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Probiotic and Antimicrobial Potential of *Enterococcus faecalis* M26 Recovered from Healthy Vietnamese Newborns' Stool

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ABSTRACT

The neonatal gut microbiota, seeded by pioneer bacteria from meconium, is fundamental to lifelong health. Meconium is an underexplored reservoir of host-adapted bacteria with probiotic potential, especially in underrepresented populations, such as those in Vietnam. This study aimed to isolate and characterise Lactic Acid Bacteria (LAB) from the meconium of healthy Vietnamese newborns to identify novel probiotic candidates. From 20 meconium samples, 30 presumptive LAB isolates were selected after phenotypic screening. These isolates underwent comprehensive in vitro assessment. We tested their tolerance to simulated gastrointestinal conditions (pH 2.0 and 0.4% bile salts) and antimicrobial activity against *Escherichia coli, Salmonella enterica*, and *Staphylococcus aureus*. Safety was evaluated through antibiotic susceptibility testing and PCR screening for virulence genes. Isolate M26 showed the highest resilience, maintaining a viability of 6.22±0.49 Log CFU/mL at pH 2.0 and 6.31±0.58 Log CFU/mL in 0.4% bile salts. The neutralised cell-free supernatant of M26 showed moderate, broad-spectrum antimicrobial activity against all pathogens tested. M26 was sensitive to clinically relevant antibiotics and lacked key virulence factors (*esp, gelE, cylA*) and antibiotic resistance genes (*vanA, vanB*). 16S rRNA gene sequencing identified M26 as *Enterococcus faecalis*. These findings suggest that neonatal meconium is a source of robust LAB. *E. faecalis* M26, with high gastrointestinal tolerance and a favourable safety profile, warrants further investigation as a potential region-specific probiotic.

1. Introduction

The establishment of a resilient gut microbiota in the neonatal period is key for human health. It profoundly influences the maturation of the immune and metabolic systems (Gensollen *et al.* 2016; Wang *et al.* 2024). This first microbial assembly trains immune cells, defends against pathogens, and helps nutrient metabolism (Zhuang *et al.* 2019). Dysbiosis—disruption of this delicate colonisation—is linked to an increased risk of diseases, including allergic, autoimmune, and metabolic disorders (Walker 2017). Therefore, understanding the origins of pioneer colonisers is critical for developing early-life interventions.

Meconium, the first feco-discharge of a newborn, represents a unique window into the fetal and early neonatal intestinal environment. While the "sterile womb" hypothesis historically posited that the fetus develops in a germ-free environment, modern molecular techniques have challenged this paradigm by detecting bacterial DNA in the placenta, amniotic fluid, and meconium (Stinson *et al.* 2019; Blaser *et al.* 2021; Banchi *et al.* 2024). Although the characterisation of the meconium microbiome remains scientifically controversial due to potential contamination issues often termed the "kitome" (Perez-Muñoz *et al.* 2017; Senn *et al.* 2020), culture-dependent studies confirm

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that meconium frequently contains viable populations of *Lactobacillus, Bifidobacterium,* and *Enterococcus* (Oktaviyani *et al.* 2021; Turunen *et al.* 2021). These pioneer bacteria are likely host-adapted and possess intrinsic mechanisms to survive the harsh conditions of the neonatal gastrointestinal tract.

Isolating Lactic Acid Bacteria (LAB) from meconium offers a distinct advantage for probiotic development, as these strains have co-evolved with the human host from the earliest stages of life. This suggests superior compatibility and safety compared to strains derived from dairy or adult sources (Henrick *et al.* 2021). Indeed, previous studies have isolated potent probiotic candidates, such as *Lactobacillus fermentum* and *Enterococcus faecalis*, from meconium (Al Atya *et al.* 2015; Zavišić *et al.* 2019). However, the composition of the neonatal microbiota is not universal; it varies significantly across geographical regions as a result of distinct maternal genetics, diets, and environmental exposures (Farinella *et al.* 2022; Lee *et al.* 2017).

Current research reveals a significant knowledge gap regarding Southeast Asian populations, particularly compared with the well-characterised Western microbiomes. Asian populations exhibit distinct microbial profiles, often with higher Prevotella levels, likely due to higher fibre intake (Govender and Ghai 2025). Further emphasizing these differences, studies in Singapore and Vietnam show that both ethnic background and living environment—rural or urban—drive gut diversity (Gounot *et al.* 2022; Kortman *et al.* 2023). Despite these findings, the meconium microbiota of Vietnamese newborns remains under-investigated. This lack of region-specific data limits the development of "precision probiotics" for local populations. Furthermore, most commercial probiotics lack standardisation, and their efficacy in diverse ethnic groups is unproven (Embleton *et al.* 2016; Athalye-Jape and Patole 2019).

To address these gaps, this study aimed to isolate and characterise LAB from the meconium of healthy, full-term Vietnamese newborns by prioritising culture-dependent methods to recover viable strains, rather than relying solely on DNA signatures. Specifically, our objectives were to (1) isolate promising LAB strains; (2) evaluate their probiotic properties, such as acid and bile tolerance, and antimicrobial activity against neonatal pathogens; and (3) rigorously assess their safety by focusing on antibiotic susceptibility and virulence factors.

2. Materials and Methods

2.1. Ethical Approval and Sample Collection

The study protocol received ethical approval from the Institutional Review Board of Tien Giang Obstetrics and Gynecology Hospital (Approval No. QĐ. 2025 BVPS). Before collecting samples, we obtained written informed consent from parents or legal guardians of all newborns. We collected 20 fresh meconium samples from healthy, full-term newborns (gestational age ≥37 weeks) delivered vaginally. Exclusion criteria included maternal antibiotic use during pregnancy, underlying chronic diseases, or meconium aspiration. We aseptically collected samples from the centre of the first passed stool with a sterile spatula. Samples were then transferred to sterile cryotubes, transported on ice, and processed within six hours of collection.

