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Screening of *Lactobacillus* from Noi chicken gut as potential probiotics against poultry pathogens

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Abstract. *Thuy NP, Trai NN. 2024. Screening of* Lactobacillus *from Noi chicken gut as potential probiotics against poultry pathogens. Biodiversitas 25: 3943-3952*. This research investigated the potential of *Lactobacillus* strains isolated from Noi chickens as probiotics for poultry health. We focused on their ability to combat major poultry pathogens: *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* sp. Thirty-two distinct *Lactobacillus* strains were successfully cultured from the digestive tracts of Noi chickens. Colony and cell morphology were diverse, confirming the presence of various *Lactobacillus* species. Biochemical tests further validated their identification. Antibacterial activity screening revealed two strains, LN11 and LN19, capable of inhibiting all three target pathogens. Thirteen isolates, LN5, LN7, LN8, LN9, LN10, LN11, LN12, LN13, LN14, LN16, LN17, LN18, LN19, LN21, LN26 displayed resistance to all four tested antibiotics: chloramphenicol, erythromycin, ampicillin, and ciprofloxacin. Key probiotic traits were assessed. Nine strains showed excellent acid tolerance, crucial for surviving the stomach's harsh environment. Ten strains demonstrated high tolerance to bile salts, essential for thriving in the intestine. LN19 exhibited particularly strong activity, highlighting its potential for probiotic development. Molecular identification using 16S rRNA gene sequencing confirmed the promising isolate LN19 as *Lactobacillus farciminis* LN19. This study provides valuable insights into the probiotic potential of Lactobacillus strains from Noi chickens. The identification of *L. farciminis* LN19 with strong antibacterial activity and robust probiotic characteristics suggests its promise for enhancing poultry health and combating infectious diseases.

Keywords: Antimicrobial activity, antibiotic resistance, *Lactobacillus*, probiotics, poultry

INTRODUCTION

Poultry production in Vietnam is a crucial economic driver, supporting both national growth and poverty alleviation. In response to rising consumer demand, the industry increasingly relies on productive breeds like the Noi chicken, recognized for its fast growth and quality meat (Ngu et al. 2016). However, the threat of bacterial infections, especially those caused by *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* sp., continues to challenge the industry. The Noi chicken, a breed native to southern Vietnam, is particularly vulnerable to these infections. Avian pathogenic *E. coli*, with its increasing antibiotic resistance, is a major concern (Nolan et al. 2008). The *Salmonella* infections, meanwhile, affect both bird health and pose a risk to humans through contaminated food (Ngoc et al. 2016; Yen et al. 2019). The global rise of antibiotic-resistant bacteria, partly fueled by overuse in poultry farming, is a serious threat to both animal and human health, creating an urgent need for alternative disease control strategies.

Probiotics, beneficial live microorganisms, offer a promising solution for boosting the immune system. They enhance host defenses, improve gut health,and compete with harmful bacteria, thereby reducing infection risk (Sood et al. 2020; El Jeni et al. 2021; Reuben et al. 2021). Research in Vietnam has isolated probiotic strains from chicken gastrointestinal tracts, with *Bacillus* species showing particular promise (Cong and Nam 2021). The interest in poultry probiotics stems from their potential to combat antibiotic resistance, promote growth, enhance feed efficiency, and prevent intestinal infections (Çapan and Bağdatl 2022).

Lactic acid bacteria (LAB), especially *Lactobacillus* spp. are well-regarded probiotics due to their safety, resilience, and proven health benefits. As natural inhabitants of the chicken gut, they enhance various aspects of poultry health and productivity. Studies demonstrated their ability to reduce mortality, inhibit harmful bacteria, improve growth performance, and support gut and immune function in broiler chickens (Kupryś-Caruk et al. 2018; Miranda et al. 2021; Wang et al. 2023). Their antimicrobial production, competition with pathogens, and immune modulation make them particularly attractive probiotic candidates (Ndaywel et al. 2023). *Lactobacillus* spp. have been shown to positively influence chicken growth and weight gain while inhibiting pathogens like *Salmonella typhimurium*, *Salmonella aureus*, and *Bacillus cereus* (Pertiwi and Mahendra 2021). Its widespread use as a poultry feed additive further underscores its potential as a safe and effective antibacterial agent (Kristianti et al. 2022).

Previous research on *Lactobacillus* strains isolated from chicken intestines highlights their probiotic potential. These strains exhibited antimicrobial activity, tolerance to harsh gut conditions, and stability under various environmental parameters (Yuksekdag et al. 2014; Ahmed

et al. 2019; Ishaq et al. 2019). Specific strains like *Lactobacillus delbrueckii* ssp. *delbrueckii* BAZ32 and *Lactobacillus acidophilus* BAZ29 show high probiotic potential due to their combined acid and bile tolerance, antimicrobial activity, and ability to form aggregates, which enhance their survival and colonization in the gut (Yuksekdag et al. 2014). Further, *Lactobacillus ingluviei* and *Lactobacillus salivarius* can modulate the gut microbiota, promoting beneficial bacteria and suppressing harmful ones (Sirisopapong et al. 2023).

