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Selection and characterization of probiotic *Enterococcus* strains isolated from Vietnamese fermented foods

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ABSTRACT

Fermented pork rolls and stinky tofu are healthy fermented foods produced in Vietnam for a long time. These foods have proven to be a rich source for probiotic isolation studies. In this study, four *Enterococcus* strains were isolated from fermented pork roll (F20BA, F26B) and stinky tofu (F53BA, F54BA) using specific selection media. Phylogenetic and ribosomal multilocus sequence typing analysis confirmed that these strains are *Enterococcus lactis*. Besides, the safety and probiotic properties of these strains were evaluated. Antimicrobial susceptibility testing and polymerase chain reaction (PCR) analysis confirmed all four strains were vancomycin-susceptible and lacked resistance genes (*vanA* and *vanB*). Hemolytic activity was assessed using blood agar plates, verifying the isolates were non-hemolytic. PCR amplification and genome sequencing further indicated that these strains do not contain common virulence-encoding genes. In addition, the isolates exhibit important probiotic properties. They can survive and grow in an de Man, Rogosa and Sharpe medium with low pH (2.5) and in the presence of 1% bile salts. Disk diffusion tests confirmed their ability to inhibit enteropathogenic bacteria, including *Escherichia coli*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, and *Listeria*. Additionally, all strains demonstrated cholesterol assimilation ability in *in-vitro* conditions. These bacterial strains are undergoing further research for potential development into probiotic products.

1. INTRODUCTION

Enterococci are a group of lactic acid bacteria commonly found in various food ingredients, including vegetables, fruits, raw milk, and dairy products like cheese and cured meats [1,2]. Among these, species such as *Enterococcus faecium* (*E. faecium*), *Enterococcus durans*, and *Enterococcus lactis* are particularly promising as biological products due to their probiotic properties, including their ability to produce bacteriocins with antibacterial effects [1,3,4]. Several Enterococci strains have been used as effective probiotics in clinical settings for many years [5,6]. For example, *E. faecium* strains such as SF68, M74, LX, and L3 have been demonstrated in multiple randomized clinical studies to be particularly effective in treating gastrointestinal diseases, including chronic gastritis, gastric ulcers, irritable bowel syndrome, pancreatitis, and chronic hepatitis [7]. The *E. lactis* has been recently studied and separated from Group B of *E. faecium* with less pathogenic potential [8]. Although the use of *E. lactis* as a commercial product is currently limited, its safety and probiotic properties have been investigated, showing promise for future probiotic applications [9].

Fermented foods and drinks, whether derived from animal or plant sources, play a crucial role in our diets. These foods typically contain lactic acid bacteria, which thrive during the fermentation process [10,11]. Lactic acid bacteria naturally produce compounds such as organic acids, ethanol, and antimicrobial substances that inhibit spoilage organisms and pathogenic bacteria in fermented foods [12]. Moreover, these bacteria are well-adapted to spontaneous fermentation and contribute significantly to the health of both humans and animals, particularly in the digestive tract, where they function as probiotics. Therefore, fermented foods are considered rich sources for isolating probiotics [10,11].

Fermented pork roll (nem chua) is a traditional Vietnamese food from which many probiotics have been isolated. According to previous studies, probiotic strains isolated from fermented pork rolls belong to the *Lactobacillus*, *Lactococcus*, and *Pediococcus* groups [13]. Stinky tofu is a special food in the Asian region that originated from China. Similar to other fermented foods, stinky tofu is also a rich source of probiotics such as *Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Pediococcus, Streptococcus,* and *Weissella* [14].

Previous studies have often isolated common probiotics such as *Lactobacillus* and *Lactococcus* from stinky tofu and fermented pork

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roll. Although *Enterococcus* bacteria are present, they were rarely isolated and studied. Therefore, in this study, four *Enterococcus* strains were isolated and analyzed for their properties. These strains were identified through gene sequencing, and their safety and probiotic properties were investigated. This study identified promising bacterial strains and provided valuable insights for the development of health-promoting probiotic products.

2. MATERIALS AND METHODS

2.1. Food materials

Samples of fermented foods, including stinky tofu and Vietnamese fermented pork rolls were collected from traditional markets in Ha Noi–Vietnam, in 2022.

2.2. Bacteria strains

Bacteria strains including *Escherichia coli* American Type Culture Collection (ATCC) 25922*, Shigellasonnei* (levine) Weldin ATCC 25931, *Staphylococcus aureus* ATCC 25923, and *Salmonella enterica* Typhimurium ATCC 14028 were purchased from ATCC. *Listeriamono cytogenes* SLR2249 was provided by Hardy Diagnostics Company.

2.3. Probiotics isolation

All food materials were crushed and immersed in a physiological saline solution (0.9% NaCl). Bacterial cells in the liquid suspension were cultured on HiCrome™ *E. faecium* Agar medium (Himedia) for 24 hours at 37°C [10]. Subsequently, target cells (green-colored colonies along with yellow coloration to the medium) were streaked to Chromatic detection agar (Liofilchem) and incubated at 37˚C for 24 hours. Then, green colonies were cultured on *Lactobacillus de Man, Rogosa and Sharpe (MRS) Agar* (Himedia) before species identification. Next, bacteria were suspended in a sodium chloride 0.45% solution to attain a density of 0.5–0.63 McF and identified using the VITEK® 2 compact system with GP Card (BioMerieux).

