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Molecular characterization and antimicrobial potential of lactic acid bacteria isolated from the Muscovy duck gut

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Abstract. *Thuy NP, Tuu NT. 2025. Molecular characterization and antimicrobial potential of lactic acid bacteria isolated from the Muscovy duck gut. Biodiversitas 26: 3590-3599.* The rise of antibiotic resistance in poultry necessitates sustainable alternatives like probiotics. The Muscovy duck (*Cairina moschata* (Linnaeus, 1758)), a vital Vietnamese agricultural breed with an uncharacterized gut microbiota, is a promising source of such beneficial bacteria. This study aimed to isolate and identify Lactic Acid Bacteria (LAB) from the gastrointestinal tract of Muscovy ducks and evaluate their in vitro antimicrobial activity against key poultry pathogens. A total of thirty LAB isolates were obtained from the crop, gizzard, small intestine, and cecum using selective media. These isolates were screened for antimicrobial activity against *Escherichia coli*, *Salmonella* sp., and *Staphylococcus aureus* using the agar well diffusion method. The most effective isolation was identified through 16S rRNA gene sequencing. Among the isolates, D11 demonstrated the most potent and broad-spectrum inhibition, producing inhibition zones of 1.90±0.35 cm against *E. coli*, 1.97±0.42 cm against *Salmonella* sp., and 2.30±0.35 cm against *S. aureus*. Molecular analysis identified isolate D11 as *Companilactobacillus farciminis*, with a 99.64% sequence identity to the reference strain. This is the first report of *C. farciminis* isolation from Muscovy ducks, expanding the known microbial biodiversity of this species. The isolate's potent antimicrobial activity positions it as a strong probiotic candidate to support sustainable and healthy duck production.

Keywords: Avian gut microbiota, *Cairina moschata*, *Companilactobacillus farciminis*, probiotic screening

INTRODUCTION

The global poultry industry must reduce its long-standing reliance on antibiotics due to the escalating crisis of antimicrobial resistance. This threat to public health, driven by the extensive use of antibiotics in livestock, necessitates a shift toward sustainable alternatives (Pérez-Sánchez et al. 2018; Patil et al. 2021). In this post-antibiotic era, probiotics, especially LAB, have emerged as a scientifically backed strategy to enhance avian gut health. Consequently, the exploration of native, host-adapted bacteria from the unique gastrointestinal tracts of poultry is a key frontier for developing next-generation probiotics.

In Vietnam, the Muscovy duck (*Cairina moschata* (Linnaeus, 1758)) is a cornerstone of the agricultural landscape, with a national population exceeding 85 million birds in 2022. This industry is vital to rural economies, particularly in the Mekong Delta, but its stability and growth potential are perpetually threatened by bacterial diseases. Pathogenic infections, especially Salmonellosis caused by *Salmonella* spp. and Colibacillosis from avian pathogenic *E. coli*, inflict severe economic damage (Abd El-Ghany 2023). These diseases result in a cascade of negative outcomes, from enteritis, septicemia, and high mortality rates in ducklings to diminished growth rates and carcass condemnation at processing (Thu et al. 2019; Sorour et al. 2023). Furthermore, *S. aureus* is a significant pathogen causing conditions like septicemia and bumblefoot (AbdelRahman and Amer 2021). The economic burden is magnified by indirect costs such as poor feed conversion, reduced egg production, and veterinary expenses (Hussein et al. 2024). The zoonotic potential of *Salmonella* and certain *E. coli* strains underscores the public health imperative to control their prevalence, a task made more difficult by the rising tide of antibiotic resistance that renders conventional treatments increasingly ineffective (Aguidissou et al. 2022; Hamed et al. 2024).

Probiotics have emerged as a leading alternative to antibiotics, defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Plaza-Diaz et al. 2019). Their efficacy stems from multiple mechanisms, including competitive exclusion of pathogens from intestinal niches, modulation of the host immune system, and the production of antimicrobial compounds. Lactic acid bacteria are exemplary probiotics, known for producing lactic acid, hydrogen peroxide, and potent antimicrobial peptides called bacteriocins, which collectively create an intestinal environment that suppresses pathogen growth (Reuben et al. 2019; Hidalgo et al. 2023; Khushboo et al. 2023). The application of probiotics in poultry has been demonstrably linked to improved growth performance and feed efficiency, aligning with the global demand for more sustainable agricultural practices (Nallala et al. 2017; Krysiak et al. 2021; Rahimoon et al. 2023).

While the benefits of LAB are well-established, their optimal performance is often host-specific, meaning probiotic

strains isolated from one species, such as chickens, may not be as effective in another (Xie et al. 2015; Alipin and Safitri 2016). This principle underscores the novelty and importance of investigating the Muscovy duck as a source for new probiotics. As waterfowl, Muscovy ducks possess distinct physiological and ecological traits compared to terrestrial poultry. Their semi-aquatic foraging behavior, adaptation to diverse wetland and agricultural habitats, and unique diet—comprising aquatic plants, invertebrates, and rice—expose them to a different spectrum of environmental microorganisms, driving the co-evolution of a specialized gut microbiota (West et al. 2022; Hemprabha and Arya 2023). Recent studies confirm this distinctiveness, showing that the duck gut microbiome has significant regional specificity, develops over time, and is influenced by rearing conditions such as water access (Ma et al. 2024). The cecal microbiota, in particular, is dominated by phyla like Bacteroidetes, Firmicutes, and Fusobacteria, with specific genera linked to host metabolic traits (Lyu et al. 2021). Furthermore, the well-developed ceca of Muscovy ducks create a unique anaerobic environment that is hypothesized to favor the colonization of novel LAB with robust antimicrobial capabilities (Kamollerd et al. 2016; Risna et al. 2020).

