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Research article

Isolation and selection of probiotic *Lactobacillus* strains from chicken intestinal tract: A potential solution for sustainable poultry production in Vietnam

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Abstract

The escalating demand for sustainable poultry production in Vietnam necessitates the exploration of alternatives to antibiotic growth promoters. This study investigated the probiotic potential of *Lactobacillus* strains isolated from the intestinal tract of healthy chickens. Thirty isolates were screened for their resilience to acidic and bile salt conditions, key characteristics for probiotic survival in the gastrointestinal tract. The strain LC16 demonstrated exceptional tolerance to both low pH and bile salts. The antimicrobial activity of the selected strains was evaluated against common poultry pathogens (*E. coli*, *Staphylococcus aureus*, and *Salmonella* sp.), and their antibiotic susceptibility was also assessed. Strain LC16 exhibited both potent antimicrobial activity and resistance to all tested antibiotics. Molecular identification through PCR and 16S rRNA gene sequencing confirmed LC16 as *Lactobacillus farciminis*. The identification of *L. farciminis* LC16, a strain possessing a combination of desirable probiotic traits, including robust antimicrobial activity, tolerance to challenging gastrointestinal conditions, and antibiotic resistance, highlights its potential as a promising candidate for further development as a probiotic for chickens.

Keywords: Antibacterial activity, Antibiotic susceptibility, Chicken, Lactobacillus, Probiotic, Stress tolerance.

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INTRODUCTION

The Vietnamese chicken industry plays a pivotal role in the national economy, providing essential protein and income for millions. Between 2011 and 2022, the chicken population grew by over 5%, reaching an estimated 316.9 million birds in 2022 and comprising 77.5% of all poultry. This growth has enabled Vietnam to expand its presence in the global market. However, this progress is not without challenges. Disease outbreaks pose a constant threat to production, while the overuse of antibiotics and hormones raises concerns about food safety and public health.

To ensure sustainable development, the industry must transition towards cleaner production methods that prioritize animal health, welfare, and food safety. This aligns with the increasing demand for poultry products raised without antibiotics or hormones, safeguarding public health (El Jeni et al., 2021; Reuben et al., 2021). One promising strategy involves supplementing animal diets with beneficial microorganisms, or probiotics, to enhance their resilience against pathogens and improve food safety (Sood et al., 2020). Research in Vietnam has focused on isolating probiotic strains from chicken gastrointestinal tracts, with encouraging results identifying *Bacillus* species demonstrating desirable probiotic characteristics (Cong and Nam, 2021). The growing interest in probiotics for poultry production is driven by concerns about antibiotic resistance and the need for natural alternatives to promote growth, improve feed conversion efficiency, and prevent intestinal infections (Çapan and Bağdatlı, 2022).

Probiotics, particularly lactic acid bacteria (LAB) like *Lactobacillus*, offer a compelling alternative to antibiotics. LAB are safe and resilient microorganisms with well-documented health benefits. Research suggests that LAB isolated from chickens' intestinal tract can be beneficial for animal health without compromising safety. Studies by Kupryś-Caruk et al. (2018) and Wang et al. (2023) demonstrated that LAB can reduce chick mortality, inhibit harmful bacteria, improve growth performance, gut health, and immune function in broiler chickens. These findings are further supported by Miranda et al. (2021), who emphasized the role of LAB in enhancing animal performance, health, and overall productivity. *Lactobacillus*, in particular, has garnered attention for its antibacterial properties in chickens, making it a potential antibiotic substitute in poultry farming (Pertiwi and Mahendra, 2021; Kristianti et al., 2022; Ndaywel et al., 2023). Research indicates that *Lactobacillus* can positively impact chicken growth and weight gain, while also inhibiting the growth of pathogenic bacteria like *Salmonella* Typhimurium*, Staphylococcus aureus*, and *Bacillus cereus* (Pertiwi and Mahendra, 2021). Additionally, the widespread use of *Lactobacillus* as a feed additive in poultry farming underscores its potential as a natural and effective antibacterial agent (Kristianti et al., 2022). Previous research has explored the potential of *Lactobacillus* strains isolated from chicken intestines as probiotics. Studies by Ishaq et al. (2019) and Ahmed et al. (2019) have shown that these strains exhibit promising characteristics, including antimicrobial activity, tolerance to acidic and bile-rich environments, and stability under varying ph and temperature conditions. Yuksekdag et al. (2014) further identified specific strains, such as *L. Delbrueckii* ssp. *Delbrueckii* BAZ32 and *L. Acidophilus* BAZ29, that display high probiotic potential due to their combined acid and bile tolerance, antimicrobial activity, and ability to form aggregates. Sirisopapong et al. (2023) built upon this research by demonstrating the efficacy of *L. Ingluviei* and *L. Salivarius* in increasing beneficial bacteria while reducing harmful bacteria in the chicken digestive tract. Collectively, these findings suggest that *Lactobacillus* strains isolated from chicken intestines hold promise as probiotics for use in both poultry feed and food preservation applications.

