BIODIVERSITAS
Volume 27, Number 2, February 2026
Pages: 10

ISSN: 1412-033X
E-ISSN: 2085-4722
https://doi.org/10.13057/biodiv/d270208

Probiotic and antimicrobial potential of *Enterococcus faecalis* CC3 isolated from traditional Vietnamese pickled *Brassica juncea*

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Manuscript received: 18 December 2025. Revision accepted: 31 January 2026.

Abstract. *Thuy NP, Anh LTL, Linh NT, Tuu NT, Phong VT. 2026. Probiotic and antimicrobial potential of* Enterococcus faecalis *CC3 isolated from traditional Vietnamese pickled* Brassica juncea*. Biodiversitas 27 (2): d270208. https://doi.org/10.13057/biodiv/d270208.* Vietnamese pickled mustard greens (*Brassica juncea*), known as *Dua cai muoi chua*, harbor diverse microbial communities with potential biotechnological applications. This study aimed to isolate and characterize Lactic Acid Bacteria (LAB) from these traditional pickles to evaluate their probiotic and antimicrobial properties. Sixty samples collected from Vinh Long province, Vietnam, yielded 64 presumptive LAB isolates. These were screened for acid and bile tolerance, antibiotic susceptibility, hemolytic activity, and antimicrobial efficacy against *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus*. Among the candidates, isolate CC3 exhibited superior resilience, maintaining high viability after 12 hours at pH 2.0 and in the presence of 1.0% bile salts. Identified as *Enterococcus faecalis* via 16S rRNA gene sequencing (GenBank Accession No. PX928037), strain CC3 demonstrated broad-spectrum antimicrobial activity, producing significant inhibition zones against *S. aureus* (22.59 ± 1.59 mm), *E. coli* (21.29 ± 1.53 mm), and *S. enterica* (20.66 ± 1.88 mm). Safety assessments confirmed CC3 as γ-hemolytic (non-hemolytic) and sensitive to clinically relevant antibiotics, including Ampicillin and Chloramphenicol. Furthermore, PCR analysis verified the absence of virulence genes (*esp, gelE, cylA, cylM*), vancomycin resistance genes (*vanA, vanB*), and biogenic amine-producing genes (*hdc1, hdc2, tdc*). These findings highlight the microbial diversity of indigenous fermented vegetables and suggest *E. faecalis* CC3 as a promising candidate for probiotic applications or as a biopreservative starter culture.

Keywords: Antimicrobial activity, *Brassica juncea*, *Enterococcus faecalis,* fermented vegetables, probiotics

INTRODUCTION

The escalating prevalence of Antimicrobial Resistance (AMR) in foodborne pathogens presents a formidable challenge to global public health and food safety (Zeng et al. 2020). This growing threat has intensified the global search for natural and effective alternatives to conventional antibiotics. Among the most promising solutions are probiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Fadare et al. 2023; Guney et al. 2025). While the clinical benefits of probiotics are well-documented, their mechanisms of action are multifaceted. These include the competitive exclusion of pathogens for nutrients and adhesion sites, the physical and chemical enhancement of the intestinal epithelial barrier, and the modulation of host immune responses through the production of various bioactive compounds (Zommiti et al. 2020).

Lactic Acid Bacteria (LAB) represent the most prominent group of microorganisms utilized as probiotics, valued for their "Generally Recognized as Safe" (GRAS) status and their long history of usage in traditional food fermentation. Beyond modulating the gut microbiome, LAB play a critical role in food preservation through the production of a range of antimicrobial metabolites, including organic acids, hydrogen peroxide, and bacteriocins. These compounds actively inhibit the growth of spoilage microorganisms and enteric pathogens, thereby extending the shelf-life of fermented products (Darbandi et al. 2022; Yaacob et al. 2022). While research has historically focused on dairy-derived strains, recent scientific attention has shifted toward plant-based fermented foods. These traditional products, particularly from the underexplored biodiversity of Southeast Asia, are now recognized as valuable reservoirs for robust probiotic strains capable of surviving the harsh environmental conditions encountered during industrial processing and gastrointestinal transit (Cao et al. 2019; Fadare et al. 2023).

Fermented vegetables, such as sauerkraut, kimchi, and pickles, harbor complex microbiota dominated by LAB (Guney et al. 2025). In Vietnam, pickled mustard greens (*Brassica juncea* (L.) Czern.), known locally as *Dua cai muoi chua*, are a staple produced through spontaneous fermentation. This process relies on indigenous microbiota to preserve the food and enhance its nutritional profile, including antioxidant activity and vitamin C content (Özer and Yıldırım 2019; Liu et al. 2024). The unique ecological niche created by high salinity and rapid acidification potentially selects for LAB strains with distinct functional adaptations compared to dairy-based counterparts (Erdoğan and Filiz 2023).

However, isolating probiotics from these sources requires careful scrutiny, especially regarding the genus *Enterococcus*. While enterococci contribute to unique food textures and flavors, they present a safety paradox due to their association with nosocomial infections and horizontal transfer of antibiotic resistance genes (Hanchi et al. 2018; Banik et al. 2023). Therefore, distinguishing between safe probiotic candidates and potentially virulent strains is critical, necessitating a dual phenotypic and genotypic assessment to ensure the absence of virulence factors and vancomycin resistance (Braïek and Smaoui 2019).

While numerous studies have characterized LAB from regional pickles, such as *Lactiplantibacillus plantarum* from Chinese pickles (Zeng et al. 2020) and *Lactobacillus fermentum* from pickled garlic (Sadeghi 2016), the specific probiotic potential and safety profile of LAB indigenous to Vietnamese *Dua cai muoi chua* remain largely uncharacterized. Existing research on Vietnamese fermented foods, notably Hussein et al. (2025) and Nguyen et al. (2023), has explored general LAB diversity; however, these studies did not simultaneously evaluate virulence determinants, biogenic amine-producing genes, and vancomycin resistance-parameters essential for the clinical safety validation of *Enterococcus* species. This highlights a clear research gap regarding the comprehensive genomic safety and functional properties of the autochthonous LAB native to this traditional Vietnamese food source.

