



Research article

***Bacillus velezensis* from the Muscovy Duck gut: A potential probiotic for controlling poultry pathogens**

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Abstract

Bacterial diseases and antimicrobial resistance threaten the poultry industry, necessitating antibiotic alternatives. Probiotics, especially *Bacillus* species, are a promising solution. This study explored the gut of the Muscovy duck (*Cairina moschata* (Linnaeus, 1758)) as an untapped source for novel probiotic candidates. The objectives were to isolate native *Bacillus* strains from Muscovy duck guts, identify the most promising candidate, and characterize its probiotic potential by assessing its resilience and antimicrobial activity against key poultry pathogens. A total of 40 bacterial strains were isolated from the gut contents of 50 Muscovy ducks using a heat-shock enrichment method. All isolates were screened *in vitro* for their tolerance to simulated gastrointestinal conditions (pH 2.0 and 0.3% bile salts). The antimicrobial activity of their cell-free supernatants was assessed against *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus* using the agar well diffusion method. The most potent isolate was definitively identified by 16S rRNA gene sequencing. Isolate BD31 demonstrated exceptional tolerance to both acidic and bile salt conditions. Furthermore, it exhibited potent, broad-spectrum antagonistic activity, producing significant inhibition zones against *E. coli* (12.5 mm), *S. enterica* (11.8 mm), and *S. aureus* (8.5 mm). Molecular analysis identified this superior isolate as *Bacillus velezensis* with 99.76% sequence identity to the type strain. The Muscovy duck gut is a valuable source for novel probiotics. *Bacillus velezensis* BD31 displays superior gastrointestinal tolerance and potent antimicrobial activity, positioning it as an excellent candidate for a direct-fed microbial to control poultry pathogens. *In vivo* validation is warranted.

Keywords: Antibacterial activity, *Bacillus velezensis*, Muscovy duck, Poultry pathogens, Probiotics.

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INTRODUCTION

Duck farming is a cornerstone of the global poultry industry and a vital component of Vietnam's agricultural economy. However, the productivity and sustainability of this sector are consistently threatened by bacterial infections. Pathogens such as *Staphylococcus aureus*, *Salmonella species*, and *Escherichia coli* are primary causes of disease, leading to significant economic losses from reduced growth rates, poor feed conversion, and increased flock mortality (Aguidissou et al., 2022; Hamed et al., 2024). Beyond animal welfare, these pathogens pose a considerable public health risk, as many are zoonotic agents responsible for foodborne illnesses in humans (Aguidissou et al., 2022). The threat is particularly acute with the emergence of multidrug-resistant strains, such as methicillin-resistant *S. aureus* (MRSA), which have been documented in duck farm environments (AbdelRahman and Amer, 2021).

The challenge of controlling these bacterial diseases is intensified by the global crisis of antimicrobial resistance (AMR). The widespread use of antibiotics in agriculture has accelerated the selection of drug-resistant bacteria, rendering many conventional treatments ineffective (Pérez-Sánchez et al., 2018; Patil et al., 2021). Recent surveillance has uncovered alarming resistance markers in duck farm environments, including fosfomycin and linezolid resistance genes in *S. aureus*, underscoring the role of duck production as a potential reservoir for AMR determinants that can transfer to humans (Hu et al., 2023; Li et al., 2024). This escalating situation underscores the urgent need for effective and sustainable alternatives to antibiotics for disease management in poultry production (Hamed et al., 2024).

In response to the diminishing efficacy of antibiotics, probiotics have emerged as one of the most promising alternative strategies. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Plaza-Diaz et al., 2019). Their beneficial effects are exerted through multiple mechanisms, including the competitive exclusion of pathogens from intestinal binding sites, the production of potent antimicrobial compounds, the enhancement of the intestinal barrier function, and the modulation of the host's immune system. In poultry, the application of probiotics has been consistently linked to improved growth performance, enhanced feed conversion ratios, and better overall health and disease resistance (Krysiak et al., 2021). The adoption of probiotics also aligns with growing consumer demand for sustainable agricultural practices that minimize chemical inputs and reduce the risk of antibiotic residues in the food chain (Rahimoon et al., 2023).

Among the diverse array of probiotic candidates, spore-forming bacteria of the genus *Bacillus* are exceptionally well-suited for application in animal nutrition. The remarkable ability of *Bacillus* species to form resilient endospores allows them to withstand the harsh acidic and enzymatic conditions of the upper gastrointestinal tract, as well as the high temperatures used in commercial feed processing, ensuring their viability and successful delivery to the gut (Ramlucken et al., 2020). Once in the intestine, these spores germinate into metabolically active cells that exert their probiotic effects. They are renowned for producing a wide array of extracellular enzymes that aid in nutrient digestion (Latorre et al., 2016; Hosseini et al., 2018) and, critically, for synthesizing a diverse arsenal of antimicrobial compounds like bacteriocins and lipopeptides that directly inhibit the growth of pathogens (Hanim et al., 2021).