2.2. Isolation and Identification of Lactic Acid Bacteria

Each meconium sample (1 g) was homogenised in 9 mL of sterile 0.9% (w/v) saline. Serial dilutions (10^{-1} to 10^{-6}) were prepared. Dilutions were spread-plated in triplicate onto de Man, Rogosa, and Sharpe (MRS) agar (Himedia, India) supplemented with 0.1% (w/v) $CaCO_3$. Plates were incubated anaerobically using the GasPak™ system at 37°C for 48 hours. Fifty-four colonies with clear zones, indicative of organic acid production, were purified by successive streaking. These were stored at -80°C in MRS broth containing 25% (v/v) glycerol (Al Atya *et al.* 2015; Kang *et al.* 2020).

Isolates were characterised by Gram staining (a method to distinguish types of bacteria based on cell wall structure), cellular morphology (shape and arrangement of cells), and biochemical assays: catalase (tests for the presence of the catalase enzyme), urease (tests for the production of the urease enzyme), and citrate utilisation (assesses the ability to use citrate as a sole carbon source), using reference strains for validation (Rahmawati *et al.* 2021). Carbohydrate fermentation capabilities—specifically the ability to ferment glucose, lactose, and sucrose—were also assessed. Only Gram-positive (bacteria that retain the crystal violet stain), catalase-negative (do not produce the catalase enzyme), urease-negative (do not produce the urease enzyme), and citrate-negative (cannot use citrate as the sole carbon source) isolates were retained. Thirty isolates meeting these criteria were selected for functional screening.

2.3. Assessment of Gastrointestinal Conditions

Survival under simulated gastrointestinal conditions was tested in triplicate using a modified protocol by Serrano-Nino *et al.* (2016). Overnight-cultured isolates in MRS broth were harvested by centrifugation (10,000 rpm, 5 min, 4°C). They were washed twice with sterile distilled water and resuspended to approximately 10^8 CFU/mL ($OD_{600} \approx 0.6$).

For acid tolerance, 1 mL of the cell suspension was inoculated into 9 mL of MRS broth adjusted to pH 2.0 or pH 3.0 using 1 M HCl. Standard MRS broth (pH 6.5) served as the control. For bile salt tolerance, 1 mL of suspension was inoculated into 9 mL of MRS broth supplemented with 0.2% or 0.4% (w/v) bile salts, with unsupplemented MRS broth serving as the control. Following a 4-hour incubation at 37°C, viable cell counts were enumerated by plating serial dilutions onto MRS agar. The survival rate was expressed as Log (N/N0), where N represents the viable count after treatment and N0 denotes the initial count. This experiment was conducted with three independent biological replicates.

2.4. Evaluation of Antimicrobial Activity

Antimicrobial activity was assessed using the agar well diffusion method (Abubakr 2018). The indicator pathogens used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella enterica* serovar Typhimurium ATCC 14028. Isolates were cultured in MRS broth for 24 hours at 37°C. The cell-free supernatant (CFS) was harvested by centrifugation (10,000 rpm, 5 min, 4°C) and neutralised to pH 7.0 with 1 M NaOH to prevent inhibition solely due to organic acids.

Pathogens were seeded into Nutrient Agar at a density of approximately 106 CFU/mL. Wells (4 mm diameter) were created in the agar and filled with 100 µL of neutralised CFS. Ampicillin (10 µg disk) served as the positive control, and neutralised sterile MRS broth as the negative control. Plates were incubated at 37°C for 24 hours. Inhibition zones were measured in millimetres (mm) and classified as follows: strong (>25 mm), moderate (13–25 mm), weak (1–12 mm), or inactive (no zone) (Rabaoui *et al.* 2023). Each assay was performed in triplicate for every isolate against each pathogen.

2.5. Safe Assessment

Antibiotic Susceptibility: The antibiotic susceptibility profiles of the 30 isolates were determined using the Kirby-Bauer disk diffusion method. Bacterial suspensions 108 CFU/mL) were spread onto MRS agar plates. Eight commercially available antibiotic disks were applied: Doxycycline (30 µg), Ampicillin (10 µg), Tetracycline (30 µg), Kanamycin (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), and Chloramphenicol (30 µg). After incubation at 37°C for 24 hours, inhibition zone diameters were measured. Results were interpreted as sensitive (≥20 mm), intermediate (15–19 mm), or resistant (≤14 mm) according to established CLSI guidelines (Alebiosu *et al.* 2017; Nguyen *et al.* 2023). All tests were conducted in triplicate.

Genotypic Screening: Genomic DNA was extracted from eight high-performing isolates using the Wizard® Genomic DNA Purification Kit (Promega). PCR was performed to detect virulence factors (*esp, gelE, fsrB, asa1, cylA, cylM*), resistance genes (vanA, vanB), and biogenic amine genes (*hdc1, hdc2, tdc*). Specific primers and expected amplicon sizes are detailed in Table 1. The PCR program consisted of: Initial denaturation at 94°C for 5 min; 30 cycles of 94°C (45 s), annealing at 55°C (60 s), and extension at 72°C (90 s); and a final extension at 72°C for 7 min. DreamTaq Green PCR Master Mix (Thermo Scientific) was used. The reaction mixture (25 µL) contained 12.5 µL Master Mix, 1 µL of each specific primer (10 µM), 1 µL of template DNA, and 9.5 µL of nuclease-free water. Known virulent *Enterococcus* strains served as positive controls, while nuclease-free water served as the negative control. Detection was confirmed via electrophoresis on 2% agarose gels (Thermo Fisher Scientific, USA) (Nguyen *et al.* 2025).