Therefore, this study aimed to isolate and characterize LAB from traditional Vietnamese pickled *B. juncea*. The primary objectives were to: (i) screen isolates for key in vitro probiotic properties using quantitative growth resilience metrics under simulated gastrointestinal stressors; (ii) evaluate safety through phenotypic assays and molecular screening for a broad panel of virulence and resistance genes; and (iii) determine antagonistic activity against foodborne pathogens. This study was guided by the hypothesis that lactic acid bacteria isolated from traditional Vietnamese pickled *B. juncea* exhibit strain-specific probiotic and antimicrobial properties, and that selected isolates can tolerate simulated gastrointestinal conditions while maintaining a favorable in vitro safety profile. Specifically, the study tested whether individual isolates differ in their acid and bile tolerance, antimicrobial activity against common foodborne pathogens, and absence of key virulence and biogenic amine-associated traits, and identified safe, indigenous strains with the potential for development as novel biopreservatives or probiotic candidates in the food industry.

MATERIALS AND METHODS

Sample collection and isolation of lactic acid bacteria

A total of 60 samples of traditional Vietnamese pickled *B. juncea* (*Dua cai muoi chua*) were collected from ten distinct local markets across Vinh Long Province, Vietnam, between October and December 2024. These markets were selected to represent a diverse geographic distribution across the province's wards. To ensure microbial diversity, samples were sourced from at least three independent artisanal producers per market. Samples were obtained from batches at peak fermentation (5-7 days) with an estimated salt concentration of 3-5% (w/v) and processed immediately upon aseptic transport to the laboratory. For isolation, 1 g of each sample was homogenized in 9 mL of sterile 0.85% (w/v) saline. The suspension was serially diluted (10^{-1} to 10^{-6}), and 100 µL aliquots were spread-plated in triplicate onto MRS agar (Himedia, India) supplemented with 0.5% $CaCO_3$ and 1% NaCl to select for acid-producing and salt-tolerant isolates. Plates were incubated anaerobically using an AnaeroGen gas pack system (Oxoid, UK) at 37°C for 48 h.

Phenotypic characterization and selection

Following incubation, 110 presumptive LAB colonies exhibiting distinct morphologies and clear acid-production halos were selected. Isolates were screened for Gram-staining reaction and catalase activity. Only Gram-positive, catalase-negative, and non-motile isolates were retained. The remaining 64 isolates were purified and subjected to biochemical assays, including urease activity, citrate utilization, and carbohydrate fermentation (glucose, lactose, and sucrose), conducted at 37°C for 24-48 h (Kang et al. 2020; Rahmawati et al. 2021). Results were compared against standard LAB profiles, and any ambiguous biochemical reactions were resolved by re-testing in triplicate. Confirmed LAB isolates were cryopreserved in MRS broth containing 20% (v/v) glycerol at -20°C.

In vitro screening for growth resilience under gastrointestinal stress

The growth resilience of the 64 isolates to simulated gastrointestinal stressors was evaluated following a modified protocol by Serrano-Nino et al. (2016). In this study, optical density (OD_{620}) served as a quantitative proxy to monitor biomass accumulation and growth trends under stress. While OD_{620} does not distinguish between live and dead cells, it provides a practical high-throughput metric for comparative growth resilience. Standardized cell suspensions (approx. 10^8 CFU/mL) were prepared by resuspending washed MRS-grown pellets in fresh broth.

Acid tolerance assessment

Stringent screening for acid resilience was conducted by inoculating 1 mL of the cell suspension into 9 mL of unmodified MRS broth adjusted to pH 2.0 and 3.0 using 1M HCl. Standard MRS broth (pH 6.5) served as the control. This 12 h incubation period was designed as a high-stress selection pressure rather than a physiological simulation of gastric transit. Growth resilience was monitored by measuring OD_{620} at 3, 6, 9, and 12 h.

Bile salt tolerance assessment

Based on the acid resilience results, the 20 most robust isolates were tested for growth in MRS broth supplemented with 0.3, 0.5, and 1.0% (w/v) oxgall bile salts (Oxoid, UK). Tolerance was quantitatively defined as the ability to maintain an $OD_{620} \geq 0.15$ throughout a 12-hour period at 1.0% bile salts, a concentration exceeding the standard physiological range.

Safety assessment

Hemolytic activity was assessed by streaking overnight cultures onto blood agar base plates (Oxoid, UK) supplemented with 5% (v/v) sterile sheep blood (Unban et al. 2021). After incubation at 37°C for 48 h, hemolysis was classified as β-hemolysis (clear zones), α-hemolysis (greenish zones), or γ-hemolysis (no change). Observations were recorded at both 24 and 48 h to ensure clarity of the reactions. Only isolates exhibiting γ-hemolysis (non-hemolytic) were considered for further validation. *Staphylococcus aureus* ATCC 25923 served as the positive control for β-hemolysis. This assay was performed in triplicate.

Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method on MRS agar. Although no official clinical breakpoints exist for enterococci on MRS, susceptibility was categorized as sensitive (≥ 20 mm), intermediate (15-19 mm), or resistant (≤ 14 mm) based on literature applying similar conditions to LAB (Alebiosu et al. 2017; Nguyen et al. 2023). Eight antibiotics were tested: Doxycycline (30 µg), Ampicillin (10 µg), Streptomycin (10 µg), Kanamycin (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Ciprofloxacin (5 µg), and Chloramphenicol (30 µg). Each test was conducted in triplicate.

