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Safety-Filtered Characterization of *Lentilactobacillus farraginis* TV3.1.2: an Autochthonous Probiotic Candidate from Vietnamese *Solanum macrocarpon* Ferments

Thuy Nguyen Phuong[1], Bui Dang Khoa[1], Nguyen Thanh Tuu[2*]

[1]*School of Agriculture and Aquaculture, Tra Vinh University, Vietnam*
[2]*College of Engineering and Technology, Tra Vinh University, Vietnam*

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ABSTRACT

Although the traditional plant-based fermented food groups are important sources of microbial biodiversity, the functional and safety properties of many autochthonous species are poorly studied. The present study aimed to characterize *Lentilactobacillus farraginis* TV3.1.2, an indigenous isolate from Vietnamese *Solanum macrocarpon* (Ca phao muoi chua) and investigate its potential as an alternative to the use of antibiotic growth promoters in livestock. Based on a systematic 5-stage screening of 62 isolates, TV3.1.2 was detected by 16S rRNA gene sequencing. The strain was found to be highly resilient, with >2 Log CFU/mL remaining viable at pH 2.0 (2.29±0.49 Log CFU/mL) and 1.0% bile salts (3.50±0.61 Log CFU/mL). Moreover, TV3.1.2 displayed broad-spectrum antimicrobial activity with an inhibition zone of 30.47 mm against *Staphylococcus aureus*, which is indicative of the production of bacteriocin-like substance. Safety testing confirmed a non-hemolytic profile and susceptibility to clinically relevant antibiotics. No virulence factors (*esp, gelE, cylA/M*), vancomycin resistance genes (*vanA/B*), and biogenic amine determinants (*hdc1/2, tdc*) were detected by genotypic analysis. Overall, these results suggest that *L. farraginis* TV3.1.2 could serve as a safe and protective starter culture in food and agriculture applications compared to pathogenic lines.

1. Introduction

Antimicrobial resistance (AMR) is a major concern in global public health and food security as it is spreading among foodborne pathogens, calling for a change of veterinary and agricultural practices. The Law on Livestock (2018) in Vietnam has banned the use of antibiotics as growth promoters (AGPs) in animal feed, which is a pressing demand for alternative sustainable methods to ensure animal well-being and food security (Zeng *et al.* 2020). Under the "One Health" umbrella, probiotics, which are defined as microorganisms that can be consumed in live form and provide health benefits when administered in sufficient quantities, have become one of the most important options to replace conventional antibiotics (Fadare *et al.* 2023). Lactic acid bacteria (LAB) are especially important for this application because they possess various therapeutic mechanisms such as competitive exclusion of pathogens and the production of bioactive metabolites like bacteriocins, which have the ability to specifically inhibit pathogens like *Staphylococcus aureus*, without causing the ecological changes that occur with the traditional use of antibiotics (Zommiti *et al.* 2020; Darbandi *et al.* 2022). In recent years, traditional plant-based fermented foods are increasingly considered as important sources of microbial diversity, e.g. Vietnamese Ca phao muoi chua (pickled *Solanum macrocarpon*). These foods serve as a niche ecosystem where changes in salinity and quick acidification have a high evolutionary impact and create indigenous and resilient microbiota (Abriouel *et al.* 2022;

*Corresponding Author
E-mail Address: nttuu@tvu.edu.vn

Sihombing *et al.* 2025). Metataxonomic and culture-based analysis of recent fermentations of vegetables have shown a predictable sequence of LAB, which can change from the early dominance of *Leuconostoc* spp. to strong *Lactiplantibacillus* spp. and *Lentilactobacillus* spp. in the later stages of fermentation (Botta *et al.* 2025). Compared with commercial starters, these autochthonous strains may have distinct technological and functional characteristics, such as increased stability of the food product, improved sensory properties, or enriched nutritional properties (Torres *et al.* 2020; Yuan *et al.* 2023).

However, the transition from artisanal tradition to industrial application requires rigorous safety validation. The "safety paradox" dictates that even generally recognized as safe (GRAS) genera may harbor acquired antibiotic resistance genes (ARGs) or virulence determinants (Lee *et al.* 2022; Kiousi *et al.* 2023). Importantly, recent metagenomic studies of pickled vegetables have shown the presence of hundreds of ARGs which have the potential to be acquired through horizontal gene transfer from environmental sources, such as Enterobacteriaceae, which are present in the environment as contaminants (Yasir *et al.* 2022). In order to guarantee clinical safety and the absence of transmissible resistance factors (Yaacob *et al.* 2022; Altavas *et al.* 2024), a "safety-filtered" screening process is required that combines phenotypic assays with genotypic validation.

