rpm for 12 minutes at 4 °C. Overnight cultures of the indicator pathogens were evenly distributed onto Mueller-Hinton agar (MHA) plates with sterile cotton swabs. Sterile paper discs (6 mm diameter) were impregnated with 100 µL of the CFS and aseptically deposited on the surface of the inoculated agar plates. The plates were incubated at 37 °C for 24 hours, and the diameters of the inhibition zones surrounding the discs were measured and recorded to determine antagonistic activity [22].

2.8. Antibiofilm assay

2.8.1. Biofilm formation assay

The selected bacterial strains (*Staphylococcus aureus*, *Shigella flexneri*, *Salmonella* sp., *Bacillus subtilis*) were simultaneously inoculated into 96-well microtiter plates containing 100 µL of Luria-Bertani (LB) broth and incubated at 37 °C for 24 hours to allow biofilm formation. After incubation, the plates were washed twice with double-distilled water, air-dried, and oven-dried at 37 °C for 1 hour. The biofilm was then fixed with 150 µL of absolute ethanol for 5 minutes, followed by staining with 0.1% crystal violet for 15 minutes. Finally, biofilm formation was quantified by measuring the optical density at 595 nm (OD_{595}).

A) Non-biofilm producer ($OD_a \leq OD_{cn}$);

B) Weak biofilm producer ($OD_{cn} < OD_a \leq 2\times OD_{cn}$);

C) Moderate biofilm producer ($2\times OD_{cn} < OD_a \leq 4\times OD_{cn}$); and

D) Strong biofilm producer ($4\times OD_{cn} < OD_a$) [23].

2.8.2. Biofilm inhibition

The impact of both isolated bacterial strains on biofilm formation was evaluated using the co-incubation method, following the previously described protocol. In this assay, the isolates were added to the pathogenic bacterial inoculum. The percentage of biofilm disruption was calculated using the following formula [24]:

Inhibition Percentage = $(OD_{control} - OD_{sample}) \times 100/OD_{control}$

2.9. Statistical analysis

All experiments were carried out in triplicate. Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance. Differences were significant if $p < 0.05$.

3. Results

3.1. Isolation of the bacteria

Fermented rice (Panta Bhat) was used to isolate *Enterococcus durans* JCM 8725 and *Enterococcus faecium* ATCC 19434. *Enterococcus durans* had a smooth surface, a round shape, a tiny size, and a creamy tint. *Enterococcus faecium*, on the other hand, was larger, creamy-looking, oval-shaped, somewhat translucent, and relatively slightly elevated.

3.2. Molecular identification

The isolates were characterized by using the 16S rRNA gene sequencing technique. The homology of the acquired sequences was assessed by comparing them to reference 16S rRNA sequences found in the nucleotide database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool BLAST). In terms of sequence similarity, isolates 1 and 2 exhibited 98.86% (**Figure 1a**) and 98.83% similarity (**Figure 1b**), respectively.

Figure 1. NCBI BLASTn results of the 16S rRNA gene sequence of the bacterial isolates. (a) Homology similarity of isolate 1 to *Enterococcus durans* JCM 8725, and (b) Homology similarity of isolate 2 to *Enterococcus faecium* ATCC 19434.

3.3. Probiotic potentiality test

3.3.1. Bile tolerance test

The isolates' bile tolerance was tested in the presence of 0.3% bile salts by measuring changes in OD_{600} over a 6-hour incubation period. Despite a slow decrease in optical density, both isolates were viable in the presence of 0.3% bile salts

throughout the period. *E. durans* had OD_{600} values of 0.467 ± 0.113, 0.328 ± 0.037, 0.263 ± 0.046, and 0.279 ± 0.036 at 0, 2, 4, and 6 hours, respectively. *E. faecium* had OD_{600} values of 0.427 ± 0.050, 0.298 ± 0.040, 0.288 ± 0.023, and 0.308 ± 0.044 at the same time points. *E. durans* had a higher overall reduction in OD_{600} (40.3%) compared to *E. faecium* (27.9%), showing that *E. faecium* was more resistant to 0.3% bile salts (**Figure 2a**). Despite the observed drop in optical density, both isolates maintained notable growth throughout the incubation period, suggesting their capacity to survive bile-induced stress, which is an important feature of potential probiotic strains.