Antagonistic activity assessment

The antimicrobial potential of the 20 selected isolates was evaluated using the agar well diffusion method (Abubakr 2018) against *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, and *Salmonella enterica* serovar Typhimurium ATCC 14028. To isolate the effects of antimicrobial compounds from organic acids, the cell-free supernatant (CFS) was collected via centrifugation (10,000 rpm, 5 min, 4°C) and neutralized to pH 7.0 with 1 M NaOH. Target pathogens (10^6 CFU/mL) were swabbed onto Nutrient Agar, and 100 µL of neutralized CFS was added to 6 mm diameter wells. A 10 µg Ampicillin disk served as the positive control, while neutralized sterile MRS broth served as the negative control. After 24 h at 37°C, inhibition zones were measured. Activity was classified as strong (>25 mm), moderate (13-25 mm), weak (1-12 mm), or inactive (Rabaoui et al. 2023). All tests were performed in triplicate.

Molecular and genotypic analysis

Genotypic safety screening was performed via PCR to detect virulence factors (*esp, gelE, fsrB, asa1, cylA, cylM*), biogenic amine genes (*hdc1, hdc2, tdc*), and vancomycin resistance genes (*vanA, vanB*). Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA). PCR was conducted using gene-specific primers (Table 1) and DreamTaq Green PCR Master Mix (Thermo Scientific), with products visualized on 2% agarose gels (Nguyen et al. 2025).

The isolate with the highest probiotic potential (CC3) was identified through 16S rRNA gene sequencing. The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Liu et al. 2020). PCR was conducted using GoTaq® Green Master Mix (Promega, USA) with the following thermal profile: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 45 s, 57°C for 60 s (optimized to ensure primer specificity), and 72°C for 90 s; followed by a final extension at 72°C for 5 min. PCR products (~1,500 bp) were confirmed via 1% agarose gel electrophoresis, purified using a QIAquick PCR Purification Kit (Qiagen, Germany), and sequenced by Next Gen Scientific Co., Ltd (Vietnam). Sequences were compared against the NCBI database using BLASTn. Phylogenetic relationships were analyzed via the Neighbor-Joining method in MEGA6 with 1,000 bootstrap replicates, incorporating closely related *Enterococcus* species and appropriate outgroups.

Table 1. Primers used for the detection of virulence factors and antibiotic resistance 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	1,03	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	1,1	Coton et al. (2004)
		Tdc R	CAAATGGAAGAAGAAGTAGG		

Statistical analysis

All assays were performed in triplicate, and data are presented as mean ± standard deviation. The significance of differences between strains and conditions was determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Statistical results were indicated in the data tables using different superscript letters to denote significance levels. Analyses were performed using SPSS software (Version 22.0, IBM Corp., USA) (Ding et al. 2017).

RESULTS AND DISCUSSION

Isolation and characterization of Lactic Acid Bacteria

From the 60 samples of *Dua cai muoi chua* collected across Vinh Long province, 110 presumptive isolates were initially retrieved. Following a primary screen for acid production on $CaCO_3$-supplemented MRS agar, 64 isolates were selected for comprehensive phenotypic profiling. As summarized in Table 2, all 64 isolates were confirmed as Gram-positive, catalase-negative, and non-motile. Furthermore, they demonstrated a uniform ability to ferment glucose, sucrose, and lactose, while testing negative for urease activity and citrate utilization. While these shared biochemical markers presumptively categorize the entire collection as Lactic Acid Bacteria (LAB), further differentiation was achieved through their varying degrees of stress tolerance and subsequent molecular identification.

Resilience to simulated gastrointestinal stress

The 64 isolates were subjected to acid and bile salt challenges to evaluate their potential for gastric survival. A quantitative selection metric was applied, ranking isolates based on their growth resilience (OD_{620}) across all stress conditions. Tables 3 and 4 summarize the performance of the top 20 candidates.

Acid tolerance: Exposure to pH 2.0 and 3.0 revealed significant variation in acid resilience among the isolates ($p < 0.05$). At the most stringent condition (pH 2.0), growth was markedly inhibited for most strains; however, isolate CC3 maintained a significantly higher OD_{620} (0.18 ± 0.10) after 12 h compared to the majority of the collection, followed by CC10 (0.23 ± 0.07) and CC6 (0.20 ± 0.06) (Table 3). These results highlight the ability of specific indigenous strains to maintain biomass under low-pH selection pressure.

Bile salt tolerance: When challenged with bile salts, exposure to 1.0% oxgall significantly inhibited the growth of most isolates. Resilience was quantitatively defined as maintaining an $OD_{620} \geq 0.15$ throughout the 12 h period. Based on this threshold, strains CC3, CC4, CC11, and CC16 demonstrated robust growth resilience at 1.0% bile salts (Table 4). By integrating the quantitative rankings from both the acid and bile assays, CC3 and CC16 were identified as the most resilient candidates for further safety and functional characterization.

Safety profiling: Phenotypic and genotypic assessment

Safety is a critical prerequisite for selecting *Enterococcus* species from food sources. Phenotypically, all 20 tested isolates exhibited γ-hemolysis on sheep blood agar, indicating a lack of hemolytic activity. However, the selective pressure of the screening process was evident in the antibiotic susceptibility profiles (Table 5). Out of the 20 isolates, 12 (60%) displayed phenotypic resistance to at least four of the tested antibiotics, with several strains (e.g., CC4, CC7, and CC13) exhibiting multidrug resistance across nearly all categories. In contrast, isolate CC3 remained sensitive or intermediate to clinically relevant agents, including Ampicillin and Chloramphenicol.