Lentilactobacillus farraginis has been gaining significant scientific attention due to its metabolic versatility and adaptability, especially within the genus *Lentilactobacillus. L. farraginis* was previously reported to exist in various environments from silage to gastrointestinal tracts of aquatic organisms, but only a few strains have been isolated from vegetable fermentation processes from Vietnam and collected in the present study (Ali *et al.* 2022; Zayed *et al.* 2022). This study will attempt to fill these research gaps by studying *L. farraginis* TV3.1.2, an autochthonous strain isolated from Ca phao muoi chua. Incorporating phenotypic stress-tolerance testing with a broad-based PCR-based screening of virulence factors, vancomycin resistance and biogenic amine determinants will transform this indigenous strain from an artisanal novelty to a standardized safe biopreservative. The main aim of this study was to: (i) assess the gastrointestinal stress resistance of L. farraginis TV3.1.2 to low pH and bile salts; (ii) perform a comprehensive safety evaluation by phenotypic and molecular screening; and (iii) investigate its antagonistic activity against the main foodborne pathogens. The integrated approach is based on science and its application serves as a scientific basis for preserving the Vietnamese microbial heritage as well as developing biological alternatives that are safe and indigenous for the food and livestock industry of the region.

2. Materials and Methods

2.1. Hierarchical Screening Pipeline

A five-stage hierarchical screening process was implemented to identify a superior probiotic candidate from 105 presumptive lactic acid bacteria (LAB) isolates. Each stage applied pre-defined criteria to ensure only isolates meeting stringent functional and safety benchmarks progressed:

Stage I (Isolation): Acid-producing, salt-tolerant LAB were enriched and selected on de Man, Rogosa, and Sharpe (MRS) agar supplemented with 1.0% (w/v) $CaCO_3$ and 1.0% (w/v) NaCl. Isolates producing visible acid-clearing halos were retained.

Stage II (Phenotypic verification): The isolates were confirmed as Gram-positive, catalase negative and non-motile. Biochemical profiles were determined by urease activity, citrate utilization and sugar fermentation (glucose, lactose and sucrose).

Stage III (Antimicrobial screening): The remaining 62 isolates were tested for antagonistic activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella enterica* serovar Typhimurium ATCC 14028. The isolates that inhibited at least two pathogens with an inhibition zone of ≥18 mm were retained.

Stage IV (Functional and safety profiling): The fifteen shortlisted isolates were subjected to gastrointestinal (GIT) resilience testing and subjected to safety screening (hemolysis and antibiotic susceptibility testing).

Stage V (Genotypic confirmation): Top-performing candidates were identified via 16S rRNA gene sequencing and subjected to PCR-based screening for virulence, vancomycin resistance, and biogenic amine-producing genes.

2.2. Sample Collection and Isolation of Lactic acid Bacteria

To collect samples of Ca phao muoi chua (traditional Vietnamese pickled *Solanum macrocarpon*), 10 local markets were chosen in Vinh Long and Dong Thap provinces, Vietnam. Samples were taken during the peak fermentation period (days 5–6) when the acidification

was stabilized, to isolate autochthonous strains adapted to the fermentation niche. All the samples were collected in sterile, chilled samples boxes (4°C) and processed within 4 h to prevent loss in viability of the indigenous microbiota.

To isolate, 1 g of the sample was homogenized in 9 mL of sterile 0.85% (w/v) saline. The serial dilutions were prepared and 100 µL was spread plated on de man, rogosa and sharpe (MRS) agar (HiMedia, India). It was supplemented with 1.0% (w/v) $CaCO_3$ for visualizing the acid production and 1.0% (w/v) NaCl for the selection of salt-tolerant lactic acid bacteria (LAB). The plates were incubated anaerobically by placing an AnaeroGen gas pack system (Oxoid, UK) at 37°C for 48 h.

2.3. Phenotypic Characterization and Selection

The first 105 lactic acid bacteria (LAB) colonies were selected based on their distinct colony morphology and acid production zone. Isolates were subcultured to check for Gram reaction and catalase activity; Gram-positive, catalase-negative and non-motile isolates were retained to exclude non-LAB contaminants. Sixty-two isolates were selected for further biochemical characterization using the following tests: urease, citrate utilization, carbohydrate fermentation (glucose, lactose, and sucrose) (Kang *et al.* 2020). Confirmed LAB isolates were stored at -20°C in MRS broth with 20% (v/v) glycerol.

2.4. *In vitro* Screening for Probiotic Properties

Gastrointestinal (GIT) survival was measured by testing the ability of the isolates to survive after a modified protocol according to Serrano-Nino *et al.* (2016) which mimics stomach and upper intestine transit. Overnight cultures were centrifuged (10,000 rpm, 10 min, 4°C), washed two times with sterile saline and re-inoculated into MRS broth at a standardized initial inoculum of 10^8 CFU/mL.

