BIODIVERSITAS
Volume 27, Number 1, January 2026
Pages: 8

ISSN: 1412-033X
E-ISSN: 2085-4722
https://doi.org/10.13057/biodiv/d270136

Biodiversity and safety profiling of indigenous *Enterococcus faecalis* from *Nem Chua,* a traditional Vietnamese fermented pork ecosystem

NGUYEN PHUONG THUY♥, NGUYEN VIET KHANH HUNG, VO NGUYEN NGOC NHU
School of Agriculture and Aquaculture, Tra Vinh University. Vinh Long Province, Vietnam. Tel./fax.: +84-294-3855692, ♥email: npthuy@tvu.edu.vn

Manuscript received: 25 November 2025. Revision accepted: 26 January 2026.

Abstract. *Thuy NP, Hung NVK, Nhu VNN. 2026. Biodiversity and safety profiling of indigenous* Enterococcus faecalis *from* Nem Chua*, a traditional Vietnamese fermented pork ecosystem. Biodiversitas 27 (1): d270136. https://doi.org/10.13057/biodiv/d270136.* Traditional Vietnamese fermented pork (*Nem Chua*) represents a specialized ecological niche, yet the microbial biodiversity and safety profiles of its non-conventional Lactic Acid Bacteria (LAB) remain insufficiently characterized for functional applications. This study explored the microbial reservoir of *Nem Chua* to isolate and evaluate indigenous LAB with biotechnological potential. Through a systematic multi-stage screening pipeline of 50 artisanal samples, an initial pool of 59 isolates was narrowed to 26 candidates based on functional and safety filters. *Enterococcus faecalis* NC5 was ultimately selected as the optimal candidate due to its superior ecological fitness and validated safety status. Molecular identification confirmed 100% identity with *E. faecalis*. NC5 demonstrated exceptional gastrointestinal resilience, maintaining viability of 4.32±0.05 Log CFU/mL at pH 2.0 and 3.82±0.02 Log CFU/mL in 1.0% bile salts. Furthermore, it exhibited potent broad-spectrum antagonism against *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus*. Safety profiling confirmed γ-hemolytic activity, suggesting the strain's potential as a protective starter culture. Molecular identification based on partial 16S rRNA gene sequencing confirmed NC5 as *E. faecalis* with 100% sequence similarity to reference strains in GenBank. These findings suggest that *E. faecalis* NC5 is a robust and safe autochthonous component of the natural *Nem Chua* microbiota. This research highlights the vital role of preserving fermented-food biodiversity to enhance the safety and functionality of traditional Southeast Asian food systems.

Keywords: Antimicrobial activity, *Enterococcus faecalis,* fermented pork, *Nem Chua,* probiotics

INTRODUCTION

The global trajectory of functional food research has shifted significantly toward the bioprospecting of novel probiotics, defined as live microorganisms that confer health benefits when managed in adequate amounts (Zommiti et al. 2020; Obayomi et al. 2024). While commercial probiotic development has traditionally depended on human intestinal isolates or dairy-derived taxa, recent scientific discourse emphasizes the ecological value of underexplored microbial reservoirs (Manassi et al. 2022). Traditional fermented ecosystems, particularly those in Southeast Asia, represent specialized niches where Lactic Acid Bacteria (LAB) have undergone rigorous natural selection through generations of artisanal production (Sionek et al. 2023). Because probiotic efficacy is strictly strain-specific, systematically exploring the microbial biodiversity of indigenous fermented foods is essential for identifying robust, stress-adapted candidates with high therapeutic and technological potential (Aponte et al. 2020).

Fermented meat products function as complex, high-pressure microbial biodiversity reservoirs where extreme environmental conditions are selected for specialized taxa (Parlindungan et al. 2021). Fermented meat products function as complex, high-pressure microbial biodiversity reservoirs where extreme environmental conditions select for specialized taxa (Chen et al. 2020; Liu et al. 2024). These microorganisms modulate the final sensory attributes of the product while simultaneously serving as a biological security system through the secretion of antimicrobial metabolites, such as organic acids and bacteriocins (Ahansaz et al. 2023; Anumudu et al. 2024). The competitive dynamics inherent in meat fermentation select for strains with exceptional ecological fitness, including tolerance to low pH and high salinity, rendering them ideal candidates for food-grade industrial applications.

Nem Chua, a traditional Vietnamese lactic-fermented pork sausage, stands as a unique ecological niche within the Southeast Asian fermented food landscape. This product is prepared from raw lean pork and boiled pig skin, homogenized with garlic, chili, and spices, and subjected to spontaneous, solid-state anaerobic fermentation (Phong et al. 2017).

Unlike most Western fermented meats, *Nem Chua* is traditionally consumed raw, without a thermal kill-step to ensure safety. This cultural practice highlights the critical role of the indigenous microbiota, which must serve as the primary defensive barrier against opportunistic foodborne pathogens (Pilasombut et al. 2015). Although the dominance of *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus* in *Nem Chua* is well-documented, the broader microbial diversity of this ecosystem, specifically the presence and functional role of non-conventional LAB, remains inadequately characterized.

The identification of *Enterococcus* species within such ecosystems presents a complex safety paradox that requires rigorous investigation. While *Enterococcus* spp. are intrinsic components of the natural *Nem Chua* microbiota and contribute significantly to proteolysis and aroma development, the genus is also scrutinized due to its association with nosocomial infections and the horizontal transfer of antibiotic resistance genes (Pumriw et al. 2021). Consequently, the exploration of indigenous Enterococci requires a comprehensive, safety-filtered approach to differentiate beneficial, food-grade strains from clinical pathotypes. The characterization of safety-validated isolates from artisanal Vietnamese sources represents a significant research gap, as these strains often possess unique stress-adaptation mechanisms evolved specifically for survival in acidified meat matrices. Hence, this study proposes the following hypothesis: A hierarchical, biodiversity-driven screening approach can identify an indigenous *Enterococcus faecalis* strain from *Nem Chua* with superior functional resilience and acceptable in vitro safety characteristics.