To distinguish food-derived candidates from pathogenic lineages, genotypic screening was performed via PCR. As detailed in Table 6, while many other isolates were excluded due to undesirable phenotypic traits, isolates CC3 and CC16 tested negative for all screened virulence determinants (*esp, gelE, fsrB, asa1, cylA, cylM*), vancomycin resistance genes (*vanA, vanB*), and biogenic amine-producing genes (*hdc1, hdc2, tdc*). The complete absence of these key genetic risk factors in CC3, compared to the high prevalence of resistance in the broader isolate pool, supports its selection for potential food-based applications.

Antimicrobial activity against foodborne pathogens

The antagonistic potential of the 20 isolates was evaluated against three indicator pathogens using neutralized Cell-Free Supernatant (CFS). While Table 7 summarizes the categorical performance of the group, specific quantitative data for the top candidates are provided in Table 8. Isolate CC3 exhibited the most potent inhibitory spectrum ($p < 0.05$), producing inhibition zones categorized as "Moderate" to "Strong" against *S. aureus* (22.59 ± 1.59 mm), *E. coli* (21.29 ± 1.53 mm), and *S. enterica* (20.66 ± 1.88 mm). The persistence of these effects after pH neutralization suggests that non-acidic antimicrobial compounds mediate the inhibition.

Table 2. Morphological, physiological, and biochemical characteristics of the 64 selected LAB isolates

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 (+)

Note: (+): Positive result, (-): Negative result. All 64 isolates exhibited identical results for the parameters listed

Table 3. Effect of acidic pH (2.0 and 3.0) on the growth of selected LAB isolates over 12 hours

Isolates	pH	3h	6h	9h	12h
CC1	2	0.04 ± 0.01	0.03 ± 0.03	0.11 ± 0.01	0.14 ± 0.05
	3	0.10 ± 0.06	0.18 ± 0.07	0.23 ± 0.08	0.33 ± 0.03
CC2	2	0.24 ± 0.04	0.09 ± 0.08	0.17 ± 0.07	0.17 ± 0.02
	3	0.11 ± 0.05	0.12 ± 0.09	0.13 ± 0.03	0.24 ± 0.06
CC3	2	0.03 ± 0.04	0.03 ± 0.01	0.11 ± 0.02	0.18 ± 0.10
	3	0.17 ± 0.04	0.13 ± 0.02	0.13 ± 0.06	0.24 ± 0.03
CC4	2	0.02 ± 0.02	0.07 ± 0.03	0.14 ± 0.08	0.15 ± 0.08
	3	0.06 ± 0.02	0.09 ± 0.03	0.08 ± 0.03	0.24 ± 0.06
CC5	2	0.07 ± 0.06	0.14 ± 0.06	0.04 ± 0.07	0.13 ± 0.08
	3	0.03 ± 0.02	0.03 ± 0.02	0.04 ± 0.03	0.16 ± 0.08
CC6	2	0.13 ± 0.04	0.20 ± 0.07	0.15 ± 0.05	0.20 ± 0.06
	3	0.25 ± 0.08	0.11 ± 0.09	0.11 ± 0.06	0.20 ± 0.04
CC7	2	0.07 ± 0.02	0.12 ± 0.03	0.09 ± 0.01	0.15 ± 0.04
	3	0.10 ± 0.09	0.12 ± 0.07	0.11 ± 0.04	0.10 ± 0.03
CC8	2	0.04 ± 0.03	0.07 ± 0.04	0.07 ± 0.01	0.11 ± 0.07
	3	0.05 ± 0.05	0.10 ± 0.06	0.13 ± 0.11	0.12 ± 0.11
CC9	2	0.05 ± 0.05	0.05 ± 0.03	0.05 ± 0.03	0.12 ± 0.03
	3	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.07	0.16 ± 0.11
CC10	2	0.17 ± 0.05	0.17 ± 0.05	0.11 ± 0.02	0.23 ± 0.07
	3	0.16 ± 0.16	0.20 ± 0.16	0.12 ± 0.02	0.27 ± 0.08
CC11	2	0.14 ± 0.05	0.12 ± 0.07	0.06 ± 0.03	0.08 ± 0.05
	3	0.19 ± 0.15	0.23 ± 0.18	0.09 ± 0.09	0.11 ± 0.08
CC12	2	0.08 ± 0.09	0.09 ± 0.07	0.09 ± 0.01	0.11 ± 0.05
	3	0.10 ± 0.04	0.12 ± 0.10	0.13 ± 0.10	0.12 ± 0.09
CC13	2	0.04 ± 0.03	0.12 ± 0.09	0.11 ± 0.04	0.11 ± 0.05
	3	0.04 ± 0.02	0.11 ± 0.05	0.11 ± 0.09	0.19 ± 0.03
CC14	2	0.09 ± 0.02	0.09 ± 0.03	0.08 ± 0.04	0.06 ± 0.06
	3	0.16 ± 0.07	0.16 ± 0.07	0.14 ± 0.09	0.12 ± 0.03
CC15	2	0.07 ± 0.06	0.10 ± 0.09	0.05 ± 0.04	0.09 ± 0.03
	3	0.01 ± 0.01	0.06 ± 0.02	0.06 ± 0.08	0.09 ± 0.08
CC16	2	0.13 ± 0.03	0.16 ± 0.06	0.12 ± 0.02	0.16 ± 0.07
	3	0.06 ± 0.03	0.04 ± 0.02	0.03 ± 0.02	0.04 ± 0.05
CC17	2	0.08 ± 0.04	0.07 ± 0.05	0.09 ± 0.04	0.06 ± 0.06
	3	0.05 ± 0.01	0.05 ± 0.03	0.06 ± 0.05	0.18 ± 0.14
CC18	2	0.10 ± 0.08	0.07 ± 0.09	0.13 ± 0.07	0.10 ± 0.06
	3	0.04 ± 0.04	0.03 ± 0.02	0.12 ± 0.09	0.09 ± 0.14
CC19	2	0.06 ± 0.03	0.08 ± 0.07	0.26 ± 0.12	0.07 ± 0.02
	3	0.04 ± 0.03	0.03 ± 0.01	0.04 ± 0.03	0.06 ± 0.02
CC20	2	0.03 ± 0.02	0.09 ± 0.06	0.11 ± 0.01	0.14 ± 0.04
	3	0.09 ± 0.04	0.14 ± 0.09	0.11 ± 0.05	0.18 ± 0.12