2.6. Molecular Identification Via 16S rRNA Gene Sequencing

The most promising isolate was identified via 16S rRNA gene sequencing. The gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Liu *et al.* 2020). PCR was performed on a Veriti thermocycler using GoTaq® Green Master Mix (Promega, USA) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 45 s, 57°C for 60 s, and 72°C for 90 s; followed by a final extension at 72°C for 5 min. The resulting ~1,500 bp product was purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced at Next Gen Scientific Co., Ltd (Ho Chi Minh City, Vietnam).

Table 1. Oligonucleotide primers used for PCR amplification of virulence, antibiotic resistance, and biogenic amine genes

Category	Target genes	Primer	Sequences (5′ → 3′)	Size (bp)	Reference
Virulence factors	*esp*	Esp F	TTGCTAATGCTAGTCCACGACC	933	(Eaton and Gasson 2001)
		Esp R	GCGTCAACACTTGCATTGCCGAA		
	gelE	GelE F	GCGTCAATCGGAAGAATCAT	213	
		GelE R	CGGGGAAAAAGCTACATCAA		
	fsrB	fsrB F	TTTATTGGTATGCGCCACAA	316	
		fsrB R	TCATCAGACCTTGGATGACG		
	asa1	asa1 F	CCAGCCAACTATGGCGGAATC	529	
		asa1 R	CCTGTCGCAAGATCGACTGTA		
	cylA	cylA F	ACTCGGGGATTGATAGGC	688	
		cylA R	GCTGCTAAAGCTGCGCTT		
	cylM	cylM F	GATTGGAATGTGGGAATCCTAA	735	
		cylM R	ACTTCCGGCAACCTTTAGTGTA		
Antibiotic resistance	*vanA*	vanA F	CCCCTTTAACGCTAATACGATCAA	1,030	(Dutka-Malen et al. 1995)
		vanA R	CATGAATAGAATAAAAGTTGCAAT		
	vanB	vanB F	GTGACAAACCGGAGGCGAGGA	433	
		vanB R	CCGCCATCCTCCTGCAAAAAA		
Biogenic amines	*hdc1*	Hdc1 F	AGATGGTATTGTTTCTTATG	367	(Le Jeune *et al.* 1995)
		Hdc1 R	AGACCATACACCATAACCTT		
	hdc2	Hdc2 F	AAYTCNTTYGAYTTYGARAARGARG	435	(De Las Rivas *et al.* 2006)
		Hdc2 R	ATNGGNGANCCDATCATYTTRTGNCC		
	tdc	Tdc F	ACATAGTCAACCATRTTGAA	1,100	(Coton *et al.* 2004)
		Tdc R	CAAATGGAAGAAGAAGTAGG		

2.7. Data and Sequence Analysis

All experiments were performed in at least three independent replicates. Data are presented as mean ± standard deviation (SD). Statistical differences were evaluated using one-way Analysis of Variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) post-hoc test for multiple comparisons. Analyses were conducted using SPSS software (version 20.0, IBM Corp., Armonk, NY, USA), with statistical significance defined as $p<0.05$ (Ding *et al.* 2017). Sequence alignment was performed using BioEdit (v. 7.0), and phylogenetic analysis was constructed via the Neighbor-Joining method in MEGA6.

Table 2. Physiological and biochemical characteristics of the 30 lactic acid bacteria (LAB) isolates obtained from neonatal meconium samples

Characteristic	Observation/result
Colony morphology	Circular, convex, smooth, milky-white
Cellular morphology	Gram-positive (+), rod-shaped or coccobacilli, typically in pairs or short chains, non-endospore-forming
Physiology	Non-motile (-)
Biochemical profile	Catalase (-), Urease (-), Citrate Utilization (-)
Carbohydrate fermentation	Glucose (+), Sucrose (+), Lactose (+)

3. Results

3.1. Isolation and Phenotypic Screening of LAB from Meconium

From the 20 meconium samples collected from healthy Vietnamese newborns, 54 presumptive Lactic Acid Bacteria (LAB) colonies were isolated based on calcium carbonate dissolution clear zones. Following purification and biochemical screening, 30 isolates were selected for further characterisation based on their confirmation as Gram-positive, catalase-negative, urease-negative, and non-endospore-forming bacteria (Table 2). Morphologically, the isolates were predominantly coccobacilli occurring in pairs or short chains. Figure 1 illustrates the typical colony and cellular morphology of the representative isolate M26, which formed circular, smooth, milky-white colonies on MRS agar. All 30 selected isolates fermented glucose, sucrose, and lactose, a metabolic profile consistent with LAB.

3.2. High Tolerance of Isolations to Simulated Gastrointestinal Conditions

The selected isolates exhibited significant variation in their ability to survive simulated gastric (low pH) and small intestinal (bile salts) conditions ($p<0.05$).