2.4. Genome sequencing

Total DNA was extracted utilizing the DNeasy Blood & Tissue Kits (Qiagen). Subsequently, 2 × 150 bp paired-end libraries were prepared employing the Nextera DNA Sample Preparation Kit (Illumina Inc., United States) following the manufacturer's instructions. Then, genome sequencing was executed using the Illumina HiSeqXten sequencing 150PE platform (Illumina Inc., United States). The quality assessment of raw sequencing data was performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and low-quality and short-length reads were filtered out by trimmomatic [15]. The high-quality pair-end reads data was de-novo assembled by SPAdes (Galaxy version 3.12.0) [16]. The completeness of a draft assembly was assessed using BUSCO scores [17].

2.5. Species identification

The assembled genome sequences underwent ribosomal multilocus sequence typing (rMLST) analysis using the online database and platform (available at https://www.pubmlst.org/species-id) [18].

2.6. Phylogenetic analysis

Gene identifications were automatically done using Prokka [19] and via aligning scaffolds to target sequences with minimap2 [20]. The Phylogenetic analysis was conducted utilizing the Molecular Evolutionary Genetic Analysis (MEGA X) software [21].

2.7. Antibiotic susceptibility

The minimum inhibitory concentration (MIC) results were generated using the Vitek 2 system with AST-GP67 test cards (bioMérieux) following the manufacturer's instructions. Antibiotic susceptibility was automatically assessed by this system according to Clinical Laboratory Standards Institute guidelines and natural resistance patterns.

2.8. Hemolytic activity

The hemolytic activity of bacteria was assessed following the protocol outlined by Angmo *et al.* [22]. Briefly, the bacteria were cultured on Columbia Agar Base (Himedia) supplemented with 5% Sheep Blood for 48 hours at 37°C.

2.9. Genotypic virulence determinants

The presence of virulence factors, biogenic amines, and antibiotic resistance genes was assessed through polymerase chain reaction (PCR) using DreamTaq green PCR master mix (Thermos Scientific) and gene-specific primers (Table 1). The PCR products were then visualized by electrophoresis on 2% agarose gels.

2.10. Acid and bile salt tolerance

Bacteria cultured in MRS (De Man – Rogosa – Sharpe) medium were harvested and washed with a 0.45% sodium chloride solution by centrifugation at 5,000 g for 10 minutes. The cell pellets were used for assessments.

For acid tolerance assessment, the cell pellets were re-suspended in an MRS liquid medium adjusted to pH 2.5, aiming for a density of 0.5–0.63 McF. These bacterial suspensions were then incubated for 0, 2, and 4 hours at 37°C. Following this treatment, the suspensions were spread onto MRS solid medium and incubated for 24 hours at 37°C. Surviving cells were quantified based on the number of colonies formed [23].

For the assessment of bile salt tolerance, the procedure was conducted similarly to the method described above. However, in this case, bacterial cells were treated with MRS liquid medium supplemented with either 0.3% or 1% ox-bile for 0 or 4 hours. To assess simultaneous acid and bile salt resistance, bacterial cells were treated with MRS liquid medium at pH 2.5, supplemented with 0.3% or 1% bile salts.

2.11. Antimicrobial activity

Antimicrobial activity was assessed following the method described by Nami *et al.* [31]. Indicator bacteria were initially cultured on MRS agar at 37°C for 24 hours. The bacteria were then suspended in water to a concentration of 1.5×10^8 CFU/ml and spread onto MRS agar plates. Wells with a diameter of 5 mm were then created in these plates. Subsequently, 50 µl of filtered cell-free supernatant obtained from the cultures of the isolates, with a cell density of 10^8 CFU/ml, was added to each well and allowed to diffuse for 4 hours at room temperature. After 24 hours of incubation at the optimal growth temperature of the indicator strains, the inhibition zones around the wells were measured using a digital caliper. Experiments were performed in triplicate, with three plates per replicate.

2.12. Cholesterol assimilation

The cholesterol assimilation was determined using the o-phthalaldehyde method described by Usman and Hosono [32]

Table 1. List of primers used in this study.