Building upon this existing research, this study aims to isolate and select probiotic *Lactobacillus* strains from the chicken intestinal tract. These selected

strains have the potential to serve as a sustainable solution for promoting animal health, welfare, and food safety within the Vietnamese poultry industry.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Fifty healthy, four-month-old Ta chickens, raised on a traditional diet of rice bran, leftover rice, and vegetables, were obtained from households across five districts in Tra Vinh Province, Vietnam between August 2023 and January 2024. Birds were humanely euthanized according to established protocols (Risa et al., 2020), and their small intestines were aseptically collected. From each Ta chicken, one gram of intestinal tissue was homogenized in 9 mL of sterile distilled water using a vortex mixer for 5 minutes. The homogenate was serially diluted tenfold, and 100 µL aliquots were spread onto de Man, Rogosa, and Sharpe (MRS) agar plates (Himedia, India) supplemented with 0.05% bromocresol green (Sigma-Aldrich) and 0.05% bile salts (Sigma-Aldrich) to select for *Lactobacillus* growth Sirisopapong et al. (2023). Following 48 hours of anaerobic incubation at 37°C, distinct colonies were isolated based on morphological characteristics. Isolates were tentatively identified as *Lactobacillus* through Gram staining and microscopic examination of cell morphology. Pure cultures were obtained by subculturing onto fresh MRS agar, and isolates were stored in 30% glycerol at -80°C for further analysis (Tsega et al., 2023).

Acid and bile salt tolerance assays

Lactobacillus isolates obtained from chicken intestinal tracts were cultured overnight in de Man, Rogosa, and Sharpe (MRS) broth (Himedia, India) at 37°C. Following this initial incubation, each culture was transferred into fresh MRS broth and incubated for an additional 24 hours at 37°C to ensure optimal growth. The cultures were then centrifuged at 7,500 x g for 5 minutes at 4°C to collect the bacterial cells. The resulting pellets were washed twice with sterile phosphate-buffered saline (PBS, pH 7.2) to remove any residual growth media. The washed cells were resuspended in fresh MRS broth and adjusted to a standardized concentration using a spectrophotometer to achieve an optical density (OD) between 0.5 and 0.7 at a wavelength of 600 nm (Reuben et al., 2019). This standardized concentration of 10^8 CFU/ml served as the starting point for tolerance assays.

To evaluate acid tolerance, 1 mL aliquots of the standardized cell suspension were added to separate tubes containing 9 mL of MRS broth adjusted to pH values of 2.0, 4.0, and 6.5 (control). These cultures were incubated at 37°C for 4 hours to assess the survival of the *Lactobacillus* isolates under acidic conditions (Jannah et al., 2014).

Bile salt tolerance was assessed by adding 1 mL aliquots of the standardized cell suspension were added to separate tubes containing 9 mL MRS broth with varying concentrations (0%, 0.15% and 0.3%) of bile salts. These cultures were incubated at 37°C for 4 hours to evaluate the ability of the *Lactobacillus* isolates to withstand the presence of bile salts, a common stressor in the intestinal environment (Tian et al., 2024).

Following incubation for both assays, serial dilutions (up to 10^{-7}) were prepared in PBS to achieve countable cell concentrations. Aliquots (100 µL) of dilutions ranging from 10^{-4} to 10^{-7} were spread onto MRS agar plates and incubated anaerobically at 37°C for 24 hours. Viable cell counts were determined by counting the colony-forming units (CFUs) on the MRS agar plates, providing a quantitative measure of survival and tolerance for each isolate under the tested conditions (Ramlucken et al., 2020).

Antimicrobial activity assay

To evaluate the potential probiotic properties of the *Lactobacillus* isolates, their inhibitory activity was assessed against three common pathogenic indicator strains: *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* sp. These indicator strains were obtained from the Biotechnology Research and Development Institute at Can Tho University and cultured in Luria-Bertani (LB) broth (Himedia, India). Concurrently, the *Lactobacillus* isolates were grown in MRS broth under anaerobic conditions at 37°C for 24 hours. Following incubation, the *Lactobacillus* cultures were centrifuged at 10,000 rpm for 5 minutes at 4°C to separate the bacterial cells from the supernatant, which would contain any secreted antimicrobial compounds. The indicator strains, prepared at a concentration of 10^8 CFU/ml, were then incorporated into molten Nutrient Agar (Neogen, USA) at a concentration of 0.2% and poured onto plates. Once the agar solidified, wells with a diameter of 4 mm were created using a sterile cork borer. 100 µL of the cell-free supernatant from each *Lactobacillus* isolate, also adjusted to 10^8 CFU/ml, was added to a separate well on the plates seeded with each indicator strain. These plates were incubated at 37°C for 24 hours to allow for potential inhibition of the indicator strains' growth. The formation of clear zones of inhibition surrounding the wells indicated antimicrobial activity. The diameter of these zones was measured in millimeters, with larger zones representing stronger antagonistic activity (Rossi et al., 2021). All analysis was conducted in triplicate. *Lactobacillus* isolates demonstrating broad-spectrum antimicrobial activity against multiple indicator strains were selected for further analysis as potential probiotic candidates.

Antibiotic susceptibility testing

To ensure the safe use of the selected *Lactobacillus* isolates as potential probiotics, their susceptibility to common antibiotics used in poultry treatment was assessed. Four antibiotics, ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), and ciprofloxacin (5 µg), were chosen for this evaluation. The susceptibility testing was conducted using the standardized disk diffusion method. Cultures of each *Lactobacillus* isolate were adjusted to a concentration of 10^8 CFU/mL. A 100 µL aliquot of each adjusted culture was spread evenly onto the appropriate agar medium. Antibiotic disks were placed on the inoculated plates after the media solidified, ensuring adequate spacing between disks. Triplicate plates were prepared for each *Lactobacillus* isolate to ensure reproducibility. The inoculated plates were incubated at the optimal temperature for *Lactobacillus* growth. After the incubation period, the diameters of the zones of inhibition surrounding each antibiotic disk were measured in millimeters. The isolates were then categorized as sensitive (≥20 mm), intermediate (15–19 mm), or resistant (≤14 mm) based on the established interpretative criteria (Makzum et al., 2023). This classification provided valuable information on the antibiotic susceptibility profile of each *Lactobacillus* isolate, guiding their potential use as safe and effective probiotics in poultry production.