Acid Tolerance: Exposure to pH 2.0 served as a stringent selection criterion; only 25 of the 30 isolates remained viable (Table 3). Isolate M26 demonstrated

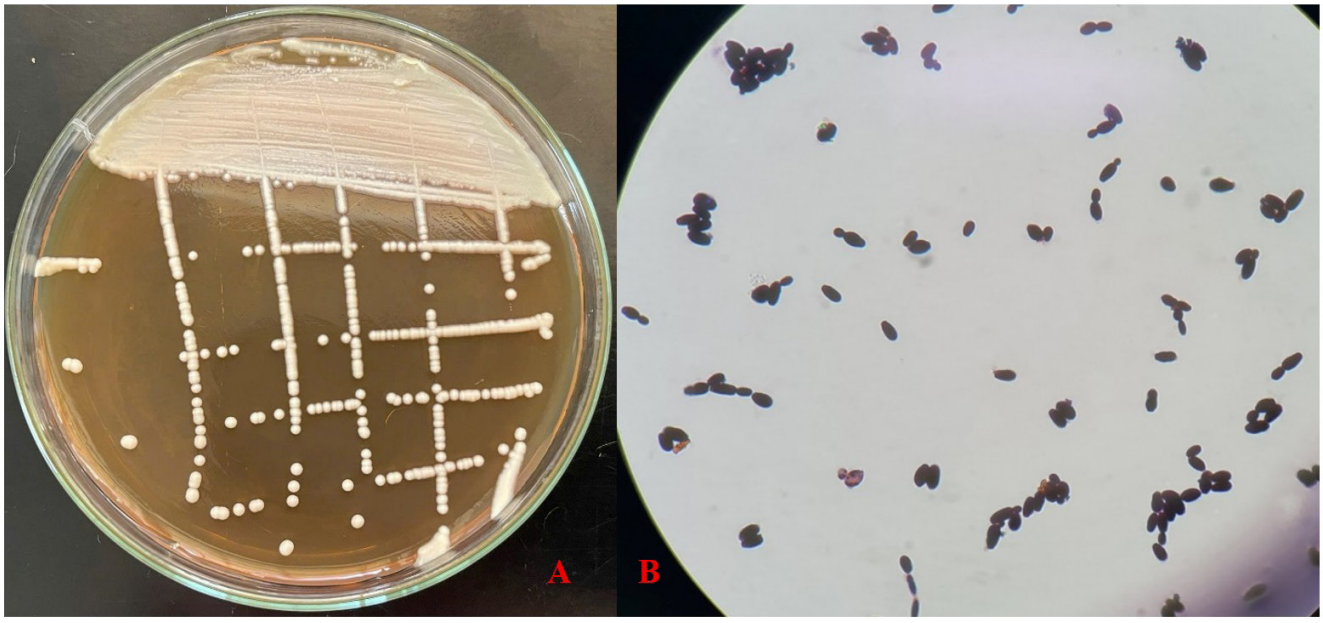

Figure 1. Representative morphology of isolate M26. (A) Colony morphology on nutrient agar, (B) gram stain revealing gram-positive, coccobacilli-shaped cells

significantly higher resilience than other candidates ($p<0.05$), maintaining a viability of 6.22±0.49 Log CFU/mL. Isolates M11 and M10 also showed robust tolerance, with survival rates of 5.89±0.39 and 5.78±0.37 Log CFU/mL, respectively. In contrast, isolates such as M1, M6, M16, M17, and M24 failed to survive at pH 2.0.

Bile Salt Tolerance: Viability was further differentiated by exposure to 0.4% bile salts (Table 4). Isolate M20 exhibited the highest tolerance (6.77±0.62 Log CFU/mL), followed by M21 (6.62±0.60 Log CFU/mL) and M26 (6.31±0.58 Log CFU/mL). Notably, Isolate M26 was the only strain to consistently rank in the top statistical tier for both acid and bile tolerance, identifying it as the most robust candidate for gastrointestinal transit.

3.3. Antimicrobial Activity Against Enteric Pathogens

The antagonistic activity of the neutralised cell-free supernatant (nCFS) of the 30 isolates was evaluated against *E. coli, S. enterica,* and *S. aureus*. The screening revealed a broad spectrum of inhibitory activity (Table 5). *Staphylococcus aureus* was the most susceptible pathogen, inhibited by 66.7% of the isolates (20/30), followed by *S. enterica* (60.0%) and *E. coli* (46.7%).

While no isolates produced "strong" inhibition zones (>25 mm), several displayed moderate activity (13–25 mm). Isolate M26 exhibited the most consistent broad-spectrum activity. As shown in Figure 2, M26

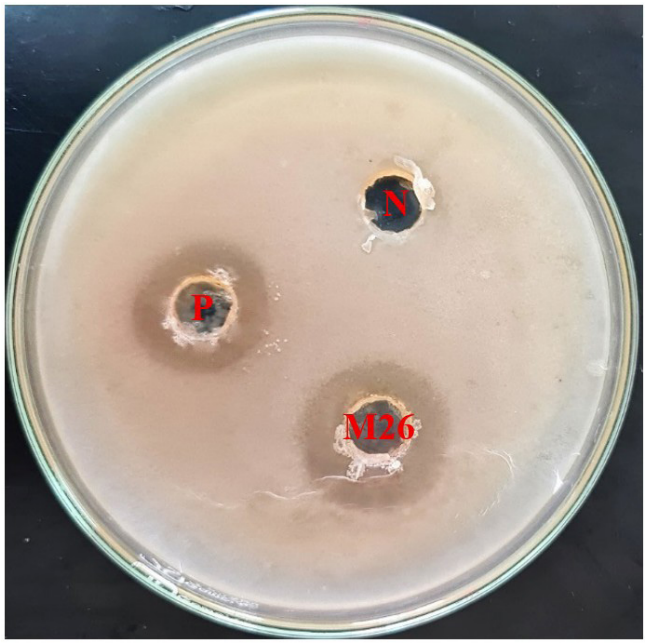

Figure 2. Representative images of the agar well diffusion assay show the antimicrobial activity of isolate M26. The plate was seeded with *S. aureus* ATCC 25923. M26: nCFS from isolate M26, P: Positive control (Ampicillin, 10 µg); and N: Negative control (sterile neutralized LB broth)

produced clear zones of inhibition against *S. aureus*. Quantitatively, the nCFS of M26 resulted in inhibition zones of 23.63±3.10 mm against *S. aureus*, 21.97±1.78 mm against *S. enterica*, and 23.33±1.15 mm against *E. coli*. These values were significantly larger than the