Bile tolerance was assessed by inoculating the standardized suspension into MRS broth containing varying concentrations of Oxgall bile salts (0.2%, 0.4%, 0.6%, 0.8%, and 1.0%; Oxoid, UK), with normal MRS broth (pH 7.2, 0% bile) serving as the control. After 4 hours of incubation at 37°C, a duration reflecting standard gastric transit time, viable cell counts (CFU/mL) were determined via the spread-plate technique. The 15 most robust isolates were subsequently subjected to acid tolerance testing in MRS broth adjusted to pH 2.0, 3.0, and 4.0 using 1 M HCl. Viability was quantified on MRS agar after 4 hours of exposure. All assays were performed in triplicate. To assess survival performance, survival rates (%) were calculated as (log CFU/mL after exposure / log CFU/mL initial) × 100, and log reductions were calculated as (log CFU/mL initial - log CFU/mL after exposure).

2.5. Safety Assessment

Hemolytic activity was tested by streaking onto blood agar base (Oxoid, UK) with 5% (v/v) sheep blood. The isolates were identified as β-hemolysis (clear zone), α-hemolysis (greenish zone) or γ-hemolysis (no change) after 24 hours incubation at 37°C. Only non-hemolytic (γ-hemolysis) strains were chosen for further validation (Unban *et al.* 2021).

Antibiotic susceptibility was evaluated using the Kirby-Bauer disk diffusion method on MRS agar. To provide a rigorous safety profile, the results were interpreted using a combined approach: Clinical and Laboratory Standards Institute (CLSI) guidelines were employed for general susceptibility testing (Alebiosu *et al.* 2017; Nguyen *et al.* 2023), while the European Food Safety Authority (EFSA) microbiological cut-off values were referenced to distinguish between acquired resistance and intrinsic, non-transmissible resistance common to the *Lentilactobacillus* genus. 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). This classification gives an indicative safety profile and restricts the use of strains for industrial application to those that do not contain transferable resistance determinants.

2.6. Antagonistic Activity Assessment

Selected isolates were tested for antimicrobial activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella enterica* serovar Typhimurium ATCC 14028 by the agar well diffusion method (Abubakr 2018). To distinguish the inhibitory effects of organic acids, hydrogen peroxide and the proteinaceous bacteriocins, the following controls were performed: Cell-Free Supernatant (CFS) was spun down (10,000 rpm for 5 min at 4°C), and then neutralized to pH 7.0 with 1 M NaOH. To remove the interference from hydrogen peroxide, catalase (5 mg/mL) was added to the neutralized CFS, when required. Positive and negative controls were Ampicillin (10 µg) and sterile neutralised MRS broth, respectively. Inhibition zones were measured after 24 hours incubation at 37°C and classified as strong (>25 mm), moderate (13–25 mm) and weak (1–12 mm).

2.7. Molecular and Genotypic Analysis

Genotypic safety was screened via PCR to identify 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 Gen DNA Purification Kit (Promega, USA). PCR was performed using DreamTaq Green PCR Master Mix (Thermo Scientific) and specific primers (Table 1), with products visualized on 1.5% agarose gels (Nguyen *et al.* 2025).

The superior isolate (TV3.1.2) was selected by the genome sequencing of 16S rRNA gene with universal primers by Liu *et al.* (2020) (5'-AGAGTTTGATCCTGGCTCAG-3') for 27F and (5'-GGTTACCTTGTTACGACTT-3') for 1492R. The thermal profile consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 60 s, and 72°C for 90 s, with a final extension at 72°C for 5 min. Sequences were compared to those available in the NCBI database using the BLASTn tool, and a phylogenetic tree was created with the Neighbor-Joining method using 1,000 bootstrap replicates in the MEGA6 software.

2.8. Statistical Analysis

All experiments were conducted in three independent biological replicates (n = 3), with technical triplicates performed for each assay to ensure reproducibility. Data are presented as mean ± standard deviation. To determine significant differences between isolates, one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference (HSD) post-hoc analysis. Statistical significance was defined as $p<0.05$. Data processing and statistical tests were executed using SPSS Version 22.0 (IBM Corp., USA).

3. Results

3.1. Isolate Isolation and Phenotypic Selection

A total of 105 presumptive lactic acid bacteria (LAB) strains were obtained in the primary screening of 53 samples of traditional Vietnamese pickled eggplant (*Solanum macrocarpon*) on MRS agar with $CaCO_3$, by the acid production halo method. After secondary screening 62 isolates were selected using morphological and biochemical profile. All the isolates selected were non-motile, catalase negative, Gram positive, and glucose, sucrose and lactose fermenters respectively (Table 2).

3.2. Resilience to Simulated Gastrointestinal Stress

The 62 isolates were challenged with acid and bile salt, to assess their gastrointestinal survivability. There was a significant difference in growth resilience

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	1,030	(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		

Table 2. Morphological, physiological, and biochemical characteristics of the 62 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
Physiology	Non-motile (-), non-endospore-forming
Biochemical profile	Catalase (-), Urease (-), Citrate Utilization (-)
Carbohydrate fermentation	Glucose (+), Sucrose (+), Lactose (+)

(+): positive, (-): negative

between the collection ($p<0.05$). The highest viable counts (Log CFU/mL) were ranked to get the best candidates for further characterization.

