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Probiotic potential and safety assessment of autochthonous *Lentilactobacillus farraginis* BDN12 from fermented okara for feed applications

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Abstract. *Thuy NP, Linh NT, Vy LT, Huong HTM, Khoa ND, Minh NN, Lam NVN, My BNT, Dan LNL, Huy NG, Chau LNB, Tien TTC, Thu NM, Diep PH. 2026. Probiotic potential and safety assessment of autochthonous* Lentilactobacillus farraginis *BDN12 from fermented okara for feed applications. Biodiversitas 27 (5): d270514. https://doi.org/10.13057/biodiv/d270514*. The escalating risk of antimicrobial resistance has intensified the search for autochthonous probiotics as sustainable alternatives to antibiotic growth promoters in livestock. This study aimed to isolate and characterize high-performance Lactic Acid Bacteria (LAB) from fermented okara, a traditional Vietnamese soybean byproduct, for specific application in poultry feed. A hierarchical five-stage pipeline was used to screen 95 initial isolates based on acid production, biochemical traits, antimicrobial activity, and gastrointestinal resilience. The lead isolate, BDN12, was identified via 16S rRNA sequencing (GenBank PX973265) as *Lentilactobacillus farraginis*, showing 99.87% identity with strain NRIC 0676. Phylogenetic analysis confirmed its placement within the *L. farraginis* clade with 100% bootstrap support. In vitro assessments showed that BDN12 maintained viability at pH 3.0 and 0.4% oxgall bile salts. BDN12 neutralized cell-free supernatant exhibited significant ($p<0.05$) inhibitory activity against *Escherichia coli* ATCC 25922 (30.33±0.58 mm) and *Salmonella enterica* ATCC 14028 (29.00±1.00 mm). This inhibitory activity may be attributed to the presence of bacteriocin-like compounds acting as non-acidic antimicrobial factors. Safety profiling confirmed the BDN12 strain was non-hemolytic and susceptible to clinically significant antibiotics. PCR safety screening further confirmed the absence of virulence factors (*esp, gelE, fsrB, asa1, cylA, cylM*), vancomycin resistance genes (*vanA, vanB*), and biogenic amine-producing genes (*hdc1, hdc2, tdc*). These results establish *L. farraginis* BDN12 as a safe, potent, and locally adapted functional feed additive capable of enhancing poultry health in antibiotic-free production systems.

Keywords: Antibiotic growth promoter, antimicrobial activity, fermented okara, PCR safety screening, poultry

INTRODUCTION

The global livestock sector faces a critical turning point due to the historical over-dependence on in-feed Antibiotic Growth Promoters (AGPs). While AGPs were traditionally used to enhance poultry production, their misuse has accelerated the emergence of resistant bacterial populations, threatening both animal health and human safety through the food chain (Coyne et al. 2020; Shah et al. 2021). In response to this crisis, Vietnam has strictly regulated the use of antibiotics in livestock. Specifically, under the Law on Livestock No. 32/2018/QH14, the use of antibiotics for growth stimulation in animals has been prohibited (National Assembly of Vietnam 2018). Despite these regulations, field surveys in Tra Vinh province indicate that 86.67% of backyard chicken farms still use antibiotics preventatively, with a significant portion applying them indiscriminately (Quyên and Hiểu 2023). This high regional Anti-Microbial Resistance (AMR) pressure underscores the urgent need for sustainable alternatives, such as probiotics, to stabilize the poultry gastrointestinal tract and reduce dependence on conventional antibiotic-based disease prevention strategies (Khan et al. 2022; Widjastuti et al. 2025).

Lactic Acid Bacteria (LAB) are prominent candidates for stabilizing the gut environment. These microorganisms enhance nutrient absorption, stimulate the host immune system, and directly inhibit enteric pathogens like *Salmonella enteritidis* and *Escherichia coli* (Harnentis et al. 2020; Jha et al. 2020). While generic LAB strains are widely available, autochthonous or locally adapted strains often exhibit superior ecological fitness within specific regional environments or feed matrices. Their functional efficacy is primarily driven by the production of bioactive metabolites, including organic acids and bacteriocins. Bacteriocins are particularly advantageous in animal feed because they offer targeted pathogen inhibition without disrupting the commensal microbiota, thereby supporting a more balanced intestinal ecosystem in poultry. This selective activity is especially important for maintaining gut stability during early growth stages (Sakandar and Zhang 2021).