3.3.2. Salt (NaCl) tolerance test

Both isolates showed growth at all tested NaCl concentrations (1–5%), demonstrating their ability to withstand salt (**Figure 2b**). *E. durans* exhibited the highest growth at 1% NaCl (OD_{600}= 1.399 ± 0.238); its growth then gradually decreased as salt concentrations increased, reaching 0.576 ± 0.078 at 5% NaCl. *E. faecium* also showed marked growth over the whole investigated range, with OD_{600} values ranging from 0.586 ± 0.160 to 0.894 ± 0.112. *E. faecium* maintained rather consistent growth between 2% and 4% NaCl and exhibited higher OD_{600} values than *E. durans* at 3% and 4% NaCl, despite the fact that growth normally declined at higher salt concentrations. The capacity of both isolates to withstand high salt concentrations was confirmed by their ability to maintain detectable growth at 5% NaCl.

3.3.3. Acid tolerance test

As can be seen in **Figure 2c** & **2d**, during the three hours of exposure, the acid tolerance test demonstrated that both isolates were viable at pH 2, 3, and 4. Bacterial growth was mostly steady at pH 2 and pH 3, with very slight variations, while pH 4 allowed for a slow rise in OD_{600} over time. After three hours, *E. durans* had the greatest OD_{600} value (0.713 ± 0.019) at pH 4, whereas *E. faecium* continued to grow at pH 4 during the incubation time, with the final growth at 3 h being OD_{600} = 0.628 ± 0.005. These findings show that both isolates are tolerant of acid, with pH 4 showing the best growth.

3.3.4. Pepsin tolerance test

In the pepsin tolerance assay, both isolates were able to withstand exposure to simulated stomach conditions (pH 2.5 with 3 mg/mL pepsin) for two hours. Following incubation, viable counts for *E. durans* and *E. faecium* were 4.544 log CFU/mL and 4.158 log CFU/mL, respectively (**Figure 2e**).