Beyond *E. coli* and *Salmonella* spp., *Staphylococcus* spp., particularly *S. aureus*, is another significant poultry pathogen. Staphylococcal infections can lead to a range of clinical syndromes in poultry, often associated with impaired immunity (Szafraniec et al. 2022). While coagulase-positive Staphylococci like *S. aureus* are the primary concern, infections by other *Staphylococcus* species, including coagulase-negative staphylococci, have also been reported (Peton and Le Loir. 2014; Wijesurendra et al. 2017; Pyzik et al. 2019; González-Martín et al. 2020). The prevalence of staphylococcal infections in poultry flocks varies, with previous studies reporting frequencies from 10.5% to 10.8% (Wieliczko et al. 2002; Marek et al. 2016). Asymptomatic *S. aureus* infections can be particularly high, reaching up to 57% in some flocks (Benrabia et al. 2020). The emergence of methicillin-resistant *S. aureus* (MRSA) in poultry is also concerning, with high prevalence rates in some regions (Richter et al. 2012).

Given these challenges, this study aims to isolate and evaluate the antibacterial activity of *Lactobacillus* spp. strains from the digestive tract of Noi chickens against key poultry pathogens*: E. coli, S. aureus*, and *Salmonella* sp. By harnessing the natural antimicrobial potential of these *Lactobacillus* strains, this research seeks to contribute to the development of sustainable and effective probiotic-based strategies for disease prevention and control in Noi chicken farming.

MATERIALS AND METHODS

Isolation and characterization of *Lactobacillus* strains

Lactobacillus strains were isolated from the gastrointestinal tracts (crop, small intestine, large intestine, and cecum) of 45 healthy, four-month-old Noi chickens raised on a traditional diet in various regions of the Ben Tre province. Following humane euthanasia in accordance with established ethical protocols (Risa et al. 2020), the gastrointestinal tracts were carefully dissected under sterile conditions, and samples were promptly collected into sterile containers.

Each gastrointestinal tract section was disinfected with 70% ethanol and rinsed twice with sterile distilled water. A one-gram sample from each washed section was then homogenized in 9 mL of sterile distilled water using a vortex mixer for 5 minutes. Serial ten-fold dilutions of these homogenates were prepared, and 100 µL aliquots from each dilution were spread onto de Man, Rogosa, and Sharpe (MRS) agar (Himedia, India) plates supplemented with 0.5% calcium carbonate and 0.05% bile salts (Sigma-Aldrich). This selective media promotes the growth of *Lactobacillus* while inhibiting other bacteria (Gupta et al. 2023; Sirisopapong et al. 2023). The plates were then incubated anaerobically at 37°C for 48 hours.

Following incubation, colonies surrounded by a clear zone, indicative of potential antibacterial activity, were selected from the highest dilution plates of each gastrointestinal tract section. These potential *Lactobacillus* colonies were further purified by repeated streaking onto fresh MRS agar plates. The purified cultures were then subjected to Gram staining, cell morphology assessment, and catalase and indole testing to confirm their identity as *Lactobacillus* spp. Isolates identified as Gram-positive, catalase-negative, and indole-negative, characteristics typical of *Lactobacillus* spp., were stored at -80°C in MRS broth containing 40% glycerol for subsequent analysis (Tsega et al. 2023).

Antimicrobial activity assay

The antimicrobial potential of the *Lactobacillus* isolates was evaluated against *E. coli*, *S. aureus*, and *Salmonella* sp. using the agar well diffusion method (Dec et al. 2016). The indicator strains, obtained from the Biotechnology Research and Development Institute at Can Tho University, were cultivated in Luria-Bertani (LB) broth.

The *Lactobacillus* isolates were grown in MRS broth under anaerobic conditions at 37°C for 24 hours. Cultures were then centrifuged at 10,000 rpm for 5 minutes at 4°C to separate the bacterial cells from the liquid supernatant, which potentially contained antimicrobial compounds. The indicator strains were mixed into molten nutrient agar at a concentration of 0.2% and poured into plates. After solidification, 4 mm diameter wells were created in the agar using a sterile tool.

Total 100 µL of the cell-free supernatant from each *Lactobacillus* isolate was then dispensed into individual wells on separate plates containing each of the indicator strains. These plates were then incubated at 37°C for 24 hours to allow for potential growth inhibition of the indicator strains. The presence of clear zones around the wells signified antimicrobial activity. The diameter of these zones was measured in millimeters, with larger zones correlating with stronger antimicrobial activity (Rossi et al. 2021). To ensure reproducibility, triplicate plates were prepared for each *Lactobacillus* isolate.

Antibiotic susceptibility testing

To ensure the safe use of the selected *Lactobacillus* isolates as potential probiotics, their susceptibility to commonly used antibiotics was determined (Sharma et al. 2024). Four antibiotics, namely 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 for 24 hours, 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.