Exposure to varying bile salt concentrations (0.2% to 1.0% w/v) demonstrated that isolate TV3.1.2 exhibited high tolerance, maintaining a significantly higher viable count (3.50±0.61 Log CFU/mL) at the 1.0% concentration compared to more sensitive isolates like TV10.1.1 (0.18±0.06 Log CFU/mL) (Table 3). Similarly, under extreme acidic conditions, TV3.1.2 maintained superior survival at pH 3.0 (5.02±0.48 Log CFU/mL) and pH 2.0 (2.29±0.49 Log CFU/mL) after 4 hours of incubation (Table 4).

3.3. Safety Profiling: Phenotypic and Genotypic Assessment

Safety is a critical prerequisite for selecting LAB for food and veterinary applications. Phenotypically, all tested isolates exhibited γ-hemolysis, indicating a lack of hemolytic activity. Antibiotic susceptibility profiles (Table 5) revealed that isolate TV3.1.2 was susceptible (S) or intermediate (I) to most clinically relevant antibiotics, including Ampicillin and Chloramphenicol, reducing the risk of contributing to antimicrobial resistance (AMR) spread.

Although specific inhibition zone diameters were measured for all replicates to ensure statistical accuracy, categories (S/I/R) are presented in Table 5 for clarity. It should be noted that the resistance observed in this study is phenotypic and does not necessarily equate to transmissible resistance without further genetic confirmation.

To distinguish this food-derived candidate from pathogenic lineages, genotypic screening via PCR was performed. As detailed in Table 6, isolate TV3.1.2 tested negative for all screened virulence determinants (*esp, gelE, fsrB, asa1, cylA, cylM*), vancomycin resistance genes (*vanA, vanB*), and biogenic amine genes (*hdc1, hdc2, tdc*). This absence of targeted genetic risk factors suggests a high safety profile, though it does not exclude novel or untargeted virulence determinants.

3.4. Antimicrobial Activity against Foodborne Pathogens

The antagonistic activity of the isolates was tested against three indicator pathogens and quantified. TV3.1.2 had a wide and strong inhibitory spectrum ($p<0.05$) as presented in Table 7. Notably, this yielded an inhibition zone that was 30.47±5.78 mm against *S. aureus* ATCC 25923. In contrast to the majority of other isolates that exhibited localized activity, TV3.1.2 had moderate to strong inhibitory activity against both Gram-positive and Gram-negative targets.

3.5. Molecular Identification and Phylogenetic Analysis

Focusing on superior stress resistance, safety profile, and antimicrobial properties. Isolate TV3.1.2 was chosen to be identified using molecular identification. The sequence of the 16S rRNA gene had been analyzed using the BLASTn algorithm, and the result showed that the sequence is 99.67% similar with *Lentilactobacillus farraginis* strain NRIC 0676 (NR_041467.1) (Table 8). TV3.1.2 was included in the *L. farraginis* clade with high bootstrap support (1,000 replications) in phylogenetic analysis by the Neighbor-Joining method (Figure 1), where it was confirmed to belong to this group. The sequence has been deposited in GenBank (PX850480).

4. Discussion

4.1. Ecological Significance and Isolation from *Solanum macrocarpon*

The microbial diversity of traditional food samples is critical and frequently found to contain LAB microbes adapted to particular local environments (Fadare *et al.* 2023). The spontaneous fermentation of Ca phao muoi chua (pickled *Solanum macrocarpon*) is a special ecological niche in Vietnam, characterized by varying salinity and strongly acidifying conditions. It is in this environment that there is significant evolutionary pressure on the resident microbiota. Some species, like *Lactiplantibacillus plantarum* and *Levilactobacillus brevis*, are widely shared in vegetable fermentation (Lee *et al.* 2021; Nguyen *et al.* 2023) but TV3.1.2 species *Lentilactobacillus farraginis* is one of the main functional species in the ecosystem of Ca phao fermentation. The results differ from the other vegetable fermented products in Southeast Asia such as Thai fermented tea leaves (miang) and Indonesian vegetable pickles, where the dominant

Table 3. Survival of selected LAB isolates (Log CFU/mL) under varying bile salt concentrations (0.2–1.0% w/v) after 4 hours