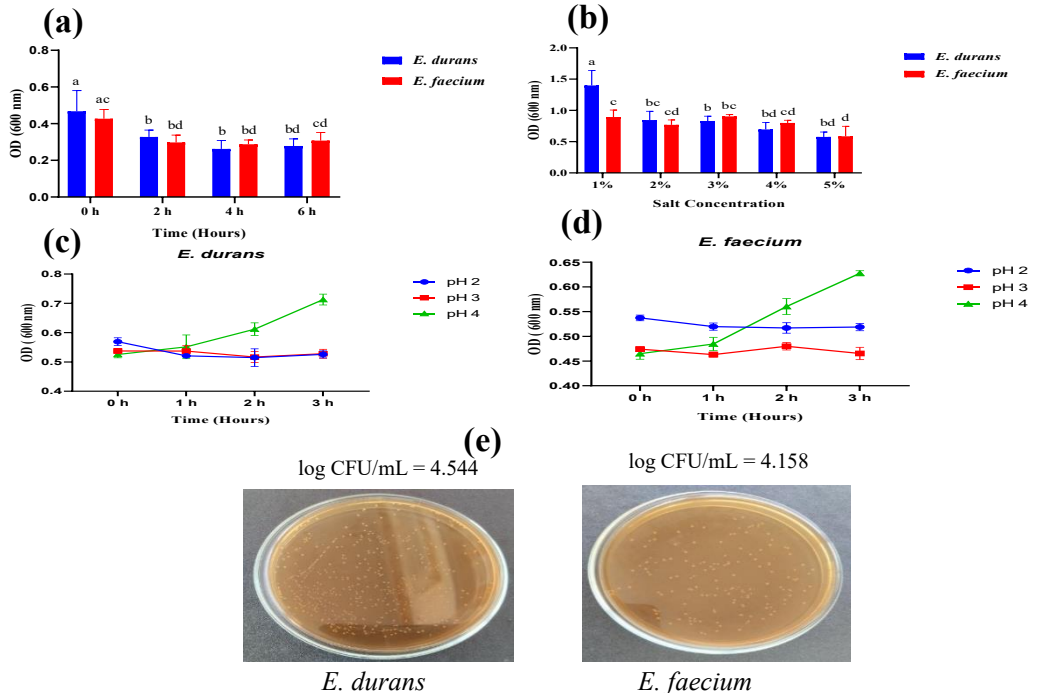


Figure 2. Probiotic potential of the bacterial isolates under stimulated gastrointestinal stress conditions. (a) Growth in 0.3% bile salts; (b) growth in 1–5% NaCl; (c) acid tolerance of *E. durans* at pH 2–4; (d) acid tolerance of *E. faecium* at pH 2–4; and (e) pepsin tolerance of the isolates. Values represent mean ± SD (n = 3). Different lowercase letters indicate statistically significant differences among treatments as determined by Tukey's HSD post hoc test ($p < 0.05$).

3.4. Phenotypic characterization

3.4.1. Amylolytic (starch hydrolysis) test

The starch hydrolysis test revealed no discernible clear zone surrounding the colonies in the current evaluation, suggesting that the isolates were amylase-negative (**Figure 3a**).

3.4.2. Hemolytic activity test

Figure 3b shows that the isolated bacterial strains exhibited γ-hemolysis as surrounding zones appeared around the bacterial growth, indicating the absence of red blood cell lysis.

3.4.3. Gelatinase activity test

In this investigation, both *E. durans* and *E. faecium* isolates demonstrated no gelatinase activity, as no obvious zone of hydrolysis was identified around the colonies (**Figure 3c**).