Traditional fermented products serve as vital reservoirs for microbial biodiversity. In Southeast Asia, fermenting agricultural by-products, such as okara the insoluble, protein-rich residue from soymilk production serves the

dual purpose of enhancing food security and reducing environmental waste. Fermented okara represents a unique ecological niche; previous studies have identified diverse LAB populations in soybean residues that have evolved to thrive under high osmotic pressure and low pH (Aritonang et al. 2017; Zheng and Xu 2017). Furthermore, autochthonous LAB isolated from these substrates can secrete enzymes like cellulase and phytase, which enhance the bioavailability of poultry feed and improve the functional value of fermented feed ingredients (Srifani et al. 2024).

Lentilactobacillus farraginis has recently emerged as a promising probiotic species within these matrices. Strains of *L. farraginis* are notably resilient to temperature fluctuations, salinity, and high bile concentrations (Zayed et al. 2022). However, selecting industrial strains involves a "safety paradox": while most LAB are Generally Recognized as Safe (GRAS), certain strains may harbor transferable resistance genes or virulence factors (Thamacharoensuk et al. 2017; Kiousi et al. 2023; Liu et al. 2023). Therefore, a stringent screening pipeline combining phenotypic and genotypic assessments is essential to prevent the horizontal transmission of toxins in mass livestock production (Wang et al. 2021; Yaacob et al. 2022).

Current literature on soybean-derived LAB often emphasizes general biodiversity but lacks the strain-level validation required for targeted feed applications (Nguyen et al. 2023). Notably, there is a lack of research characterizing *L. farraginis* strains from Vietnamese fermented okara regarding their probiotic potential against regional poultry pathogens. To address this gap, this study characterizes *L. farraginis*, an autochthonous strain isolated from fermented okara. The objectives of this study were to: (i) isolate LAB isolates; (ii) assess antimicrobial potency against major foodborne pathogens; (iii) evaluate resilience to simulated gastrointestinal stressors; and (iv) validate safety through molecular screening for virulence and resistance genes. This research provides a scientific framework for utilizing regional microbial heritage to support antibiotic-free poultry production in Vietnam.

MATERIALS AND METHODS

Stepwise screening pipeline

A five-stage hierarchical screening pipeline was implemented to systematically evaluate isolates against performance and safety criteria.

Stage I: Preliminary isolation of acid-producing colonies. Stage II: Identification of isolates as LAB through phenotypic and biochemical assays. Stage III: Antimicrobial filtering, selecting isolates with inhibition zones ≥18 mm against at least two pathogens. Stage IV: Functional and safety evaluation of selected finalists. Isolates were prioritized based on their ability to maintain viability ($\geq 10^3$ CFU/mL) under simulated gastrointestinal stress (pH 3.0 and 0.4% bile salts), lack of hemolytic activity, and susceptibility to clinically relevant antibiotics. Stage V: Molecular identification and genotypic safety characterization of the lead isolate.

Sample collection, laboratory fermentation, and primary isolation

Fresh okara (soybean byproduct) was obtained from local tofu and soy milk production facilities in Vinh Long Province, Vietnam. To ensure sample representativeness, fresh batches were collected from various traditional producers and transported to the laboratory at 4°C for immediate processing. The fresh okara was then subjected to a controlled fermentation process. Specifically, the substrate was inoculated with selected Lactic Acid Bacteria (LAB) strains, including *L. farraginis*, to initiate the fermentation. The process was monitored until the peak phase (3-7 days), reaching a target pH range of 3.8-4.5 and salt levels of 3-5% (w/v), ensuring an optimal environment for probiotic enrichment.