This study addresses this research gap by evaluating the microbial biodiversity of *Nem Chua* to isolate and characterize indigenously pre-adapted and safety-vetted LAB. The novelty of this work lies in the systematic integration of biodiversity exploration with safety-filtered functional profiling. Specifically, the research objectives were to: (i) employ a multi-step screening pipeline to identify isolates with superior resilience to simulated gastrointestinal stressors, (ii) quantify the broad-spectrum antagonistic potential of the isolates against prevalent foodborne pathogens, and (iii) conduct an exhaustive safety profiling through phenotypic assays. By framing the characterization of *E. faecalis* NC5 within this biodiversity and safety framework, this study aims to provide a scientific basis for the preservation and application of indigenous microbial heritage in modern food safety systems.

MATERIALS AND METHODS

Stepwise screening pipeline

A four-stage hierarchical pipeline was implemented to ensure only the most resilient isolates were characterized: (i) Stage I: Initial isolation of 80 putative acid-producing colonies. (ii) Stage II: Primary confirmation of 59 LAB isolates. (iii) Stage III: Functional and safety filtering of 26 candidates based on antagonistic and γ-hemolytic status. (iv) Stage IV: Definitive characterization of the isolate, NC5.

Sample collection and Lactic Acid Bacteria isolation

Fifty samples of artisanal *Nem Chua* were purchased from local markets in Vinh Long Province, Vietnam. Samples were transported under aseptic conditions to the laboratory at 4°C and processed within 2 hours. 1 g of each homogenized sample was suspended in 9 mL sterile physiological saline (0.9% w/v NaCl). Following serial dilution to 10^{-6}, 100 µL aliquots were spread-plated in triplicate onto de Man, Rogosa, and Sharpe (MRS) agar (Himedia, India) supplemented with 0.5% (w/v) $CaCO_3$ and 1% (w/v) NaCl to select for acid-producing and salt-tolerant strains. Plates were incubated anaerobically using an AnaeroGen system (Oxoid, UK) for 48 hours at 37°C.

Eighty putative LAB colonies were randomly selected based on distinctive morphology and clear zones of acidification. Purified isolates were initially characterized via Gram-staining and cellular morphology, followed by biochemical verification (Kang et al. 2020; Rahmawati et al. 2021). These tests included catalase, urease, and citrate utilization assays using appropriate positive and negative control strains. For the catalase test, *Staphylococcus aureus* ATCC 25923 (positive control) and *L. plantarum* ATCC 14917 (negative control) were used. For the urease assay, *Proteus mirabilis* ATCC 12453 (positive control) and *Escherichia coli* ATCC 25922 (negative control) were employed. Citrate utilization was assessed using *E. coli* ATCC 25922 (negative control) and *Enterobacter aerogenes* ATCC 13048 (positive control). Fermentation assays confirmed the capability to metabolize glucose, lactose, and sucrose. Fifty-nine isolates meeting the LAB criteria (Gram-positive, catalase-negative, urease-negative, and citrate-negative) were selected as candidate strains and maintained as glycerol stocks (20% v/v) at -80°C.

Antagonistic activity assessment

The antimicrobial activity of the 59 selected isolates was performed using agar well diffusion method against three reference foodborne pathogens: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (Abubakr 2018). The indicator strains were attained from the American Type Culture Collection (ATCC) and maintained on Nutrient Agar slants. LAB isolates were cultured in MRS broth for 24 hours at 37°C. To determine whether inhibition was mediated by bacteriocins or bacteriocin-like inhibitory substances (BLIS), cell-free supernatants (CFS) were obtained via centrifugation (10,000 rpm, 10 min, 4°C) and neutralized to pH 7.0 using 1 M NaOH.

Pathogen cultures were standardized to 10^6 CFU/mL and seeded into Nutrient Agar plates. Wells (6 mm diameter) were punched with a sterile cork bore in the solidified agar and filled with 100 µL of the neutralized CFS. A 10 µg ampicillin disk served as a positive control for inhibition, and neutralized sterile MRS broth was used as a negative control. After incubation at 37°C for 24 hours, the inhibition zone diameters were calculated in millimeters (mm). The assay was performed three times for each isolate against each pathogen, and the mean inhibition zone diameter was used for analysis. Activity was classified as strong (>25 mm), moderate (13-25 mm), weak (1-12 mm), or inactive (no zone) (Rabaoui et al. 2023).

Safety profiling: Hemolytic activity

As a primary safety filter, hemolytic activity was evaluated for the 26 isolates showing superior antagonistic properties. Isolates were streaked in triplicate onto Columbia agar base (Himedia, India) supplemented with 5% (v/v) sterile sheep blood (Unban et al. 2021), and incubated at 37°C for 24 hours. Hemolytic activity was determined by observing the zones around the colonies: β-hemolysis (clear

zones, indicating complete lysis), α-hemolysis (greenish zones, indicating partial lysis), or γ-hemolysis (no change, indicating non-hemolytic activity). Only strains exhibiting γ-hemolysis were considered non-pathogenic and selected for further in-depth safety and probiotic testing.

In vitro probiotic property screening

The resilience of the safe, high-efficacy isolates to simulated gastrointestinal stress was evaluated by measuring survival under acidic and bile salt conditions (Serrano-Nino et al. 2016). For each assay, isolates were grown overnight in MRS broth, harvested by centrifugation (10,000 rpm for 10 minutes at 4°C), washed twice with sterile phosphate-buffered saline (PBS, pH 7.4), and resuspended in MRS broth to a final cell density of approximately 10^8 CFU/mL ($OD_{600} \approx 0.6$).