Category	Target genes	Primer name	Primer sequences (5′ → 3′)	Amplicon size (bp)	References
Virulence factors	*Esp*	Esp F	TTGCTAATGCTAGTCCACGACC	933	[24]
		Esp R	GCGTCAACACTTGCATTGCCGAA		
	SprE	SprE F	GCGTCAATCGGAAGAATCAT	233	[25]
		SprE R	CGGGGAAAAAGCTACATCAA		
	fsrB	fsrB F	TTTATTGGTATGCGCCACAA	316	[25]
		fsrB R	TCATCAGACCTTGGATGACG		
	asa1	asa1 F	CCAGCCAACTATGGCGGAATC	529	[25]
		asa1 R	CCTGTCGCAAGATCGACTGTA		
	CylA	cylA F	ACTCGGGGATTGATAGGC	688	[26]
		cylA R	GCTGCTAAAGCTGCGCTT		
	CylM	cylM F	GATTGGAATGTGGGAATCCTAA	735	[27]
		cylM R	ACTTCCGGCAACCTTTAGTGTA		
Vancomycin resistance genes	*VanA*	vanA F	CCCCTTTAACGCTAATACGATCAA	1,030	[28]
		vanA R	CATGAATAGAATAAAAGTTGCAAT		
	VanB	vanB F	GTGACAAACCGGAGGCGAGGA	433	[28]
		vanB R	CCGCCATCCTCCTGCAAAAAA		
Histidine decarboxylase	*Hdc1*	Hdc1 F	AGATGGTATTGTTTCTTATG	367	[29]
		Hdc1 R	AGACCATACACCATAACCTT		
	Hdc2	Hcd2 F	AAYTCNTTYGAYTTYGARAARGARG	534	[29]
		Hdc2 R	ATNGGNGANCCDATCATYTTRTGNCC		
Tyrosine decarboxylase	*Tdc*	Tdc F	ACATAGTCAACCATRTTGAA	1,100	[30]
		Tdc R	CAAATGGAAGAAGAAGTAGG		
		EntP R	ATGTCCCATACCTGCCAAAC		

and Asan-Ozusaglam and Gunyakti [33] with some modifications. Briefly, the bacteria were cultured in MRS broth supplemented with 0.3% ox gall (Merk, Germany) and cholesterol (150 µg/ml; Sigma-Aldrich) at 37°C for a day. After incubation, the cells were removed by centrifugation at 10,000 g for 15 minutes. Subsequently, a mixture consisting of 1 ml of cell-free broth, 1 ml of KOH (33% *w*/*v*), and 2 ml of 96% ethanol was heated at 60°C for 15 minutes. After cooling to room temperature, 2 ml of water and 3 ml of hexane were added and mixed for 1 minute. One ml of the hexane layer was transferred into a glass tube and evaporated in a water bath at 80°C. The residue was dissolved in 2 ml of 0.05% (*w*/*v*) o-phthalaldehyde reagent (Merck, Germany). After standing for 10 minutes, 0.5 ml of concentrated sulfuric acid was added. The absorbance was measured using a spectrophotometer at 550 nm.

Cholesterol assimilation was calculated using the following equation: A = 100−[(B/C) × 100], where A represents the percentage of cholesterol assimilation (%), B is the amount of cholesterol in the inoculated medium, and C is the amount of cholesterol in the non-inoculated (control) medium.

2.13. Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Duncan's multiple range tests, in SPSS software (version 20). Statistical significance was set at $p < 0.05$. Data are presented as mean ± standard deviation, based on three biological replicates.

Table 2. Isolation of *Enterococcus* strains in traditional fermented foods.

No.	Strains	Source of isolation	Species identification
1	F20BA	Fermented pork roll	*Enterococcus lactis*
2	F26BA	Fermented pork roll	*Enterococcus lactis*
3	F53BA	Stinky tofu	*Enterococcus lactis*
4	F54BA	Stinky tofu	*Enterococcus lactis*

3. RESULTS

3.1. Isolation of *Enterococcus* strains from traditional fermented foods

To isolate probiotics, samples were collected from two sources of stinky tofu and two sources of fermented pork rolls. Bacterial suspensions from these foods were cultured and selected on *E. faecium* HiCrome™ agar, Chromatic detection agar, and *Lactobacillus* MRS agar. As a result, four bacterial strains including F20BA, F26BA, F53BA, and F54BA were isolated, each corresponding to a different food sample (Table 2). The F20BA and F26BA strains were isolated from fermented pork rolls, while the F53BA and F54BA strains were isolated from stinky tofu samples. The identification results using the VITEK® 2 system indicated that all four strains were identified as *E. faecium* with accuracy ranging from 96% to 98% (data not shown).

3.2. Species identification of new isolates

Some *Enterococcus* species are closely related, making them difficult to distinguish using conventional taxonomic methods [34,35]. Therefore, analysis of rMLST and *Rhomboid protease* (*GluP*) gene sequences was utilized for species identification.

Whole-genome sequencing and MLST analysis had demonstrated efficacy in species identification of *Enterococcus* spp [35]. In this study, genome sequencing of four isolated strains was conducted utilizing the Illumina Next-Generation Sequencing platform. More than 10.5 million qualified reads were obtained. The assembled

Supplementary Table 1. Genome assembly of bacteria strains.

Parameter	F20BA	F26BA	F53BA	F54BA
No. of qualified read	11157864	11354700	10528202	11152760
Mean of coverage	591	599	533	578
Total assembly length (Mb)	2.68	2.63	2.77	2.75
No. of contigs	124	57	76	99
Genome completeness	99.2%	98.4%	98.4%	98.4%
GC content (%)	38.35	38.29	38.1	38.17
No. of coding sequences	2560	2521	2635	2673
GenBank accession no.	JARWSG000000000	JARGGM000000000	JARJOX000000000	JARDYX000000000

Supplementary Table 2. Species identification by rMLST analysis.