Molecular identification of *Lactobacillus* isolates

Genomic DNA was extracted from overnight LC6 strain cultures using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol, including lysozyme treatment and proteinase K digestion. DNA concentration and purity were assessed using a NanoDrop spectrophotometer. The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3') (James, 2010) with GoTaq Green Master Mix (Promega, USA) in a conventional thermocycler (Veriti, Applied Biosystems, USA). Cycling conditions included an initial denaturation at 94°C for 3 minutes, followed by 29 cycles of 94°C for 45 seconds, 53°C for 60 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 5 minutes. Amplified products were visualized on a 2% agarose gel. PCR products were purified and

the bacteria DNA sequences were done by Next Gen Scientific Co., Ltd (Ho Chi Minh City). The region gene sequence was analyzed with BioEdit software (version 7.0). Consensus sequences were compared against the GenBank database using NCBI BLAST to confirm species-level identification of the isolates (Mudawaroch et al., 2023).

Statistical analysis

Results are presented as mean ± standard deviation of three independent experiments. One-way analysis of variance (ANOVA) was performed for acid, bile tolerance, and Antimicrobial Activity Assay data using SPSS (Statistics 22, IBM) with significance set at $p < 0.05$.

RESULTS

Isolation and morphological characterization of *Lactobacillus* strains

Thirty potential probiotic *Lactobacillus* strains were successfully isolated from 50 free-range chickens collected from 10 households. Isolation was achieved using de Man, Rogosa, and Sharpe (MRS) agar supplemented with 0.05% bromocresol green and 0.05% bile salts. After incubation at 37°C for 48 hours, 30 distinct colonies were selected based on their morphological characteristics, including colony size, shape, color, and texture.

Morphological characterization revealed considerable diversity among the isolated bacterial colonies. Colony color, size, margin, and elevation varied. Two distinct colony shapes were observed: circular (93.3%) and irregular (6.7%). Colony color distribution was as follows: white (60.0%), milky-white (23.3%), translucent white (10.0%), and opaque white (6.7%). The predominance of white colonies suggests a potential dominance of specific *Lactobacillus* species or strains known for this pigmentation.

Microscopic examination of the isolates revealed two primary cell morphologies: rod-shaped (63.3%) and coccobacilli (36.7%) (Figure 1). All isolates were Gram-positive, further supporting their identification as *Lactobacillus*. Detailed morphological characteristics of each isolate are presented in Table 1.

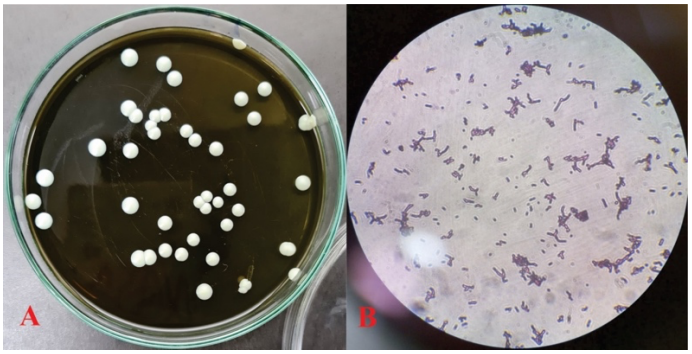

Figure 1 Morphological and Gram staining of isolated strains. (**A**): colony morphology of LC16 strain in MRS agar (with 0.15% bile salts). (**B**): Gram staining of LC16 strain. Cells are purple, rod shape, and without spores.

Table 1 Colony characteristics and cell morphology observed under the microscope of 60 presumptive *Lactobacillus* isolates.

Isolates ID	Colony Morphology					Cell morphology
	Shape	Pigmentation	Size	Elevation	Margin	
LC1	Circular	Milky-white	Large	Raised	Undulate	Gram-positive rods
LC2	Circular	Translucent white	Small	Raised	Entire	Gram-positive rods
LC3	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC4	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC5	Circular	White	Large	Raised	Entire	Gram-positive rods
LC6	Circular	White	Large	Raised	Entire	Gram-positive rods
LC7	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC8	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC9	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC10	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC11	Circular	Milky-white	Large	Convex	Entire	Gram-positive coccobacilli
LC12	Circular	Opaque white	Small	Flat	Undulate	Gram-positive rods
LC13	Circular	White	Large	Raised	Entire	Gram-positive rods
LC14	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC15	Circular	White	Large	Raised	Entire	Gram-positive rods
LC16	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC17	Circular	White	Large	Raised	Entire	Gram-positive rods
LC18	Circular	Milky-white	Moderate	Raised	Entire	Gram-positive rods
LC19	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC20	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC21	Circular	Translucent white	Moderate	Raised	Entire	Gram-positive rods
LC22	Circular	White	Large	Raised	Entire	Gram-positive rods
LC23	Circular	Translucent white	Small	Raised	Entire	Gram-positive rods
LC24	Circular	Milky-white	Moderate	Raised	Entire	Gram-positive rods
LC25	Irregular	Opaque white	Small	Flat	Undulate	Gram-positive rods
LC26	Circular	White	Large	Raised	Entire	Gram-positive coccobacilli
LC27	Circular	Milky-white	Small	Raised	Entire	Gram-positive coccobacilli
LC28	Irregular	Milky-white	Moderate	Raised	Entire	Gram-positive coccobacilli
LC29	Circular	White	Large	Raised	Entire	Gram-positive rods
LC30	Circular	Milky-white	Large	Raised	Entire	Gram-positive coccobacilli

Assessment of probiotic potential

Acid and bile tolerance

The probiotic potential of the 30 isolated *Lactobacillus* strains was evaluated by assessing their tolerance to acidic conditions and bile salts, key stressors encountered in the chicken gastrointestinal tract.