Despite the Muscovy duck's agricultural significance and unique biology, its native LAB microbiota remains a critically underexplored resource, especially within the Vietnamese context. While preliminary studies have identified beneficial *Lactobacillus*, *Enterococcus*, and *Pediococcus* strains in other duck species (Kamollerd et al. 2016; Herdian et al. 2018; Risna et al. 2022; Maquiné et al. 2024), a comprehensive characterization of LAB from Muscovy ducks is lacking. This knowledge gap represents a missed opportunity for developing host-adapted probiotics tailored for this important poultry sector. This study was therefore designed to test the hypothesis that the gastrointestinal tract of healthy Vietnamese Muscovy ducks harbors a unique reservoir of LAB with potent antimicrobial activity. The primary aims were to isolate and molecularly identify LAB strains from these ducks and to evaluate their in vitro antimicrobial efficacy against key poultry pathogens *Salmonella* spp., *E. coli*, and *S. aureus*, with the ultimate goal of identifying promising candidates for developing a native probiotic to support a more sustainable and secure Muscovy duck industry.

MATERIALS AND METHODS

Isolation and characterization of LAB

Under approved ethical protocols (Risna et al. 2020), Gastrointestinal Tract (GIT) sections, crop, gizzard, small intestine, and cecum were aseptically collected from 50 healthy, market-age Muscovy ducks (25 males, 25 females) from farms in Tra Vinh Province, Vietnam. Following euthanasia, the samples were immediately transported on ice to the laboratory.

To prevent surface contamination, each GIT section was disinfected with 70% ethanol and rinsed with sterile water. A 1-g sample of tissue from each section was then homogenized, serially ten-fold dilutions in sterile saline, and 100 µL aliquots from appropriate dilutions were spread plated in triplicate onto de Man, Rogosa, and Sharpe (MRS) agar (Himedia, India) supplemented with 0.05% bromocresol green and 0.05% bile salts. Plates were incubated anaerobically at 37°C for 48 hours (Tavakoli et al. 2017). From the incubated plates, 50 presumptive LAB colonies exhibiting yellow halos were selected for purification by successive streaking (Nurcahyo et al. 2019).

Each pure isolate was first confirmed as a presumptive LAB through Gram staining and microscopic examination of cellular morphology (Kandi 2015; Reuben et al. 2020). Isolates were then subjected to a panel of key biochemical tests to exclude non-LAB species. The validity of each biochemical assay was rigorously confirmed by including known positive and negative control strains, which were run in parallel with the isolates in every batch of tests. For the catalase test, *Staphylococcus aureus* ATCC 25923 was used as the positive control, and *Lactobacillus plantarum* ATCC 14917 served as the negative control. Similarly, for the urease test, *Proteus mirabilis* ATCC 12453 (positive) and *Escherichia coli* ATCC 25922 (negative) were used. Citrate utilization was validated using *Enterobacter aerogenes* ATCC 13048 (positive) and *E. coli* ATCC 25922 (negative).

The final selection criteria for candidate LAB strains were a Gram-positive reaction and negative results for catalase, urease, and citrate tests. A total of 30 isolates that met these criteria were selected for subsequent antimicrobial activity assessment. These isolates were preserved as stock cultures at -20°C in MRS broth supplemented with 30% (v/v) sterile glycerol (Pisano et al. 2014).

Antimicrobial activity assay

The antimicrobial potential of 30 LAB isolates was evaluated against pathogenic indicator strains, including *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *Salmonella enterica* serovar Typhimurium ATCC 14028. Pathogenic strains were cultured aerobically in Luria-Bertani (LB) broth (Himedia, India) at 37°C for 18-24 hours. The reference strain *Lactobacillus plantarum* ATCC 14917 was used as a positive control to produce antimicrobial compounds.

Each of the 30 LAB isolates and the positive control was grown in MRS broth at 37°C for 24 hours under anaerobic conditions. The cultures were centrifuged at 10,000 rpm for 5 min at 4°C to pellet the cells. The resulting Cell-Free Supernatant (CFS) was carefully collected. To specifically assess the antimicrobial potential of metabolites other than organic acids, the CFS was neutralized to a pH of 7.0±0.2 using 1 M NaOH.

Antimicrobial activity was determined using the agar well diffusion method. A suspension of each indicator pathogen was prepared and adjusted to approximately 10^6 CFU/mL in molten Nutrient Agar (Neogen, USA) maintained at 45-50°C. This seeded agar was poured into sterile Petri plates and allowed to solidify completely. Wells with a diameter of 4 mm were then created in the agar using a sterile cork borer.