No.	rMLST locus	Alleles			
		F20BA	F26BA	F53BA	F54BA
1	BACT000001 (rpsA)	31679	31679	1840	1840
2	BACT000002 (rpsB)	6834	6834	1634	121706
3	BACT000003 (rpsC)	1527	1527	1476	1527
4	BACT000004 (rpsD)	130070	130070	18399	829
5	BACT000005 (rpsE)	776	776	776	16275
6	BACT000006 (rpsF)	1386	1386	1386	1386
7	BACT000007 (rpsG)	1412	1412	1412	1412
8	BACT000008 (rpsH)	717	717	717	717
9	BACT000009 (rpsI)	1480	1480	1480	1480
10	BACT000010 (rpsJ)	815	815	815	815
11	BACT000011 (rpsK)	783	783	783	783
12	BACT000012 (rpsL)	770	770	770	770
13	BACT000013 (rpsM)	775	775	1311	775
14	BACT000014 (rpsN)	750	750	4533	750
15	BACT000014 (rpsN)	4533	4533	750	4533
16	BACT000015 (rpsO)	748	748	748	748
17	BACT000016 (rpsP)	760	760	760	1363
18	BACT000017 (rpsQ)	735	735	735	735
19	BACT000018 (rpsR)	1262	1262	1262	1262
20	BACT000019 (rpsS)	717	717	717	717
21	BACT000020 (rpsT)	834	834	834	834
22	BACT000021 (rpsU)	590	590	590	590
23	BACT000030 (rplA)	883	883	883	883
24	BACT000031 (rplB)	1174	1174	6799	1174
25	BACT000032 (rplC)	1552	1552	1552	1552
26	BACT000033 (rplD)	780	780	6096	780
27	BACT000034 (rplE)	810	810	5648	810
28	BACT000035 (rplF)	1511	1511	1466	2486
29	BACT000036 (rplL)	759	759	759	759
30	BACT000038 (rplI)	1571	1571	4181	1571
31	BACT000039 (rplJ)	1455	1455	1455	1455
32	BACT000040 (rplK)	1431	1431	1431	1431
33	BACT000042 (rplM)	892	892	892	892
34	BACT000043 (rplN)	771	771	771	771
35	BACT000044 (rplO)	771	771	771	771
36	BACT000045 (rplP)	753	753	753	753
37	BACT000046 (rplQ)	1403	1403	860	860
38	BACT000047 (rplR)	698	698	4875	698
39	BACT000048 (rplS)	1400	1400	1400	7272
40	BACT000049 (rplT)	784	784	1462	34821
41	BACT000050 (rplU)	736	736	1317	736
42	BACT000051 (rplV)	749	749	749	749
43	BACT000052 (rplW)	1006	1006	1006	1006
44	BACT000053 (rplX)	727	727	727	727
45	BACT000056 (rpmA)	975	975	883	1344
46	BACT000057 (rpmB)	662	662	662	662
47	BACT000058 (rpmC)	646	646	646	646
48	BACT000059 (rpmD)	1042	1042	718	718
49	BACT000060 (rpmE)	1152	1152	1152	1152
50	BACT000061 (rpmF)	713	713	713	713
51	BACT000062 (rpmG)	714	714	714	714
52	BACT000062 (rpmG)	4383	4383	4383	4383
53	BACT000063 (rpmH)	653	653	653	653
54	BACT000064 (rpmI)	656	656	656	20322
55	BACT000065 (rpmJ)	629	629	629	629

genome sequences ranged from 2.63 to 2.77 Mb in length, with coverage between 533 and 599. Genome completeness was from 98.4% to 99.2% (Supplementary Table 1). Species identifications were performed using rMLST analysis with the assembled genome sequences. Analysis of data from 55 genes encoding bacterial ribosome protein subunits revealed that all four isolates were *Enterococcuslactis* (Table 2, Supplementary Table 2).

The GluP has been identified as the best candidate gene for distinguishing between *E. faecium* and *E. lactis* [36]. In this study, *GluP* sequences from isolated strains, retrieved from genome sequencing data, were compared with their orthologs in the Enterococcus genome using phylogenetic analysis (Fig. 1). The analysis revealed that all four *GluP* sequences from the isolates exhibited high similarity with those of *E. lactis* and showed distinct distances from other orthologous genes. This result is consistent with the rMLST analysis, which also identified all four isolates as *E. lactis*.

3.3. Safety assessment of isolated strains

3.3.1. Hemolytic activity

The bacteria were cultured on Columbia Agar with 5% Sheep Blood to assess their hemolytic capacity. After 24 hours of incubation, no discernible change was observed in the medium under and around the colonies (Fig. 2). Consequently, all four isolates did not induce hemolysis. This result suggests that the isolates are non-hemolytic or exhibit γ-hemolysis.