Upon exposure to varying pH levels (2.0, 4.0, and 6.5) for 4 hours at 37°C, significant differences ($p < 0.05$) were observed in the survival rates of the *Lactobacillus* isolates (Table 2). At pH 2.0, four strains (LC3, LC14, LC17, and LC21)

were not viable. Among the surviving strains, LC4, LC9, and LC15 displayed the lowest tolerance, with viable cell counts of 4.73, 4.73, and 4.77 Log CFU/mL, respectively. Conversely, strains LC16, LC20, LC25, LC28, LC22, and LC27 demonstrated superior acid tolerance, with viable cell counts ranging from 6.54 to 6.77 Log CFU/mL. The survival of most isolates improved at pH 4.0 and 6.5.

The isolates were further evaluated for their ability to withstand varying concentrations of bile salts (0%, 0.15%, and 0.3%). Resistance to bile salts varied significantly among the strains ($p < 0.05$) (Table 3). At 0.3% bile salts, strains LC20, LC16, LC13, LC10, LC19, and LC24 exhibited the highest tolerance, with viable cell counts ranging from 5.08 to 5.26 Log CFU/mL. However, ten strains (LC27, LC25, LC4, LC3, LC6, LC2, LC5, LC21, LC7, and LC12) did not survive under these conditions. All strains showed dramatically improved survival at 0.15% bile salts, with viable cell counts ranging from 5.08 Log CFU/mL (LC29) to 6.22 Log CFU/mL (LC30). Notably, strains LC20 and LC16 consistently demonstrated superior tolerance to both low pH and bile salts, highlighting their potential as promising probiotic candidates.

Table 2 Selected *Lactobacillus* isolates pH tolerance

Isolates ID	Viable *Lactobacillus* bacteria isolates (Log CFU/mL)								
	pH 6.5 (Control)			pH 4			pH 2		
LC1	7.59	±	0.11	7.20	±	0.17	5.10	±	0.17
LC2	7.66	±	0.10	7.26	±	0.24	5.20	±	0.17
LC3	8.97	±	0.01	6.49	±	0.20	0.00	±	0.00
LC4	7.83	±	0.13	6.82	±	0.04	4.73	±	0.05
LC5	7.76	±	0.15	6.33	±	0.35	5.10	±	0.17
LC6	8.34	±	0.05	7.95	±	0.16	5.86	±	0.03
LC7	7.53	±	0.21	7.06	±	0.06	5.26	±	0.24
LC8	7.79	±	0.10	7.10	±	0.17	5.26	±	0.01
LC9	7.65	±	0.16	7.33	±	0.35	4.73	±	0.05
LC10	7.30	±	0.30	7.26	±	0.24	5.80	±	0.02
LC11	7.69	±	0.09	7.52	±	0.07	6.28	±	0.02
LC12	9.07	±	0.01	8.98	±	0.03	6.18	±	0.03
LC13	8.99	±	0.01	8.09	±	0.10	5.82	±	0.07
LC14	7.54	±	0.28	6.55	±	0.13	0.00	±	0.00
LC15	7.40	±	0.35	6.53	±	0.21	4.77	±	0.07
LC16	8.10	±	0.07	6.82	±	0.07	6.54	±	0.02
LC17	8.69	±	0.01	8.09	±	0.08	0.00	±	0.00
LC18	8.69	±	0.02	8.49	±	0.02	5.40	±	0.17
LC19	8.63	±	0.08	7.20	±	0.35	5.55	±	0.13
LC20	8.31	±	0.01	8.11	±	0.06	6.54	±	0.01
LC21	8.86	±	0.03	7.23	±	0.40	0.00	±	0.00
LC22	7.40	±	0.17	6.20	±	0.35	6.65	±	0.04
LC23	8.19	±	0.02	7.16	±	0.28	6.18	±	0.04
LC24	8.95	±	0.01	7.84	±	0.10	6.19	±	0.04
LC25	7.77	±	0.12	7.10	±	0.17	6.54	±	0.01
LC26	8.71	±	0.05	7.90	±	0.05	5.10	±	0.17
LC27	8.32	±	0.06	6.20	±	0.17	6.77	±	0.00
LC28	8.42	±	0.05	6.16	±	0.28	6.63	±	0.00
LC29	8.96	±	0.01	7.82	±	0.07	5.56	±	0.07
LC30	9.02	±	0.01	7.30	±	0.30	5.77	±	0.07