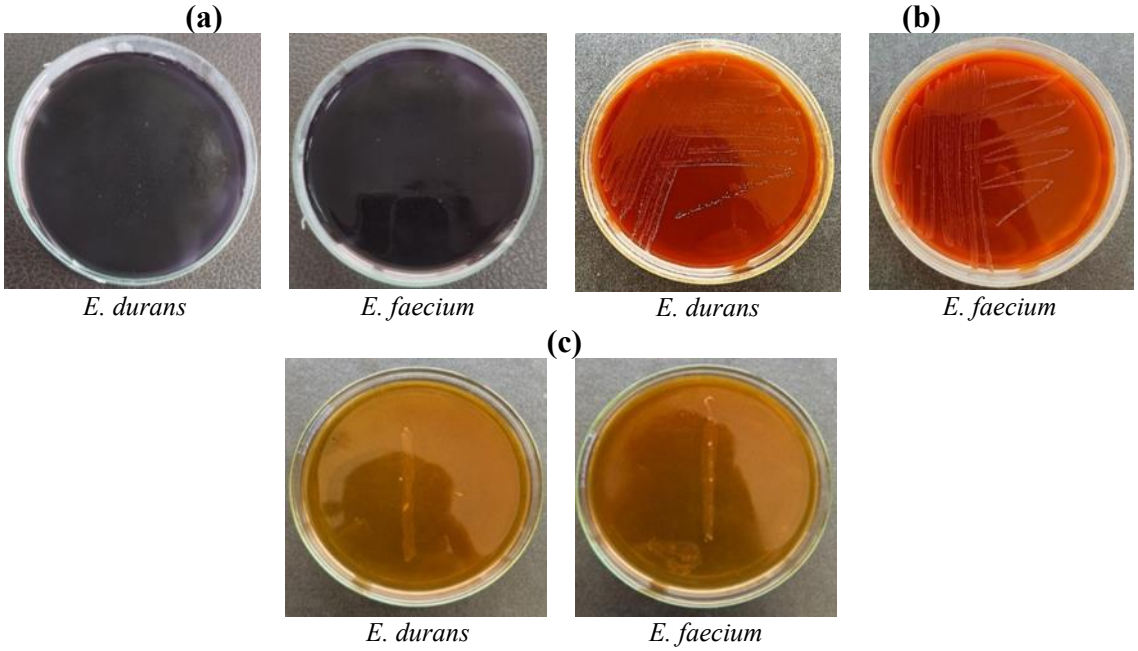


Figure 3. Phenotypic characterization of the isolates. (a) Starch hydrolysis activity, (b) hemolytic activity test, (c) gelatinase activity test.

3.5. Antibiotic sensitivity test

Antibiotic susceptibility of the isolates was tested against 15 different antibiotics (**Figure 4**) to determine their resistance patterns. *E. durans* was sensitive to five antibiotics, intermediate to three, and resistant to seven of the tested antibiotics. In comparison, *E. faecium* was susceptible to three antibiotics, intermediately resistant to one, and resistant to eleven antibiotics (**Table 2**). Interpretation according to CLSI guidelines: S, susceptible; IR, intermediately resistant; R, resistant.

Table 2. Zone of inhibition measured in mm and standard range for antibiotic sensitivity test.

Antibiotic name	**Zone (in mm)**	
	Enterococcus durans	*Enterococcus faecium*
Chloramphenicol	20 ± 0.577 (S)	16 ± 1.00 (IR)
Gentamicin	8 ± 0.50 (R)	11 ± 0.50 (R)
Ampicillin	34 ± 1.00 (S)	7 ± 0.577(R)
Kanamycin.	7± 0.50 (R)	7 ± 0.577 (R)
Doxycycline	23 ± 0.577 (S)	22 ± 0.50 (S)
Cefixime	8 ± 0.50 (R)	-
Cefuroxime	10 ± 0.50 (R)	-
Aztreonam	-	8 ± 1.00 (R)
Ciprofloxacin	16 ± 1.00 (IR)	11 ± 0.577 (R)
Ceftazidime	-	7 ± 0.577 (R)
Vancomycin	17 ± 0.50 (S)	16 ± 1.00 (S)
Erythromycin	9 ± 1.00 (R)	7 ± 0.50 (R)
Penicillin	27 ± 1.00 (IR)	19 ± 0.50 (R)
Amoxicillin	29 ± 1.00 (S)	28 ± 1.00 (S)
Cefotaxime	14 ± 0.50 (IR)	7 ± 0.50 (R)

3.6. Antibacterial activity test

The antimicrobial activity assay with untreated cell-free supernatants (CFS) revealed that both isolates inhibited the development of *Bacillus subtilis* and *Pseudomonas* sp. (**Figure 5**). The inhibition zones expanded with increasing CFS volume (50-120 µL), showing a concentration-dependent action. Overall, *E. faecium* had slightly higher antimicrobial activity than *E. durans*, with maximum inhibition at 120 µL CFS (**Table 3**).