For microbial isolation, 10 g of the laboratory-fermented okara was homogenized in 90 mL of 0.1% (v/v) peptone water (Oxoid, UK). Serial dilutions (up to 10^{-6}) were performed, and 100 µL aliquots were spread-plated in triplicate on de Man, Rogosa, and Sharpe (MRS) agar (HiMedia, India) supplemented with 1.0% (w/v) $CaCO^3$. The plates were incubated anaerobically at 37°C for 48 h to selectively isolate acid-producing colonies (Makhlouf et al. 2024).

Phenotypic and biochemical characterization

The isolated colonies with different halos in the production of acid were selected to undergo additional screening. The isolates were carefully selected based on the presence of typical LAB traits: Gram-positive staining, non-motility, and no catalase activity (3% H_2O_2). This rule was done to exclude any possible non-LAB colonies (Gram-negative or catalase-positive). The rest of 52 purified isolates were identified by fermentation profile of carbohydrates (glucose, lactose, and sucrose) (Kang et al. 2020) and cryopreserved in MRS broth with 20% glycerol at -80°C (Guney et al. 2025).

Assessment of antagonistic activity

Antimicrobial potential was evaluated against *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Salmonella enterica Typhimurium* ATCC 14028 using a standardized agar well diffusion assay (Abubakr 2018). Indicator strains were prepared at a standardized inoculum density of 10^6 CFU/mL. To isolate non-acidic effects, the Cell-Free Supernatant (CFS) was neutralized to pH 7.0±0.1 using 1 M NaOH, with values confirmed via a digital pH meter. Neutralized CFS (nCFS, 100 µL) was introduced into 6 mm wells in Nutrient Agar (agar thickness standardized to 4 mm). Ampicillin (10 µg) and sterile neutralized MRS broth served as positive and negative controls, respectively. Following 24 h of incubation at 37°C, the diameters of inhibition zones were measured from the edge of the well to the edge of the zone in triplicate.

Functional probiotic potential and resilience

The selected finalists were evaluated for their ability to survive simulated gastrointestinal transit. Overnight cultures were harvested (10,000 rpm, 10 min, 4°C), washed

with Phosphate-Buffered Saline (PBS, pH 7.2), and resuspended to a standardized OD_{620} of 0.6 (≈10^8 CFU/mL) (Lu et al. 2024).

Salinity tolerance was assessed in MRS broth containing 2.0%, 4.0%, 6.0%, and 8.0% NaCl over 72 h at 37°C. Acid and bile tolerance were evaluated by adding suspensions to MRS broth adjusted to pH 2.0 and 3.0, or supplemented with oxgall bile salts (0.2%, 0.4%, and 0.8% w/v; Oxoid, UK). Standard MRS (pH 7.0, 0% bile) served as the control. After 4 h of incubation at 37°C, growth was recorded using semi-quantitative categories based on final viable counts: Excellent (+++, >10^7 CFU/mL), moderate moderate (++, 10^4-10^6 CFU/mL), weak (+, 10^1-10^3 CFU/mL), and no growth (-) (Ou et al. 2022; Banik et al. 2023). Results were pooled from three biological replicates.

Safety profiling and antibiotic susceptibility

Hemolytic activity was assessed on 5% sheep blood agar at 37°C for 24 h. Strains were categorized as β-hemolytic (clear zones), α-hemolytic (greenish zones), or γ-hemolytic (no zones/non-hemolytic). Only γ-hemolytic strains were considered for further assessment (Unban et al. 2021). Antibiotic susceptibility was determined via the Kirby-Bauer disc diffusion method on MRS agar against eight antibiotics: 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). Antibiotic susceptibility was determined via the Kirby-Bauer disk diffusion method on MRS agar against eight antibiotics. Due to the lack of universal CLSI standards for LAB, interpretive breakpoints were adapted from established literature (Alebiosu et al. 2017; Nguyen et al. 2023): Susceptible ≥20 mm; Intermediate 15-19 mm; Resistant ≤14 mm. Actual inhibition zone diameters (mm) were recorded for BDN12 to support qualitative assessments.