Table 3 Selected *Lactobacillus* isolates Bile salt tolerance

Isolates ID	Viable *Lactobacillus* bacteria isolates (Log CFU/mL)								
	0% (Control)			0.15%			0.30%		
LC1	7.59	±	0.11	5.40	±	0.17	4.43	±	0.51
LC2	7.66	±	0.10	5.79	±	0.10	0.00	±	0.00
LC3	8.97	±	0.01	5.63	±	0.06	0.00	±	0.00
LC4	7.83	±	0.13	5.10	±	0.17	0.00	±	0.00
LC5	7.76	±	0.15	5.59	±	0.11	0.00	±	0.00
LC6	8.34	±	0.05	5.20	±	0.35	0.00	±	0.00
LC7	7.53	±	0.21	4.23	±	0.40	0.00	±	0.00
LC8	7.79	±	0.10	5.36	±	0.10	4.43	±	0.51
LC9	7.65	±	0.16	5.62	±	0.15	4.16	±	0.28
LC10	7.30	±	0.30	5.77	±	0.07	5.09	±	0.05
LC11	7.69	±	0.09	5.72	±	0.12	4.84	±	0.10
LC12	9.07	±	0.01	5.40	±	0.02	0.00	±	0.00
LC13	8.99	±	0.01	5.95	±	0.05	5.19	±	0.66
LC14	7.54	±	0.28	5.58	±	0.17	4.57	±	0.51
LC15	7.40	±	0.35	5.69	±	0.09	4.63	±	0.85
LC16	8.10	±	0.07	5.98	±	0.09	5.21	±	0.55
LC17	8.69	±	0.01	5.63	±	0.13	4.79	±	0.71
LC18	8.69	±	0.02	4.84	±	0.06	4.73	±	0.51
LC19	8.63	±	0.08	5.88	±	0.35	5.09	±	0.60
LC20	8.31	±	0.01	5.86	±	0.07	5.26	±	0.67
LC21	8.86	±	0.03	5.84	±	0.12	0.00	±	0.00
LC22	7.40	±	0.17	5.72	±	0.12	4.59	±	0.53
LC23	8.19	±	0.02	5.70	±	0.17	4.43	±	0.51
LC24	8.95	±	0.01	5.87	±	0.11	5.08	±	0.54
LC25	7.77	±	0.12	5.32	±	0.28	0.00	±	0.00
LC26	8.71	±	0.05	5.58	±	0.17	4.43	±	0.51
LC27	8.32	±	0.06	5.23	±	0.40	0.00	±	0.00
LC28	8.42	±	0.05	5.82	±	0.19	4.79	±	0.10
LC29	8.96	±	0.01	3.93	±	3.41	4.73	±	0.51
LC30	9.02	±	0.01	6.22	±	0.03	4.69	±	0.65

Antimicrobial activity of selected *Lactobacillus* isolates

The antimicrobial activity of the *Lactobacillus* isolates was evaluated against three common poultry pathogens: *E. coli*, *Salmonella* sp., and *S. aureus*. The isolates demonstrated varying degrees of inhibitory activity against the tested pathogens (Table 4 and Figure 2).

All 30 isolates demonstrated the ability to inhibit the growth of *E. coli*, with inhibition zones ranging from 1.30 ± 0.17 cm (LC23) to 5.33 ± 3.51 cm (LC11). Against *S. aureus*, LC28 exhibited the largest inhibition zone (4.33 ± 3.51 cm). In contrast, LC20 and LC22 showed no inhibitory activity against this pathogen. For *Salmonella* sp., LC16 exhibited the strongest inhibition, with an inhibition zone of 4.00 ± 2.65 cm. Several other isolates also demonstrated notable activity against *Salmonella* sp., while LC26 was the only isolate that did not exhibit any inhibitory effect.

Three strains, LC8, LC12, and LC16, demonstrated broad-spectrum antimicrobial activity, effectively inhibiting the growth of all three tested pathogens. LC8 produced inhibition zones of 3.10 ± 2.52 cm, 3.10 ± 1.65 cm, and 1.90 ± 0.46 cm against *E. coli*, *S. aureus*, and *Salmonella* sp., respectively. LC12 showed similar activity, with inhibition zones of 3.67 ± 3.79 cm, 4.00 ± 4.36 cm, and 3.00 ± 2.65 cm against the respective pathogens. Lastly, LC16 exhibited inhibition zones of 3.00 ± 1.73 cm, 2.80 ± 1.06 cm, and 4.00 ± 2.65 cm.

The selected *Lactobacillus* isolates possess varying degrees of antimicrobial activity against common poultry pathogens. Notably, strains LC8, LC12, and LC16

demonstrated the most promising broad-spectrum potential for use as probiotics in poultry production.