Note: Data are presented as mean optical density (OD_{620}) ± standard deviation (n=3)

Molecular identification and phylogenetic analysis

Based on its superior resilience, safety profile, and antimicrobial activity, isolate CC3 was selected for molecular identification. 16S rRNA gene sequencing yielded a 1,500 bp amplicon. BLASTn analysis confirmed a 99.47% sequence identity with *Enterococcus faecalis* (NR_114782.1) (Table 8). Phylogenetic analysis using the Neighbor-Joining method clustered CC3 within the *E. faecalis* clade with a high bootstrap value, confirming its identity as *E. faecalis* CC3 (GenBank accession: PX928037). The phylogenetic tree (Figure 1) illustrates its close relationship to reference strains while remaining distinct within its ecological clade.

Discussion

Ecological significance of autochthonous Lactic Acid Bacteria

Traditional fermented foods are increasingly recognized as vital reservoirs of microbial diversity, often harboring Lactic Acid Bacteria (LAB) with functional adaptations specific to their local ecological niches (Fadare et al. 2023). Unlike commercial fermentations that rely on standardized starter cultures, spontaneous processes such as the production of Vietnamese *Dua cai muoi chua* exert intense selective pressure that favors strains capable of rapid adaptation to fluctuating salinity and rapid acidification. While species such as *L. plantarum* and *Levilactobacillus brevis* are commonly documented as the dominant microbiota in vegetable fermentations (Lee et al. 2021; Lin et al. 2023; Nguyen et al. 2023).

Table 4. Effect of bile salt concentrations (0.3, 0.5, and 1.0%) on the growth of selected LAB isolates