Bacillus velezensis, in particular, has garnered significant attention for its robust probiotic and biocontrol capabilities. This species is known for its high tolerance to bile salts and its ability to successfully colonize the intestinal mucosa, where it helps regulate the gut microbiota by promoting beneficial microorganisms and suppressing pathogens (Su et al., 2023). Genomic and metabolomic studies have revealed that *B. velezensis* produces a diverse arsenal of bioactive compounds, including bacteriocins and lipopeptides like surfactin, fengycin, and

difficidin, which exhibit broad-spectrum antagonistic activity against bacteria and fungi (Liu et al., 2017; Rabbee et al., 2019; Perini et al., 2024). Strains of *B. velezensis* have demonstrated potent inhibitory effects against critical poultry pathogens, including *S. aureus*, *E. coli*, and *Salmonella Typhimurium*, both in vitro and in animal models (Afroj et al., 2021; Husna et al., 2023; Wang et al., 2024).

An effective strategy for discovering novel probiotics is to isolate them directly from the gastrointestinal tract of healthy, locally adapted animals, as these native microorganisms are already adapted to thrive in that specific gut environment (Menconi et al., 2013). While research has successfully identified robust *Bacillus* probiotics from chickens (Mazanko et al., 2022), the microbial biodiversity of indigenous duck breeds remains largely unexplored. The Muscovy duck (*Cairina moschata* (Linnaeus, 1758)), a native breed in Vietnam, represents a valuable and untapped reservoir of such beneficial microbes. We hypothesized that the unique gut microbiome of the Muscovy duck hosts *Bacillus* strains with superior antimicrobial capabilities adapted to control pathogens common in waterfowl. Therefore, the present study aimed to isolate native *Bacillus* strains from the gastrointestinal tract of healthy Muscovy ducks, identify them to the species level, and characterize their antagonistic activity against key bacterial pathogens affecting the poultry industry. While other studies have focused on *Bacillus* from chickens, the novelty of this work lies in the bioprospecting of the indigenous Muscovy duck, an under-explored niche, leading to the identification of a native *B. velezensis* strain with potent, broad-spectrum anti-pathogen activity.

MATERIALS AND METHODS

Ethical approval and sample collection

All experimental procedures involving animals were conducted in strict accordance with the ethical guidelines established by the Institutional Ethical Committee of the School of Agriculture and Aquaculture of Tra Vinh University, Vietnam (Approval No. 5887/QĐ-DHTV). Gastrointestinal (GI) tract samples were collected from 50 healthy, market-age Muscovy ducks, comprising 25 males and 25 females, sourced from a commercial farm in Vinh Long Province, Vietnam. Immediately following humane euthanasia, the entire GI tract of each bird was aseptically excised. The luminal contents were carefully collected from four distinct anatomical regions: the crop, gizzard, small intestine, and cecum, and placed into sterile containers for immediate microbiological analysis.

Isolation and presumptive identification of *Bacillus* strains

To selectively isolate endospore-forming bacteria, one gram of luminal content from each GI section was homogenized in 9 mL of sterile phosphate-buffered saline (PBS; pH 7.2). The resulting suspension was subjected to heat shock treatment in a water bath at 80°C for 15 minutes. This step is designed to eliminate non-spore-forming vegetative bacteria and enrich heat-resistant *Bacillus* endospores (Hellany et al., 2024). Following heat treatment, the suspension was serially diluted (10^{-2} to 10^{-7}) in PBS. Aliquots (100 μ L) from each dilution were then spread-plated in triplicate onto Nutrient Agar (NA). The plates were incubated aerobically at 37°C for 24 to 48 hours (Phan et al., 2025). Colonies displaying morphologies characteristic of the *Bacillus* genus, typically large, opaque, and with irregular margins, were selected and purified by repeated streaking onto fresh NA plates to ensure axenic cultures (Al Azad et al., 2020).

Purified isolates were presumptively identified as *Bacillus* based on key phenotypic traits. Microscopic examination following Gram staining was performed to confirm the presence of Gram-positive, rod-shaped cells and the formation of endospores (Tripathi and Sapra, 2020). Further characterization involved a panel of

standard biochemical tests, including catalase and urease production, motility, and citrate utilization, as well as the fermentation of glucose, sucrose, and lactose (Shen and Zhang, 2022; Khushboo et al., 2023). These tests were validated using reference strains: *Staphylococcus aureus* ATCC 25923 (catalase-positive), *Lactobacillus plantarum* ATCC 14917 (catalase-negative), *Proteus mirabilis* ATCC 12453 (urease-positive), and *Escherichia coli* ATCC 25922 (urease-negative). Only isolates confirmed as Gram-positive, spore-forming, and catalase-positive were retained. The purified isolates were cryopreserved at -20°C in LB broth supplemented with 30% (v/v) sterile glycerol for long-term storage.