Table 4 Antimicrobial activity of *Lactobacillus* strains against pathogens

Isolates ID	*Escherichia coli* (cm)			*S. aureus* (cm)			*Salmonella* sp. (cm)		
LC1	2.00	±	0.40	1.53	±	0.21	1.77	±	0.21
LC2	1.57	±	0.21	1.50	±	0.17	1.63	±	0.45
LC3	1.90	±	0.66	2.60	±	0.61	1.63	±	0.06
LC4	1.80	±	0.20	1.53	±	0.21	1.63	±	0.32
LC5	1.57	±	0.67	0.73	±	0.06	1.27	±	0.42
LC6	3.00	±	1.73	2.67	±	1.15	1.23	±	0.06
LC7	2.00	±	0.30	1.90	±	0.17	1.53	±	0.31
LC8	3.10	±	2.52	3.10	±	1.65	1.90	±	0.46
LC9	1.60	±	0.20	1.57	±	0.25	1.40	±	0.50
LC10	1.93	±	0.12	1.30	±	0.10	1.67	±	0.31
LC11	5.33	±	3.51	1.67	±	0.58	3.67	±	3.79
LC12	3.67	±	3.79	4.00	±	4.36	3.00	±	2.65
LC13	1.97	±	0.31	1.50	±	0.36	1.90	±	0.36
LC14	1.60	±	0.20	1.87	±	0.23	1.50	±	0.10
LC15	1.57	±	0.06	2.33	±	0.21	2.33	±	0.38
LC16	3.00	±	1.73	2.80	±	1.06	4.00	±	2.65
LC17	1.90	±	0.46	1.60	±	0.17	1.60	±	0.44
LC18	1.70	±	0.46	2.43	±	0.72	1.70	±	0.36
LC19	1.73	±	0.21	2.03	±	0.21	1.37	±	0.15
LC20	4.00	±	2.65	0.00	±	0.00	3.33	±	2.08
LC21	1.83	±	0.21	1.77	±	0.21	1.90	±	0.53
LC22	3.67	±	2.52	0.00	±	0.00	3.33	±	2.08
LC23	1.30	±	0.17	1.47	±	0.12	1.83	±	0.45
LC24	1.60	±	0.17	1.73	±	0.12	1.97	±	0.40
LC25	2.67	±	2.08	1.00	±	0.00	3.67	±	2.52
LC26	1.67	±	0.58	2.00	±	2.65	0.00	±	0.00
LC27	1.50	±	0.17	2.00	±	0.10	1.37	±	0.35
LC28	1.33	±	0.31	4.33	±	3.51	3.33	±	2.08
LC29	1.43	±	0.50	1.63	±	0.32	2.13	±	0.31
LC30	1.43	±	0.51	1.53	±	0.12	1.93	±	0.47

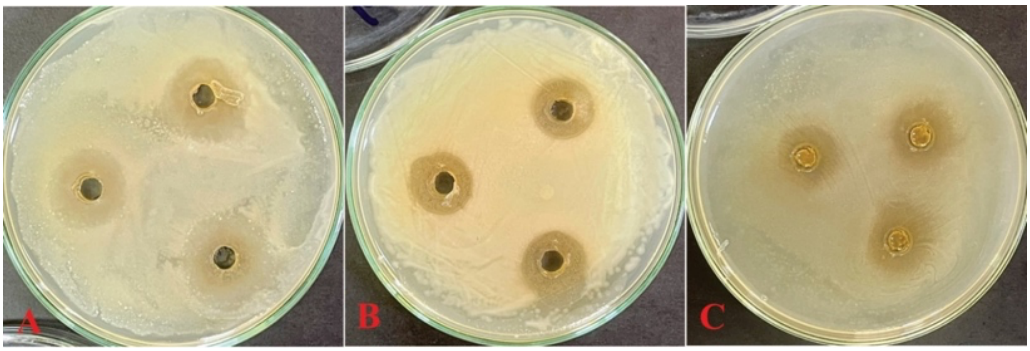

Figure 2 The inhibition zones of the strain LC16 against pathogenic bacteria. (**A**): *E. coli*; (**B**): *Samonella* sp.; (C): *S. aureus*.

Antibiotic susceptibility of selected *Lactobacillus* isolates

The antibiotic susceptibility of 30 presumptive *Lactobacillus* isolates was evaluated using four common antibiotics utilized in livestock for gastrointestinal disease prevention and treatment: chloramphenicol, erythromycin, ampicillin, and ciprofloxacin. These antibiotics are listed in Circular 06-2016/TT/BNNPTNT and the list of licensed veterinary drugs in Vietnam (as of December 31, 2020).

The majority of the isolates demonstrated resistance to the tested antibiotics (Table 5). Specifically, 53.3%, 56.6%, and 56.6% of the isolates were resistant to chloramphenicol, erythromycin, and ampicillin, respectively. A concerning 90.0% of the isolates were resistant to ciprofloxacin. Thirteen isolates (LC2, LC3, LC4, LC5, LC7, LC11, LC13, LC14, LC16, LC17, LC24, LC28, and LC30) showed resistance to all four tested antibiotics.

Based on a combination of desirable characteristics, including resistance to the tested antibiotics, robust antimicrobial activity, and superior tolerance to both low pH and bile salts, LC16 emerges as a particularly promising candidate for further probiotic development in chickens.

Table 5 Antibiotic susceptibily of *Lactobacillus* strains

Isolates ID	Antibiotic designation				Isolates ID	Antibiotic designation			
	C	E	AMP	CIP		C	E	AMP	CIP
LC1	S	I	R	R	LC16	R	R	R	R
LC2	R	R	R	R	LC17	R	R	R	R
LC3	R	R	R	R	LC18	I	I	S	R
LC4	R	R	R	R	LC19	S	R	S	R
LC5	R	R	R	R	LC20	S	I	S	I
LC6	S	S	S	I	LC21	I	I	S	R
LC7	R	R	R	R	LC22	I	I	S	R
LC8	S	S	I	R	LC23	S	I	R	R
LC9	S	I	S	R	LC24	R	R	R	R
LC10	R	S	S	R	LC25	R	R	S	R
LC11	R	R	R	R	LC26	S	R	R	R
LC12	I	I	S	I	LC27	S	R	S	R
LC13	R	R	R	R	LC28	R	R	R	R
LC14	R	R	R	R	LC29	I	I	R	R
LC15	R	I	I	R	LC30	R	R	R	R

* Values are reported as the means of triplicates. C: Chloramphenicol (30 µg), E: Erythromycin (15 µg), AMP: Ampicillin (10 µg), CIP: Ciprofloxacin (5 µg). R: resistant, I: intermediate, S: sensitivity.