Isolates	Bile (%)	3h	6h	9h	12h
CC1	0.3%	0.16 ± 0.04	0.19 ± 0.04	0.17 ± 0.07	0.31 ± 0.12
	0.5%	0.25 ± 0.01	0.28 ± 0.02	0.34 ± 0.02	0.50 ± 0.12
	1.0%	0.21 ± 0.04	0.19 ± 0.05	0.23 ± 0.14	0.32 ± 0.17
CC2	0.3%	0.18 ± 0.02	0.17 ± 0.03	0.14 ± 0.03	0.38 ± 0.05
	0.5%	0.23 ± 0.02	0.23 ± 0.03	0.25 ± 0.04	0.25 ± 0.06
	1.0%	0.22 ± 0.03	0.20 ± 0.02	0.19 ± 0.04	0.19 ± 0.02
CC3	0.3%	0.17 ± 0.04	0.18 ± 0.06	0.21 ± 0.03	0.34 ± 0.07
	0.5%	0.22 ± 0.02	0.23 ± 0.02	0.32 ± 0.03	0.42 ± 0.05
	1.0%	0.20 ± 0.01	0.20 ± 0.02	0.28 ± 0.05	0.34 ± 0.08
CC4	0.3%	0.19 ± 0.07	0.22 ± 0.10	0.23 ± 0.15	0.54 ± 0.06
	0.5%	0.21 ± 0.02	0.20 ± 0.03	0.21 ± 0.08	0.54 ± 0.05
	1.0%	0.19 ± 0.01	0.18 ± 0.03	0.18 ± 0.01	0.46 ± 0.07
CC5	0.3%	0.16 ± 0.02	0.18 ± 0.03	0.18 ± 0.08	0.24 ± 0.05
	0.5%	0.10 ± 0.24	0.25 ± 0.01	0.27 ± 0.01	0.30 ± 0.03
	1.0%	0.18 ± 0.02	0.17 ± 0.02	0.19 ± 0.03	0.18 ± 0.08
CC6	0.3%	0.16 ± 0.06	0.17 ± 0.06	0.14 ± 0.14	0.35 ± 0.11
	0.5%	0.25 ± 0.01	0.28 ± 0.01	0.31 ± 0.02	0.48 ± 0.17
	1.0%	0.19 ± 0.02	0.20 ± 0.04	0.20 ± 0.04	0.30 ± 0.06
CC7	0.3%	0.14 ± 0.05	0.17 ± 0.02	0.16 ± 0.06	0.34 ± 0.02
	0.5%	0.26 ± 0.00	0.28 ± 0.02	0.29 ± 0.02	0.49 ± 0.10
	1.0%	0.19 ± 0.01	0.21 ± 0.01	0.21 ± 0.00	0.29 ± 0.07
CC8	0.3%	0.14 ± 0.02	0.14 ± 0.00	0.17 ± 0.01	0.35 ± 0.02
	0.5%	0.21 ± 0.02	0.23 ± 0.03	0.26 ± 0.03	0.45 ± 0.02
	1.0%	0.17 ± 0.01	0.18 ± 0.00	0.19 ± 0.01	0.33 ± 0.05
CC9	0.3%	0.18 ± 0.01	0.19 ± 0.01	0.22 ± 0.03	0.43 ± 0.02
	0.5%	0.21 ± 0.04	0.21 ± 0.02	0.23 ± 0.02	0.46 ± 0.03
	1.0%	0.19 ± 0.02	0.20 ± 0.03	0.21 ± 0.04	0.37 ± 0.08
CC10	0.3%	0.18 ± 0.01	0.23 ± 0.07	0.23 ± 0.06	0.32 ± 0.06
	0.5%	0.26 ± 0.02	0.26 ± 0.02	0.28 ± 0.02	0.47 ± 0.11
	1.0%	0.17 ± 0.05	0.19 ± 0.04	0.21 ± 0.05	0.32 ± 0.17
CC11	0.3%	0.16 ± 0.03	0.17 ± 0.03	0.18 ± 0.01	0.34 ± 0.12
	0.5%	0.22 ± 0.02	0.23 ± 0.03	0.26 ± 0.03	0.40 ± 0.01
	1.0%	0.19 ± 0.01	0.20 ± 0.02	0.21 ± 0.03	0.39 ± 0.07
CC12	0.3%	0.07 ± 0.11	0.10 ± 0.11	0.08 ± 0.06	0.01 ± 0.14
	0.5%	0.34 ± 0.12	0.37 ± 0.12	0.36 ± 0.07	0.28 ± 0.10
	1.0%	0.07 ± 0.13	0.01 ± 0.22	0.03 ± 0.08	0.03 ± 0.08
CC13	0.3%	0.14 ± 0.04	0.19 ± 0.05	0.08 ± 0.07	0.10 ± 0.05
	0.5%	0.36 ± 0.07	0.38 ± 0.10	0.38 ± 0.07	0.35 ± 0.13
	1.0%	0.17 ± 0.11	0.11 ± 0.23	0.04 ± 0.03	0.10 ± 0.11
CC14	0.3%	0.17 ± 0.03	0.20 ± 0.03	0.08 ± 0.07	0.16 ± 0.02
	0.5%	0.40 ± 0.14	0.40 ± 0.16	0.37 ± 0.13	0.39 ± 0.13
	1.0%	0.19 ± 0.03	0.20 ± 0.03	0.04 ± 0.04	0.09 ± 0.12
CC15	0.3%	0.14 ± 0.02	0.19 ± 0.01	0.03 ± 0.12	0.09 ± 0.08
	0.5%	0.35 ± 0.09	0.35 ± 0.11	0.33 ± 0.10	0.30 ± 0.05
	1.0%	0.19 ± 0.01	0.12 ± 0.05	0.06 ± 0.14	0.03 ± 0.17
CC16	0.3%	0.21 ± 0.03	0.25 ± 0.04	0.22 ± 0.09	0.25 ± 0.06
	0.5%	0.24 ± 0.05	0.26 ± 0.06	0.28 ± 0.08	0.35 ± 0.11
	1.0%	0.22 ± 0.03	0.21 ± 0.03	0.22 ± 0.06	0.32 ± 0.14
CC17	0.3%	0.22 ± 0.08	0.22 ± 0.12	0.16 ± 0.01	0.21 ± 0.11
	0.5%	0.33 ± 0.14	0.35 ± 0.16	0.30 ± 0.13	0.31 ± 0.15
	1.0%	0.22 ± 0.02	0.21 ± 0.01	0.16 ± 0.06	0.06 ± 0.13
CC18	0.3%	0.18 ± 0.03	0.20 ± 0.02	0.13 ± 0.05	0.20 ± 0.03
	0.5%	0.26 ± 0.05	0.30 ± 0.01	0.26 ± 0.07	0.25 ± 0.04
	1.0%	0.20 ± 0.02	0.22 ± 0.02	0.20 ± 0.07	0.13 ± 0.04
CC19	0.3%	0.19 ± 0.03	0.24 ± 0.09	0.21 ± 0.25	0.20 ± 0.08
	0.5%	0.33 ± 0.08	0.34 ± 0.07	0.35 ± 0.07	0.29 ± 0.08
	1.0%	0.22 ± 0.04	0.23 ± 0.05	0.20 ± 0.06	0.12 ± 0.06
CC20	0.3%	0.13 ± 0.03	0.11 ± 0.06	0.12 ± 0.09	0.54 ± 0.06
	0.5%	0.18 ± 0.02	0.17 ± 0.01	0.17 ± 0.02	0.45 ± 0.07
	1.0%	0.15 ± 0.02	0.15 ± 0.01	0.14 ± 0.01	0.44 ± 0.10

Note: Data are presented as mean optical density (OD_{620}) ± standard deviation (n=3)

Table 5. Antibiotic susceptibility profiles of the 20 selected LAB isolates

Isolates	CIP	DO	CN	STR	AMP	E	C	K
CC1	S	S	S	R	I	S	S	I
CC2	S	S	S	I	S	S	I	I
CC3	S	S	S	S	S	I	I	I
CC4	R	R	R	R	R	S	R	R
CC5	S	R	R	R	R	S	R	R
CC6	R	R	R	R	S	R	R	R
CC7	R	R	R	R	R	R	R	R
CC8	I	R	R	R	R	R	R	R
CC9	S	S	S	I	I	S	S	I
CC10	I	R	I	R	R	R	R	R
CC11	S	R	I	R	S	R	R	R
CC12	I	R	R	R	R	R	R	R
CC13	R	R	R	R	R	R	R	R
CC14	R	R	R	R	S	S	I	R
CC15	S	S	I	R	R	R	R	R
CC16	S	S	I	I	S	S	S	I
CC17	R	R	R	R	I	R	R	R
CC18	R	R	R	R	R	R	R	R
CC19	S	R	I	R	S	R	R	R
CC20	I	R	R	R	I	R	I	R

Note: Susceptibility was determined by disk diffusion. AMP: Ampicillin (10 µg), C: Chloramphenicol (30 µg), CIP: Ciprofloxacin (5 µg), CN: Gentamicin (10 µg), DO: Doxycycline (30 µg), E: Erythromycin (15 µg), K: Kanamycin (30 µg), STR: Streptomycin (10 µg). Classification: R: Resistant, I: Intermediate, S: Susceptible