Selection for low pH and bile salt tolerance

Lactobacillus strains were initially cultured overnight in MRS broth at 37°C with shaking at 120 rpm. The bacterial cells were then collected by centrifugation at 7,500×g for 5 minutes at 4°C. The resulting pellets were washed twice with sterile distilled water and resuspended in fresh MRS broth. The cell suspensions were then standardized to an optical density (OD) between 0.5 and 0.7 at 600 nm using a spectrophotometer (Reuben et al. 2021). This standardized concentration, approximately 10^8 CFU/mL, was used for subsequent tolerance assays. To evaluate acid tolerance, one mL aliquots of the standardized cell suspension were inoculated into 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 to separate tubes containing 9 mL MRS broth supplemented with varying concentrations (0%, 0.15%, and 0.3%) of bile salts. These cultures were also incubated at 37°C for 4 hours to evaluate the ability of the *Lactobacillus* isolates to withstand bile salts, a common challenge in the intestinal environment (Tian et al. 2024).

Following incubation for both assays, serial dilutions (up to 10^{-7}) were prepared in sterile distilled water to achieve countable cell concentrations. Aliquots (100 µL) of appropriate dilutions 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).

Molecular identification of the potential antimicrobial strain

The strain exhibiting the most promising antimicrobial and probiotic potential based on previous assays was selected for further identification. Genomic DNA was extracted from overnight cultures of this strain using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions, which included lysozyme treatment and proteinase K digestion. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Haendiges et al. 2020).

The 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3') (Swacita et al. 2022) with GoTaq Green Master Mix (Promega, USA) in a conventional thermocycler (Veriti, Applied Biosystems, USA). The PCR cycling conditions consisted of 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. The PCR products were then purified, and the bacterial DNA sequences were determined by Next Gen Scientific Co., Ltd (Ho Chi Minh City). The resulting 16S rRNA gene sequences were analyzed using BioEdit software (version 7.0). Consensus sequences were compared against the GenBank database using NCBI BLAST to confirm the species-level identification of the isolate (Mudawaroch et al. 2023).

Statistical analysis

Experimental data were analyzed using SPSS software. Results are presented as the mean ± standard deviation of three independent replicates. One-way analysis of variance (ANOVA) was employed to compare the data for acid tolerance,bile tolerance, and antimicrobial activity. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Isolation and phenotypic characterization of *Lactobacillus* strains

Thirty-two potential probiotic *Lactobacillus* strains were successfully isolated from the digestive tracts of 45 Noi chickens. Selective culturing on MRS agar supplemented with calcium carbonate and bile salts yielded 32 colonies with distinct morphological characteristics. The majority of colonies exhibited a circular shape, convex elevation, and an opaque white or clear white color (Figure 1). A clear zone surrounding the colonies on MRS agar supplemented with calcium carbonate indicated potential acid production, a hallmark of *Lactobacillus*.

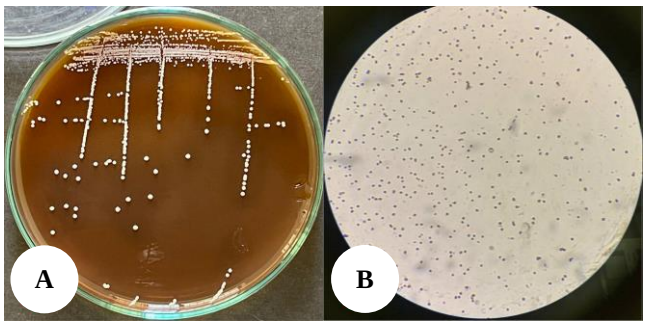

Figure 1. Morphological and Gram staining of isolated strains. A. Colony morphology of LN19 strain in MRS agar (with 0.15% bile salts); B. Gram staining of LN19 strain. Cells are purple, coccobacilli-shaped, and without spores

The morphological characteristics of the isolated *Lactobacillus* spp. were diverse. Colonies exhibited various shapes, including circular and irregular forms, and a range of colors, from translucent white to opaque white. Colony size varied significantly, from very small to large, and elevation was predominantly raised, convex, or flat. Microscopic examination revealed that the isolates were predominantly rod-shaped or coccobacilli. All isolates were Gram-positive and non-spore-forming. Biochemical tests, including negative results for indole and catalase production, further confirmed their identification as *Lactobacillus* spp.

Antimicrobial activity of *Lactobacillus* strains

All 32 *Lactobacillus* isolates demonstrated the ability to inhibit at least one of the three major poultry pathogens: *E. coli*, *S. aureus*, and *Salmonella* sp. in the well diffusion assay (Table 1, Figure 2). The zones of inhibition, which indicate the strength of the antimicrobial effect, varied across strains and pathogens. Against *E. coli*, inhibition zones ranged from 0.87 to 2.37 cm, with LN18, LN11, LN8, LN16, LN12, LN14, and LN19 showing the largest zones. For *S. aureus*, the zones ranged from 0.67 to 2.40 cm, with the strongest inhibition observed for LN15, LN17, LN25, LN6, LN11, and LN19. Against *Salmonella* sp., inhibition zones spanned from 1.27 to 2.60 cm, with LN11, LN29, LN7, LN22, LN25, and LN19 exhibiting the most potent activity.