Table 3. Acid tolerance of presumptive probiotic LAB isolates pH tolerance

Isolated ID	Viable bacteria isolates (Log CFU/mL)		
	pH 6.5 (control)	pH 3	pH 2
M1	9.27±0.17	7.04±0.20	0.00±0.00
M2	9.73±1.40	7.78±0.14	4.51±0.15
M3	8.49±0.14	6.21±0.11	4.61±0.17
M4	9.45±1.02	7.40±0.13	4.35±0.13
M5	10.30±0.89	8.00±0.17	5.47±0.32
M6	7.57±2.95	4.31±2.10	0.00±0.00
M7	7.28±2.71	4.26±2.02	2.18±0.29
M8	8.50±0.49	6.49±0.13	4.51±0.15
M9	7.25±2.77	4.12±2.02	2.14±0.29
M10	9.91±0.90	7.72±0.18	5.78±0.37
M11	10.14±0.34	7.62±0.13	5.89±0.39
M12	9.02±0.96	7.01±0.17	2.06±0.32
M13	9.65±0.27	6.65±0.54	5.59±0.34
M14	10.12±0.18	7.17±0.71	5.38±0.29
M15	10.37±1.36	8.24±0.16	5.55±0.33
M16	9.47±0.13	7.01±0.12	0.00±0.00
M17	9.36±0.29	0.00±0.00	0.00±0.00
M18	9.82±0.40	7.37±0.13	1.95±0.33
M19	9.97±0.76	7.69±0.15	5.64±0.35
M20	9.27±0.51	6.61±0.13	2.02±0.31
M21	9.13±0.79	6.38±0.13	5.63±0.35
M22	9.90±1.09	7.81±0.17	2.07±0.31
M23	8.79±0.29	6.35±0.11	4.61±0.17
M24	7.44±2.84	4.31±2.10	0.00±0.00
M25	10.57±0.92	8.21±0.17	4.96±0.23
M26	9.74±0.05	7.19±0.13	6.22±0.49
M27	10.55±0.68	8.08±0.14	5.58±0.22
M28	9.39±0.64	6.99±0.37	5.56±0.34
M29	9.33±0.80	7.10±0.17	1.69±0.37
M30	9.51±1.60	7.69±0.14	5.51±0.33

Viable counts (Log CFU/mL, mean ± SD, n = 3) after 4-hour exposure to MRS broth at pH 6.5 (control), pH 3.0, and pH 2.0

Table 4. Bile salt tolerance of presumptive probiotic LAB isolates

Isolated ID	Viable bacteria isolates (Log CFU/mL)		
	0% (Control)	0.2%	0.4%
M1	9.36±0.29	6.91±0.59	5.78±0.53
M2	9.82±0.40	7.15±0.66	5.85±0.61
M3	10.01±0.56	6.72±0.61	2.55±0.26
M4	10.55±0.68	7.72±0.72	5.61±0.51
M5	10.30±0.89	7.04±0.64	6.32±0.58
M6	7.57±2.95	7.04±0.64	6.26±0.57
M7	8.49±0.14	7.62±0.69	4.76±0.44
M8	9.73±1.40	6.86±0.63	5.49±0.50
M9	7.25±2.77	6.66±0.61	3.21±0.31
M10	10.57±0.92	6.90±0.62	6.01±0.55
M11	9.74±0.05	7.32±0.67	6.23±0.58
M12	9.02±0.96	2.97±0.27	0.00±0.00
M13	9.65±0.27	5.78±0.53	5.01±0.46
M14	10.12±0.18	6.76±0.56	0.00±0.00
M15	10.37±1.36	6.84±0.63	5.61±0.51
M16	9.47±0.13	6.85±0.63	5.74±0.53
M17	9.33±0.80	7.09±0.65	5.61±0.51
M18	9.39±0.64	7.49±0.69	2.90±0.27
M19	9.97±0.76	7.74±0.71	5.18±0.47
M20	9.27±0.51	7.59±0.69	6.77±0.62
M21	9.13±0.79	7.34±0.67	6.62±0.60
M22	9.90±1.09	6.94±0.63	6.23±0.57
M23	8.79±0.29	7.14±0.65	3.02±0.27
M24	7.44±2.84	7.11±0.65	6.19±0.56
M25	9.91±0.90	6.92±0.63	5.99±0.55
M26	9.27±0.17	7.74±0.71	6.31±0.58
M27	9.45±1.02	6.51±0.60	5.84±0.53
M28	9.51±1.60	6.49±0.59	0.00±0.00
M29	7.28±2.71	7.53±0.69	5.75±0.53
M30	8.50±0.49	6.91±0.65	5.29±0.48

Viable counts (Log CFU/mL, mean ± SD, n = 3) after 4-hour exposure to MRS broth supplemented with %, 0.2%, and 0.4% (w/v) bile salts

Table 5. Antimicrobial activity of neutralized cell-free supernatants (nCFS) from LAB isolates against indicator pathogens

Indicator pathogen	Strong	Moderate	Weak	Inactive	Total active isolates (%)
E. coli ATCC 25922	0	8	6	16	14 (46.7 %)
S. enterica ATCC 14028	0	7	11	12	18 (60.0%)
S. aureus ATCC 25923	0	13	7	10	20 (66.7 %)

Inhibition was categorized as: Strong (>25 mm); Moderate (13–25 mm); Weak (1–12 mm); or Inactive (No inhibition zone)

negative control (0 mm) ($p<0.05$), though smaller than the Ampicillin positive control.