Screening for probiotic potential

Acid and bile salt tolerance

Isolates were evaluated for their ability to survive simulated GI conditions. Strains were cultured overnight in LB broth (37°C , 120 rpm), and cells were harvested by centrifugation (8,000 rpm, 5 min, 4°C), washed twice with sterile distilled water, and resuspended in fresh LB broth. The cell density was adjusted to an optical density (OD_{600}) of 0.5–0.7 (approximately 10^8 CFU/mL).

For acid tolerance, 1 mL of the standardized cell suspension was inoculated into 9 mL of LB broth adjusted to either pH 2.0 or pH 4.0. LB broth at pH 6.5 served as the control. Cultures were incubated at 37°C for 4 hours (Söylemez-Milli et al., 2025). For bile salt tolerance, 1 mL of the cell suspension was inoculated into 9 mL of LB broth supplemented with either 0.15% or 0.3% (w/v) bile salts, with 0% bile salt broth as the control, and incubated under the same conditions (Nair and Dubhashi, 2016).

Following incubation, viable cell counts were determined. Briefly, samples from each treatment were serially diluted (10^{-1} to 10^{-8}) in sterile PBS. Aliquots (100 μL) of appropriate dilutions were spread-plated in triplicate onto NA and incubated at 37°C for 24 hours. Colonies were counted and expressed as log-transformed colony-forming units per milliliter (Log CFU/mL). All experiments were performed in triplicate.

Antimicrobial activity

The antagonistic activity of each isolate was assessed using the agar well diffusion method (El-Banna and Qaddoumi, 2016). The indicator pathogens used were *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *Salmonella enterica* serovar Typhimurium ATCC 14028. To prepare cell-free supernatant (CFS), each *Bacillus* isolate was grown in LB broth at 37°C for 24 hours. The culture was centrifuged (10,000 rpm, 5 min, 4°C), and the pH of the resulting supernatant was adjusted to 7.0 ± 0.2 with 1 M NaOH to eliminate antimicrobial effects due to acidity.

For the assay, molten NA was seeded with an overnight culture of an indicator pathogen to a final concentration of approximately 1×10^6 CFU/mL and poured into sterile petri plates. Wells (4 mm diameter) were cut into solidified agar and filled with 100 μL of the neutralized CFS. Ampicillin (10 μg) and sterile, neutralized LB broth were used as positive and negative controls, respectively. Plates were incubated at 37°C for 24 hours, and antimicrobial activity was quantified by measuring the diameter (mm) of the inhibition zone. The assay was performed in triplicate.

Molecular identification by 16S rRNA gene sequencing

Isolates demonstrating the most potent, broad-spectrum antimicrobial activity were selected for definitive molecular identification. Genomic DNA was extracted using a modified phenol-chloroform protocol (De et al., 2010). The nearly full-length 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3') (Swacita et al., 2022). The PCR reaction was prepared using GoTaq® Green Master Mix (Promega, USA) and performed in a Veriti thermocycler under the

following conditions: initial denaturation at 94°C for 3 min; 29 cycles of 94°C for 45 s, 53°C for 60 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. The presence of the expected ~1,500 bp amplicon was verified by 2% agarose gel electrophoresis. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced via the Sanger method at Next Gen Scientific Co., Ltd (Ho Chi Minh City, Vietnam). The 16S rRNA gene sequence of isolate BD31 has been deposited in the NCBI GenBank database under accession number PX467063.

Statistical and sequence analysis

The raw 16S rRNA gene sequences were edited and assembled using BioEdit software (v. 7.0). Species-level identification was achieved by querying the sequences against the NCBI nucleotide database using the BLASTn algorithm. A sequence identity and query coverage of $\geq 99\%$ were used as the thresholds for a definitive match (Tamura et al., 2021). Data from the antimicrobial assays were expressed as the mean $\pm$ standard deviation (SD) of three independent replicates. Statistical analysis was performed using SPSS software. A one-way Analysis of Variance (ANOVA) followed by Tukey's HSD post-hoc test was used to determine significant differences between inhibition zones. A p-value of <0.05 was considered statistically significant.

RESULTS

Isolation and phenotypic characterization of *Bacillus* isolates

A total of 40 distinct bacterial isolates were recovered from the luminal contents of the crop, gizzard, small intestine, and cecum of healthy Muscovy ducks. On Nutrient Agar, all isolates formed colonies consistent with the genus *Bacillus*, appearing large, milky-white, opaque, and circular with a dry texture and either entire or undulate margins (Figure 1A).