Acid tolerance assay: 1 mL of the standardized cell suspension was inoculated into 9 mL of MRS broth adjusted to pH 2.0, 3.0, and 4.0 using 1 M HCl. A culture in standard MRS broth (pH 6.5) served as the control. The inoculated broths were incubated at 37°C for 4 hours. These pH values were selected to simulate the dynamic acidity of the human stomach (pH 2.0-3.0) and the transition to the small intestine (pH 4.0), while the 4-hour duration mimics the typical gastric transit time.

Bile salt tolerance assay: 1 mL of the cell suspension was inoculated into 9 mL of MRS broth supplemented with 0.1, 0.2, 0.3, 0.5, or 1.0% (w/v) oxgall bile salts (Sigma-Aldrich). MRS broth without bile salts served as the control. The inoculated broths were incubated at 37°C for 4 hours. The concentration range included physiological levels (0.3%) to assess survival in the small intestine, as well as higher concentrations (up to 1.0%) to evaluate tolerance to extreme bile stress.

For both assays, surviving cells were quantified by spread-plating serial dilutions onto MRS agar. Plates were incubated at 37°C for 24 hours, and then colonies were counted. Survival rate was calculated as the ratio of the final viable count to the initial viable count, expressed as Log_{10} CFU/mL. Each experiment was conducted in triplicate, with results shown as the mean ± standard deviation (SD).

Molecular identification and phylogenetic analysis

The lead candidate (NC5), selected for its superior resilience and safety profile, was identified via 16S rRNA gene sequencing. We extracted Genomic DNA using the Wizard® Genomic DNA Purification Kit (Promega, USA). The 16S rRNA gene was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Liu et al. 2020). PCR was performed on a Veriti thermocycler with GoTaq® Green Master Mix (Promega, USA) under standard conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. The ~1,500 bp PCR product was confirmed by 1% agarose gel electrophoresis, purified using a QIAquick PCR Purification Kit (Qiagen, Germany), and sequenced at Next Gen Scientific Co., Ltd (Ho Chi Minh City, Vietnam). Raw sequences were edited in BioEdit (v. 7.0) and identified using the NCBI BLASTn tool (requiring ≥99% identity). A phylogenetic tree was constructed using the Neighbor-Joining method in MEGA6 with 1,000 bootstrap replicates (Ding et al. 2017).

Statistical and bioinformatic analysis

All experimental assays were performed in triplicate (n=3). Quantitative results are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post-hoc test. This statistical framework was explicitly applied to evaluate the significance of antimicrobial zones, acid tolerance viability, and bile salt survival data. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Isolation and phenotypic characterization of autochthonous Lactic Acid Bacteria

The primary isolation phase from 50 artisanal *Nem Chua* samples yielded 80 presumptive colonies identified by clear zones of acidification on selective MRS agar. Subsequent purification and biochemical screening confirmed 59 isolates as Lactic Acid Bacteria (LAB). As summarized in Table 1, these isolates exhibited a consistent phenotypic profile, characterized as Gram-positive, non-motile coccobacilli or rods that were negative for catalase, urease, and citrate utilization. Additionally, all 59 isolates successfully fermented glucose, sucrose, and lactose, establishing a verified pool of LAB for functional evaluation.

Table 1. Morphological, physiological, and biochemical characteristics of the 59 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: (+) indicates a positive result; (-) indicates a negative result. All 59 isolates exhibited identical results for these parameters

Antagonistic activity against foodborne pathogens

The 59 confirmed LAB isolates were evaluated for their ability to inhibit key foodborne pathogens using neutralized cell-free supernatants. The distribution of antimicrobial activity is summarized in Table 2. Inhibition was most frequently observed against *S. aureus* (44.07% of isolates), followed by *E. coli* (40.68%) and *S. enterica* (25.42%). While the majority of active isolates produced weak inhibition zones (<12 mm), isolates NC4, NC11, and NC22 demonstrated significantly stronger ($p<0.05$) broad-spectrum antagonism, consistently inhibiting all three indicator pathogens. Based on these efficacy results and preliminary resilience screening, 26 isolates were prioritized for safety and gastrointestinal survival assessments.

Safety profiling: Hemolytic activity

A preliminary safety screening was conducted on the 26 prioritized high-efficacy isolates. In the hemolytic assay, 100% of the tested isolates exhibited γ-hemolysis, showing no clearing or discoloration of the blood agar. This confirmed the non-hemolytic status of all 26 candidates, allowing their progression to simulate gastrointestinal stress testing.

In vitro resilience to gastrointestinal stressors

The survival capacity of the 26 safe isolates under simulated gastric and intestinal conditions revealed distinct resilience patterns among the strains.

Acid tolerance

As shown in Table 3, viability was maintained at high levels (typically >8.0 Log CFU/mL) at pH 4.0; however, population counts decreased significantly as the acidity reached pH 2.0 ($p<0.05$). Isolate NC4 demonstrated the highest acid resilience, maintaining a viable count of 5.44±0.07 Log CFU/mL at pH 2.0. Six other notable candidates (NC1, NC3, NC9, NC11, NC16, and NC19) also maintained survival thresholds exceeding 4.0 Log CFU/mL, while several isolates (e.g., NC2, NC6, NC7) showed no detectable survival at this extreme pH.