Isolation ID	Viable bacteria isolates (Log CFU/mL)					
	0.00%	0.20%	0.40%	0.60%	0.80%	1.00%
HT14.3	9.36±1.53^{ab}	9.30±1.56^{ab}	7.40±2.00^{ab}	5.81±0.78^{ab}	4.81±0.77^{ab}	2.94±0.75^{abc}
TV16.2	11.60±1.52^{a}	10.21±1.55^{a}	9.81±1.05^{a}	6.39±1.51^{ab}	5.57±1.12^{ab}	4.26±0.74^{a}
NH12.2	10.33±1.51^{ab}	9.29±1.54^{ab}	8.93±0.76^{ab}	5.10±1.52^{ab}	3.61±0.02^{bc}	1.69±0.52^{cd}
TV3.1.2	10.32±1.50^{ab}	8.94±1.54^{ab}	7.10±2.09^{ab}	5.94±0.60^{ab}	3.61±0.03^{bc}	3.50±0.61^{ab}
TV3.1.2.1	7.59±0.62^{b}	6.19±1.02^{b}	4.63±1.18^{b}	3.21±1.01^{b}	2.55±0.73^{c}	0.34±0.36^{de}
TV10.1.1	7.97±0.58^{b}	6.60±0.99^{ab}	5.41±2.49^{ab}	5.27±2.10^{ab}	2.59±1.00^{c}	0.18±0.06^{e}
DH25.1	9.37±1.54^{ab}	9.28±1.60^{ab}	6.73±1.08^{ab}	6.34±1.58^{ab}	4.68±0.47^{ab}	1.98±0.50^{c}
CN53.1	11.14±1.51^{ab}	10.09±1.55^{a}	9.41±1.05^{ab}	5.93±1.51^{ab}	4.42±0.01^{abc}	2.87±0.14^{abc}
PN1.2	9.99±0.57^{ab}	8.61±0.99^{ab}	6.78±1.71^{ab}	5.92±0.61^{ab}	4.93±0.57^{ab}	2.23±0.51^{bc}
TV14.3	10.07±0.63^{ab}	8.66±1.01^{ab}	7.10±1.18^{ab}	6.35±1.55^{ab}	5.02±0.72^{ab}	3.48±0.74^{ab}
TV17.1	10.02±0.61^{ab}	8.96±1.19^{ab}	6.73±1.09^{ab}	6.30±1.56^{ab}	4.97±0.74^{ab}	2.22±0.51^{bc}
MT1.1	10.02±0.57^{ab}	8.65±0.99^{ab}	7.44±2.48^{ab}	7.32±2.10^{a}	4.97±0.55^{ab}	2.07±0.14^{bc}
GC6.2	10.81±0.57^{ab}	9.45±0.99^{ab}	8.25±2.49^{ab}	6.77±0.58^{ab}	5.76±0.56^{a}	2.62±0.18^{bc}
MP2.2	9.13±1.51^{ab}	7.41±0.99^{ab}	5.54±1.72^{ab}	4.75±0.59^{ab}	3.74±0.56^{bc}	0.36±0.31^{de}
MT8.2	8.16±1.53^{ab}	7.76±1.52^{ab}	5.50±0.97^{ab}	4.59±0.78^{ab}	3.60±0.75^{bc}	2.52±0.46^{bc}

Values are mean ± SD (n = 3). Different superscript letters in the same column indicate significant differences ($p<0.05$)

Table 4. Survival of selected LAB isolates (Log CFU/mL) under varying acidic pH (2.0, 3.0, 4.0) after 4 hours

Isolation ID	Viable bacteria isolates (Log CFU/mL)			
	pH 7.2	pH 4	pH 3	pH 2
HT14.3	9.36±1.53^{ab}	7.60±2.50	5.27±0.56^{a}	2.34±0.67^{abc}
TV16.2	11.60±1.52^{a}	7.69±2.58	5.36±0.63^{a}	2.06±0.40^{abcd}
NH12.2	10.33±1.51^{ab}	9.18±0.77	4.02±0.07^{ab}	2.34±0.52^{abc}
TV3.1.2	10.32±1.50^{ab}	7.02±1.09	5.02±0.48^{a}	2.29±0.49^{abc}
TV3.1.2.1	7.59±0.62^{b}	6.35±0.48	3.85±0.02^{ab}	1.97±0.50^{abcde}
TV10.1.1	7.97±0.58^{b}	6.95±0.98	5.11±0.75^{a}	1.38±0.45^{bcdef}
DH25.1	9.37±1.54^{ab}	7.37±2.05	4.03±0.10^{ab}	1.44±0.67^{bcdef}
CN53.1	11.14±1.51^{ab}	7.39±1.12	5.39±0.84^{a}	2.70±0.40^{ab}
PN1.2	9.99±0.57^{ab}	6.42±2.48	3.76±0.97^{ab}	1.17±0.54^{cdef}
TV14.3	10.07±0.63^{ab}	4.24±1.21	2.24±0.73^{b}	0.12±0.11^{f}
TV17.1	10.02±0.61^{ab}	7.02±1.72	5.36±0.53^{a}	3.27±0.54^{a}
MT1.1	10.02±0.57^{ab}	7.06±1.76	5.39±0.61^{a}	2.38±0.76^{abc}
GC6.2	10.81±0.57^{ab}	5.78±0.95	2.78±0.46^{b}	0.57±0.40^{def}
MP2.2	9.13±1.51^{ab}	7.74±2.05	5.24±0.77^{a}	2.55±0.29^{abc}
MT8.2	8.16±1.53^{ab}	6.89±1.01	2.72±1.14^{b}	0.47±0.45^{ef}