Molecular identification and genotypic analysis

Genotypic safety was screened via PCR for 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 performed using gene-specific primers (Table 1) and DreamTaq Green PCR Master Mix (Thermo Scientific). Amplicons were visualized via 1.5% agarose gel electrophoresis (Nguyen et al. 2025).

Selected isolate was identified through 16S rRNA gene sequencing using universal 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Liu et al. 2020). The optimized 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. Specific annealing temperatures for each primer set are detailed in Table 1. Purified amplicons (QIAquick, Qiagen) were sequenced by Ktest Co., Ltd. (Vietnam). Sequences were analyzed using NCBI BLASTn, and a phylogenetic tree was constructed using the Neighbor-Joining method in MEGA6 with 1,000 bootstrap replicates.

Statistical analysis

All assays were performed using three independent biological replicates as the experimental unit. Significant differences ($p<0.05$) between isolates were determined using One-Way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test. Statistical analysis was performed for each pathogen separately to compare isolate efficacy using SPSS Version 22.0. In data tables, values represent mean±SD (n = 3); different superscript letters in the same column indicate statistically significant differences (Ding et al. 2017).

Table 1. Primers used for the detection of virulence factors, antibiotic resistance genes, and biogenic amine-producing genes

Categories	Target genes	Primers	Sequences (5′ → 3′)	Size (bp)	Annealing temp (°C)	References
Virulence factors	*esp*	Esp F	TTGCTAATGCTAGTCCACGACC	933	55	(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	54	(Dutka-Malen et al. 1995)
		vanA R	CATGAATAGAATAAAAGTTGCAAT			
	vanB	vanB F	GTGACAAACCGGAGGCGAGGA	433		
		vanB R	CCGCCATCCTCCTGCAAAAAA			
Biogenic amines	*hdc1*	Hdc1 F	AGATGGTATTGTTTCTTATG	367	52	(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			

RESULTS AND DISCUSSION

Isolation and preliminary characterization

A total of 95 initial isolates were obtained from fermented okara. During phenotypic screening, 43 isolates were excluded due to Gram-negative staining (n = 30), catalase-positive reactions (n = 9), or motility (n = 4). The remaining 52 isolates (54.7% of the total) were confirmed as Lactic Acid Bacteria (LAB), matching the classic phenotypic profile: Gram-positive, non-motile, and catalase-negative. Morphologically, these isolates formed smooth, circular, milky-white colonies on MRS agar. Microscopic examination revealed rod-shaped or coccobacilli cells arranged in short chains or pairs, without endospore formation.

Antagonistic potential against foodborne pathogens

The antimicrobial efficacy of the finalists varied significantly ($p<0.05$) across the three indicator pathogens (Table 2). Out of 52, only strain BDN12 emerged as a potent antagonist, exhibiting broad activity against the tested pathogens. Notably, the neutralized Cell-Free Supernatant (nCFS) of BDN12 maintained high inhibitory activity at pH 7.0, yielding substantial zones of inhibition against *E. coli* ATCC 25922 (30.33±0.58 mm) and *S. enterica* ATCC 14028 (29.00±1.00 mm). While the activity against *S. aureus* ATCC 25923 was lower (17.00±1.00 mm), it remained statistically significant compared to other candidates ($p<0.05$). The variability observed in some isolates, such as BDN1 against *S. enterica* (29.67±5.51 mm), was attributed to biological variation across independent replicates. The retention of activity at a neutral pH (Figure 1) suggests that the antagonistic effect was independent of organic acid production.

Resilience to simulated gastrointestinal stress

All the 12 isolates were evaluated for their ability to survive salinity, acid, and bile salt stressors (Table 3). Growth was recorded using operational definitions for internal comparison:

Salinity: All 12 isolates showed excellent growth (+++) at 2.0% and 4.0% NaCl; however, growth was attenuated at 6.0%, and no isolates survived at 8.0%.

Gastric simulation: No isolates remained viable at pH 2.0, five candidates (BDN3, BDN4, BDN8, BDN10, and BDN12) maintained moderate growth (++) at pH 3.0.