Table 2. Distribution of antimicrobial activity of 59 bacterial isolates against key foodborne pathogens

Indicator pathogen	Strong (>25 mm)	Moderate (13-25 mm)	Weak (1-12 mm)	Inactive (No zone)	Total active isolates (%)
Escherichia coli ATCC 25922	0	11	13	35	24 (40.68%)
Salmonella enterica ATCC 14028	1	7	7	44	15 (25.42%)
Staphylococcus aureus ATCC 25923	0	12	14	33	26 (44.07%)

Note: Activity is categorized based on the diameter of the inhibition zone, including the well diameter (6 mm)

Table 3. Viability of 26 selected isolates under simulated gastric conditions (pH 4.0, 3.0, and 2.0)

ID Isolate	Viable bacteria isolates (Log CFU/mL)			
	pH 6.50 (Control)	pH 4.00	pH 3.00	pH 2.00
NC1	9.86±0.07	8.33±0.03	5.54±0.06	4.39±0.03
NC2	8.86±0.10	8.00±0.07	5.58±0.07	0.00±0.00
NC3	8.84±0.04	8.43±0.02	7.57±0.08	5.39±0.11
NC4	**10.09±0.02**	**8.15±0.03**	**6.56±0.07**	**5.44±0.07**
NC5	8.82±0.02	7.32±0.02	7.31±0.21	4.32±0.05
NC6	8.81±0.03	8.44±0.05	0.00±0.00	0.00±0.00
NC7	9.87±0.09	8.28±0.02	6.50±0.03	0.00±0.00
NC8	8.82±0.03	8.25±0.11	0.00±0.00	0.00±0.00
NC9	11.14±0.04	8.01±0.07	6.58±0.08	4.38±0.06
NC10	7.82±0.05	7.20±0.14	4.49±0.03	1.43±0.05
NC11	9.11±0.06	8.42±0.02	6.61±0.08	5.39±0.05
NC12	8.80±0.04	8.11±0.07	2.54±0.07	0.00±0.00
NC13	9.83±0.04	8.41±0.02	6.55±0.03	3.33±0.02
NC14	9.83±0.04	9.37±0.06	6.58±0.08	0.00±0.00
NC15	9.85±0.07	9.32±0.05	8.49±0.02	3.38±0.05
NC16	10.82±0.08	9.49±0.03	5.06±0.06	4.37±0.04
NC17	11.09±0.03	9.25±0.02	5.11±0.03	0.00±0.00
NC18	10.85±0.08	9.18±0.05	5.10±0.04	3.43±0.08
NC19	8.86±0.08	8.44±0.03	5.08±0.09	4.34±0.04
NC20	9.10±0.02	7.46±0.06	0.00±0.00	0.00±0.00
NC21	10.82±0.04	8.33±0.11	1.53±0.07	1.48±0.03
NC22	8.84±0.04	8.13±0.03	6.44±0.06	2.33±0.02
NC23	8.80±0.04	6.43±0.06	6.35±0.25	2.40±0.06
NC24	9.97±0.05	8.35±0.09	4.56±0.05	0.00±0.00
NC25	9.86±0.05	8.41±0.06	7.56±0.07	2.43±0.08
NC26	9.08±0.02	8.15±0.00	8.53±0.03	1.35±0.02

Note: Data are presented as Mean ± Standard Deviation (n=3). Values are expressed in Log CFU/mL. Indicates statistically significant superiority at pH 2.0 ($p<0.05$)

Bile salt tolerance

The survival rates under varying bile concentrations (0.2 to 1.0%) are presented in Table 4. While most isolates survived physiological concentrations (0.3%), a sharp decline in viability was observed at 1.0% bile stress ($p<0.05$). Only isolates NC5, NC22, and NC25 maintained populations above 3.80 Log CFU/mL at the 1.0% concentration. Notably, isolate NC5 exhibited no statistically significant population reduction between the 0.5 and 1.0% bile concentrations, maintaining a viable count of 3.82±0.02 Log CFU/mL.

Molecular identification of isolate NC5

Isolate NC5 was selected as the optimal candidate for definitive identification due to its balanced profile of superior bile resilience, competitive acid tolerance, and non-hemolytic status. BLASTn analysis of the 1,500 bp 16S rRNA gene sequence (Table 5) revealed that isolate NC5 shared 100.00% identity with *E. faecalis* strain ATCC 19433. Phylogenetic analysis using the Neighbor-Joining method (Figure 1) confirmed this placement, showing NC5 clustering within a well-supported clade of *E. faecalis* reference strains. The sequence has been deposited in GenBank under accession number PX924374.

Discussion

Ecological niche of Nem Chua *and the isolation of* Enterococcus faecalis *NC5*

Contemporary probiotic research emphasizes the value of traditional fermented ecosystems as reservoirs for stress-adapted microorganisms evolved within competitive, nutrient-rich environments (Fijan 2016). In this study, *E. faecalis* NC5 was successfully isolated from *Nem Chua*, a traditional Vietnamese fermented pork product representing a specialized ecological niche. The *Nem Chua* matrix is characterized by high salinity, antimicrobial spices, and rapid acidification—conditions that select for a robust autochthonous microbiota (Phong et al. 2017). Our isolation strategy, utilizing $CaCO_3$-supplemented selective media, effectively identified acid-producing taxa within this complex fermented ecosystem, consistent with methodologies used to survey microbial biodiversity in similar fermented matrices (Nguyen et al. 2020; Yelnetty et al. 2020).

The prevalence of *E. faecalis* in *Nem Chua* is consistent with its established role in international fermented meats, such as Turkish sucuk and salami, where Enterococci contribute to ripening and sensory development (Erdoğmuş et al. 2021). However, the isolation of strain NC5 from artisanal Vietnamese sources suggests it possesses specific ecological fitness evolved under local processing conditions. Prioritizing such indigenous isolates supports the preservation of fermented-food biodiversity and provides a scientific rationale for their potential application in local food systems. Furthermore, the capacity of *E. faecalis* to modulate sensory profiles underscores its technological value within traditional food microbiology (Worsztynowicz et al. 2019; Margalho et al. 2020).