Values are mean ± SD (n = 3). Different superscript letters in the same column indicate significant differences ($p<0.05$)

Table 5. Antibiotic susceptibility profiles of the 15 selected LAB isolates

Isolates	CIP	DO	CN	STR	AMP	E	C	K
HT14.3	I	I	S	R	I	R	R	R
TV16.2	S	S	S	S	R	R	R	R
NH12.2	I	I	R	R	S	R	I	R
TV3.1.2	S	S	S	S	I	I	I	I
TV3.1.2.1	S	S	S	S	S	I	I	I
TV10.1.1	I	I	R	R	I	S	I	I
DH25.1	I	R	S	S	I	S	S	S
CN53.1	S	I	S	R	I	R	I	I
PN1.2	I	R	R	I	S	S	S	I
TV14.3	S	I	S	I	R	R	R	R
TV17.1	S	R	S	R	I	R	R	I
MT1.1	I	I	S	R	S	R	I	S
GC6.2	S	S	I	R	I	S	R	R
MP2.2	S	I	S	S	S	R	I	I
MT8.2	I	R	S	R	I	R	I	R

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). R: Resistant; I: Intermediate; S: Susceptible

Table 6. PCR screening results for target virulence, antibiotic resistance, and biogenic amine genes

Category	Target gene	TV3.1.2	TV3.1.2.1	DH25.1	MP2.2
Virulence factors	*esp, gelE, fsrB, asa1, cylA, cylM*	-	-	-	-
Antibiotic resistance	*vanA, vanB*	-	-	-	-
Biogenic amines	*hdc1, hdc2, tdc*	-	-	-	-

(-) indicates the absence of the target gene (negative PCR result). No amplicons were detected for any tested genes in these strains

Table 7. Mean inhibition zones (mm) of top LAB isolates against indicator pathogens

Isolation ID	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*S. enterica* ATCC 14028
HT14.3	29.70±6.07^{ab}	0.00±0.00^{d}	17.90±2.89^{a}
TV16.2	0.00±0.00^{c}	21.37±7.05^{abc}	0.00±0.00^{b}
NH12.2	33.63±5.45^{a}	16.73±1.16^{abc}	27.63±8.52^{a}
TV3.1.2	23.90±9.59^{ab}	30.47±5.78^{a}	25.73±4.01^{a}
TV3.1.2.1	18.87±3.93^{ab}	14.90±1.37^{bc}	20.07±6.29^{a}
TV10.1.1	31.93±10.90^{ab}	19.00±1.41^{abc}	0.00±0.00^{b}
DH25.1	20.13±3.20^{ab}	22.53±6.07^{abc}	22.73±3.46^{a}
CN53.1	18.03±2.92^{ab}	20.80±6.63^{abc}	20.80±3.89^{a}
PN1.2	0.00±0.00^{c}	27.73±10.35^{ab}	0.00±0.00^{b}
TV14.3	20.77±4.36^{ab}	17.13±1.22^{abc}	22.50±6.42^{a}
TV17.1	15.63±0.49^{bc}	15.20±1.57^{bc}	0.00±0.00^{b}
MT1.1	0.00±0.00^{c}	0.00±0.00^{d}	17.37±2.06^{a}
GC6.2	26.10±9.25^{ab}	23.10±6.53^{abc}	27.50±3.52^{a}
MP2.2	17.47±0.85^{ab}	16.83±0.93^{abc}	0.00±0.00^{b}
MT8.2	30.23±5.42^{ab}	13.37±1.11^{cd}	24.17±8.46^{a}

Values are mean ± SD (n = 3). Significant differences ($p<0.05$) are indicated by superscript letters

Table 8. BLASTn alignment statistics for the 16S rRNA gene sequence of isolate TV3.1.2

Closely related species	Max score	Query coverage (%)	E-value	Identity (%)	Accession No.
Lentilactobacillus farraginis strain NRIC 0676 16S ribosomal RNA, partial sequence	2769	91	0.0	99.67	NR_041467.1