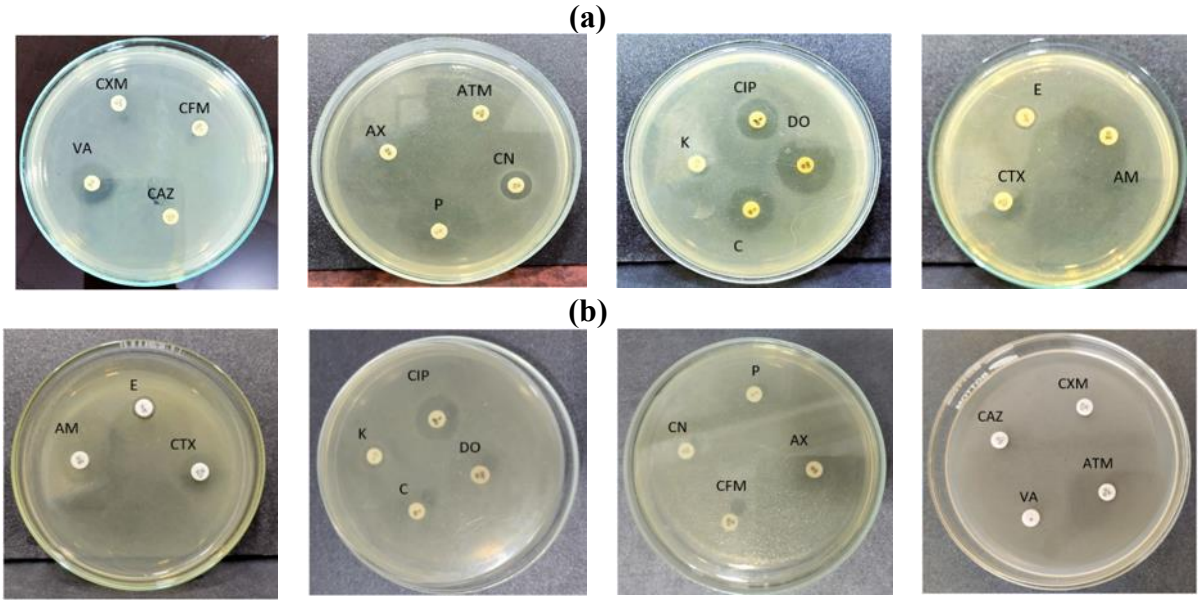
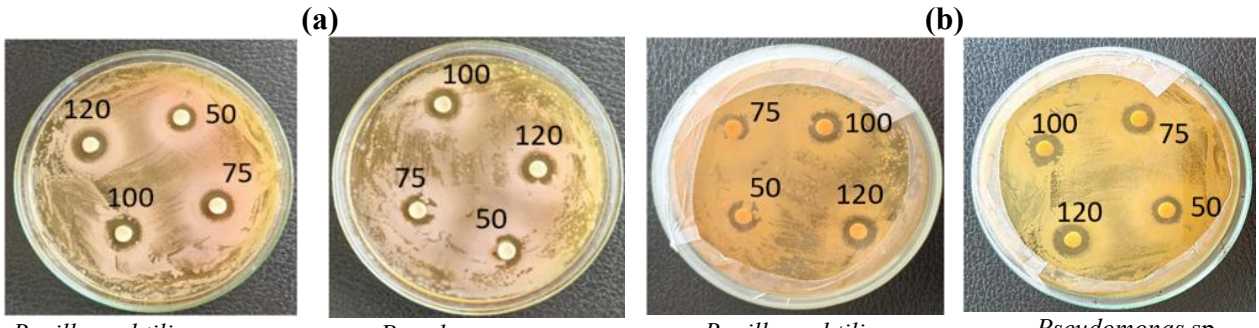


Figure 4. Antibiotic sensitivity tests of the two isolates, where (a) indicates antibiotic sensitivity tests of *Enterococcus durans* and (b) indicates antibiotic sensitivity tests of *Enterococcus faecium*.

Table 3. Antagonistic activity of cell-free supernatants of the isolates on *Bacillus subtilis* and *Pseudomonas* sp.

Isolate Name	Concentration (µL)	Zone of Inhibition (mm)	
		Bacillus subtilis	***Pseudomonas* sp.**
E. durans	50	6 ± 0.50	7 ± 0.50
	75	7 ± 0.50	8 ± 0.00
	100	10 ± 0.00	11 ± 0.50
	120	13 ± 0.50	13 ± 1.00
E. faecium	50	8 ± 0.50	7 ± 0.577
	75	9 ± 0.577	9 ± 0.50
	100	11 ± 0.00	13 ± 0.00
	120	15 ± 0.50	14 ± 0.50



Bacillus subtilis *Pseudomonas* sp. *Bacillus subtilis* *Pseudomonas* sp.