Bile tolerance: BDN12 demonstrated robust tolerance within the tested in vitro conditions, retaining viability in concentrations up to 0.4% oxgall bile salts (10^1-10^3 CFU/mL), while most other candidates showed no growth (-). Specifically, BDN12 showed a viable count of 3.57×10^3±0.15 CFU/mL after 4 h of exposure to 0.4% bile.

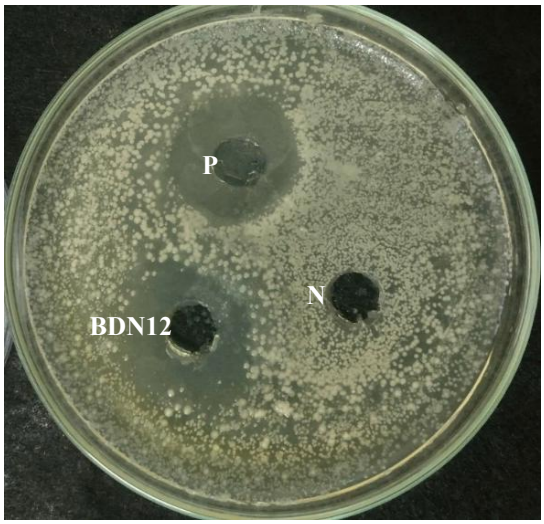

Figure 1. Antagonistic effect of *Lentilactobacillus farraginis* BDN12 against *Escherichia coli* ATCC 25922. BDN12: nCFS from *L. farraginis* BDN12, P: Positive control (Ampicillin, 10 µg), N: Negative control (Neutralized MRS broth)

Table 2. Antimicrobial activity (inhibition zone diameters) of the top LAB isolates against indicator pathogens

Isolates	*Escherichia coli* ATCC 25922	*Staphylococcus aureus* ATCC 25923	*Salmonella enterica* ATCC 14028
BDN1	19.33±6.66bc	16.67±3.51ab	29.67±5.51a
BDN2	26.00±3.61ab	16.40±1.64ab	22.33±1.53ab
BDN3	27.67±2.52ab	18.00±2.00ab	30.33±2.52a
BDN4	25.33±2.52ab	19.67±3.51a	15.33±3.51b
BDN5	26.33±1.53ab	19.33±1.15a	19.00±7.94ab
BDN6	23.00±2.00abc	21.00±1.00a	19.33±2.52ab
BDN7	24.33±1.53ab	17.67±2.52ab	23.33±12.10ab
BDN8	21.33±1.15abc	19.33±5.69a	30.33±3.51a
BDN9	27.33±2.52ab	20.67±2.89a	15.67±1.15b
BDN10	23.33±4.16abc	18.67±3.21ab	19.33±1.15ab
BDN11	21.67±2.89abc	16.33±3.51ab	30.33±4.16a
BDN12	30.33±0.58a	17.00±1.00ab	29.00±1.00a

Note: Values are expressed as mean diameter (mm)±SD (n = 3). Different superscript letters in the same column indicate statistically significant differences ($p<0.05$)

Table 3. Resilience of 12 selected LAB isolates to NaCl, pH, and bile salt stressors

Isolates	NaCl tolerance				pH tolerance				Bile tolerance			
	2.0%	4.0%	6.0%	8.0%	2.0	3.0	4.0	7.0	0%	0.20%	0.40%	0.80%
BDN1	+++	+++	+	-	-	+	++	+++	+++	++	-	-
BDN2	++	++	+	-	-	+	+	+++	+++	++	+	-
BDN3	+++	+++	+	-	-	+	+	+++	+++	+	+	-
BDN4	++	++	-	-	-	++	++	+++	+++	+	-	-
BDN5	+++	+++	-	-	-	+	+	++	++	+	+	-
BDN6	++	+	+	-	-	+	+	+++	+++	+	+	-
BDN7	+++	++	+	-	-	+	+	++	++	++	+	-
BDN8	+++	+++	-	-	-	++	+	+++	++	+	+	-
BDN9	+++	+++	+	-	-	+	+	++	++	++	-	-
BDN10	++	++	+	-	-	++	++	+++	+++	+	+	-
BDN11	+++	+++	+	-	-	+	+	+++	+++	+	-	-
BDN12	**+++**	**+++**	**+**	**-**	**-**	**++**	**++**	**+++**	**+++**	**+**	**+**	**-**