A 100 µL of nCFS from each LAB isolate was dispensed into separate wells. Each plate included a negative control

well containing 100 µL of sterile, neutralized MRS broth, and a positive control well containing 100 µL of nCFS from the reference strain *Lactobacillus plantarum* ATCC 14917. All assays were performed in triplicate.

The plates were incubated at 37°C for 24 hours. Following incubation, antimicrobial activity was quantified by measuring the diameter of the clear zone of inhibition in millimeters (mm), inclusive of the 4 mm well diameter. Based on criteria adapted from Rossi et al. (2021), the activity was classified as strong (>15 mm), moderate (10-15 mm), or weak/absent (<10 mm). Isolate demonstrating inhibitory activity against all three indicator pathogens were selected for molecular identification.

Genomic DNA isolation

Genomic DNA was extracted from the LAB isolates using a modified phenol-chloroform method. First, frozen isolates were revived by inoculating them into sterile MRS broth and incubating at 37°C for 24 hours under anaerobic conditions. Following this, 10 µL of the active culture was transferred to 10 mL of fresh MRS broth and incubated at 37°C for 10 hours to reach the active log phase.

LAB cultures (2 mL) were treated with sodium ampicillin (2-3 µL of a 50 mg/mL solution) and incubated at 37°C for 1 hour. Subsequently, the bacterial cells were collected by centrifugation at 5000 rpm for 5 minutes at 4°C. The supernatant was removed, and the resulting pellet was washed three times with 1 mL of NaCl-EDTA solution (30 mM NaCl, 2 mM EDTA, pH 8.0). The washed pellet was then resuspended in 100 µL of the same NaCl-EDTA solution, followed by the addition of 100 µL of a freshly prepared lysozyme solution (10 mg/mL in NaCl-EDTA). This mixture was incubated at 37°C for 1 hour with intermittent shaking. To eliminate RNA, 4 µL of an RNase-A solution (100 µg/mL) was added prior to further incubation. The volume was adjusted to 500 µL with NaCl-EDTA, and then 50 µL of 10% SDS solution and 10 µL of proteinase K solution (20 mg/mL) were added. This mixture was incubated at 55°C for 1 hour. Following incubation, an equal volume of Tris-saturated phenol (pH 8.0) was added, mixed, and centrifuged at 10,000 rpm at 22°C for 10 minutes to separate the phases. The upper aqueous phase, containing the DNA, was carefully collected. This extraction step was repeated using an equal volume of phenol-chloroform (1:1), and the supernatant was collected again. DNA precipitation was achieved by adding 0.8 volumes of isopropanol and 0.3 M sodium acetate (pH 5.2). The precipitated DNA was pelleted by centrifugation at 10,000 rpm at 4°C for 5 minutes, washed with 70% ethanol, allowed air-dry, and finally dissolved in 50 µL of Tris-EDTA buffer (10:1, pH 8.0). The concentration and purity of the extracted DNA were then assessed using a NanoDrop spectrophotometer (De et al. 2010).

16S rRNA gene amplification and molecular identification

The 16S rRNA gene was amplified from the extracted genomic DNA using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR was performed in a Veriti thermocycler (Applied Biosystems, USA) using GoTaq® Green Master Mix (Promega, USA). The PCR cycling protocol consisted of an initial denaturation at 94°C for 3 minutes, followed by 29 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 60 seconds, and extension at 72°C for 90 seconds. A final extension step was performed at 72°C for 5 minutes (Swacita et al. 2022). The presence of 1,500 bp amplicons was confirmed by electrophoresis on a 2% agarose gel. The PCR products were purified, and sequences by Next Gen Scientific Co. Ltd (Ho Chi Minh City).

Data analysis

Experimental data were analyzed using SPSS software (IBM Corp. Armonk, NY, USA). Results are presented as the mean±standard deviation of three independent replicates. One-Way Analysis of Variance (ANOVA) was employed to compare the antimicrobial activity data. A p-value of less than 0.05 was considered statistically significant.

The 16S rRNA gene sequences were manually checked for quality and edited using BioEdit software (v. 7.0). To identify the bacterial isolates, the edited consensus sequences were queried against the National Center for Biotechnology Information (NCBI) database using the BLASTn (Basic Local Alignment Search Tool for nucleotides) algorithm. A species-level identification was assigned based on a percent identity and query coverage of >99% to the closest matching type strain sequence in the database (Tamura et al. 2021).

RESULTS AND DISCUSSION

Identification of presumptive lactic acid bacteria

Thirty bacterial isolates were obtained from the gastrointestinal tract of Muscovy ducks and cultured on selective MRS agar containing bromocresol green and bile salts. The successful growth of presumptive LAB was indicated by the formation of a yellow halo around colonies and a concurrent color shift in the media (Figure 1). This observation suggests the production of acid, a characteristic feature of LAB, due to the fermentation of sugars within the medium.