3.4. Safety Assessment: Antibiotic Susceptibility and Virulence Factors

To ensure suitability for human application, a comprehensive safety assessment was conducted on the eight best-performing isolates.

Phenotypic antibiotic susceptibility: All eight tested isolates, including M26, were 100% sensitive to the panel of eight clinically relevant antibiotics, including Ampicillin, Tetracycline, Gentamicin, and Chloramphenicol (Table 6). No intermediate or resistant profiles were detected.

Genotypic screening: PCR analysis reinforced the phenotypic safety data. As summarised in Table 7, the four top candidates (M5, M11, M24, and M26) tested negative for all screened virulence genes (*esp, gelE, fsrB, asa1, cylA, cylM*), biogenic amine-producing genes (*hdc1, hdc2, tdc*), and vancomycin resistance genes (vanA, vanB). The detection was confirmed by the absence of bands matching the target amplicon sizes listed in Table 1.

Table 6. Antibiotic susceptibility profile of the eight shortlisted LAB isolates determined by the Kirby-Bauer disk diffusion method

Antibiotics	Concentration (µg/disc)	Sensitive (%)	Intermediary (%)	Resistance (%)
Doxycycline	30	100	0	0
Ampicillin	10	100	0	0
Tetracycline	30	100	0	0
Kanamycin	30	100	0	0
Gentamicin	10	100	0	0
Erythromycin	15	100	0	0
Ciprofloxacin	5	100	0	0
Chloramphenicol	30	100	0	0

Zone diameters (mm) are interpreted as Sensitive (S), Intermediate (I), or Resistant (R) according to CLSI guidelines

Table 7. Genotypic safety screening of the eight shortlisted LAB isolates. PCR results for the presence of virulence genes (*esp, gelE, fsrB, asa1, cylA, cylM*), vancomycin resistance genes (*vanA, vanB*), and biogenic amine genes (*hdc1, hdc2, tdc*)

Total active isolates (%)	Target gene	M5	M11	M24	M26
Virulence factors	*esp*	-	-	-	-
	gelE	-	-	-	-
	fsrB	-	-	-	-
	asa1	-	-	-	-
	cylA	-	-	-	-
	cylM	-	-	-	-
Antibiotic resistance	*vanA*	-	-	-	-
	vanB	-	-	-	-
Biogenic amines	*hdc1*	-	-	-	-
	hdc2	-	-	-	-
	tdc	-	-	-	-

(-) Negative results (no amplicon detected by PCR)

3.5. Molecular Identification of Isolate M26 as *Enterococcus faecalis*

Isolate M26, selected as the most promising probiotic candidate due to its superior stress tolerance, antimicrobial activity, and safety profile, was identified via 16S rRNA gene sequencing. PCR amplification yielded a single, clear band at approximately 1,500 bp (Figure 3).

BLASTn analysis of the sequenced amplicon revealed a 99.70% identity with *Enterococcus faecalis* strain NBRC 100480 (Table 8). This identification was confirmed by phylogenetic analysis using the Neighbor-Joining method. The resulting tree (Figure 4) shows that isolate M26 clusters tightly within the *Enterococcus faecalis* clade, supported by a high bootstrap value. Consequently, the isolate was

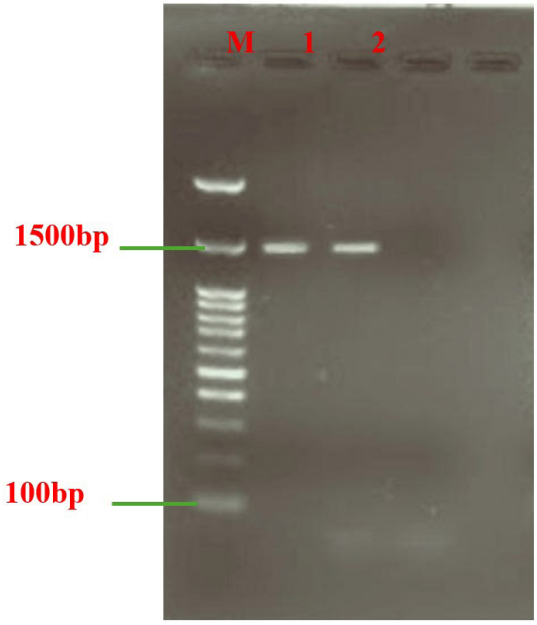

Figure 3. Agarose gel (2%) electrophoresis of PCR-amplified 16S rRNA gene from isolate M26. Lane M: 100 bp DNA ladder. Lane 1: Amplicon of approximately 1500 bp corresponding to the 16S rRNA gene of isolate M26

Table 8. 16S rRNA gene sequence similarity of isolate M26 to closely related type strains in the NCBI GenBank database, as determined by BLASTn analysis

Subject description	Max score	Query cover	E-value	Percent identity	Accession number
Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA, partial sequence	1201	100%	0.0	99.70%	NR_113901.1