Two strains, LN11 and LN19, stood out for their ability to inhibit all three tested pathogens. LN11 produced inhibition zones of 1.93 cm against both *E. coli* and *Salmonella* sp., and 2.07 cm against *S. aureus*. LN19 displayed even stronger broad-spectrum activity, with zones of 2.27 cm, 2.03 cm, and 2.60 cm against *E. coli*, *Salmonella* sp., and *S. aureus*, respectively.

These findings underscore the diverse antimicrobial potential within the *Lactobacillus* isolates. While most strains showed some level of inhibitory activity, a few, such as LN21 and LN22, had limited or no effect against certain pathogens. This variability highlights the strain-specific nature of antimicrobial traits in *Lactobacillus*.

The broad-spectrum activity of strains LN11 and LN19 marks them as particularly promising candidates for further research into their use as probiotics in poultry production. Their ability to combat multiple key pathogens suggests they could contribute to improved gut health and disease resistance in poultry.

Antibiotic susceptibility of *Lactobacillus* strains

The antibiotic susceptibility profiles of the 32 *Lactobacillus* isolates were assessed against four commonly used antibiotics in poultry production: chloramphenicol, erythromycin, ampicillin, and ciprofloxacin. The results indicated a prevalence of antibiotic resistance among these strains (Table 2).

Table 1. Antimicrobial activity of *Lactobacillus* strains against pathogens

Isolates ID	*Escherichia coli* (cm)	*Staphylococcus aureus* (cm)	*Salmonella* sp. (cm)
LN1	1.50±0.17	1.53±0.12	2.40±0.35
LN2	2.37±0.06	2.10±0.46	1.67±0.29
LN3	1.37±0.06	1.63±0.12	1.50±0.17
LN4	1.63±0.31	1.70±0.17	1.87±0.23
LN5	1.70±0.26	1.50±0.26	1.80±0.35
LN6	1.80±0.17	2.07±0.06	1.60±0.35
LN7	1.50±0.10	1.87±0.38	1.97±0.12
LN8	1.97±0.25	1.67±0.232	1.77±0.29
LN9	0.97±0.84	1.53±0.12	1.53±0.12
LN10	1.63±0.21	1.47±0.12	1.63±0.12
LN11	1.93±0.06	2.07±0.45	1.93±0.23
LN12	2.03±0.15	1.80±0.17	1.33±0.06
LN13	1.83±0.58	1.53±0.06	1.87±0.23
LN14	2.07±0.21	1.53±0.12	1.80±0.17
LN15	1.47±0.12	1.93±0.15	2.40±0.35
LN16	2.03±0.06	1.27±0.12	2.40±0.10
LN17	1.90±0.44	1.97±0.25	1.40±0.00
LN18	1.93±0.40	1.87±0.25	1.73±0.40
LN19	2.27±0.31	2.60±0.36	2.03±0.45
LN20	1.57±0.15	1.60±0.00	1.60±0.00
LN21	1.87±0.12	1.37±0.21	0.67±1.15
LN22	0.87±0.76	1.77±0.25	1.97±0.40
LN23	1.80±0.10	1.63±0.12	1.80±0.35
LN24	1.70±0.20	1.70±0.17	1.60±0.35
LN25	1.73±0.31	2.07±0.06	1.97±0.12
LN26	1.53±0.25	1.87±0.38	1.77±0.29
LN27	1.60±0.10	1.80±0.17	1.53±0.12
LN28	1.43±0.45	1.53±0.06	1.63±0.12
LN29	1.73±0.50	1.53±0.12	1.93±0.23
LN30	1.50±0.30	1.87±0.38	1.63±0.45
LN31	1.57±0.15	1.80±0.17	1.43±0.15
LN32	1.40±0.40	1.53±0.06	1.43±0.31

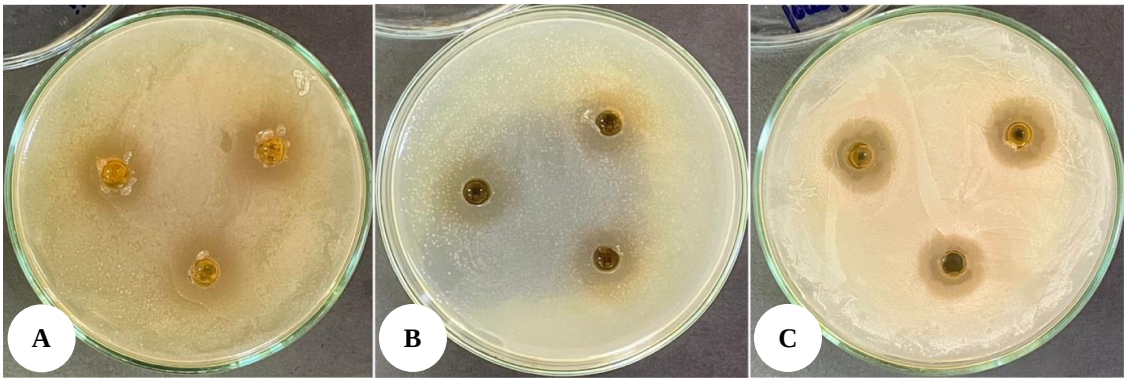

Figure 2. The inhibition zones of the strain LN19 against pathogenic bacteria. A. *E. coli*; B. *Samonella* sp.; C. *S. aureus*