Table 6. PCR screening results for virulence factors, antibiotic resistance determinants, and biogenic amine-producing genes

Category	Target gene	CC2	CC3	CC16
Virulence factors	*esp*	-	-	-
	gelE	-	-	-
	fsrB	-	-	-
	asa1	-	-	-
	cylA	-	-	-
	cylM	-	-	-
Antibiotic resistance	*vanA*	-	-	-
	vanB	-	-	-
Biogenic amines	*hdc1*	-	-	-
	hdc2	-	-	-
	tdc	-	-	-

Note: (-) indicates the absence of the target gene (negative PCR result). No amplicons were detected for any of the tested genes in strains CC2, CC3, or CC16

Table 7. Spectrum of antimicrobial activity of the 20 LAB isolates against indicator foodborne pathogens

Indicator pathogen	Strong	Moderate	Weak	Inactive	Total active isolates (%)
Escherichia coli ATCC 25922	0	6	3	11	9 (45.00 %)
Salmonella enterica ATCC 14028	0	3	8	9	11 (55.00 %)
Staphylococcus aureus ATCC 25923	0	5	3	12	8 (40.00 %)

Note: Inhibition was categorized based on zone diameter: Strong (>25 mm), Moderate (13-25 mm), Weak (1-12 mm) or Inactive (No inhibition zone)

Table 8. BLASTn alignment statistics for the 16S rRNA gene sequence of isolate CC3 against the NCBI database

Closely related species	Max score	Query coverage (%)	E-value	Identity (%)	Accession No.
Enterococcus faecalis strain LMG 7937 16S ribosomal RNA, partial sequence	2771	97 %	0.0	99.47%	NR_114782.1

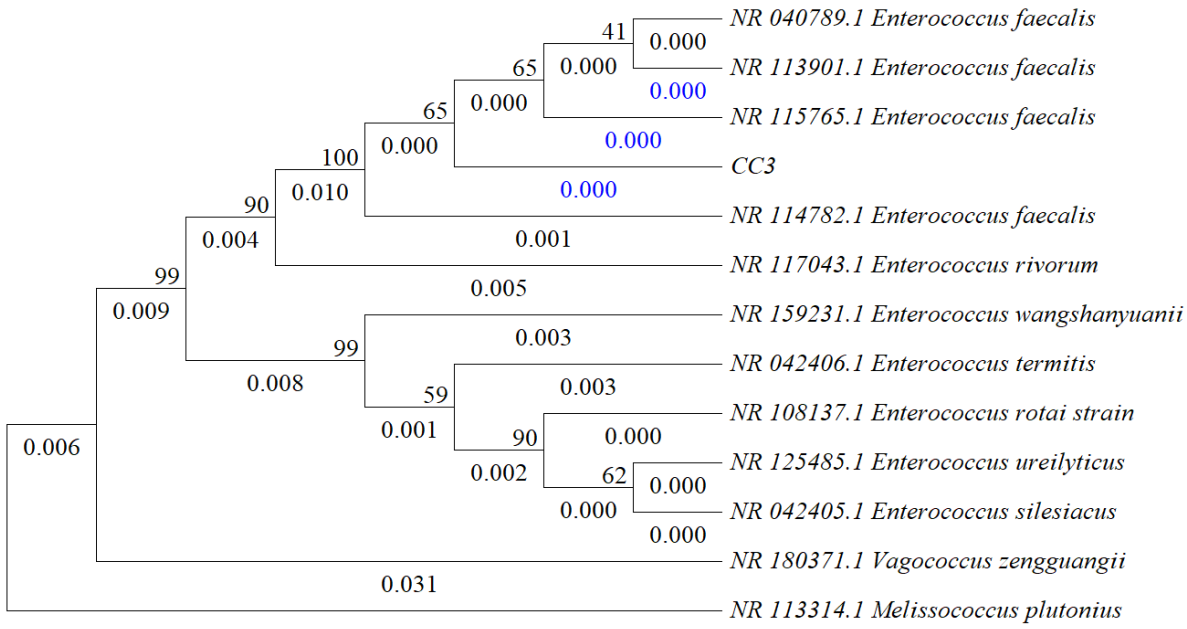

Figure 1. Phylogenetic relationships of isolate CC3 and related *Enterococcus faecalis* based on 16S rRNA gene sequences. The tree was constructed using the Neighbor-Joining method. Bootstrap values (expressed as percentages of 1,000 replicates) are shown at the nodes. The scale bar represents the number of nucleotide substitutions per site

Our findings confirm that *Enterococcus* species also constitute a significant component of this microbial ecosystem. The isolation of *E. faecalis* CC3 suggests that this strain is autochthonous to the pickled *B. juncea* matrix, likely having evolved specific metabolic traits to survive the harsh environment of the pickle. This presence aligns with studies on traditional Asian ferments (Li and Gu 2019; Banik et al. 2023), which suggests that enterococci contribute to the fermentation process, particularly regarding acidification and the development of unique flavor profiles. However, while its isolation is noteworthy, it is important to tone down claims about an "integral role" in the fermentation process itself, as direct evidence of its functional contribution to the *Dua cai muoi chua* sensory or chemical profile was not provided by this study; its role remains a hypothesis based on its proven in vitro attributes and presence at peak fermentation.