Figure 5. Antimicrobial activity of the isolates against indicator pathogens, expressed as the diameter of inhibition zones (mm) determined by the disc diffusion assay where (a) indicate antibacterial activity of *E. durans* and (b) indicate antibacterial activity of *E. faecium*.

3.7. Antibiofilm activity test

All pathogenic bacteria could form strong biofilms, as shown in **Table 4**. **Figure 6** shows the biofilm inhibitory potential of *E. durans* and *E. faecium* against the four bacterial pathogens. The lowest anti-biofilm activity was seen against *Staphylococcus aureus*, with inhibition rates of 37.26 ± 3.298% and 43.55 ± 6.398% for *E. durans* and *E. faecium*, respectively. This was followed by moderate inhibition against *Bacillus subtilis* (47.66 ± 14.766% and 52.02 ± 11.565%) and *Salmonella* sp. (51.28 ± 11.314% and 56.96 ± 9.651%). The strongest anti-biofilm activity was seen against *Shigella flexneri*, with inhibition percentages of 66.10 ± 3.322% and 79.05 ± 4.707% for *E.durans* and *E. faecium*, respectively.

Table 4. Biofilm formation of the selected pathogenic bacteria.

Name of the bacteria	Result
S. aureus	Strong
B. subtilis	Strong
S. flexneri	Strong
Salmolella sp.	Strong

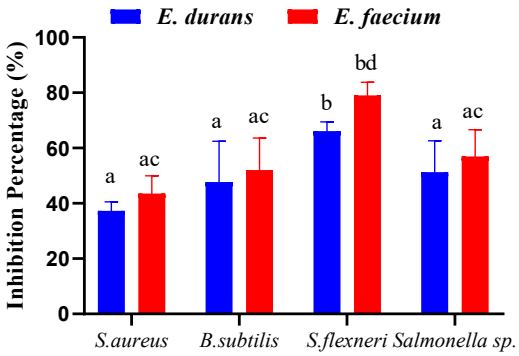


Figure 6. Anti-biofilm activity of *E. durans* and *E. faecium* against selected pathogenic bacteria, expressed as percentage biofilm inhibition (%). Different lowercase letters indicate statistically significant differences among treatments as determined by Tukey's HSD post hoc test ($p < 0.05$).

4. Discussion

The increased interest in functional foods and microbiome-based treatments has accelerated the pursuit of novel probiotic candidates from a variety of ecological niches, particularly those linked with traditional fermented foods that have long been safe for human use. Fermented cereal-based preparations are an underexplored reservoir of lactic acid bacteria (LAB) diversity compared to the more frequently studied dairy-derived fermented foods such as yogurt, kefir, and cheese [2]. Panta bhat, a traditional South Asian fermented rice preparation commonly consumed in Bangladesh, India, and Myanmar, is one such substrate. It is made by spontaneous overnight fermentation of cooked rice in water under ambient conditions. This procedure promotes a moderately acidic, starch-rich milieu that selectively increases LAB populations potentially predisposed to gastrointestinal resilience and glucose metabolism [4]. The microbial ecology of Panta bhat is relatively unexplored in the scientific literature, and the current study contributes to filling this knowledge gap by isolating and characterizing functionally active LAB from this traditional substrate. Among the obtained isolates, *Enterococcus durans* JCM 8725 and *Enterococcus faecium* ATCC 19434 demonstrated a variety of beneficial probiotic characteristics, indicating their potential use in functional food and health-related formulations. Enterococcus is a controversial genus in probiotic science because it is known to be both opportunistic pathogens with potential virulence determinants and beneficial commensals with well-established fermentative utility. However, new evidence supports the probiotic applicability of certain strains, especially those that originate from traditional fermented foods with a long history of safe human consumption [10] [11]. An important type of ethnopharmacological evidence that the LAB communities residing in this substrate are unlikely to harbour overt pathogenicity is the generations-long traditional consumption of Panta Bhat by diverse South Asian populations. This provides additional contextual plausibility to the probiotic candidacy of the isolates described here [4][25].