Note: +: Indicates growth (10^1-10^3 CFU/mL), ++: Indicates moderate growth (10^4-10^6 CFU/mL), +++: Indicates excellent growth (>10^7 CFU/mL), -: Indicates no growth

Table 4. Antibiotic susceptibility profiles of 12 selected LAB isolates

Isolates	CIP	DO	CN	STR	AMP	E	C	K
BDN1	S	S	S	S	I	I	S	I
BDN2	S	S	R	S	S	R	R	R
BDN3	R	R	R	R	R	I	I	I
BDN4	S	I	I	R	S	R	R	R
BDN5	S	R	R	R	R	S	R	R
BDN6	I	S	I	S	I	I	S	S
BDN7	S	S	I	S	I	S	I	S
BDN8	I	R	R	S	S	R	I	R
BDN9	R	R	R	R	I	R	R	S
BDN10	I	R	I	R	R	R	R	R
BDN11	S	S	I	S	I	I	S	S
BDN12	I	I	I	S	S	S	S	S

Note: S: Susceptible, I: Intermediate, R: Resistant, 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)

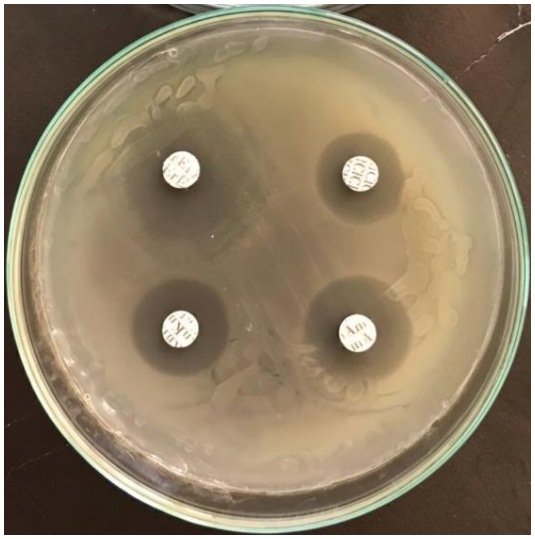

Figure 2. Kirby-Bauer disc diffusion assay for *Lentilactobacillus farraginis* BDN12

Safety profiling and antibiotic susceptibility

The safety test ensured that there were no pathogenic characteristics in the 12 finalists that were made. All the isolates had a γ-hemolysis (non-hemolytic) phenotype. The testing of antibiotic susceptibility (Table 4) showed that BDN12 was Susceptible (S) to Ampicillin, Chloramphenicol and Erythromycin. The inhibition zone diameters of the BDN12 were as follows: Ampicillin (25.33±0.58 mm), Erythromycin (22.67±1.15 mm), and Chloramphenicol (24.00±1.00 mm), which was larger than the susceptibility threshold of 20 mm and above (Figure 2).

BDN12 demonstrated Intermediate (I) responses to Ciprofloxacin and Doxycycline and multi-drug resistant patterns in other isolates caused them to be excluded in the Stage IV filter. Although these phenotypic findings can meet the preliminary safety standards of feed usage, they offer the qualitative measure of resistance.

Genotypic safety was further corroborated via PCR for the top functional performers, BDN4 and BDN12 (Table 5). Both strains 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*). While the absence of these specific markers is promising, it did not exclude all undesirable traits, and Whole-Genome Sequencing (WGS) remains a definitive requirement for future safety verification.