Morphological characteristics of bacterial isolates

All isolates exhibited circular colony morphology when grown on solid media, indicating consistent and uniform growth patterns (Table 1, Figure 2). While colony shape was consistent, variations were noted in colony size, margin, elevation, pigmentation, optical properties, and texture. Colony sizes ranged from punctiform (D2, D7, D8, D15-D22, D25, D26) to small (D3-D6, D9-D11, D23, D24, D29, D30) and moderate (D1, D12-D14, D27, D28). Colony margins were predominantly smooth (D2, D5-D8, D10-D22, D24-D29), suggesting even radial growth, while a subset of isolates displayed wavy margins (D1, D3, D4, D9, D23, D30). All isolates presented a convex elevation and consistent milky white pigmentation. Colonies were uniformly opaque, indicating dense cellular growth, and primarily exhibited a dry texture, with the exception of isolate D1, which was muscoid (slimy). Gram staining confirmed that all isolates were Gram-positive (Table 1,

Figure 2). Microscopic examination revealed that the majority of isolates were coccobacillus (D1-D10, D12-D30), except for isolates D11 as rod, and D21 identified as short rod.

Biochemical and physiological characteristics

Biochemical profiling of the isolates included tests for catalase, urease, and citrate utilization, and all thirty isolates tested negative for catalase activity. Similarly, all isolates were urease-negative and did not utilize citrate as a sole carbon source, as evidenced by negative results in the respective biochemical assays. Fermentation assays demonstrated that all isolates were capable of fermenting glucose, lactose, and sucrose, as indicated by acid production in the test media. Furthermore, the motility assessment revealed that all isolates were non-motile (Table 1).

Antimicrobial activity of LAB isolates

The antimicrobial potential of nCFS from 30 LAB isolates (D1-D30) was quantitatively evaluated against three significant pathogenic bacteria. The inhibitory effect was determined by measuring the diameter of the Zone of Inhibition (ZOI). A substantial majority of the isolates, 26 out of 30 (86.7%), demonstrated antimicrobial activity against at least one of the indicator pathogens. The detailed quantitative results for all isolates are presented in Table 2.

To validate the assay, both negative and positive controls were included and performed as expected. The negative control, consisting of sterile neutralized MRS broth, produced no inhibition zone (0.00 mm), confirming that the medium itself had no antimicrobial properties. Conversely, the positive control, using nCFS from the reference strain *Lactobacillus plantarum* ATCC 14917, yielded significant inhibition zones against *E. coli* ATCC 25922 (18.50±1.40 mm), *S. enterica* serovar Typhimurium ATCC 14028 (16.80±1.90 mm), and *S. aureus* ATCC 25923 (21.20±2.10 mm). These results confirm the validity and sensitivity of the experimental method.

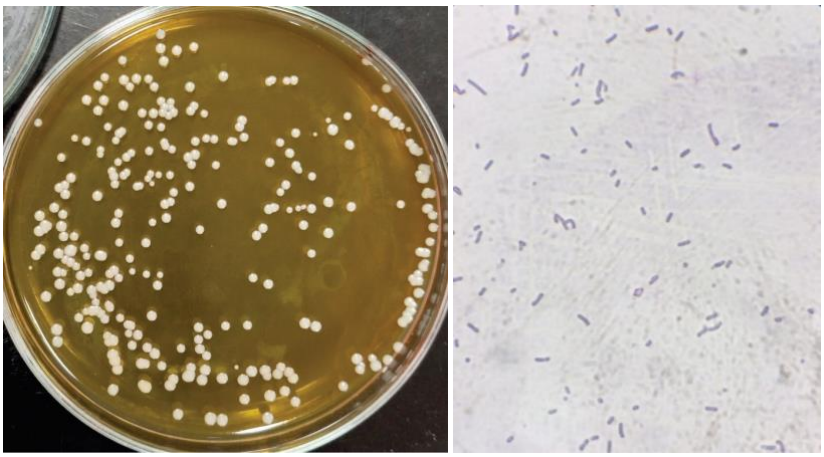

Figure 2. Representative images of colony morphology and Gram staining of bacterial isolates

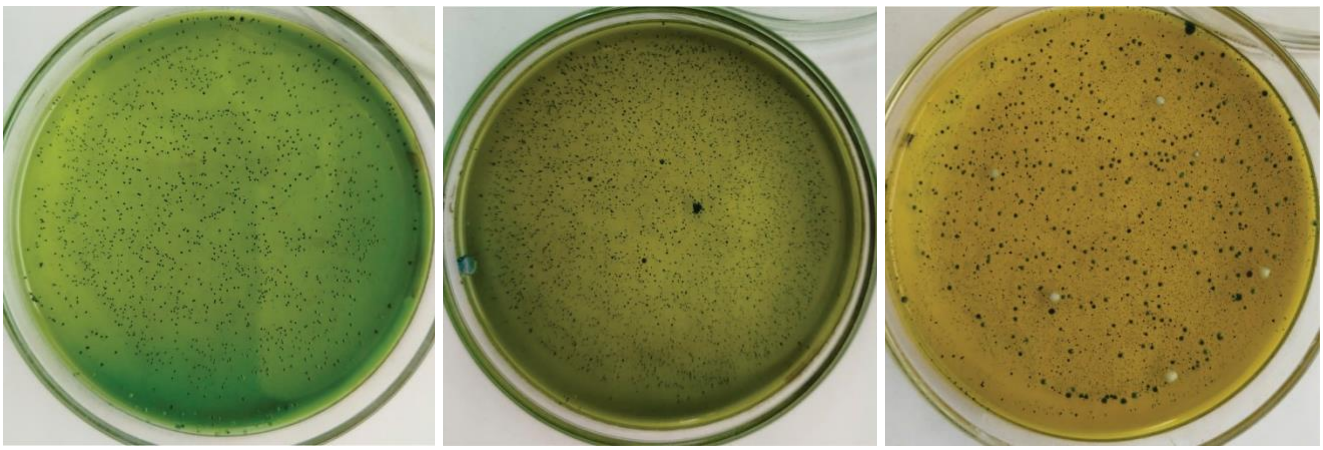

Figure 1. Characteristic green-to-yellow color change indicating presumptive Lactic Acid Bacteria (LAB) on MRS agar supplemented with bromocresol green and bile salts