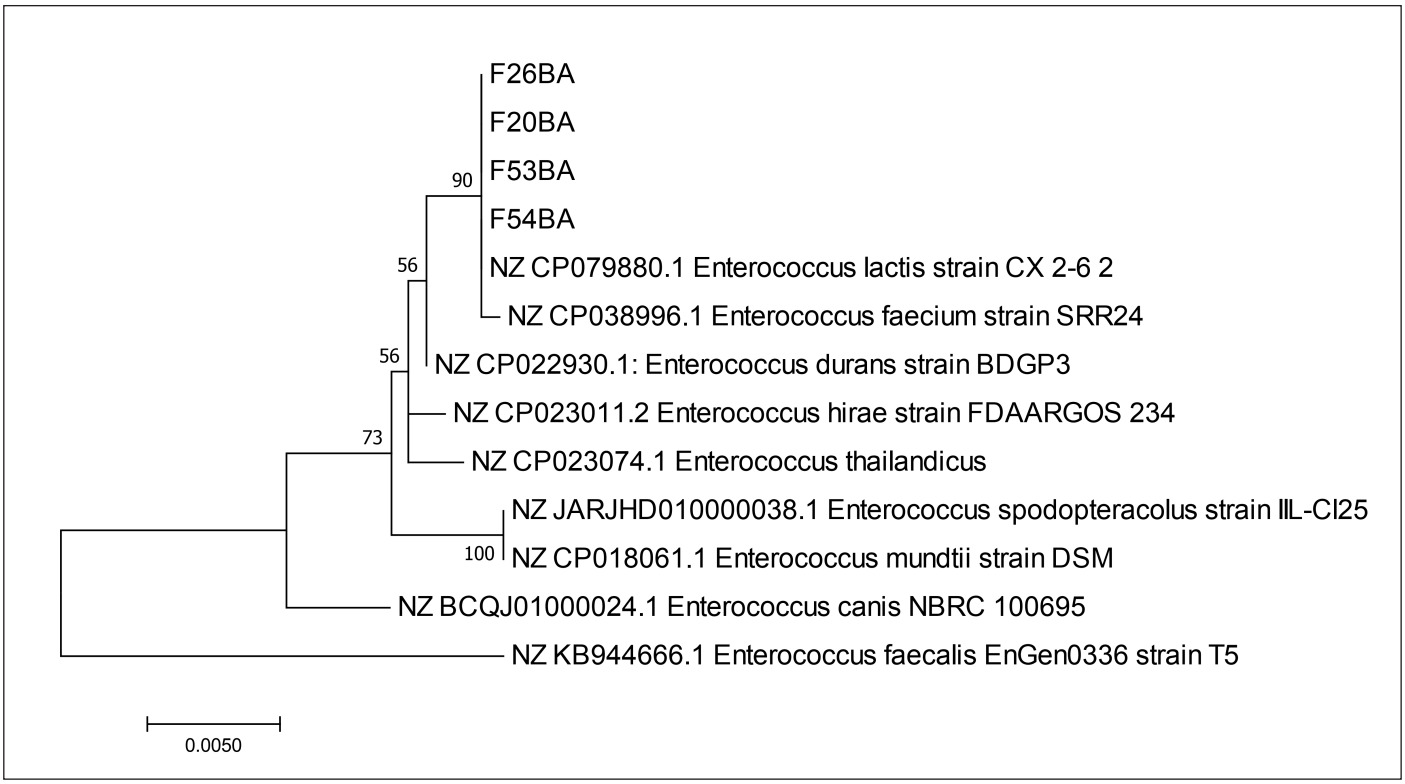

Figure 1. Dendrogram illustrating the relationship between *GluP* gene sequences from isolated strains and *Enterococcus spp.* The tree was constructed by MEGA X using maximum likelihood method and JTT matrix-based model. Numbers at nodes indicate the percentage bootstrap scores from 10,000 replicates. The scale bar represents 0.05 estimated number of substitution events per site.

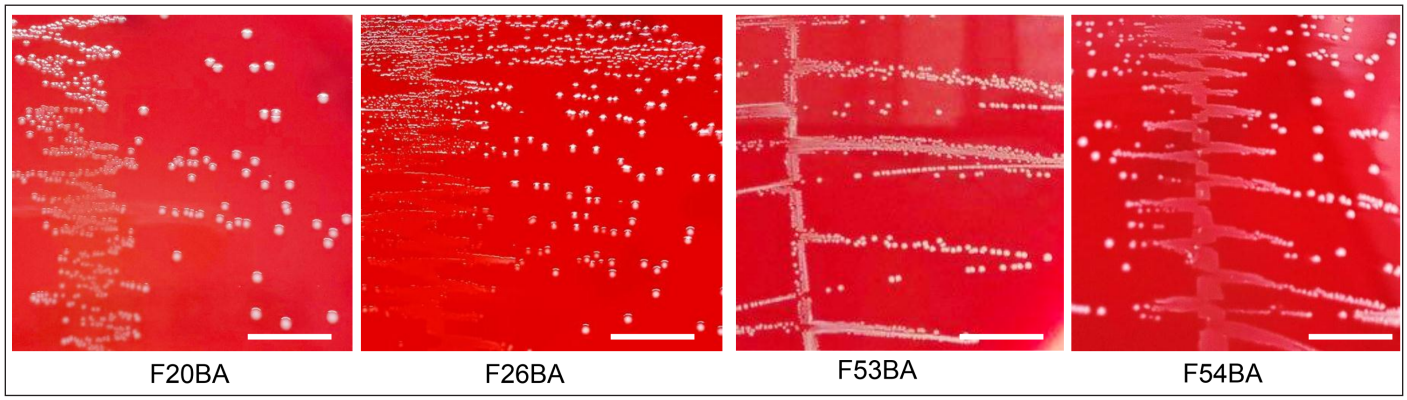

Figure 2. Hemolytic assays of isolated strains. The bacteria was grown on Columbia Agar supplemented with 5% sheep blood for 24 hours at 37˚C. Scale bar: 1 cm

3.3.2. Virulence factors

The presence of common virulence factors in *Enterococcus* genomes was assessed via PCR analysis. Consequently, the virulence-encoding genes including *Enterococcal surface protein* (*Esp*), *serine protease* (*SprE), fsrB, surface aggregating protein* (*asa1*), *cytolysin A* (*cylA*), *cytolysinM (CylM),* as well as the bacterial toxin-encoding genes *Histidine decarboxylase (Hdc1, Hdc2), Tyrosine decarboxylase (Tdc)* were negative for PCR (Table 3). Furthermore, these coding sequences were found to be absent in the assembled genome (data not shown).