Accurate molecular identification is a crucial precondition in probiotic characterization, given that strain-specificity strongly affects both probiotic effectiveness and safety profiles [26]. In this investigation, 16S rRNA gene sequencing validated the taxonomic identity of both isolates with strong sequence similarity (>98%) to previously deposited type strains, corresponding with the threshold values typically recognized for species-level identification in LAB [27]. This molecular validation supports the experimental findings and establishes a traceable genotypic foundation for future genomic and regulatory studies. Genetically verified LAB strains are increasingly being preferred for industrial and medicinal research, since established identification enhances reproducibility and allows regulatory approval under frameworks such as the European Food Safety Authority's Qualified Presumption of Safety (QPS) evaluation [28][14].

A key prerequisite for probiotic activity is the ability to survive transit through the gastrointestinal tract. Both isolates showed significant resistance to acidic conditions (pH 2.0-4.0) and 0.3% bile salts, which are similar to those seen in the human stomach and proximal small intestine. These features are mechanistically linked to proton pump modulation, bile salt hydrolase (BSH) activity, and adaptive membrane lipid remodeling, all of which allow LAB to maintain intracellular homeostasis during physiological stress [29] [30]. The strong gastrointestinal resistance seen in the recovered isolates may be explained by the starch-rich, somewhat acidic fermentation environment of Panta Bhat, which may act as a selection pressure favouring organisms with innate acid tolerance. Potential probiotics, *Enterococci* isolated from fermented foods and intestinal habitats show similar tolerance profiles; these modifications have been linked to improved intestinal persistence and host-microbe interaction [31].

Another technologically significant characteristic is salt tolerance, as probiotic microbes are frequently added to food matrices that are exposed to fluctuating osmotic conditions. At 1% NaCl, both isolates grew at their best; at greater concentrations, their proliferation gradually decreased, although they continued to develop noticeably at higher salinities. The addition of these strains to fermented and processed foods, where osmotic stress may jeopardize bacterial survival, benefits from their mild halotolerance. The buildup of suitable solutes, activation of osmoprotectant transporters, and modifications to cell wall architecture all contribute to salt adaptation in LAB [32]. During industrial fermentation operations, these mechanisms have been linked to increased storage stability and flexibility [33][31].

Among the most clinically noteworthy findings were the isolates' strong antibiofilm activity against a group of clinically and food-relevant pathogens such as *Staphylococcus aureus*, *Shigella flexneri*, *Pseudomonas* sp., and *Bacillus* sp. Biofilm-associated infections are a serious global health problem because structured biofilm communities provide significant resistance to antimicrobial drugs and immune effectors while enhancing microbial persistence and pathogenicity [34][35]. The antibiofilm action demonstrated in this study is most likely due to the secretion of bacteriocins, organic acids, hydrogen peroxide, or biosurfactants, which are well-known substances among *Enterococcus* spp. that can interfere with quorum sensing and impede pathogen attachment [11]. It has been demonstrated that enterocins produced by E. faecium and similar species have broad-spectrum action against foodborne pathogens that are both Gram-positive and Gram-negative [10], [36], [37]. Since bacteriocin-producing LAB from fermented cereal substrates are becoming more widely acknowledged for their potential in food biopreservation and natural antibacterial applications, the isolation of enterocin-producing strains from a grain fermentation like Panta Bhat is significant [38] [39] [40].