Table 1. Morphological, Gram staining, biochemical, fermentation, and motility characteristics of bacterial isolates

Isolates ID	Colony morphology							Gram stain	Biochemical			Fermentation of carbohydrates			Motility
	Shape	Size	Margin	Elevation	Pigmentation	Optical properties	Texture		Catalase	Urease	Citrate	Glucose metabolism	Lactose metabolism	Sucrose metabolism	
D1	Circular	Moderate	Wavy	Convex	Milky white	Opaque	Muscoid	Positive, coccobacillus	-	-	-	+	+	+	-
D2	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D3	Circular	Small	Wavy	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D4	Circular	Small	Wavy	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D5	Circular	Small	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D6	Circular	Small	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D7	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D8	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D9	Circular	Small	Wavy	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D10	Circular	Small	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D11	Circular	Small	Smooth	Convex	Milky white	Opaque	Dry	Positive, rod	-	-	-	+	+	+	-
D12	Circular	Moderate	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D13	Circular	Moderate	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D14	Circular	Moderate	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D15	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D16	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D17	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D18	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D19	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D20	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D21	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, short rod	-	-	-	+	+	+	-
D22	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D23	Circular	Small	Wavy	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D24	Circular	Small	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D25	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D26	Circular	Punctiform	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D27	Circular	Moderate	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D28	Circular	Moderate	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D29	Circular	Small	Smooth	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-
D30	Circular	Small	Wavy	Convex	Milky white	Opaque	Dry	Positive, coccobacillus	-	-	-	+	+	+	-

Table 2. Antimicrobial activity of neutralized cell-free supernatants from LAB isolates against pathogenic indicator strains. Data are presented as the mean zone of inhibition in millimeters (mm)±Standard Deviation (SD), inclusive of the 4 mm well diameter

Isolates ID	*E. coli* ATCC 25922 (mm)	*S. enterica* serovar Typhimurium ATCC 14028 (mm)	*S. aureus* ATCC 25923 (mm)
D1	15.00±4.00	10.33±2.08	12.67±2.08
D2	10.67±2.08	10.00±1.73	0.00±0.00
D3	14.00±6.56	18.67±5.03	11.33±0.58
D4	13.00±2.00	0.00±0.00	11.33±3.21
D5	0.00±0.00	2.33±0.58	7.67±4.16
D6	16.67±2.89	16.33±2.31	0.00±0.00
D7	15.00±3.00	14.00±1.73	10.33±3.06
D8	0.00±0.00	0.00±0.00	0.00±0.00
D9	11.00±2.00	10.67±2.52	9.00±5.00
D10	14.33±1.15	8.00±1.00	11.67±3.06
D11	19.00±5.29	19.67±4.16	23.00±3.46
D12	19.00±4.00	18.67±4.73	17.33±3.21
D13	14.67±3.06	10.00±3.61	14.00±3.61
D14	11.00±2.00	13.67±2.31	10.00±1.00
D15	0.00±0.00	0.00±0.00	0.00±0.00
D16	9.67±3.06	23.00±10.58	7.33±0.58
D17	14.00±4.58	11.00±1.73	11.00±4.36
D18	12.00±4.58	19.67±4.16	0.00±0.00
D19	0.00±0.00	15.33±2.08	8.67±1.53
D20	19.00±2.65	0.00±0.00	18.33±2.08
D21	13.33±2.08	12.67±2.08	14.00±5.29
D22	0.00±0.00	0.00±0.00	0.00±0.00
D23	8.00±1.73	9.67±1.15	13.33±4.51
D24	11.00±1.73	0.00±0.00	14.67±4.04
D25	0.00±0.00	0.00±0.00	0.00±0.00
D26	0.00±0.00	17.00±2.65	0.00±0.00
D27	10.00±1.73	15.00±1.00	0.00±0.00
D28	0.00±0.00	19.33±3.51	18.33±2.08
D29	9.33±5.03	0.00±0.00	16.33±3.06
D30	9.33±5.13	10.33±1.15	14.33±4.73
Positive control	18.50±1.40	16.80±1.90	21.20±2.10
Negative control	0.00	0.00	0.00

The LAB isolates exhibited a broad and varied spectrum of inhibitory activity (Table 2). Against the Gram-negative *E. coli*, the most potent activity was observed from isolates D11 (19.00±5.29 mm), D12 (19.00±4.00 mm), and D20 (19.00±2.65 mm). In the case of *S. enterica*, isolate D16 produced the largest mean inhibition zone (23.00±10.58 mm), while isolates D11 (19.67±4.16 mm) and D18 (19.67±4.16 mm) also showed strong and consistent inhibition. The most effective activity against the Gram-positive pathogen *S. aureus* was recorded for isolate D11, which generated the largest and one of the most consistent inhibition zones in the entire study (23.00±3.46 mm).