Specifically, resistance was most common against ciprofloxacin, with all 32 isolates (100%) exhibiting resistance. High levels of resistance were also observed for erythromycin and ampicillin, with 59.4% of the isolates resistant to each. Chloramphenicol resistance was slightly lower, with 53.1% of isolates showing resistance. A finding was that 13 isolates: LN5, LN7, LN8, LN9, LN10, LN11, LN12, LN13, LN14, LN16, LN17, LN18, LN19, LN21, LN26 displayed resistance to all four tested antibiotics. This multi-drug resistance phenotype raises concerns about the potential transfer of resistance genes to pathogenic bacteria, posing a risk to both animal and human health.

Acid tolerance

The probiotic potential of the 32 isolated *Lactobacillus* strains was initially assessed by evaluating their ability to survive in acidic conditions, simulating the harsh environment of the chicken's stomach. Survival rates varied significantly among the isolates when exposed to different pH levels 2.0, 4.0, and 6.5 s for 4 hours at 37°C (Table 3).

Table 2. Antibiotic susceptibility of *Lactobacillus* strains

Isolates ID	Antibiotic designation			
	C	E	AMP	CIP
LN1	R	R	I	R
LN2	S	R	R	R
LN3	S	R	I	R
LN4	S	S	R	R
LN5	R	R	R	R
LN6	S	S	S	R
LN7	R	R	R	R
LN8	R	R	R	R
LN9	R	R	R	R
LN10	R	R	R	R
LN11	R	R	R	R
LN12	R	R	R	R
LN13	R	R	R	R
LN14	R	R	R	R
LN15	S	S	S	R
LN16	R	R	R	R
LN17	R	R	R	R
LN18	R	R	R	R
LN19	R	R	R	R
LN20	S	S	R	R
LN21	R	R	R	R
LN22	S	S	S	R
LN23	S	I	S	R
LN24	R	S	S	R
LN25	I	I	I	R
LN26	R	R	R	R
LN27	S	I	S	R
LN28	S	R	S	R
LN29	S	I	I	R
LN30	I	I	S	R
LN31	I	I	S	R
LN32	S	S	R	R

Notes: *: 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

At the highly acidic pH of 2.0, nine strains LN2, LN3, LN9, LN10, LN12, LN15, LN16, LN19, and LN21 demonstrated superior acid tolerance, maintaining viable cell counts ranging from 5.59 to 6.28 Log CFU/mL. This ability to withstand low pH is crucial for probiotic bacteria to transit through the stomach and reach the intestine, where they can exert their beneficial effects. In contrast, five strains LN4, LN7, LN22, LN27, and LN32 were unable to survive at this pH, indicating their limited potential as probiotics in poultry. As expected, the survival of most isolates improved at the less acidic pH levels of 4.0 and 6.5. These findings highlight the inherent variability in acid tolerance among different *Lactobacillus* strains, underscoring the importance of selecting acid-tolerant strains for probiotic applications in poultry

Bile salt tolerance

Equally important for probiotic functionality is the ability to tolerate bile salts, which are present in the intestinal environment and can disrupt bacterial cell membranes. The isolates' tolerance to bile salts was assessed at concentrations of 0%, 0.15%, and 0.3%.