Table 4. Viability of 26 selected isolates under simulated intestinal conditions (0.2% - 1.0% bile salts)

ID Isolate	Viable bacteria isolates (Log CFU/mL)				
	0.00% (Control)	**0.20%**	**0.30%**	**0.50%**	**1.00%**
NC1	9.86±0.07	8.69±0.12	6.46±0.04	4.45±0.02	1.86±0.07
NC2	8.86±0.10	8.63±0.03	6.38±0.03	2.42±0.12	0.00±0.00
NC3	8.84±0.04	8.61±0.05	6.50±0.09	3.38±0.18	2.84±0.04
NC4	9.09±0.02	6.64±0.04	5.39±0.03	3.33±0.12	3.09±0.02
NC5	**8.82±0.02**	**7.67±0.07**	**5.46±0.02**	**4.45±0.03**	**3.82±0.02**
NC6	8.81±0.03	6.61±0.07	5.45±0.09	0.00±0.00	0.00±0.00
NC7	8.87±0.09	7.64±0.03	6.49±0.04	2.31±0.12	1.87±0.09
NC8	8.82±0.03	7.63±0.06	6.45±0.02	0.00±0.00	0.00±0.00
NC9	11.11±0.04	4.74±0.12	3.49±0.04	2.39±0.09	2.14±0.04
NC10	9.82±0.05	7.67±0.06	6.40±0.02	2.82±0.05	2.47±0.02
NC11	9.11±0.06	7.64±0.03	6.53±0.13	4.53±0.14	3.11±0.06
NC12	8.80±0.04	8.69±0.16	6.42±0.02	3.80±0.04	3.45±0.02
NC13	9.83±0.04	8.65±0.09	6.37±0.03	3.47±0.03	0.00±0.00
NC14	9.83±0.04	7.69±0.11	6.41±0.02	2.83±0.04	2.46±0.20
NC15	9.85±0.07	6.62±0.04	6.47±0.03	2.46±0.02	0.00±0.00
NC16	10.82±0.08	9.59±0.04	6.44±0.02	2.48±0.02	1.82±0.08
NC17	11.09±0.03	9.66±0.08	3.40±0.05	2.49±0.07	2.09±0.03
NC18	10.85±0.08	9.64±0.04	3.35±0.07	0.00±0.00	0.00±0.00
NC19	8.86±0.08	6.66±0.05	5.46±0.17	4.44±0.03	2.86±0.08
NC20	9.10±0.02	4.63±0.06	3.48±0.03	2.28±0.00	0.00±0.00
NC21	8.82±0.04	6.59±0.07	6.49±0.04	3.31±0.01	2.82±0.04
NC22	**8.84±0.04**	**8.54±0.13**	**5.48±0.06**	**4.53±0.07**	**3.84±0.04**
NC23	8.80±0.04	8.60±0.08	6.48±0.03	2.80±0.04	2.50±0.02
NC24	9.83±0.04	9.71±0.14	6.53±0.06	2.48±0.02	1.97±0.05
NC25	**9.86±0.05**	**7.59±0.08**	**6.40±0.05**	**4.45±0.01**	**3.83±0.04**
NC26	9.08±0.02	6.68±0.10	5.45±0.03	3.25±0.03	0.00±0.00

Note: Data are presented as Mean ± Standard Deviation (n=3). Values are expressed in Log CFU/mL. Indicates the highest statistical tolerance group at 1.0% bile ($p<0.05$)

Table 5. BLASTn identification results for the 16S rRNA gene sequence of isolate NC5

Subject description	Max score	Query cover	E-value	Percent identity	Accession number
Enterococcus faecalis strain ATCC 19433 16S ribosomal RNA, partial sequence	2739	90 %	0.0	100.00 %	NR_115765.1

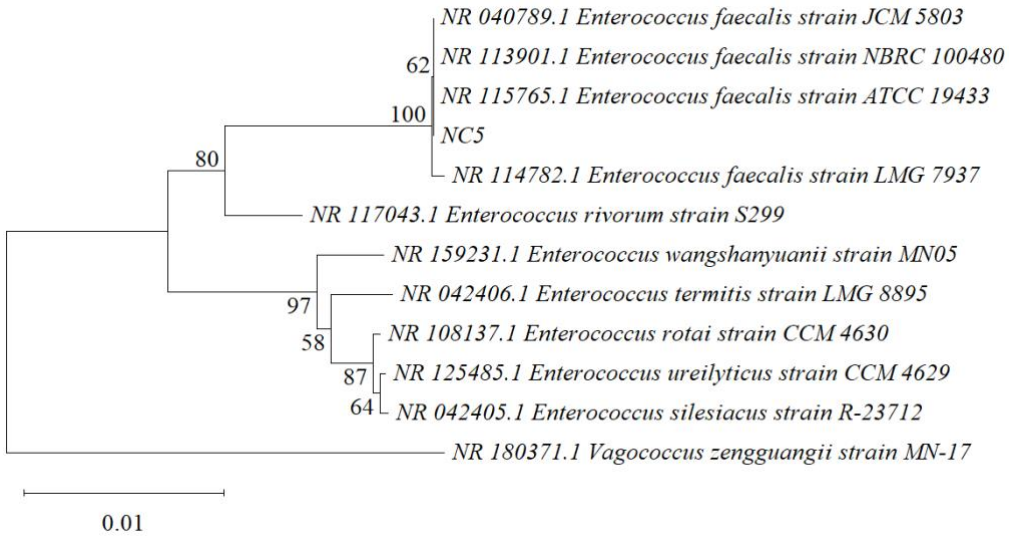

Figure 1. Phylogenetic tree constructed using the Neighbor-Joining method based on 16S rRNA gene sequences, showing the evolutionary relationship between isolate NC5 and related *E. faecalis* strains. Bootstrap values (expressed as percentages of 1,000 replications) are shown at the branching points

Gastrointestinal resilience and potential probiotic efficacy

A fundamental prerequisite for any probiotic candidate is the ability to survive transit through the upper gastrointestinal (GI) tract (Obayomi et al. 2024). *E. faecalis* NC5 demonstrated significant in vitro resilience to simulated GI stressors, maintaining viability at pH 2.0 and robust tolerance to 1.0% bile salts. Survival at pH 2.0 is particularly critical, as gastric acidity represents a primary biological barrier against non-adapted bacteria (Fijan 2016). The survival of NC5 indicates an intrinsic acid tolerance response, likely pre-adapted through natural selection within the acidic environment of *Nem Chua*.