A key finding was the identification of several isolates with broad-spectrum activity, defined as the ability to inhibit all three indicator pathogens. Notably, isolates D11 and D12 consistently demonstrated strong, broad-spectrum inhibition. The potent activity of the most promising isolation, D11, is visually documented in Figure 3. In contrast, four isolates, D8, D15, D22, and D25, were completely inactive against all tested pathogens.

6S rRNA gene amplification and molecular identification

Based on antimicrobial activity screening, isolate D11 was selected for molecular identification. While both D11 and D12 demonstrated strong, broad-spectrum inhibition against *E. coli*, *Salmonella* sp., and *S. aureus*, isolate D11 exhibited the highest activity against *S. aureus* (2.30 cm inhibition zone) and slightly higher activity against *Salmonella* sp. compared to D12, indicating particularly promising overall antimicrobial potential.

Genomic DNA was extracted from isolate D11 and subjected to PCR amplification targeting the 16S rRNA gene. Successful amplification was confirmed by gel electrophoresis, which revealed a distinct DNA fragment of the expected size, approximately 1500 base pairs (Figure 4). This result verified the presence of the targeted 16S rRNA gene sequence in isolate D11, suitable for subsequent sequencing and identification. The purified PCR product was sequenced, and the resulting sequence was deposited into the GenBank database under the accession number PV992727.

To identify the isolate, the 16S rRNA sequence was analyzed using BLASTn against the NCBI nucleotide database. The results, summarized in Table 3, show that the sequence from isolate D11 shares a 99.81% identity with the *Companilactobacillus farciminis* strain BCRC 14043 (accession no. NR_114398.1), with 100% query coverage and an E-value of 0.0.

To further confirm the taxonomic position of isolate D11 and visualize its evolutionary relationship with closely related species, a phylogenetic tree was constructed using 16S rRNA gene sequences (Figure 5). The analysis shows that isolate D11 forms a distinct monophyletic clade with the type strain of *C. farciminis*, supported by a high bootstrap value. This robust phylogenetic evidence, in conjunction with the BLAST analysis, provides definitive confirmation of the identification of isolate D11 as *C. farciminis*.

Table 3. Top BLASTn hit for the 16S rRNA gene sequence for isolate D11

Subject description	Max score	Query cover	E-value	Percent identity	Accession number
Companilactobacillus farciminis strain BCRC 14043 16S ribosomal RNA, partial sequence	1982	100 %	0.0	99.81 %	NR_114398.1

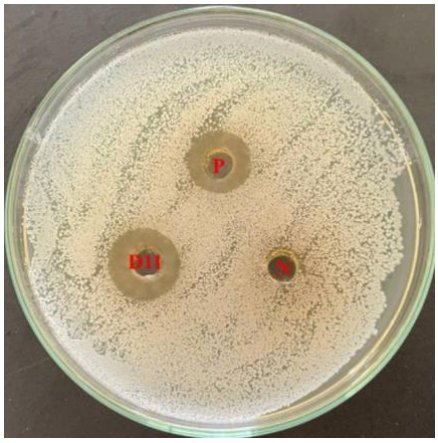

Figure 3. Representative images of the agar well diffusion assay show the antimicrobial activity of isolate D11. The plate was seeded with *S. enterica* serovar Typhimurium ATCC 14028. Wells contain: D11: nCFS from isolate D11, P: nCFS from the positive control train *Lactobacillus plantarum* ATCC 14917; and N: Sterile neutralized MRS broth as the negative control. Clear zones around the wells indicate bacterial growth inhibition

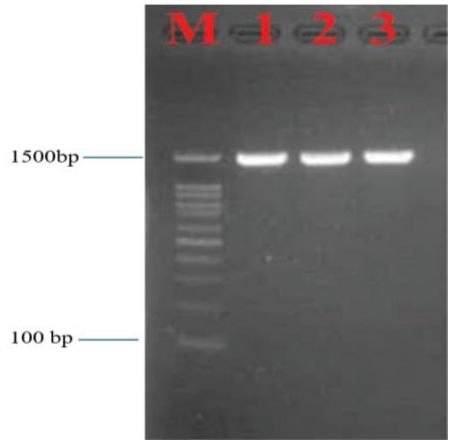

Figure 4. Agarose gel electrophoresis of the 16S rRNA gene amplified from isolate D11. M: 100 bp DNA ladder, Lane 1: Positive control, Lanes 2-3: PCR product from isolate D11 showing a clear band at approximately 1500 bp