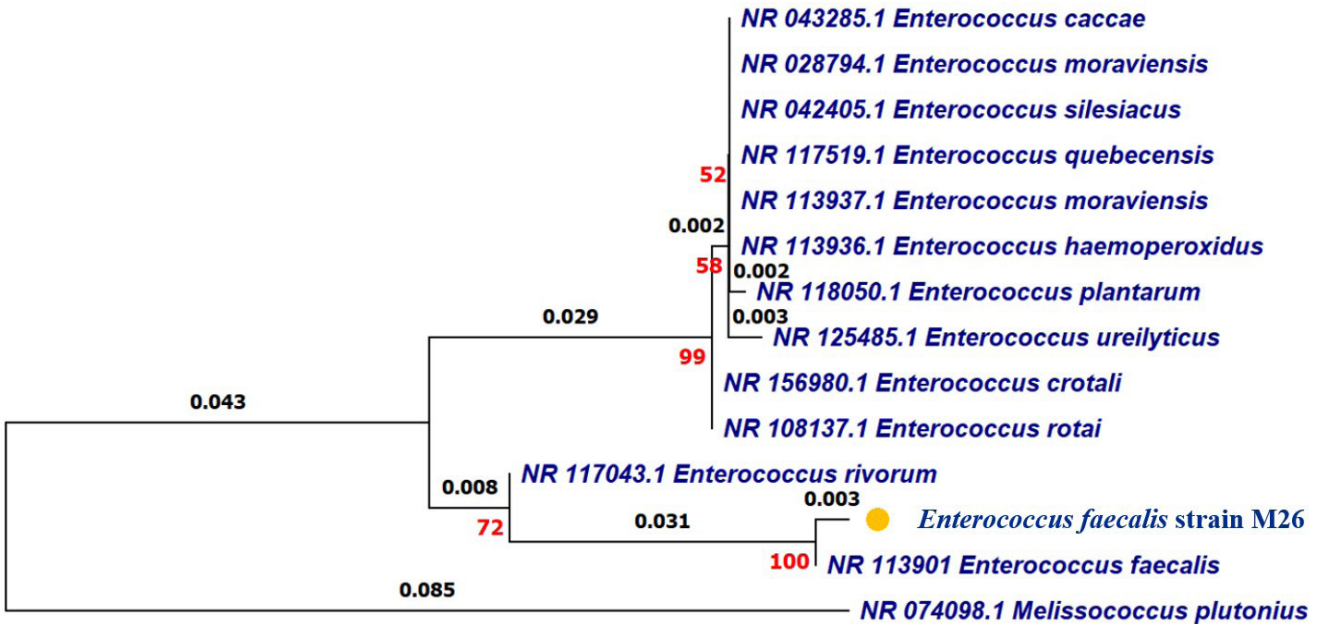

Figure 4. Phylogenetic tree based on 16S rRNA gene sequences, constructed using the Neighbor-Joining method. The tree positions *Enterococcus faecalis* M26 (highlighted with a black circle) within a clade containing other *E. faecalis* reference strains. Bootstrap values (based on 1,000 replicates) are shown at branch nodes. The scale bar indicates 0.005 substitutions per nucleotide position

designated as *Enterococcus faecalis* M26, and the sequence was deposited in GenBank under accession number PM112821.

4. Discussion

The transition from a protected uterine environment to the microbial exposure of the postnatal world is a pivotal phase in human development. Our study demonstrates that meconium, far from being biological waste, serves as a rich, indigenous reservoir of Lactic Acid Bacteria (LAB) with significant functional potential. By isolating *Enterococcus faecalis* M26, we bridge the gap between early-life microbial seeding and the practical application of "precision probiotics". The following sections detail how the physiological resilience and antimicrobial profile of M26 validate its role as a pioneer colonizer capable of supporting neonatal health within the specific context of the Vietnamese population.

4.1. Meconium as a Reservoir for Region-Specific Probiotics

The establishment of the neonatal gut microbiota is a critical determinant of long-term immune and metabolic health (Gensollen *et al.* 2016; Wang *et al.* 2024). Challenging the historical "sterile womb" paradigm, recent evidence suggests that bacterial colonisation may begin in utero (Perez-Muñoz *et al.* 2017; Blaser *et al.* 2021). As the first feco-discharge, meconium serves as the primary inoculum for the neonatal gut, containing pioneer bacteria uniquely adapted to the immune-naive intestinal environment (Stinson *et al.* 2019; Turunen *et al.* 2021).

In this study, the isolation of 30 distinct Lactic Acid Bacteria (LAB) from the meconium of healthy Vietnamese newborns validates meconium as an underexplored reservoir of probiotic candidates. This finding is particularly significant given the underrepresentation of Southeast Asian populations in global microbiome research (Farinella *et al.* 2022). Since the vertical transmission of microbial consortia is influenced by maternal genetics and the high-fibre, rice-based diet typical of Vietnam (Kortman *et al.* 2023; Govender and Ghai 2025), isolating region-specific strains such as *Enterococcus faecalis* M26 is essential for developing "precision probiotics" with enhanced host compatibility and ecological fitness.

4.2. Gastrointestinal Resilience: A Prerequisite for Efficacy

Survival during transit through the stomach and duodenum is a fundamental requirement for any oral probiotic. The acidic environment of the stomach (pH 1.5–3.5) and the detergent properties of bile salts in the small intestine constitute severe physiological barriers

that eliminate many exogenous microorganisms (Fijan 2016; Caillard and Lapointe 2017). Unlike commercial probiotic powders, which often suffer massive viability losses up to 99% reduction during gastric transit (Venema *et al.* 2019; Han *et al.* 2021), isolate M26 exhibited exceptional resilience.