Molecular identification of *Lactobacillus* isolates

To confirm the species-level identity of the selected *Lactobacillus* isolate LC16, the 16S rRNA gene was amplified using universal primers 27F and 1492R. PCR amplification successfully yielded a single product of approximately 1,500 base pairs (Figure 3), confirming the presence of the target gene region and its suitability for sequencing. The amplified PCR product was sequenced and the resulting sequence was deposited in the GenBank database under the accession number OP420797. A BLAST search of this sequence revealed a high similarity (99.64%) to the 16S rRNA gene of *Lactobacillus farciminis*, with a maximum score of 2,045 and an E-value of 0.0. The alignment covered 647 out of 1,121 nucleotides. Based on this molecular identification, the isolated strain LC16 was definitively identified as *Lactobacillus farciminis*, and was named *Lactobacillus farciminis* LC16.

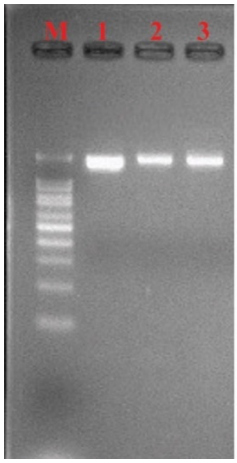

Figure 3 Amplification of DNA barcodes from LC16 strain. Product of the 16S rDNA region from 1500 bp on 2% agarose gel with 100 bb ladder; M: DNA marker; Lanes 1: Positive control; Lanes 2-3: Samples; Lanes 4: Negative control without DNA.

DISCUSSION

Isolation and characterization of potentially beneficial *Lactobacillus* strains

The use of probiotics, particularly those derived from the host animal's natural environment, has emerged as a promising and sustainable alternative to antibiotics in poultry production (Ahmad et al., 2022; Bhogoju and Nahashon, 2022). These beneficial microorganisms have been shown to positively impact various aspects of poultry health and production, including growth performance, bone health, meat and eggshell quality, immune response, gut microbiota balance, and disease resistance. Research in both ruminants and non-ruminants has established the positive effects of probiotics on gut health, immunity, and overall production (Mahesh et al., 2021).

The efficacy of probiotics is not uniform, however, as strain selection and host specificity significantly influence their effectiveness (Cameron and McAllister, 2019). This highlights the importance of developing host-specific probiotics to optimize animal health and production outcomes (Dowarah et al., 2018). In the context of poultry, numerous studies have identified *Lactobacillus* species as promising probiotic candidates for the chicken intestinal tract (Shokryazdan et al., 2014; Wang et al., 2014; Ahmed et al., 2019).

In this study, we successfully isolated 30 lactic acid bacteria (LAB) strains from the intestinal tracts of healthy chickens using bromocresol purple-supplemented MRS agar, a method that allows for visual identification of LAB based on the formation of yellow halos around colonies (Sobrun et al., 2012). Microscopic examination revealed that these isolates were Gram-positive, non-spore-forming rods or coccobacilli, consistent with the typical morphology of *Lactobacillus* species commonly found in the chicken digestive tract (Schuster et al., 2019). These findings align with previous research, which has also emphasized the inherent variability in aggregation ability and gastrointestinal stress tolerance among *Lactobacillus* strains Aziz et al. (2019), underscoring the need for careful strain selection in probiotic development.

Assessment of probiotic potential

Acid and bile tolerance

The isolation and initial characterization of these *Lactobacillus* strains represent a crucial first step in identifying potential probiotic candidates that could contribute to sustainable and antibiotic-free poultry production in Vietnam. Further evaluation of their probiotic properties and *in vivo* efficacy will be essential to determine their suitability for application in poultry farming practices.

The ability of probiotic bacteria to survive the harsh conditions of the gastrointestinal (GI) tract, notably the low pH of gastric acid and the presence of bile salts, is essential for their colonization and beneficial effects within the host. Successful navigation of these challenges is a critical factor in the selection and evaluation of probiotic candidates.

Previous research has established pH 2.0-3.0 and 0.3% bile salts as benchmarks for assessing acid and bile tolerance in probiotic strains (Jannah et al., 2014; Yuksekdag et al., 2014; Hu et al., 2018). In pigs, for instance, various strains of *Lactobacillus* spp., *Streptococcus* spp., and *Bifidobacterium* spp. demonstrating tolerance to these conditions have shown promise as probiotics (Ryu et al., 2009). Similar findings have been reported in poultry, where *Lactobacillus* spp. strains exhibiting tolerance to pH 2.0 and 0.3% bile salts, along with additional probiotic properties such as adhesion to intestinal cells and antimicrobial activity, have shown efficacy (Akpa et al., 2022; Kéhi et al., 2022). These findings are further supported by research specifically focused on chickens, which has confirmed the probiotic potential of *Lactobacillus* species based on their high acid and bile salt tolerance (Ahmed et al., 2019).

In the present study, two isolates, LC20 and LC16, displayed good survivability under pH 2.0 and 0.3% bile salt conditions for 4 hours. This result aligns with prior studies showing good acid and moderate bile tolerance in *Lactobacillus* strains isolated from the chicken intestine, particularly the caecum (Jin et al., 1998). The literature also documents the ability of various *Lactobacillus* isolates, including *L. plantarum* and *L. casei* subsp. *casei*, to tolerate bile and acid, albeit with strain-specific variations in tolerance levels (Singhal et al., 2010).