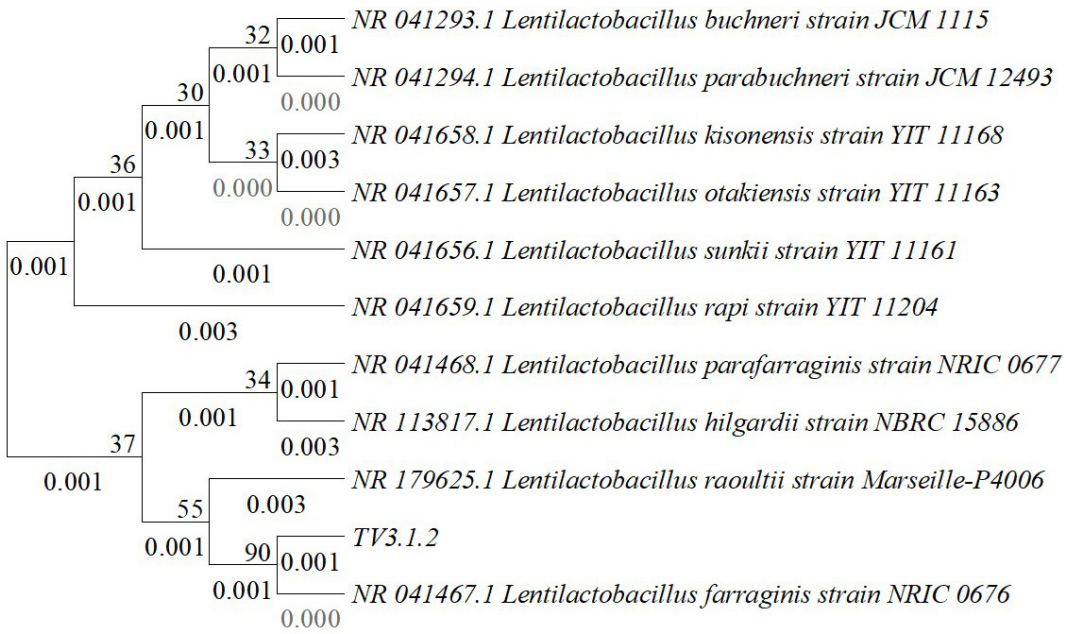

Figure 1. Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences. TV3.1.2 clusters firmly with the *L. farraginis* clade with a bootstrap value of 100%

species are typically *Lactobacillus, Pediococcus,* and *Weissella* species (Unban *et al.* 2021; Sihombing *et al.* 2025). The differences between regions indicate that the composition of communities in plant-based ferments is driven by matrix specific selective pressures and not a single pathway of a succession of LAB. Therefore, *L. farraginis* TV3.1.2 is a new contribution from Vietnam to this ecosystem, which has not been reported.

TV3.1.2 isolated from *Solanum macrocarpon* highlights its autochthonous nature. The glycoalkaloids and phenolic compounds present in *Solanum* genus plants are known for their antimicrobial activity (Masih 2025) and are present in plants in distinct chemical profiles, enabling niche-specific genomic plasticity in indigenous strains, which can tolerate these peculiar chemical profiles (Abriouel *et al.* 2022; Fiesel *et al.* 2022). The continuity of TV3.1.2 during the period of heavy fermentation indicates ecological specialisation. *L. farraginis* was recovered here, although this species is not as widely used in commercial starter cultures as *L. plantarum*, the recovery here demonstrates an underappreciated role of this species in the microbial complexity and stability of traditional food systems in Vietnam (Sihombing *et al.* 2025).

4.2. Gastrointestinal Competence: Survival Under Acid and Bile Stress

Tolerance to gastrointestinal (GIT) conditions is a fundamental prerequisite for any probiotic candidate, since the health benefits conferred by LAB are often diminished by declining viable counts during transit through the stomach and upper intestine (Han *et al.* 2021), and characterizing this survival remains a persistent challenge in strain selection (Ayyash *et al.* 2021). *L. farraginis* TV3.1.2 retained measurable viability at pH 2.0 (2.29±0.49 Log CFU/mL) and in the presence of 1.0% bile salts (3.50±0.61 Log CFU/mL) after 4 hours of exposure. To place this performance in context, the well-characterized reference strain *Lacticaseibacillus rhamnosus* GG typically retains viable counts within 1–2 Log units of the initial inoculum after 2–3 hours at pH 2.0–3.0, while *Lactiplantibacillus plantarum* and *Limosilactobacillus reuteri* strains generally tolerate bile salt concentrations in the range of 0.3–0.5% with limited loss of viability; While direct comparison with reference strains was not performed, the survival rates of TV3.1.2 at 1.0% bile salts are comparable to those reported for established probiotic benchmarks in literature; confirming this would require parallel testing of TV3.1.2 alongside reference strains under a standardized protocol. These findings are nonetheless consistent with reports on ruminant-derived *L. farraginis* strains recommended as probiotic feed additives owing to their limited growth reduction under bile and salt stress (Zayed *et al.* 2022), and with aquaculture studies in which *L. farraginis* enhanced serum lysozyme activity and other immune markers, improving disease resistance in rainbow trout (Ali *et al.* 2022). The phenotypic resilience observed in TV3.1.2 is plausibly attributable to adaptive mechanisms such as the acid tolerance response (ATR) or bile salt hydrolase (BSH) activity; LAB more generally regulate internal pH homeostasis through the F1F0-ATPase system or amino acid decarboxylation (Zommiti *et al.* 2020; Page *et al.* 2023). As these molecular determinants were not directly assayed in this study, the survival phenotypes reported here should be regarded as confirmed empirical traits, while the underlying genetic mechanisms remain literature-supported hypotheses that warrant direct investigation.