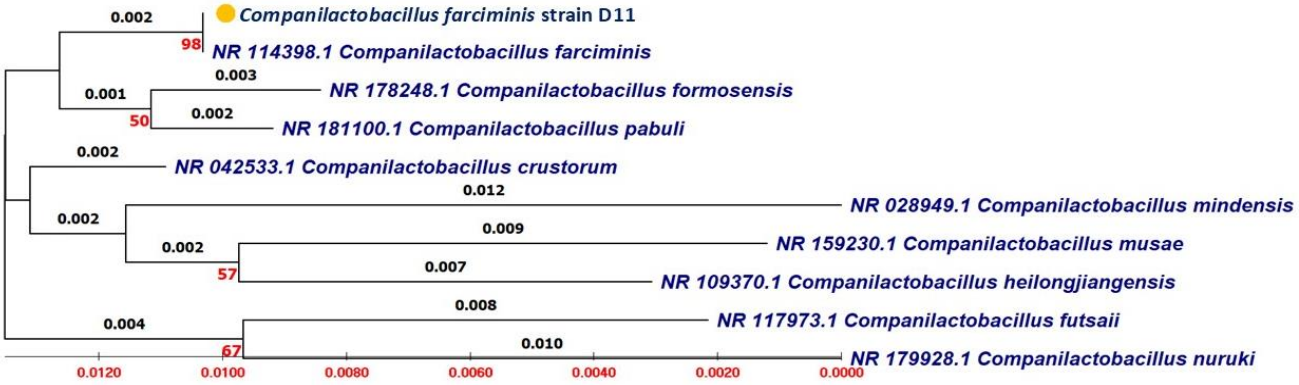

Figure 5. Phylogenetic tree constructed using the Neighbor-Joining method, illustrating the evolutionary relationship of isolate D11. The analysis was based on 16S rRNA gene sequences and involved related *Companilactobacillus* species. Bootstrap values (from 1000 replicates) are indicated at the nodes. The scale bar represents the number of nucleotide substitutions per site

Discussion

The global effort to curtail antibiotic use in livestock production necessitates the development of effective alternatives, with host-adapted probiotics representing a premier strategy. In Vietnam, where Muscovy ducks are an economically vital part of the poultry sector, infectious diseases pose a significant threat to productivity. This study addressed this challenge by exploring the gastrointestinal tract of healthy Muscovy ducks as a reservoir for novel LAB with probiotic potential. Our investigation successfully yielded 30 distinct LAB isolates, evaluated their antimicrobial capabilities against key poultry pathogens, and culminated in the molecular identification of a highly potent strain, *C. farciminis* D11.

Phenotypic traits as indicators of probiotic fitness

The initial screening on bile-supplemented MRS agar was strategically designed to select for isolates already adapted to the harsh environment of the GIT. Bile tolerance is a critical prerequisite for probiotic survival and efficacy, ensuring that bacteria can transit the upper GIT to colonize the intestines (Akalu et al. 2017). Our findings align with research by Jose et al. (2015), which demonstrated that LAB from animal gut sources possess superior bile tolerance compared to those from dairy environments, underscoring the principle that host origin is a key determinant of this functional trait. Concurrently, the acid production indicated by yellow halos on bromocresol green medium is a hallmark of LAB. This metabolic activity contributes to competitive exclusion by lowering the intestinal pH, creating an environment that inhibits the growth of acid-sensitive pathogens like *Salmonella* and *E. coli* (Nurcahyo et al. 2019; Risna et al. 2022).

Further characterization revealed functional diversity relevant to probiotic action. Most isolates displayed typical LAB colony morphologies (Khalil and Anwar 2016), but the unique mucoid texture of isolate D1 suggested the production of Exopolysaccharides (EPS). EPS are

multifunctional polymers that can enhance probiotic performance by facilitating adhesion to the intestinal mucosa, promoting biofilm formation, and modulating the host immune response (Gomand et al. 2019). Microscopically, the confirmation of all 30 isolates as Gram-positive and the observation of varied cell shapes, including coccobacilli and distinct rods (D11), reflect the known morphological heterogeneity within key LAB genera (Yerlikaya et al. 2020).

Biochemically, the universal absence of catalase activity distinguishes these isolates from other Gram-positive bacteria like *Staphylococcus* and confirms their adaptation to the anaerobic gut (El Ahmadi et al. 2025). The uniform inability to utilize citrate may suggest a metabolic specialization to the nutrient profile of the Muscovy duck gut, differentiating this microbial community from those in other niches where citrate metabolism is more advantageous (Eicher et al. 2024). Together, these phenotypic and biochemical traits confirm the isolates as authentic LAB and provide strong indicators of their adaptation to and potential function within the avian host.

Antimicrobial spectrum and mechanistic insights

A central goal of this study was to assess the antimicrobial activity of the isolates, a key mechanism for pathogen control. By using neutralized Cell-Free Supernatants (nCFS), we could specifically evaluate the effects of secreted antimicrobial compounds, independent of organic acid inhibition. The results revealed a spectrum of activity, from broad-spectrum inhibition to pathogen-specific antagonism, highlighting the strain-specific nature of this crucial probiotic function (Chen et al. 2023).

Several isolates, particularly D11, D12, and D20, demonstrated potent inhibition against *E. coli*, a primary agent of colibacillosis in poultry. This aligns with studies in broiler chickens where specific *Lactobacillus* strains were shown to reduce gut colonization by avian pathogenic *E. coli*, although efficacy varied significantly by strain (Sirisopapong et al. 2023). The strong activity of our top isolates suggests they secrete highly effective molecules targeting this Gram-negative pathogen. In contrast, activity against *Salmonella* sp. was more specialized. Isolate D16 exhibited exceptionally strong inhibition, while many other isolates were inactive. This potent but narrow-spectrum activity is particularly valuable for targeted biocontrol of salmonellosis and echoes findings in other waterfowl, where specific LAB strains effectively reduced *Salmonella* shedding (Tumbarski et al. 2025).