Molecular identification and phylogenetic analysis

Molecular characterization of the 16S rRNA gene sequence (GenBank Accession No. PX973265) revealed a 99.87% identity with *L. farraginis* strain NRIC 0676 (Table 6). Phylogenetic reconstruction clustered BDN12 firmly within the *L. farraginis* clade (Figure 3). The 16S rRNA gene sequence of isolate BDN12 has been deposited in GenBank under Accession No. PX973265.

Discussion

Isolation and ecological adaptation

The isolation of 95 initial strains from fermented okara, with 52 (54.7%) confirmed as Lactic Acid Bacteria (LAB), underscores traditional fermentation systems as vital

reservoirs for microbial biodiversity. The predominance of rod-shaped, Gram-positive, and catalase-negative isolates in this study is consistent with the microbial profiles typically observed in soybean byproduct fermentations. These matrices act as specialized ecological niches; the high organic acid concentrations and osmotic pressure select for resilient taxa capable of thriving under low pH conditions (Aritonang et al. 2017; Zheng and Xu 2017). The successful identification of *L. farraginis* BDN12 aligns with previous reports of robust LAB being harbored in similar plant-based substrates, such as fermented soy and vegetable residues (Gudisa and Yenasew 2022; Meanti et al. 2024).

Antagonistic potential against foodborne pathogens

A primary finding of this study is the broad-spectrum inhibitory activity exhibited by *L. farraginis* BDN12 against major avian pathogens. In the present assays, the neutralized Cell-Free Supernatant (nCFS) maintained significant zones of inhibition at pH 7.0, with the highest efficacy observed against *E. coli* ATCC 25922 (30.33±0.58 mm) and *S. enterica* ATCC 14028 (29.00±1.00 mm). This retention of activity at a neutral pH strongly suggests that the antagonistic effect is independent of organic acid production and may be attributed to the secretion of specialized non-acidic metabolites.

Previous studies have reported that bacteriocin-like compounds produced by various LAB strains show significant inhibitory effects against Gram-negative foodborne pathogens (Chauhan and Singh 2019; Cai et al. 2022; Hatem et al. 2024). While the presence of such compounds in BDN12 is a strong possibility, other factors, including hydrogen peroxide or thermolabile proteins, may contribute to the observed inhibition. Interestingly, the relatively lower activity against *S. aureus* (17.00±1.00 mm) compared to the Gram-negative indicators suggests a targeted inhibitory spectrum. Such differences in efficacy are often linked to variations in cell wall architecture or the specific mechanism of the secreted metabolites (Harnentis et al. 2020).

Resilience to simulated gastrointestinal stress

Survival during transit through the animal Gastro-Intestinal Tract (GIT) is a fundamental probiotic requirement. In the present study, BDN12 maintained moderate growth at pH 3.0, no isolates remained viable at pH 2.0. This lack of survival at pH 2.0 represents a limitation that may restrict applications in animal species with extremely low gastric pH. The avian gastric environment presents a significant challenge, with gizzard pH typically ranging from 2.0 to 3.5 (Lorenzo-Rebenaque et al. 2021). The survival observed at pH 3.0 is consistent with a known Acid Tolerance Response (ATR), potentially involving the F_1F_0-ATPase proton pump system (Baloch et al. 2019).

Furthermore, BDN12 demonstrated robust tolerance to 0.4% oxgall bile salts, a concentration exceeding the typical avian physiological range of 0.2% to 0.3% (Ruiz et al. 2013; Hu et al. 2018). This resilience is frequently associated with Bile Salt Hydrolase (BSH) activity (Lee et al. 2024). However, it should be noted that viability at 0.4% bile remains in the 10^1-10^3 CFU/mL range based on our operational definitions.

Table 5. PCR screening results for target virulence, antibiotic resistance, and biogenic amine genes.