4.3. Antimicrobial Potential and Bioprotective Applications

The capacity to inhibit foodborne pathogens is central to the functional value of indigenous LAB. *L. farraginis* TV3.1.2 displayed a broad inhibitory spectrum, most prominently against *Staphylococcus aureus* ATCC 25923, with an inhibition zone exceeding 30 mm. A notable observation was that antimicrobial activity in the cell-free supernatant (CFS) persisted after neutralization to pH 7.0, indicating that inhibition is not attributable solely to organic acid production and may involve additional bioactive metabolites. Related work on *L. parafarraginis* reported inhibitory activity against multidrug-resistant pathogens attributed to bacteriocin structural genes (Allen-McFarlane *et al.* 2019), and *L. plantarum* strains from Chinese pickled vegetables have similarly shown broad-spectrum antibacterial activity linked to lactic acid production (Zeng *et al.* 2020). Candidate metabolites in TV3.1.2 could plausibly include bacteriocins, ribosomally synthesized peptides that typically disrupt the membranes of competing bacteria (Darbandi *et al.* 2022). The observed antimicrobial activity after neutralization and catalase treatment suggests the presence of non-acid, non-peroxide inhibitory metabolites. Further studies, including proteinase K sensitivity assays and molecular screening, are required to characterize these active compounds. Definitive characterization requires proteinase K sensitivity testing, bioassay-guided fractionation, and molecular screening, with rigorous controls to exclude residual hydrogen peroxide or diacetyl, to confirm the nature of the inhibitory agent (Hatem *et al.* 2024).

4.4. Safety-Filtered Characterization: A Prerequisites for Application

Despite the Generally Recognized as Safe (GRAS) status broadly associated with LAB, individual strains can harbor virulence genes or transmissible antibiotic resistance determinants (Kiousi *et al.* 2023), a discrepancy that necessitates strain-specific screening rather than reliance on genus-level assumptions (Altavas *et al.* 2024). This study addressed that requirement through combined phenotypic and genotypic screening. Phenotypically, TV3.1.2 was γ-hemolytic, satisfying a primary safety criterion for food-associated microorganisms (Unban *et al.* 2021), and its susceptibility to clinically relevant antibiotics such as ampicillin and chloramphenicol indicates a low likelihood of contributing to the horizontal transfer of antimicrobial resistance, consistent with safety profiles reported for other *Lactobacillus* strains (Anisimova and Yarullina 2019).

Genotypic screening provided further validation: the absence of virulence determinants (*esp, gelE, cylA/M*) distinguishes TV3.1.2 from pathogenic clinical lineages (Kanak *et al.* 2023). Furthermore, the absence of vancomycin resistance (*vanA, vanB*) and biogenic amine-producing genes (*hdc1/2, tdc*) is particularly important, ensuring the strain is unlikely to generate toxic amines like histamine or tyramine in fermented products (Câmara *et al.* 2020). It is important to note, however, that PCR-based screening has inherent limitations; a negative result confirms only the absence of the specific targeted genes and does not exclude novel or untested virulence factors or mobile genetic elements. Therefore, the safety profile established for TV3.1.2 represents the confirmed absence of targeted risks rather than an exhaustive guarantee. Future work should incorporate whole-genome sequencing (WGS) to provide a definitive safety map and support regulatory compliance for Qualified Presumption of Safety (QPS) or GRAS status.

4.5. Limitations, Translational Considerations, and Future Directions

The scope of this study was primarily *in vitro*. While these phenotypic assays establish a necessary baseline, they cannot fully replicate complex host–microbe interactions. Consequently, *in vivo* validation using animal models is required to confirm whether these probiotic benefits, such as immunomodulation or pathogen exclusion, are retained in situ. Additionally, the colonization capacity of TV3.1.2, including adhesion and biofilm formation, remains to be determined.

The 16S rRNA gene sequence analysis provides a putative taxonomic classification of isolate TV3.1.2 as *Lentilactobacillus farraginis* (99.67% identity), though definitive species resolution will require future whole-genome sequencing (WGS), to validate safety in comprehensive ways, and to identify biosynthetic gene clusters that explain the antimicrobial activity detected. Finally, the performance of TV3.1.2 in scaled-up fermentation process and its ability to survive during lyophilization and/or stability in the final product will have to be assessed. Bridge these translational gaps will help indigenous strains such as TV3.1.2 enter into the food and livestock sectors as standardised applications, and preserve and make use of Vietnam's microbial heritage.

In conclusion, In this study, *Lentilactobacillus farraginis* TV3.1.2 is identified as a potent probiotic strains for use in traditional Vietnamese product – Ca phao muoi chua. The strain exhibited an outstanding ability to tolerate gastrointestinal stressors and exhibit strong antimicrobial activity against major foodborne pathogens. A thorough safety filter was used to verify that no transmissible resistance/virulence genes were present, making TV3.1.2 a safe and effective potential starter culture. The results confirm the need to preserve the Vietnamese microbial heritage and also offer a scientific basis for the production of indigenous biological alternatives to traditional antibiotics in food and veterinary applications.

Conflict of Interest

The authors declare that there is no conflict of interest.

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