Against the Gram-positive pathogen *S. aureus*, isolates D11, D12, D20, and D28 were highly effective. This is significant given the increasing prevalence of antibiotic-resistant *S. aureus* in veterinary medicine (Pato et al. 2021). The robust inhibition observed strongly suggests the production of bacteriocins, ribosomally synthesized antimicrobial peptides. Bacteriocins produced by LAB are often most effective against other Gram-positive bacteria, as they typically act by disrupting the cell membrane integrity of target cells (Simons et al. 2020; Li et al. 2024). The potent anti *S. aureus* activity observed here is consistent with studies identifying bacteriocin-producing LAB from chickens (Veettil and Chitra 2022) and points to a primary mechanism for the inhibitory effects seen in our most active isolates. The broad-spectrum activity of D11 and D12 makes them ideal candidates for general-purpose probiotics, while the targeted potency of D16 against *Salmonella* highlights its potential for specialized applications. The lack of activity in certain strains (D8, D15) is also informative, suggesting their probiotic benefits may derive from other mechanisms not measured here, such as resource competition or immunomodulation.

Companilactobacillus farciminis *D11: A novel isolate with high probiotic potential*

Isolate D11 was selected for molecular identification due to its superior, broad-spectrum antimicrobial activity against all three tested pathogens. Sequencing the 16S rRNA gene, the standard for bacterial phylogenetic analysis (Church et al. 2020), yielded an approximately 1500 bp amplicon. A BLAST search revealed a 99.81% sequence identity to *Companilactobacillus farciminis*. This molecular identification was strongly corroborated by its phenotype: A Gram-positive, catalase-negative, non-motile, fermentative rod, consistent with the characteristics of the genus *Companilactobacillus*.

The identification of *C. farciminis* in this context is a key finding. While this species is a known inhabitant of diverse environments, including fermented foods and the GIT of chickens (Jung et al. 2021; Bampidis et al. 2023), this study reports the first isolation and identification of *C. farciminis* from the Muscovy duck gastrointestinal tract. This discovery expands our understanding of the microbial biodiversity within this avian host and suggests a potential co-evolutionary relationship. Species within the recently reclassified *Companilactobacillus* genus are increasingly recognized for their probiotic attributes, which include reinforcing the gut barrier and modulating host immunity, in addition to antimicrobial production (Wang et al. 2021, 2022; Tabanelli et al. 2024). The potent in vitro activity demonstrated by *C. farciminis* D11 solidifies its status as an exceptionally promising candidate for development as a host-specific probiotic for Muscovy ducks.

Implications, limitations, and future directions

This research provides a direct pathway toward developing sustainable, non-antibiotic solutions for the Vietnamese poultry industry. The identification of native, host-adapted LAB, particularly the novel and potent *C. farciminis* D11, offers a tangible opportunity to enhance gut health and disease resistance in Muscovy duck farming. However, these promising in vitro findings represent the first step. The critical next phase requires a rigorous progression from laboratory to application.

Future work must prioritize in vivo trials to confirm the safety and efficacy of *C. farciminis* D11 in Muscovy ducks, assessing its ability to reduce pathogen colonization and improve health parameters under farm conditions. Mechanistic studies are also essential to isolate and characterize the specific antimicrobial compounds, likely bacteriocins, responsible for its potent activity. Furthermore, whole-genome sequencing of the D11 isolate is imperative. This

will not only provide deeper insight into its probiotic gene repertoire but is also crucial for a comprehensive safety assessment to rule out the presence of transferable antibiotic resistance genes or virulence factors before any commercial application (Nami et al. 2018). This integrated approach will be vital for translating a promising laboratory finding into an effective and safe tool for the poultry industry.

In conclusion, this research successfully isolated and characterized a community of Lactic Acid Bacteria from the Muscovy duck gut, leading to a significant finding with direct implications for both local biodiversity and sustainable poultry production: the first reported identification of *Companilactobacillus farciminis* from this avian host. The potent, broad-spectrum antimicrobial activity of the isolate *C. farciminis* D11 against key pathogens such as *E. coli*, *Salmonella* sp., and *S. aureus* establishes it as a powerful candidate for a host-specific probiotic feed additive. For animal health, its application offers a targeted strategy to enhance gut integrity, reduce pathogen load, and mitigate disease outbreaks in Muscovy duck farming, providing a direct alternative to antibiotics. From a biodiversity perspective, the discovery of *C. farciminis* as a native inhabitant of the Vietnamese Muscovy duck GIT expands our knowledge of this host's unique microbiome and provides a valuable genetic resource from a previously underexplored ecological niche. Therefore, *C. farciminis* D11 represents a tangible asset for developing next-generation probiotics tailored to the local poultry industry. Prioritizing its progression into *in vivo* trials and genomic safety assessments is the crucial next step in translating this foundational discovery into a practical agricultural application.

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