Categories	Target genes	BDN4	BDN12
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 tested genes in these strains

Table 6. BLASTn alignment statistics for the 16S rRNA gene sequence of isolate BDN12

Closely related species	Max score	Query coverage (%)	E-value	Identity (%)	Accession no.
Lentilactobacillus farraginis strain NRIC 0676 16S ribosomal RNA, partial sequence	2785	91 %	0.0	99.87 %	NR_041467.1

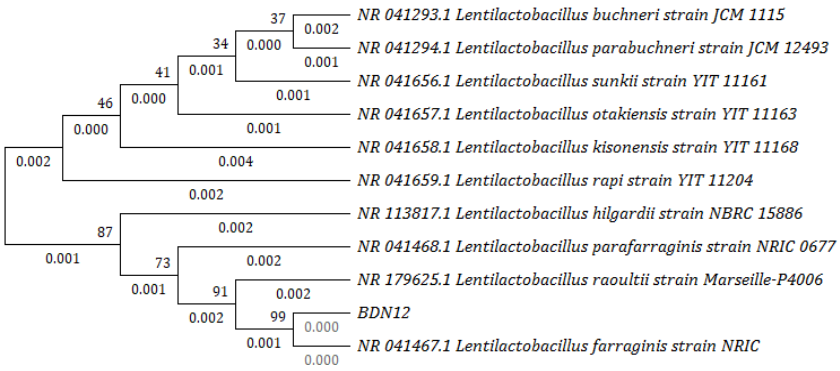

Figure 3. Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences. BDN12 (highlighted) clusters firmly with the *Lentilactobacillus farraginis* clade with a bootstrap value of 100%

Molecular identification and phylogenetic analysis

Molecular characterization of the 16S rRNA gene sequence revealed that isolate BDN12 shares 99.87% identity with *L. farraginis* strain NRIC 0676. Phylogenetic reconstruction firmly clustered BDN12 within the *L. farraginis* clade with 100% bootstrap support. While *L. farraginis* was originally characterized from shochu residue compost (Endo and Okada 2007), our findings demonstrate its successful adaptation to fermented okara. This high genetic identity with the type strain, supported by robust phylogenetic clustering, justifies the taxonomic placement of BDN12. In the context of Vietnamese agriculture, utilizing a strain isolated from local okara provides a distinct ecological advantage, as the isolate is already adapted to the regional feed matrices and environmental conditions prevalent in the Mekong Delta (Tâm et al. 2025).

Safety profile and suitability for feed applications

Safety validation confirmed the absence of pathogenic traits in the top candidates. Phenotypically, BDN12 exhibited γ-hemolysis and susceptibility to clinically relevant antibiotics. This susceptibility pattern is critical, as it suggests the strain is unlikely to serve as a reservoir for transferring resistance genes to the poultry gut microbiota.

Genotypically, PCR screening confirmed the absence of all screened virulence and biogenic amine genes, including *esp*, *gelE*, *fsrB*, *asa1*, *cylA*, *cylM*, *hdc1*, *hdc2*, and *tdc*. The absence of vancomycin resistance genes (*vanA*, *vanB*) is particularly significant given vancomycin's status as a "last-resort" antibiotic in human medicine (Toğay et al. 2014; Kiousi et al. 2023). While the current results are highly promising, comprehensive Whole-Genome Sequencing (WGS) and in vivo safety studies are essential to meet future regulatory standards and provide a high-resolution map of the strain's full metabolic and genetic potential (Wang et al. 2021; Liu et al. 2023).

In conclusion, this study demonstrates that fermented okara serves as a vital reservoir for autochthonous lactic acid bacteria with high functional potential. Through a systematic five-stage screening, *L. farraginis* BDN12 was identified as a superior probiotic candidate, exhibiting robust ecological fitness under significant gastrointestinal stressors and broad activity against major poultry pathogens. Rigorous safety assessment confirmed that BDN12 was non-hemolytic, antibiotic-susceptible, and devoid of key virulence or biogenic amine-producing genes. By leveraging regional microbial heritage, BDN12 provides a foundation for stabilizing the poultry gut and reducing reliance on synthetic growth promoters. These results establish BDN12 as a promising candidate, its practical application requires further validation through comprehensive in vivo trials to evaluate its efficacy in improving growth performance and modulating gut microbiota in Vietnamese poultry farms. Future research should prioritize Whole Genome Sequencing (WGS) to provide a high-resolution map of the strain's genetic safety and metabolic potential.

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