Safety assessment is crucial in the probiotic characterization of *Enterococcus* isolates, considering the genus' reported link with nosocomial infections, transferable antibiotic resistance, and virulence gene carriage [41] [42]. In the present investigation, both isolates were negative for hemolytic and gelatinase activities, two phenotypic markers typically connected with *Enterococcus* virulence, indicating the absence of key pathogenicity-associated characteristics [43][44]. These findings are consistent with prior studies showing that non-hemolytic, gelatinase-negative enterococci may be considered substantially safer candidates for potential probiotic use [45]. Panta bhat's historic consumption setting reinforces a favourable safety background, since spontaneously fermented foods consumed without recorded adverse health effects across generations implicitly imply a non-pathogenic microbial population [4]. However, phenotypic screening is insufficient to fully verify safety. Before any commercial or clinical deployment, whole-genome sequencing (WGS) and comparative genomic analysis must be performed to identify virulence gene clusters, transferable antibiotic resistance determinants, and mobile genetic elements like as plasmids, transposons, and pathogenicity islands [46].

Overall, the current findings show that *Enterococcus durans* JCM 8725 and *Enterococcus faecium* ATCC 19434, isolated from the traditional fermented rice preparation Panta Bhat, have several desirable probiotic properties, including gastrointestinal tolerance, moderate halotolerance, antimicrobial and antibiofilm activity, amylase production, and preliminary safety profiles consistent with non-pathogenic phenotypes. These multifunctional properties make the isolates excellent candidates for development into probiotic supplements, functional cereal-based meals, and natural

biopreservatives. Nonetheless, more research, such as whole-genome sequencing, metabolomic profiling, mucous adhesion and epithelial adhesion assays, and in vivo validation in appropriate animal models, is required to better understand their mechanisms of action, long-term safety, and therapeutic efficacy.

5. Conclusion

The current work demonstrates that Panta Bhat, a historically fermented rice dish with profound cultural roots in South Asian culinary traditions, is a viable and hitherto unexplored ecological source of functionally active, possibly probiotic, lactic acid bacteria (LAB). Using 16S rRNA gene sequencing, two isolates isolated from this substrate were identified with high sequence similarity (>98%) to reference bacteria as *Enterococcus durans* JCM 8725 and *Enterococcus faecium* ATCC 19434. Both strains showed high tolerance to gastrointestinal transit conditions, indicating their ability to survive in the human digestive environment, a property that may be influenced by Panta bhat's mildly acidic, carbohydrate-rich fermentation conditions. Safety testing indicated no haemolytic or gelatinase activity, which is consistent with non-virulent phenotypes and compatible with the source food's long history of safe ingestion. Furthermore, both isolates exhibited a variety of functional characteristics, including broad-spectrum antibacterial activity, antibiofilm effectiveness against clinically relevant pathogens, amylase production, and a sensitivity profile to clinically essential antibiotics. Taken together, these findings show that the isolated *Enterococcus* strains have favourable probiotic and functional properties and are viable candidates for the formulation of cereal-based probiotic products and functional foods, which is especially important in regions where dairy-based probiotic vehicles are scarce.

Author contributions: Conceptualization: RT, MAS, and SZ; methodology: RT, DDH, UAN, MAU, MA, MNJ; software: RT, DDH, and MAS; validation: MAEE, SU, MAS, and SZ; formal analysis: RT, DDH, UAN, MAU, MA, MNJ; investigation: RT, DDH, and MAU; resources: MAEE, SU, MAS, and SZ ; data curation RT, DDH, UAN, MAU, MA, MNJ; writing original draft preparation: RT, DDH.; writing review and editing: MAS, and SZ; supervision: MAS and SZ; project administration: MAEE, SU, MAS, and SZ. All authors have read and agreed to the published version of the manuscript.

Acknowledgements: N/A

Data availability statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical statement: The article does not contain any studies with human participants performed by any of the authors.

Funding: The authors received no external funding from any sources.

Conflict of interest statement: The authors declared that there were no conflicts of interest concerning this manuscript.

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