The strain's ability to withstand 1.0% bile salts, exceeding the typical physiological range of 0.3%, further highlights its ecological adaptation (Suwannaphan 2021; Repoila et al. 2022). Similar levels of gastrointestinal resilience have been observed in *E. faecalis* strains isolated from diverse fermented matrices, such as Nigerian dairy and Tunisian cheeses, suggesting that the fermented food environment often harbors robust probiotic candidates (Oguntoyinbo et al. 2012; Baccouri et al. 2019). While the performance of NC5 fulfills the critical in vitro prerequisites for potential colonization, these results must be considered as potential until validated through in vivo studies (Aponte et al. 2020).

Antimicrobial potential as a protective culture

A notable find regarding food safety is the broad-spectrum antagonistic activity exhibited by *E. faecalis* NC5. The strain inhibited key pathogens, including the Gram-positive *S. aureus* and the Gram-negative *E. coli* and *S. typhimurium*. Since *Nem Chua* is traditionally consumed raw, the indigenous microbiota must function as the primary defensive barrier against foodborne pathogens (Pilasombut et al. 2015). Because inhibition was confirmed using neutralized cell-free supernatants, our data suggest this activity is likely mediated by proteinaceous metabolites, such as bacteriocins, rather than organic acids alone (Waheed et al. 2021).

The inhibition of Gram-negative bacteria by NC5 is particularly significant, as many Lactic Acid Bacteria (LAB) metabolites are primarily effective against Gram-positive targets (Selman et al. 2021; Hatem et al. 2024). These antagonistic properties suggest that NC5 has potential as a protective culture or biopreservative to enhance the biological security of raw-consumed fermented products. Further exploration of these potential applications could help mitigate risks associated with zoonotic pathogens in traditional food systems (Nami et al. 2019).

Safety assessment and *Enterococcus* paradox

The application of *Enterococcus* species in food requires a rigorous safety assessment due to their dual nature as beneficial commensals and potential opportunistic pathogens (Hanchi et al. 2018). To address this paradox, we conducted a safety-filtered screening focusing on non-pathogenic traits. Regulatory frameworks prioritize the absence of acquired virulence factors for strains intended for food use (Mull et al. 2020). *E. faecalis* NC5 demonstrated a favorable

safety profile, characterized by γ-hemolysis (non-hemolytic activity), a primary indicator of non-pathogenicity.

By confirming a non-hemolytic status, this research distinguishes the indigenous strain NC5 from clinical pathotypes. These results suggest that NC5 is a safe component of the natural *Nem Chua* microbiota, aligning with safety profiles of Enterococci found in other traditional fermented foods like Brazilian artisanal cheeses and Korean soybean paste (Margalho et al. 2020; Han et al. 2024). However, it is essential to delimit these claims to in vitro findings. The potential for horizontal gene transfer remains a general concern for the genus, highlighting that safety must be interpreted within the specific context of traditional food fermentation (Franz et al. 2003).

Limitations and future perspectives

While this study establishes a strong functional baseline for *E. faecalis* NC5, certain limitations must be acknowledged. Our findings are based on in vitro models, which do not fully replicate the complex physiological interactions within a host immune system (Obayomi et al. 2024). Additionally, the absence of Whole Genome Sequencing (WGS) is a methodological limitation that prevents a definitive assessment of cryptic virulence factors or mobile antibiotic resistance genes (Nami et al. 2019; Sionek et al. 2023).

Future research should prioritize genomic characterization to provide the high-resolution safety data required for regulatory consideration. Additionally, in vivo trials in animal models or controlled human intervention studies are necessary to validate the strain's colonization efficiency, persistence, and actual immunomodulatory effects under physiological conditions (Aponte et al. 2020). Exploring the performance of NC5 within diverse food matrices beyond pork would also determine its versatility as a multi-functional biopreservative for the broader food industry.

In conclusion, this study establishes that the traditional Vietnamese fermented pork product, *Nem Chua*, serves as a significant microbial reservoir for stress-adapted Lactic Acid Bacteria (LAB) with functional potential. From 59 presumptive *Enterococcus* isolates obtained from *Nem Chua*, 26 strains exhibited antagonistic activity against foodborne pathogens, and successive gastrointestinal resilience screening reduced the candidates to a single superior strain (NC5). Strain NC5 maintained viable counts above 6.0 log CFU/mL under acidic conditions (pH 2.5-3.0) and tolerated bile salts at 0.3-1.0%, indicating strong functional robustness. NC5 demonstrated broad antagonistic activity against *E. coli*, *Salmonella* spp., and *S. aureus*, and displayed γ-hemolysis, confirming the absence of hemolytic activity. Molecular identification based on partial 16S rRNA sequencing showed 100% sequence similarity to *E. faecalis* reference strains, validating its taxonomic identity. These findings validate *E. faecalis* NC5 as a promising candidate for further evaluation as a protective starter culture or biopreservative in fermented meat products. Ultimately, this research underscores the critical necessity of bioprospecting and preserving indigenous microbial biodiversity to develop natural food safety systems and enhance the functional value of traditional Southeast Asian fermented foods.

ACKNOWLEDGEMENTS

This research was fully funded by Tra Vinh University, Vietnam, under grant contract number 59/2025/HĐ.HĐKH &ĐT-ĐHTV.

REFERENCES

Abubakr MAS. 2018. Antimicrobial activities of lactic acid bacteria strains isolated from human breast milk against human pathogenic strains. Intl J Clin Dev Anat 4 (1): 27-31. https://doi.org/10.11648/j.ijcda.20180401.14.

Ahansaz N, Tarrah A, Pakroo S, Corich V, Giacomini A. 2023. Lactic acid bacteria in dairy foods: Prime sources of antimicrobial compounds. Fermentation 9 (11): 964. https://doi.org/10.3390/fermentation9110964.