Table 3. Selected *Lactobacillus* isolates pH tolerance

Isolates ID	Viable *Lactobacillus* bacteria isolates (Log CFU/mL)		
	pH 6.5 (Control)	pH 4	pH 2
LN1	7.87±0.48	7.78±0.47	4.50±0.71
LN2	8.16±0.54	8.09±0.68	5.59±0.02
LN3	8.14±0.19	8.12±0.16	5.84±0.20
LN4	7.32±0.03	7.28±0.03	0.00±0.00
LN5	7.70±0.78	7.60±0.73	2.57±3.64
LN6	7.79±0.46	7.84±0.59	5.39±0.12
LN7	7.50±0.29	7.34±0.48	0.00±0.00
LN8	7.75±0.78	7.51±1.15	2.00±2.83
LN9	7.89±0.16	7.12±0.16	5.64±0.45
LN10	7.76±0.71	6.65±0.49	5.67±0.46
LN11	8.46±0.40	8.40±0.42	5.55±0.32
LN12	6.89±0.83	6.65±0.92	5.70±0.54
LN13	8.21±0.29	8.15±0.31	2.35±3.32
LN14	8.66±0.47	8.65±0.27	5.07±0.32
LN15	7.63±0.10	7.56±0.20	6.28±0.47
LN16	8.13±0.31	8.10±0.29	5.71±0.10
LN17	7.12±0.16	6.89±0.58	2.30±3.25
LN18	8.05±0.17	7.85±0.69	5.46±0.02
LN19	8.11±0.30	8.01±0.45	5.95±0.49
LN20	8.53±0.60	8.49±0.58	5.54±0.43
LN21	8.70±0.34	8.68±0.24	5.63±0.73
LN22	7.51±0.47	0.00±0.00	0.00±0.00
LN23	7.90±0.54	7.72±0.60	2.24±3.17
LN24	7.47±0.18	7.28±0.28	2.35±3.32
LN25	7.04±0.80	3.24±4.58	2.45±3.47
LN26	6.85±0.21	6.85±0.21	4.65±0.49
LN27	7.19±0.41	3.24±4.58	0.00±0.00
LN28	6.65±0.92	6.50±0.71	4.74±0.37
LN29	8.26±0.37	8.18±0.47	4.65±0.49
LN30	6.89±0.83	3.15±4.46	2.42±3.43
LN31	6.80±0.28	6.65±0.49	4.74±0.37
LN32	7.19±0.41	3.24±4.58	0.00±0.00

Table 4. Selected *Lactobacillus* isolates bile salt tolerance

Isolates ID	Viable *Lactobacillus* bacteria isolates (Log CFU/mL)		
	0% (Control)	0.15%	0.30%
LN1	7.87±0.48	5.97±0.58	5.47±0.18
LN2	8.16±0.54	6.16±0.54	5.48±0.17
LN3	8.14±0.19	5.90±0.60	5.43±0.49
LN4	7.32±0.03	6.10±0.37	5.31±0.23
LN5	7.70±0.78	5.96±0.35	5.67±0.04
LN6	7.79±0.46	6.66±0.50	5.97±0.47
LN7	7.50±0.29	6.48±0.52	5.44±0.06
LN8	7.75±0.78	6.44±0.60	2.74±3.87
LN9	7.89±0.16	6.66±0.48	4.89±0.58
LN10	7.76±0.71	6.12±0.25	5.85±0.21
LN11	8.46±0.40	6.32±0.50	5.89±0.16
LN12	6.89±0.83	2.56±3.62	0.00±0.00
LN13	8.21±0.29	4.98±0.71	4.74±0.37
LN14	8.66±0.47	5.79±0.55	0.00±0.00
LN15	7.63±0.10	5.89±0.15	5.30±0.00
LN16	8.13±0.31	5.79±0.41	2.39±3.38
LN17	7.12±0.16	5.55±0.08	5.31±0.75
LN18	8.05±0.17	6.06±0.08	5.98±0.32
LN19	8.11±0.30	6.06±0.16	5.92±0.54
LN20	8.53±0.60	6.53±0.57	6.41±0.64
LN21	8.70±0.34	6.32±0.40	6.26±0.55
LN22	7.51±0.47	6.01±0.24	5.89±0.27
LN23	7.90±0.54	6.15±0.99	2.85±4.03
LN24	7.47±0.18	6.57±0.61	4.50±0.71
LN25	7.04±0.80	5.91±0.24	5.19±1.26
LN26	6.85±0.21	5.74±0.37	3.03±4.29
LN27	7.19±0.41	5.62±0.82	5.52±0.80
LN28	6.65±0.92	5.59±0.58	0.00±0.00
LN29	8.26±0.37	5.93±0.11	5.68±0.32
LN30	6.89±0.83	5.86±0.20	5.00±1.41
LN31	6.80±0.28	5.54±0.34	2.98±4.21
LN32	7.19±0.41	5.62±0.82	5.52±0.80

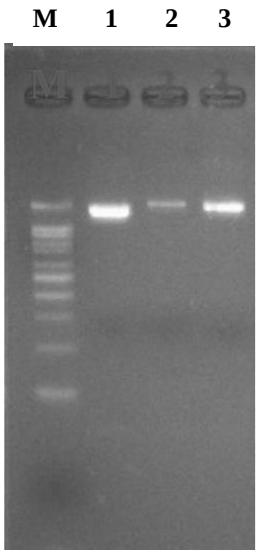

Figure 3. Amplification of DNA barcodes from LN19 strain. Product of the 16S rDNA region from 1500 bp on 2% agarose gel with 100 bb ladder; M: DNA marker; Lanes 1: Positive control; Lanes 2-3: Samples; Lanes 4: Negative control without DNA

All strains demonstrated robust growth in the absence of bile salts (0%). However, their tolerance varied significantly ($p<0.05$) at higher concentrations (Table 4). At 0.3% bile salts, ten strains LN5, LN6, LN10, LN11, LN18, LN19, LN20, LN21, LN22, and LN29 exhibited the highest tolerance. In contrast, three strains LN12, LN14, and LN28 were unable to survive under these conditions. The remaining isolates showed intermediate tolerance, with viable cell counts decreasing as the bile salt concentration increased.