Our results highlight significant strain-specific variations in stress tolerance. While several isolates failed to survive at pH 2.0, isolate M26 maintained a viability of 6.22±0.49 Log CFU/mL after 4 hours. This robustness aligns with recent studies demonstrating the intrinsic durability of *E. faecalis* isolated from harsh environments, such as camel milk (Kudaibergenova *et al.* 2025) and fermented foods (Mubarak and Soraya 2018). Furthermore, M26 demonstrated high survival in 0.4% bile salts (6.31±0.58 Log CFU/mL), a concentration exceeding physiological levels typically found in the human intestine (0.3%). This dual resistance suggests that M26 possesses intrinsic stress-adaptation mechanisms, such as upregulation of proton pumps or Bile Salt Hydrolase (BSH) activity, which are critical for successful colonisation (Ayyash *et al.* 2021).

4.3. Antimicrobial Potential and Pathogen Exclusion

A defining characteristic of functional probiotics is the ability to inhibit pathogenic colonisation. The neutralised cell-free supernatant (nCFS) of isolate M26 demonstrated broad-spectrum inhibitory activity (13-25 mm) against *S. aureus, E. coli,* and *S. enterica* as categorized in our methodology. Crucially, because the supernatant was neutralised to pH 7.0, this inhibition cannot be attributed to organic acids; instead, it strongly implies the production of antimicrobial peptides, likely bacteriocins such as enterocins (Imade *et al.* 2021; Lee *et al.* 2024).

This antimicrobial profile is clinically relevant, as *S. aureus* and *E. coli* are primary causative agents of neonatal sepsis and gastrointestinal infections. Our findings corroborate previous research by Mojgani and Khalkhali (2017) and Yi *et al.* (2021), who reported that *E. faecalis* strains isolated from human milk effectively inhibited *Staphylococcus* spp. and *Escherichia* spp. Isolate M26 may therefore play a "policing" role, stabilising the developing microbiota by preventing the overgrowth of opportunistic pathogens and contributing to colonisation resistance in the developing infant gut (Wang *et al.* 2021; Abdulhussein and Isa 2024).

4.4. Safety Assessment: Addressing the *Enterococcus paradox*

The identification of isolate M26 as *Enterococcus faecalis* requires a rigorous safety evaluation. *Enterococcus* species occupy a paradoxical status: they are ubiquitous gut commensals and commercial probiotics (e.g., Symbioflor®), yet they are also opportunistic nosocomial pathogens associated with multi-drug resistance (Franz *et al.* 2011; Hanchi *et al.* 2018). Consequently, the European Food Safety Authority (EFSA) excludes *Enterococcus* from the Qualified Presumption of Safety (QPS) list, mandating strain-specific confirmation of safety (Herman *et al.* 2019).

Isolate M26 distinguishes itself from clinical strains through a favourable safety profile. Phenotypically, it exhibited 100% sensitivity to clinically relevant antibiotics, including ampicillin, vancomycin, and gentamicin. This contrasts with clinical isolates, which are often characterised by high resistance rates to erythromycin and tetracycline (Sanlibaba and Senturk 2018; Guan *et al.* 2024; Yang *et al.* 2025). Genotypically, PCR screening confirmed the absence of the *vanA* and *vanB* operons (responsible for Vancomycin-Resistant Enterococci, VRE) and major virulence factors (*esp, gelE, cylA*). This aligns with findings by Han *et al.* (2024) and Wang *et al.* (2020), who demonstrated that probiotic *E. faecalis* strains of infant origin are often genetically "cleaner" than clinical isolates, lacking transmissible resistance genes and cytolysins. These results suggest that M26 is a safe commensal candidate, distinct from virulent nosocomial lineages.

4.5. Novelty, Limitations, and Future Applications

The novelty of this study lies in the identification of a host-adapted, robust probiotic candidate specifically from the meconium of Vietnamese newborns, providing a foundation for region-specific "precision probiotics". However, there are limitations to consider. While in vitro results are promising, they do not fully replicate the complex microbial interactions and host immune responses *in vivo*. Furthermore, while we screened for major virulence genes, the inherent genomic plasticity of *Enterococcus necessitates* more comprehensive analysis.

Given the regulatory stringency regarding human use, M26 also holds immediate value in livestock applications, specifically in poultry. The poultry gut

shares similarities with the human infant gut regarding the need for rapid colonization to prevent *Salmonella* infection. Future research will prioritize Whole Genome Sequencing (WGS) to screen for any remaining mobile genetic elements and in vivo trials to validate efficacy in microbiome restoration or as a functional feed additive to reduce antibiotic dependency (Sanlibaba and Senturk 2018).

In conclusion, this study identifies *Enterococcus faecalis* M26, isolated from the meconium of healthy Vietnamese newborns, as a robust probiotic candidate characterised by exceptional acid and bile tolerance and broad-spectrum antimicrobial activity against neonatal pathogens. These findings validate that neonatal meconium in Southeast Asian populations acts as a reservoir for pioneer bacteria with distinct functional adaptations. Crucially, M26 presents a pristine safety profile regarding antibiotic susceptibility and virulence factors, addressing major regulatory concerns associated with the genus. While promising, the genomic plasticity of enterococci necessitates further investigation. Future research will prioritise Whole Genome Sequencing (WGS) to screen for mobile genetic elements and *in vivo* trials to validate its efficacy in microbiome restoration or as a functional feed additive to reduce antibiotic dependency in livestock.

Conflict of Interest

The authors declare that there is no conflict of interest.

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