Anumudu CK, Miri T, Onyeaka H. 2024. Multifunctional applications of lactic acid bacteria: Enhancing safety, quality, and nutritional value in foods and fermented beverages. Foods 13 (23): 3714. https://doi.org/10.3390/foods13233714.

Aponte M, Murru N, Shoukat M. 2020. Therapeutic, prophylactic, and functional use of probiotics: A current perspective. Front Microbiol 11: 562048. https://doi.org/10.3389/fmicb.2020.562048.

Baccouri O, Boukerb AM, Farhat LB, Zébré A, Zimmermann K, Domann E, Cambronel M, Barreau M, Maillot O, Rincé I, Muller C, Marzouki MN, Feuilloley M, Abidi F and Connil N. 2019. Probiotic potential and safety evaluation of *Enterococcus faecalis* OB14 and OB15, isolated from traditional Tunisian Testouri cheese and Rigouta, using physiological and genomic analysis. Front Microbiol 10: 881. https://doi.org/10.3389/fmicb.2019.00881.

Chen Y, Yu L, Qiao N, Xiao Y, Tian F, Zhao J, Zhang H, Chen W, Zhai Q. 2020. *Latilactobacillus curvatus*: A candidate probiotic with excellent fermentation properties and health benefits. Foods 9 (10): 1366. https://doi.org/10.3390/foods9101366.

Ding W, Shi C, Chen M, Zhou J, Long R, Guo X. 2017. Screening for lactic acid bacteria in traditional fermented Tibetan yak milk and evaluating their probiotic and cholesterol-lowering potentials in rats fed a high-cholesterol diet. J Funct Foods 32: 324-332. https://doi.org/10.1016/j.jff.2017.03.021.

Erdoğmuş SF, Erişmiş UC, Uğuz C. 2021. Isolation and identification of lactic acid bacteria from fermented meat products and evaluation of their antimicrobial effect. Czech J Food Sci 39 (4): 289-296. https://doi.org/10.17221/222/2020-CJFS.

Fijan S. 2016. Antimicrobial effect of probiotics against common pathogens. In: Rao V, Rao LG (eds.). Probiotics and Prebiotics in Human Nutrition and Health. InTech, Rijeka. https://doi.org/10.5772/63141.

Franz CM, Stiles ME, Schleifer KH, Holzapfel WH. 2003. Enterococci in foods-a conundrum for food safety. Intl J Food Microbiol 88 (2-3): 105-22. https://doi.org/10.1016/s0168-1605(03)00174-0.

Han KI, Shin HD, Lee Y, Baek S, Moon E, Park YB, Cho J, Lee JH, Kim TJ, Manoharan RK. 2024. Probiotic and postbiotic potentials of *Enterococcus faecalis* EF-2001: A safety assessment. Pharmaceuticals 17 (10): 1383. https://doi.org/10.3390/ph17101383.

Hanchi H, Mottawea W, Sebei K, Hammami R. 2018. The genus *Enterococcus*: Between probiotic potential and safety concerns-an update. Front Microbiol 9: 1791. https://doi.org/10.3389/fmicb.2018.01791.

Hatem EA, Hashad A, Bayomi EB, Kamal AA. 2024. Antimicrobial effect of isolated lactic acid bacteria and *Bacillus* spp. Sci J Fac Sci Menoufia Univ 28 (2): 49-67. https://doi.org/10.21608/sjfsmu.2024.326749.1009.

Kang W, Pan L, Peng C, Dong L, Cao S, Cheng H, Wang Y, Zhang C, Gu R, Wang J. 2020. Isolation and characterization of lactic acid bacteria from human milk. J Dairy Sci 103 (11): 9980-9991. https://doi.org/10.3168/jds.2020-18704.

Liu W, Chen M, Duo L, Wang J, Guo S, Sun H, Menghe B, Zhang H. 2020. Characterization of potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum. J Dairy Sci 103 (5): 4013-4025. https://doi.org/10.3168/jds.2019-17602.

Liu Y, Gao S, Cui Y, Wang L, Duan J, Yang X, Liu X, Zhang S, Sun B, Yu H. 2024. Characteristics of lactic acid bacteria as potential probiotic starters and their effects on the quality of fermented sausages. Foods 13 (2): 198. https://doi.org/10.3390/foods13020198.

Manassi CF, de Souza SS, de Souza Hassemer G, Sartor S, Lima CMG, Miotto M, Lindner JDD, Rezzadori K, Pimentel TC, Ramos GLPA. 2022. Functional meat products: Trends in pro-, pre-, syn-, para- and post-biotic use. Food Res Intl 154: 111035. https://doi.org/10.1016/j.foodres.2022.111035.

Margalho LP, van Schalkwijk S, Bachmann H, Sant'Ana AS. 2020. *Enterococcus* spp. in Brazilian artisanal cheeses: Occurrence and assessment of phenotypic and safety properties of a large set of strains through the use of high-throughput tools combined with multivariate statistics. Food Control 118: 107425. https://doi.org/10.1016/j.foodcont.2020.107425.

Mull RW, Brennan AA, Russ BR, Tal-Gan Y. 2020. Attenuating the selection of vancomycin resistance among enterococci through the development of peptide-based vancomycin antagonists. ACS Infect Dis 6 (11): 2913-2925. https://doi.org/10.1021/acsinfecdis.0c00319.

Nami Y, Vaseghi Bakhshayesh R, Mohammadzadeh Jalaly H, Lotfi H, Eslami S, Hejazi MA. 2019. Probiotic properties of *Enterococcus* isolated from artisanal dairy products. Front Microbiol 10: 300. https://doi.org/10.3389/fmicb.2019.00300.

Nguyen VP, Bien TLT, Pham TH, Dang TNM, Nguyen TTN. 2020. Isolation and identification of lactic acid bacteria from vegetable-growing soils in Da Lat, Lam Dong. J Agric Dev 19 (4): 1-9. https://doi.org/10.52997/jad.1.04.2020.