Molecular identification of the potential antimicrobial *Lactobacillus* strain

Genomic DNA was extracted from all *Lactobacillus* isolates and subjected to PCR amplification of the 16S rDNA region. Gel electrophoresis confirmed successful amplification, with products of approximately 1500 base pairs observed for all isolates (Figure 3).

To pinpoint the species identity of the promising isolate LN19, its 16S rRNA gene was amplified and sequenced. The resulting sequence was deposited in GenBank (accession number PQ357285) and compared to existing sequences using BLAST. This analysis revealed a high similarity (99.81%) to the 16S rRNA gene of *Lactobacillus farciminis.* Based on this molecular identification, isolate LN19 was definitively classified as *Lactobacillus farciminis* LN19.

This molecular characterization provides valuable information for understanding the diversity and potential applications of *Lactobacillus* strains isolated from Noi chickens. The identification of *L. farciminis* LN19 adds to the growing body of knowledge on the probiotic potential of this species, particularly in the context of poultry production.

Discussion

Isolation and characterization of potentially beneficial Lactobacillus *strains*

The utilization of probiotics, particularly those sourced from the host animal's natural environment, has emerged as a promising and sustainable strategy to mitigate the reliance on antibiotics in poultry production (Bhogoju and Nahashon 2022). These beneficial microorganisms have demonstrated a positive influence on various aspects of poultry health and production, encompassing growth performance, bone health, meat and eggshell quality, immune response, gut microbiota balance, and disease resistance. Research spanning both ruminants and non-ruminants has firmly established the positive effects of probiotics on gut health, immunity, and overall production (Mahesh et al. 2021).

However, the efficacy of probiotics is not uniform. Strain selection and host specificity play critical roles in determining their effectiveness (Cameron and McAllister 2019). This underscores 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 32 *Lactobacillus* strains from the intestinal tracts of healthy Noi chickens. We employed bromocresol purple-supplemented MRS agar, a method that facilitates visual identification of lactic acid bacteria (LAB) based on the formation of clear halos around colonies due to acid production (Sobrun et al. 2012). Microscopic examination revealed that these isolates were Gram-positive, non-spore-forming rods or coccobacilli, aligning with the typical morphology of *Lactobacillus* species commonly found in the chicken digestive tract (Schuster et al. 2019).

Our findings are consistent with previous research that has highlighted the inherent variability in aggregation ability and gastrointestinal stress tolerance among *Lactobacillus* strains (Aziz et al. 2019), emphasizing the critical need for careful strain selection in probiotic development. The successful isolation and initial characterization of these 32 *Lactobacillus* strains represent a crucial first step in identifying potential probiotic candidates that could contribute to sustainable and antibiotic-free poultry production. Further evaluation of their probiotic properties and in vivo efficacy will be essential to determine their suitability for application in poultry farming practices.

Previous studies have also reported the successful isolation of *Lactobacillus* from chicken intestines, demonstrating characteristic colony morphologies and utilizing selective media for isolation (Shamsudin et al. 2019; Ahmad et al. 2022). These studies, along with ours, highlight the abundance and diversity of *Lactobacillus* species within the chicken gut, providing a rich source for potential probiotic discovery.

Antimicrobial activity of Lactobacillus *strains*

Our study revealed the promising antimicrobial potential of *Lactobacillus* spp. isolated from Noi chickens against key poultry pathogens, namely *E. coli*, *S. aureus*, and *Salmonella* sp. All 32 isolates demonstrated the ability to inhibit at least one of these pathogens, with varying degrees of efficacy. Notably, strains LN11 and LN19 exhibited broad-spectrum activity, effectively inhibiting all three pathogens tested. This finding is significant, as it suggests the potential of these strains as probiotics for promoting gut health and disease resistance in poultry.

The observed antimicrobial activity aligns with existing literature demonstrating the ability of *Lactobacillus* strains to inhibit a range of pathogenic bacteria, including *E. coli*, *S. typhimurium*, *S. aureus*, *C. perfringens*, *Klebsiella* spp., and *Proteus* spp. (Cisek et al. 2022). This inhibitory effect is often attributed to competitive exclusion and the production of antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins (Jose et al. 2015). The broad-spectrum activity observed in some of our isolates is consistent with previous reports of *Lactobacillus* strains capable of inhibiting multiple pathogens (Cisek et al. 2022).

Furthermore, our study supports previous findings that chicken-derived *Lactobacillus* isolates can produce active compounds that directly antagonize pathogens like *E. coli* and *S. aureus* (Dec et al. 2016; Shamsudin et al. 2019). The inhibitory activity we observed against *E. coli* and *S. enterica* aligns with these previous reports. It's worth noting that the antimicrobial potential of *Lactobacillus* is not limited to poultry isolates; research has also demonstrated the inhibitory activity of isolates from other sources, such as camel milk, against pathogens like *S. aureus* (Muhammad et al. 2019).