3.3.3. Antibiotic susceptibility

Antibiotic susceptibility is a key criterion for evaluating the safety of probiotics. In this study, antibiotic susceptibility was assessed using the Vitek 2 Compact system. As a result, all bacterial strains were susceptible to at least 10 of the 13 antibiotics used (Table 4). All strains showed moderate to high resistance to erythromycin. But, only F20BA and F54 are resistant to tetracycline, and F26BA and F53BA are resistant to nitrofurantoin. Notably, all four strains were sensitive to vancomycin.

3.4. Determination of probiotic potential

3.4.1. Acid and bile salt tolerance

Acid tolerance of the strains was assessed by incubating bacteria in an MRS medium with a pH of 2.5 for 1 to 4 hours. The results showed that, except for F54BA, which had a survival rate between 88.7% ± 0.3% and 92.2% ± 0.4%, the other three strains demonstrated higher tolerance, with survival rates exceeding 95.5% (Table 5).

To assess bile salt tolerance, bacteria were incubated in MRS medium containing either 0.3% or 1% bile salt. The probiotic strains continued to survive and grow in both bile salt concentrations, with survival rates ranging from 84% to 99.2% (Table 5). In 0.3% bile salt, strains F20BA and F53BA demonstrated higher survival rates (98.2% to 99.2%) compared to the other two strains, which ranged from 85.3% to 90%. Notably, strain F53BA maintained high tolerance in 1% bile salt, with survival rates of approximately 98.2% to 98.3%. These results indicate that F53BA exhibits better bile salt tolerance than the other strains.

The combined effect of acid and bile salt on bacterial survival was also investigated. Strain viability was assessed in MRS medium (pH 2.5) supplemented with 0.3% and 1% bile salt. The results showed that in both 0.3% and 1% bile salt environments, the probiotic strains continued to survive and grow, with a survival ratio ranging from 85% to 99.2% (Table 5). In particular, the survival rate of strain F54BA (85% to 88.8%) was lower compared to the other three strains, which had survival rates ranging from 89.9% to 99.2%.

3.4.2. Antimicrobial activity

Four bacterial strains were co-cultured with other pathogenic microorganisms capable of transmission through the human digestive tract, including *E. coli, Salmonella, Shigella, Staphylococcus aureus,* and *Listeria*. After 24 hours of co-cultivation, inhibition zones emerged surrounding all wells (Table 6). These findings indicate that all four isolated strains effectively suppressed the growth of the tested bacterial pathogens.

Table 3. PCR detection of virulence factors, vancomycin resistance genes, and biogenic amines encoding genes.

Genes	Isolated *Enterococcus lactis* strains			
	F20BA	F26BA	F53BA	F54BA
Esp	-	-	-	-
SprE	-	-	-	-
fsrB	-	-	-	-
asa1	-	-	-	-
cylA	-	-	-	-
cylM	-	-	-	-
vanA	-	-	-	-
vanB	-	-	-	-
Hdc1	-	-	-	-
Hdc1	-	-	-	-
Tdc	-	-	-	-

Table 4. Antibiotic susceptibility of isolated strains.

Antibiotic	MIC (mg/l)	F20BA	F26BA	F53BA	F54BA
Ampicillin	≤2	S	S	S	S
Vancomycin	4	S	S	S	S
Gentamycin(*)	SYN-S	S	S	S	S
Erythromycin	1	I	I	R	I
Tetracycline	≥ 16	R	S	S	R
Benzylpenicillin	0.25	S	S	S	S
Streptomycin (*)	SYN-S	S	S	S	S
Ciprofloxacin	≤0.5	S	S	S	S
Levofloxacin	2	S	S	S	S
Quinupristin/ Dalfopristin	1	S	S	S	S
Linezolid	2	S	S	S	S
Tigecycline	≤0.12	S	S	S	S
Nitrofurantoin	32	S	I	I	S

Notes: (S) susceptible; (R) resistance; (I) intermediate; SYN: synergy; (*) high level, synergy. Parameter Set: CLSI-based and natural resistance.

Remarkably, all four strains inhibit *Escherichia coli*, *Salmonella*, and *Shigella* more effectively as evidenced by the presence of larger inhibition zones exceeding 20 mm in diameter. Additionally, F26BA exhibited superior effectiveness in inhibiting all five tested pathogens among the isolates.

3.4.3. Cholesterol assimilation

To assess cholesterol assimilation, bacterial strains were cultured in a medium containing cholesterol. After 24 hours of incubation, varying degrees of reduction in cholesterol levels were observed, ranging from 10% to 38% (Fig. 3). Therefore, all four strains have cholesterol assimilation ability. The F20BA strain exhibited the highest assimilation rate at 38.8% ± 0.3%. Subsequently, strains F26BA and F54BA followed with assimilation rates of 24.5% ± 1.4% and 20.8% ± 0.6%, respectively. In contrast, the F53BA strain displayed the lowest assimilation ability, recording only 10% ± 1%.