Obayomi OV, Olaniran AF, Owa SO. 2024. Unveiling the role of functional foods with emphasis on prebiotics and probiotics in human health: A review. J Funct Foods 119: 106337. https://doi.org/10.1016/j.jff.2024.106337.

Oguntoyinbo, FA, Okueso O. 2013. Prevalence, distribution, and antibiotic resistance pattern among enterococci species in two traditional fermented dairy foods. Ann Microbiol 63: 755-761. https://doi.org/10.1007/s13213-012-0530-8.

Parlindungan E, Lugli GA, Ventura M, van Sinderen D, Mahony J. 2021. Lactic acid bacteria diversity and characterization of probiotic candidates in fermented meats. Foods 10 (7): 1519. https://doi.org/10.3390/foods10071519.

Phong HX, Van TH, Thanh NN, Long BHD, Dung NTP. 2017. Antifungal activity of lactic acid bacteria isolated from *Nem Chua*. Chem Biomol Eng 1 (2): 49. https://doi.org/10.11648/j.cbe.20160102.14.

Pilasombut K, Rumjuankiat K, Ngamyeesoon N, Duy LND. 2015. In vitro characterization of bacteriocin produced by lactic acid bacteria isolated from *Nem Chua*, a traditional Vietnamese fermented pork. Korean J Food Sci Anim Resour 35 (4): 473-478. https://doi.org/10.5851/kosfa.2015.35.4.473.

Pumriw S, Luang-In V, Samappito W. 2021. Screening of probiotic lactic acid bacteria isolated from fermented Pak-Sian for use as a starter culture. Curr Microbiol 78 (7): 2695-2707. https://doi.org/10.1007/s00284-021-02521-w.

Rabaoui G, Sánchez-Juanes F, Tebini M, Naghmouchi K, Bellido JLM, Ben-Mahrez K, Réjiba S. 2023. Potential probiotic lactic acid bacteria with anti-*Penicillium expansum* activity from different species of Tunisian edible snails. Probiotics Antimicrob Proteins 15 (1): 82-106. https://doi.org/10.1007/s12602-021-09882-5.

Rahmawati N, Syukri M, Darmawi D, Zachreini I, Yusuf M, Idroes R. 2021. Identification of lactic acid bacteria from etawa goat milk kopelma Darussalam Village, Banda Aceh. IOP Conf Ser: Earth Environ Sci 667: 012022. https://doi.org/10.1088/1755-1315/667/1/012022.

Repoila F, Le Bohec F, Guérin C, Lacoux C, Tiwari S, Jaiswal AK, Santana MP, Kennedy SP, Quinquis B, Rainteau D, Juillard V, Furlan S, Bouloc P, Nicolas P, Miyoshi A, Azevedo V, Serror P. 2022. Adaptation of the gut pathobiont *Enterococcus faecalis* to deoxycholate and taurocholate bile acids. Sci Rep 12: 8485. https://doi.org/10.1038/s41598-022-12552-3.

Selman HM, Mahdi AA, El Rofaei NA, Mutwali EM. 2021. Antibacterial activity of the bacteriocins producing-lactic acid bacteria isolated from some processed meat products against selected indicator bacterial strains. World J Adv Res Rev 12 (2): 640-645. https://doi.org/10.30574/wjarr.2021.12.2.0643.

Serrano-Nino JC, Solis-Pacheco JR, Gutierrez-Padilla JA, Cobian-Garcia A, Cavazos-Garduno A, González-Reynoso O, Aguilar-Uscanga BR. 2016. Isolation and identification of lactic acid bacteria from human milk with potential probiotic role. J Food Nutr Res 4 (3): 170-177. https://doi.org/10.12691/jfnr-4-3-7.

Sionek B, Szydłowska A, Zielińska D, Neffe-Skocińska K, Kołożyn-Krajewska D. 2023. Beneficial bacteria isolated from food in relation to the next generation of probiotics. Microorganisms 11 (7): 1714. https://doi.org/10.3390/microorganisms11071714.

Suwannaphan S. 2021. Isolation, identification, and potential probiotic characterization of lactic acid bacteria from Thai traditional fermented food. AIMS Microbiol 7 (4): 431-446. https://doi.org/10.3934/MICROBIOL.2021026.

Unban K, Chaichana W, Baipong S, Abdullahi AD, Kanpiengjai A, Shetty K, Khanongnuch C. 2021. Probiotic and antioxidant properties of lactic acid bacteria isolated from indigenous fermented tea leaves (Miang) of north Thailand and promising application in synbiotic formulation. Fermentation 7 (3): 195. https://doi.org/10.3390/fermentation7030195.

Waheed S, Rasool MH, Aslam B, Muzammil S, Waseem M, Shahid M, Saqib M, Hayat S, Naeem M, Taj Z, Kabir S, Saqalein M, Nisar MA, Khurshid M. 2021. Antagonistic potential of dairy-origin *Enterococcus faecium* against multidrug-resistant foodborne pathogens. Rom Biotechnol Lett 26 (2): 2406-2415. https://doi.org/10.25083/rbl/26.2/2406.2415.

Worsztynowicz P, Olejnik-Schmidt A, Białas W, Grajek W. 2019. Identification and partial characterization of proteolytic activity of *Enterococcus faecalis* relevant to their application in the dairy industry. Acta Biochim Pol 66 (1): 61-69. https://doi.org/10.18388/abp.2018_2714.

Yelnetty A, Purwadi, Tallei TE. 2020. Indigenous lactic acid bacteria isolated from spontaneously fermented goat milk as potential probiotics. Pak J Biol Sci 23 (7): 883-890. https://doi.org/10.3923/pjbs.2020.883.890.

Zommiti M, Feuilloley MGJ, Connil N. 2020. Update of probiotics in the human world: A nonstop source of benefits till the end of time. Microorganisms 8 (12): 1907. https://doi.org/10.3390/microorganisms8121907.