In our study, we employed the agar well diffusion method to evaluate the antimicrobial activity of the isolated *Lactobacillus* strains against common poultry pathogens. Most strains exhibited strong to moderate antimicrobial activity against *S. aureus*, *Salmonella* sp., and *E. coli*. Interestingly, Khyralla et al. (2022) reported similar inhibitory effects of *Lactobacillus* isolates from camel milk against a range of pathogens, including *S. aureus*, *E. coli*, and *Salmonella typhimurium*. Likewise, Pooja et al. (2024) observed the inhibitory activity of *Lactobacillus* strains from chicken intestines against *E. coli*. However, our results diverge from those of Ahmed et al. (2019), who reported higher activity against *Salmonella* than *E. coli*. This discrepancy could be attributed to factors such as strain-specific differences, growth conditions, and the particular antimicrobial compounds produced. Among different *Lactobacillus* species, *L. salivarius* isolated from chicken showed better antagonism in vitro against various poultry pathogens, including *Salmonella* spp. and *E. coli* (Filho et al. 2015; Aazami et al. 2016). The antagonism observed in our study may be an outcome of immunomodulatory responses triggered by antimicrobial metabolites produced by the isolated strains (Mashak 2016).

The variability in inhibitory activity among the different *Lactobacillus* strains and against different pathogens underscores the strain-specific nature of antimicrobial traits within this genus. This highlights the importance of careful strain selection in the development of probiotic interventions for poultry.

Significance of acid and bile tolerance in probiotic selection

The ability of probiotic bacteria to survive the challenges of the gastrointestinal tract, such as the low pH of gastric acid and the presence of bile salts, is essential for their efficacy. Successful navigation of these hurdles allows probiotic strains to colonize the gut and exert their beneficial effects on the host. Previous research has identified 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 the present study, two isolates, LN19 and LN21, demonstrated good survivability under both 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 (Jin et al. 1998; Akpa et al. 2022; Kéhi et al. 2022). The ability of probiotic strains to survive in the presence of bile acids is particularly crucial due to their role in lipid absorption and their impact on the gut microbiota composition and function (Schmid et al. 2016; Ahmed et al. 2019).

The acid and bile salt tolerance exhibited by LN19 and LN21, in combination with their other potential probiotic

attributes, such as antimicrobial activity, suggests their promising candidacy for further exploration and development as effective poultry probiotics.

Molecular identification of the potential antimicrobial Lactobacillus *strains*

This study focused on the molecular characterization of a promising *Lactobacillus* isolate, LN19, which was chosen from an initial collection of 34 strains obtained from the digestive tracts of Noi chickens. The 16S rDNA region of LN19 was amplified using PCR, and successful amplification was confirmed by gel electrophoresis, yielding a product of approximately 1500 base pairs. This size is consistent with the expected range for 16S rDNA fragments in various *Lactobacillus* species (Aazami et al. 2016; Jeyagowri et al. 2023).

To accurately identify LN19, its 16S rRNA gene was sequenced and submitted to GenBank (accession number PQ357285). A BLAST search of this sequence against existing databases showed a high degree of similarity (99.81%) to the 16S rRNA gene of *Lactobacillus farciminis*, allowing us to confidently classify LN19 as *Lactobacillus farciminis* LN19.

This molecular characterization offers valuable insights into the diversity and potential applications of *Lactobacillus* strains found within the gut microbiota of Noi chickens. The identification of *L. farciminis* LN19 is particularly noteworthy, as it expands our understanding of the probiotic potential of this species, underscores the importance of further exploring the gut microbiota of indigenous poultry breeds for novel probiotic strains that can contribute to sustainable and antibiotic-free poultry production.

This study successfully isolated 32 potential probiotic *Lactobacillus* strains from Noi chickens, exhibiting diverse morphological and biochemical characteristics. Notably, strains LN11 and LN19 demonstrated broad-spectrum antimicrobial activity against *E. coli*, *Salmonella* sp., and *S. aureus*, highlighting their potential as probiotics for poultry. A finding was that 13 isolates: LN5, LN7, LN8, LN9, LN10, LN11, LN12, LN13, LN14, LN16, LN17, LN18, LN19, LN21, LN26 displayed resistance to all four tested antibiotics: chloramphenicol, erythromycin, ampicillin, and ciprofloxacin. Several isolates, including LN19 and LN21, displayed promising probiotic traits such as tolerance to acidic conditions and bile salts. Particularly, nine strains exhibited remarkable acid tolerance at pH 2.0, while ten strains showed high tolerance to 0.3% bile salts. Further investigation revealed that strain LN19, identified as *Lactobacillus farciminis* through 16S rRNA gene sequencing, possesses potent antimicrobial activity and robust probiotic properties, underscoring the value of exploring indigenous poultry breeds for novel probiotic strains.

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