The ability of probiotic strains to survive in the presence of bile acids is of particular importance due to the role of bile acids in lipid absorption and their impact on the gut microbiota composition and function (Schmid et al., 2016). As not all *Lactobacillus* strains possess the same level of tolerance to these harsh conditions, careful strain selection is imperative to identify those with the resilience necessary to thrive in the GI tract (Reyes-Nava et al., 2016). The acid and bile salt tolerance exhibited by LC20 and LC16, along with their other potential probiotic attributes, suggests their promising candidacy for further exploration and development as effective poultry probiotics.

Antimicrobial activity of selected *Lactobacillus* isolates

The antagonistic activity of probiotic microorganisms against pathogens is a crucial characteristic for maintaining a balanced gut microbiota and protecting the host from harmful bacteria. Probiotics inhibit pathogenic growth through the production of various antimicrobial compounds, including organic acids (such as lactic acid), hydrogen peroxide, and bacteriocins (Jose et al., 2015).

In this study, the isolated *Lactobacillus* strains demonstrated varying degrees of inhibitory activity against *E. coli*, *Salmonella* sp., and *S. aureus*. All isolates exhibited some level of inhibition against *E. coli*, with LC12 and LC16 demonstrating the most substantial inhibitory effects against all three tested pathogens. This broad-spectrum antimicrobial activity aligns with previous research demonstrating that different *Lactobacillus* strains can inhibit the growth of various pathogenic bacteria, including *E. coli*, *S.* Typhimurium, *S. aureus*, *C. perfringens*, *Klebsiella* spp., and *Proteus* spp. These inhibitory effects are often mediated through competitive exclusion, whereby the probiotic bacteria compete

with pathogens for nutrients and attachment sites, as well as through the production of antimicrobial compounds that directly inhibit pathogen growth (Cisek et al., 2022). Jannah et al. (2014) also reported the inhibitory activity of various *L. salivarius* strains against *E. coli* and *S.* Enteritidis, further supporting the potential of *Lactobacillus* species as effective antimicrobial agents.

Furthermore, *Lactobacillus* isolates from chickens have been shown to produce active compounds that directly antagonize pathogens like *E. coli* and *S. aureus*. Shamsudin et al. (2019) identified three such isolates from chicken intestines, demonstrating both probiotic potential and the ability to inhibit the growth of *E. coli*. Similarly, Dec et al. (2016) found that chicken-derived *Lactobacillus* isolates produced compounds that effectively inhibited *E. coli*, *Salmonella enterica*, and *Clostridium perfringens*. Our study further supports these findings, as the chicken-derived *Lactobacillus* isolates we examined demonstrated clear inhibitory activity against *E. coli* and *Salmonella enterica*. It's important to note that the antimicrobial potential of *Lactobacillus* is not limited to poultry isolates. Research has shown that isolates from other sources, such as camel milk, can also inhibit the growth of pathogens. For instance, Muhammad et al. (2017) reported that *Lactobacillus* isolates from camel milk, particularly *L. plantarum*, exhibited the ability to inhibit the growth of *S. aureus*.

These findings collectively highlight the potential of *Lactobacillus* isolates from various sources, particularly the chicken intestinal tract, as natural alternatives to antibiotics in poultry production. The ability of these isolates to inhibit the growth of multiple pathogens supports their use as probiotics to maintain gut health, reduce the need for antibiotic intervention, and contribute to more sustainable poultry farming practices.

Antibiotic susceptibility testing of selected *Lactobacillus* isolates

In this study, the majority of *Lactobacillus* isolates exhibited resistance to the tested antibiotics, aligning with previous research on *Lactobacillus* strains from chicken gastrointestinal tracts (Chin et al., 2005; Saleem et al., 2018). Notably, resistance was highest against ciprofloxacin (90%), a fluoroquinolone antibiotic. While resistance to ampicillin, a cell wall inhibitor, was also common (56.6%), all *Lactobacillus* strains isolated from domestic geese in a previous study were sensitive to this antibiotic (Dec et al., 2015). This disparity could be attributed to inherent differences between *Lactobacillus* species or variations in antibiotic exposure in different poultry populations.

The observed antibiotic resistance in these isolates is likely intrinsic and non-transferable. This is a crucial characteristic for probiotic strains, as it ensures safety in feed and food applications while enhancing their survival in the gastrointestinal tract during antibiotic therapy (Jose et al., 2015; Khalil et al., 2018). The presence of antibiotic resistance genes, such as *tetW*, *ermB*, and *cat*, has been reported in *Lactobacillus* isolates from chickens (Dec et al., 2017), further supporting the notion of intrinsic resistance.

Isolate LC16 emerged as a particularly promising probiotic candidate due to its combined traits of antibiotic resistance, antimicrobial activity, and tolerance to low pH and bile salts. These attributes are essential for probiotic strains to survive the harsh conditions of the gastrointestinal tract and exert beneficial effects on the host.

The high prevalence of antibiotic resistance among *Lactobacillus* isolates highlights the need for prudent antibiotic use in poultry farming. By reducing antibiotic pressure, we can foster a gut environment that favors the colonization and persistence of beneficial probiotic strains, thereby minimizing the need for antibiotic intervention and promoting sustainable poultry production.

CONCLUSIONS

In conclusion, this study successfully isolated 30 *Lactobacillus* strains from free-range chickens in Vietnam, highlighting the rich diversity of these beneficial bacteria in the local poultry population. Among these isolates, *Lactobacillus farciminis* LC16 consistently demonstrated superior tolerance to low pH and bile salts, broad-spectrum antimicrobial activity against common poultry pathogens, and resistance to the tested antibiotics. These findings underscore the potential of *L. farciminis* LC16 as a promising probiotic candidate for enhancing chicken health and productivity in sustainable poultry production systems.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare

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