Table 5. Viability of isolated *E. lactis* strains after exposure to low pH and bile salt.

Treatment	Time of treatment	Survival ratio (%)			
		F20BA	F26BA	F53BA	F54BA
pH 2.5	0 hour	99.1 ± 0.8^{a}	95.1 ± 0.5^{ab}	97.6 ± 0.2^{a}	92.2 ± 0.4^{bc}
	2 hours	98.6 ± 0.7^{a}	95.5 ± 0.5^{ab}	97.2 ± 0.5^{a}	88.7 ± 0.3^{cdefg}
	4 hours	98.1 ± 0.7^{a}	98.6 ± 4.6^{a}	96.5 ± 0.9^{a}	89.2 ± 1.1^{cdef}
0.3% ox-bile	0 hour	99.2 ± 0.4^{a}	85.3 ± 1.5^{fgh}	98.6 ± 0.3^{a}	89.7 ± 1.2c^{de}
	4 hours	98.2 ± 0.5^{a}	89.1 ± 0.2^{cdef}	98.8 ± 0.3^{a}	90.0 ± 0.9^{cde}
1% ox-bile	0 hour	89.0 ± 0.5^{cdef}	84.1 ± 0.3^{h}	98.2 ± 1.0^{a}	89.1 ± 1^{cdef}
	4 hours	90.9 ± 0.2^{cd}	84.0 ± 0.5^{h}	98.3 ± 0.4^{a}	87.4 ± 0.5^{defgh}
pH 2.5, 0.3% ox-bile	0 hour	97 ± 0.6^{a}	89.9 ± 3.2^{cde}	97.5 ± 0.6^{a}	86.5 ± 2.4^{efgh}
	4 hours	97.5 ± 0.6^{a}	99.2 ± 2^{a}	98.9 ± 0.6^{a}	85 ± 1.9^{gh}
pH 2.5, 1% ox-bile	0 hour	97.5 ± 1.2^{a}	98.9 ± 1.6^{a}	98.8 ± 0.4^{a}	88.8 ± 6.9^{cdefg}
	4 hours	98.1 ± 1^{a}	98.6 ± 2.7^{a}	98.8 ± 0.7^{a}	86.6 ± 2.5^{efgh}

Notes: Data are presented as mean ± SD of three replicates. Different letters indicate significant differences based on one-way ANOVA analysis with Duncan's test ($p < 0.05$).

Table 6. Antimicrobial activity of isolated strains against pathogenic microorganisms.

NNo.	Bacteria strain	Diameter of inhibition zone (mm)			
		F20BA	F26BA	F53BA	F54BA
1	*E. coli* ATCC 25922	26.7 ± 1.2^{b}	26.7 ± 1.9^{b}	17.7 ± 0.5^{gfed}	21.7 ± 1.2^{dc}
2	*Shigella* ATCC® 25931TM	33.7 ± 1.9^{a}	33.7 ± 3.4^{a}	21.3 ± 0.5^{dc}	21 ± 1.6^{edc}
3	*Staphylococcus aureus* ATCC® 25923	9.7 ± 0.5^{h}	16.7 ± 0.9^{gf}	10^{h}	17 ± 1.6^{gfe}
4	*Salmonella entericatyphy* ATCC 14028	29.7 ± 1.7^{b}	26.7 ± 3.9^{b}	21.3 ± 0.5^{dc}	22.3 ± 3.3^{c}
5	*Listeria monocytogenes* SLR2249	15.3 ± 0.5^{g}	20.3 ± 2.6^{fedc}	16 ± 0.8^{g}	18.7 ± 1.2^{gfedc}

Notes: Data are presented as mean ± SD of three replicates. Different letters indicate significant differences based on one-way ANOVA analysis with Duncan's test ($p < 0.05$).

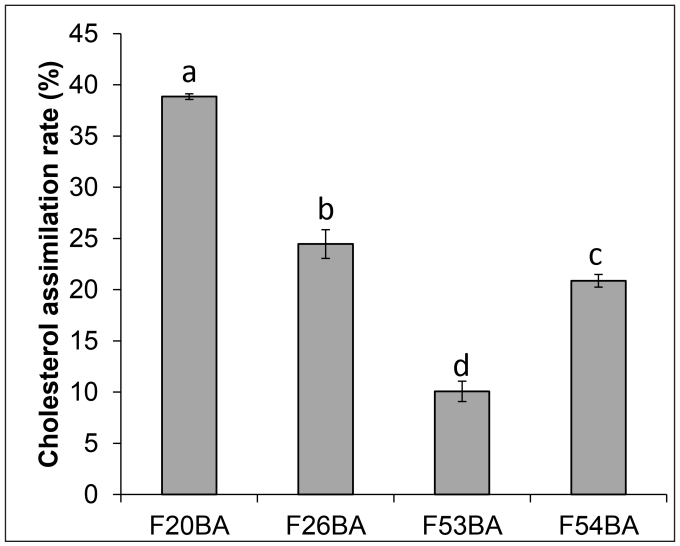

Figure 3. Cholesterol assimilation ability of isolated strains. Data are presented as mean ± SD of three replicates. Different letters indicate significant differences based on one-way ANOVA analysis with Duncan's test ($p < 0.05$).