Gastrointestinal competence: Acid and bile tolerance

To confer a health benefit, a potential probiotic must survive the harsh physicochemical barriers of the upper gastrointestinal (GI) tract (Fijan 2016). The primary barrier is the gastric environment, where pH levels can drop to 2.0. Our results demonstrate that *E. faecalis* CC3 possesses exceptional acid tolerance, maintaining high viability after 12 hours of exposure to pH 2.0. This resilience may be associated with an intrinsic Acid Tolerance Response (ATR)-like mechanism, where bacteria alter their cell membrane composition and proton pump activity to maintain pH homeostasis (Tokatl et al. 2015). Given the strain's origin in a high-acid pickle environment, it is plausible that the niche selects for a robust ATR, allowing the strain to thrive where others fail. Following gastric transit, probiotics must withstand the detergent-like action of bile salts (Kushnir 2020; Luo et al. 2022; Kathiriya et al. 2023). Remarkably, *E. faecalis* CC3 demonstrated growth in the presence of 1.0% bile salts, a concentration significantly exceeding the physiological range of 0.3% (Ayyash et al. 2021). The hyper-tolerance observed in CC3 may indicate the presence of active Bile Salt Hydrolase (BSH) activity. In LAB, BSH activity is a vital trait for intestinal survival and is often linked to host health benefits, such as cholesterol reduction (Castorena-Alba et al. 2018; Suwannaphan 2021). However, as neither ATR genes nor BSH enzymatic activity were directly measured, these mechanisms are currently inferred from the literature rather than established as confirmed traits for strain CC3.

Antimicrobial activity and biopreservation potential

The ability to inhibit foodborne pathogens is a critical criterion for selecting probiotic and biopreservative agents. *E. faecalis* CC3 demonstrated broad-spectrum antagonistic activity, significantly inhibiting *S. enterica*, *E. coli*, and *S. aureus*. Crucially, the Cell-Free Supernatant (CFS) was neutralized to pH 7.0 prior to testing. This confirms that the observed inhibition was not merely due to organic acid accumulation, but rather suggests the involvement of non-acidic compounds, potentially bacteriocins or enterocins (Hatem et al. 2024). These findings align with recent work validating the antibacterial efficacy of enterococci from fermented foods (Han et al. 2024; Nguyen et al. 2025). The significant inhibition of *S. aureus* (22.59 mm zone) is particularly promising for biopreservation. As *S. aureus* is a common contaminant in handled foods, strain CC3 likely produces metabolites that target the cell wall synthesis of Gram-positive pathogens (Tatsaporn and Kornkanok 2020). However, concluding that this activity is definitely due to bacteriocins remains preliminary. While the retention of activity after neutralization is indicative of such

compounds, confirmation requires proteinase sensitivity tests and proteomic analysis to rule out other metabolites.

Safety assessment

The use of *Enterococcus* species in food requires a rigorous safety assessment due to their association with nosocomial infections and antibiotic resistance transfer (Hanchi et al. 2018). This study provides an initial in vitro safety evaluation of a food-derived strain, not a clinical validation. Phenotypically, *E. faecalis* CC3 is γ-hemolytic, ensuring it does not possess hemolysins (Unban et al. 2021). Genotypically, PCR screening confirmed the absence of key virulence determinants, including surface proteins (*esp*), gelatinase (*gelE*), and cytolysin (*cylA/cylM*) (Kanak et al. 2023). Furthermore, the antibiotic resistance profile of CC3 distinguishes it from the broader isolate pool; while 60% of our isolates displayed multi-drug resistance, CC3 remained sensitive to clinically relevant agents like Ampicillin and Chloramphenicol (Nami et al. 2019). The absence of virulence and vancomycin resistance genes (*vanA, vanB*) genetically demarcates strain CC3 from hospital-adapted pathotypes (Dutka-Malen et al. 1995). Additionally, the absence of decarboxylase genes (*hdc1, hdc2, tdc*) confirms its inability to produce toxic biogenic amines (Câmara et al. 2020). While CC3 exhibits traits consistent with safe food-associated strains, these results are preliminary. Future research must prioritize Whole Genome Sequencing (WGS) to definitively map genetic safety and mobile elements, alongside *in vivo* studies and adhesion tests to fully substantiate its status as a probiotic.

This study successfully isolated and characterized the indigenous strain *E. faecalis* CC3 from traditional Vietnamese pickled *B. juncea* (*Dua cai muoi chua*). Among 36 isolates screened, CC3 showed superior tolerance to simulated gastrointestinal conditions, maintaining stable growth at pH 2.0-3.0 and in the presence of 0.3-0.5% bile salts. Our findings demonstrate that strain CC3 possesses robust in vitro resilience to simulated gastrointestinal stressors, maintaining biomass accumulation at pH 2.0 and showing hyper-tolerance to 1.0% bile salts. Furthermore, CC3 exhibited broad-spectrum antimicrobial activity against *S. aureus*, *E. coli*, and *S. enterica*. Importantly, the persistence of antagonism following supernatant neutralization suggests the presence of non-acidic antimicrobial metabolites. The preliminary safety profile, characterized by γ-hemolysis and the absence of key virulence (*esp, gelE, cylA, cylM*), biogenic amine (*hdc1, hdc2, tdc*), and vancomycin resistance genes (*vanA, vanB*), distinguishes CC3 from pathogenic clinical lineages. These functional and safety attributes indicate that *E. faecalis* CC3 shows promise as a candidate protective starter culture for enhancing the safety of fermented vegetables. However, to fully validate its biotechnological application, future research must include Whole-Genome Sequencing (WGS) to definitively map mobile genetic elements, alongside in vivo trials and sensory quality assessments to substantiate its efficacy and safety in actual food systems. Limitations of this study include its reliance on in vitro assays, the use of optical density as a proxy for bacterial growth, and safety evaluation limited to targeted gene screening.

ACKNOWLEDGEMENTS

We acknowledge the support of time and facilities from Tra Vinh University (TVU), Vietnam, for this study.

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