4. DISCUSSION

4.1. Four *E. lactis* trains from local traditional fermented foods

Certain *Enterococcus* species pose challenges in differentiation through conventional methods due to their close genetics [35]. In this study, *Enterococcus* strains were isolated from local fermented foods using a specific selection medium, and species identification was conducted utilizing rMLST. Consequently, these strains were identified as belonging to *E. lactis* (Supplementary Table 2). The *E. lactis* was proposed as an independent species in 2012 [2], although the earlier strains were isolated from milk samples [37]. Within the *Enterococcus* genus, *lactis* and *faecium* are closely related species. Recently, based on genome studies, clade B of *E. faecium* has been proposed to be reclassified as *E. lactis* [8]. *gluP* was identified as the most promising candidate for distinguishing between these two species [36]. The results indicate that the *gluP* gene sequences of all four strains exhibit high similarity when compared to those of *E. lactis* (Fig. 1). The data presented above demonstrate that the isolated strains belong to *E. lactis*.

The *E. lactis* is a significant probiotic strain that has been isolated from diverse sources. Among these, fermented foods stand out as

rich reservoirs of lactic acid bacteria. Similar to this study, numerous strains have been recovered from this source, including those found in dairy products and rice wine koji [38], radish pickle fermentation [39], and raw milk cheeses [2]. Besides, *E. lactis* has also been isolated from other sources such as human gut [31,38,40], and raw shrimps [41]. However, studies on isolating *E. lactis* from fermented foods are limited. This study is the first to report the isolation and evaluation of *E. lactis* in Vietnamese fermented pork rolls and local stinky tofu.

4.2. Probiotic potential of isolates

In addition to being used as probiotics, some *Enterococci* strains are known to be pathogenic and can cause clinical diseases such as such as bacteremia, infectious endocarditis, and urinary tract infections [42,43]. Therefore, safety is the primary requirement for *Enterococcus* spp. strains intended for probiotic production [44]. The strains must be susceptible to key antibiotics used in treating intestinal pathogens such as vancomycin, should not contain genes expressing major virulence factors such as *cylA*, *cylB*, *cylM*, *esp*, and *gelE*, and must not be hemolytic [9,31,45]. Moreover, biogenic amines produced by Enterococci are known for their harmful effects on human health [46]. Therefore, selecting *Enterococcus* strains that lack major virulence factors, are vancomycin-susceptible, and are incapable of producing biogenic amines has become a popular procedure for their use as probiotics [31,46–48]. In this study, all four isolated strains tested negative for PCR detection of vancomycin resistance genes (*VanA, VanB*), virulence factor encoding genes (*Esp, SprE, fsrB, asa1, CylA, CylM)* and biogenic amine metabolism genes (*Hdc1, Hdc2, Tdc)*. Additionally, they are sensitive to numerous antibiotics, including vancomycin (Table 4), and are non-hemolytic (Fig. 2). Furthermore, compared to *E. faecium*, *E. lactis* strains also cause human infections, but to a much lesser extent [36,38]. Based on genetic analysis, *E. lactis* contains fewer antibiotic-resistance genes than *E. faecium* [38] and lacks hospital infection-associated markers [8]. These data indicate the basic safety parameters of the isolates.

In this study, the isolated strains showed inhibitory effects on enteropathogenic bacteria including *E. coli*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, and *Listeria (*Table 6*)*. These abilities of *E. lactis* were also reported in previous studies [9,41,49]. Additionally, these strains can survive and grow in bile and low-pH environments (Table 5). These properties are essential for bacteria to thrive in the gut and compete with other microbial species [46]. In addition, the ability to assimilate cholesterol provides the potential for developing cardiovascular support products, especially isolated strain F20BA.

In conclusion, the initial *in-vitro* tests in this study provided preliminary evidence that the isolated bacterial strains have potential as probiotics. However, additional studies are necessary to progress toward commercial applications. For instance, the studies include assessing the activities of isolated strains in simulated gastrointestinal environments and conducting clinical trials.

5. CONCLUSION

In this study, two *E. lactis* strains, F20BA and F26BA, were isolated from fermented pork rolls, while two other strains, F53BA and F54BA, were isolated from stinky tofu. Those are vancomycin susceptible, lacking common virulence-encoding genes, and non-hemolytic bacteria. Besides, they exhibit potential probiotic properties such as acid and bile salt tolerance, antimicrobial activity, and cholesterol assimilation. However, further studies are needed to support the development of new probiotic products from these strains.

6. SUPPLEMENTARY INFORMATION

Supplementary Table 1: Genome assembly of bacteria strains. Supplementary Table 2: Species identification by rMLST analysis.

7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data are available with the authors and shall be provided upon reasonable request. The genome sequencing data have been deposited in GenBank (NCBI) under the following accession numbers: JARWSG000000000 (F20BA), JARGGM000000000 (F26BA), JARJOX000000000 (F53BA), and JARDYX000000000 (F54BA).

12. PUBLISHER'S NOTE

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