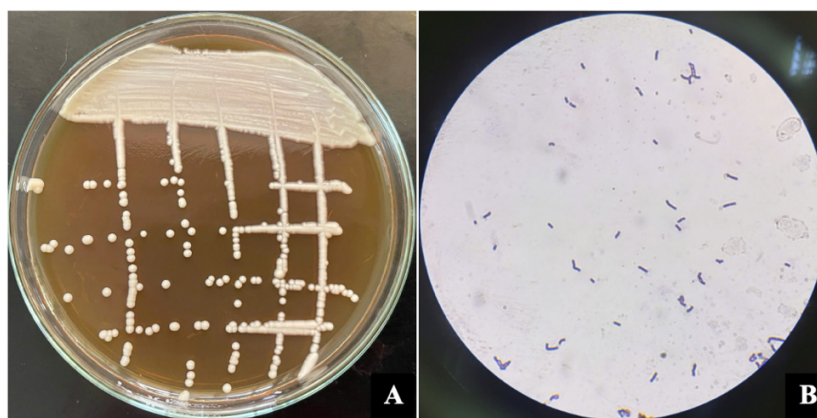


Figure 1. Representative colony morphology (A) and Gram stain (B) of isolate BD31. The isolate forms characteristic milky-white, circular colonies on Nutrient Agar and consists of Gram-positive, endospore-forming rods (1000x magnification).

Microscopic and biochemical analyses confirmed this presumptive identification. Gram staining revealed that all 40 isolates were Gram-positive, endospore-forming rods (Figure 1B). The isolates exhibited a uniform biochemical profile: all were positive for motility, catalase, and citrate utilization, but negative for urease activity. Furthermore, they fermented glucose and sucrose but not

lactose. These consistent morphological and physiological characteristics, summarized in Table 1, provided strong evidence for classifying all 40 isolates as members of the *Bacillus* genus, warranting their inclusion in subsequent functional screenings.

Table 1 Summary of key morphological and biochemical characteristics of the 40 bacterial isolates from the Muscovy duck gastrointestinal tract.

Characteristic	Observation/Result
Colony morphology	
Shape	Circular
Margin	Entire or undulate
Elevation	Convex
Color and Opacity	Milky-white, opaque
Cellular morphology	
Gram Stain	Positive (+), rod-shaped
Spore Formation	Positive (+)
Biochemical tests	
Motility	Positive (+)
Catalase	Positive (+)
Urease	Negative (–)
Citrate Utilization	Positive (+)
Carbohydrate fermentation	
Glucose	Positive (+)
Sucrose	Positive (+)
Lactose	Negative (–)

Tolerance to simulated avian gastrointestinal conditions

The ability of the isolates to survive the harsh physicochemical conditions of the avian GI tract was assessed. Significant strain-dependent variation in tolerance to both acid and bile salts was observed.

When exposed to a highly acidic environment (pH 2.0) for four hours, nine isolates (BD8, BD10, BD12, BD13, BD14, BD21, BD24, BD25, and BD31) demonstrated superior resilience by maintaining high cell viability, whereas many other strains were rendered completely non-viable at this pH (Table 2). As expected, survival rates were significantly higher at the less acidic pH of 4.0. Similarly, tolerance to a physiologically relevant concentration of 0.3% bile salts varied significantly among the isolates ($p < 0.05$). Ten isolates showed high survival rates, with BD10 and BD31 being particularly robust. In contrast, isolates BD2, BD17, BD29, and BD39 failed to grow under these conditions (Table 3).

Table 2 Summary of key morphological and biochemical characteristics of the 40 bacterial isolates from the Muscovy duck gastrointestinal tract.

Isolates ID	Viable <i>Bacillus</i> bacteria isolates (Log CFU/mL)					
	pH 6.5 (Control)		pH 4		pH 2	
BD1	10.32	± 0.61	9.51	± 0.87	4.78	± 1.41
BD2	10.51	± 0.79	7.80	± 0.71	0.00	± 0.00
BD3	10.10	± 0.68	7.35	± 0.71	0.00	± 0.00
BD4	10.35	± 0.63	0.00	± 0.00	0.00	± 0.00
BD5	10.30	± 0.77	7.85	± 0.21	0.00	± 0.00
BD6	10.07	± 0.79	7.50	± 0.71	0.00	± 0.00
BD7	10.15	± 0.66	0.00	± 0.00	0.00	± 0.00
BD8	10.08	± 0.68	7.98	± 0.71	6.85	± 1.20
BD9	10.22	± 0.71	0.00	± 0.00	0.00	± 0.00
BD10	10.04	± 0.77	8.22	± 0.32	7.50	± 0.71
BD11	10.03	± 0.69	8.99	± 1.19	0.00	± 0.00
BD12	10.04	± 0.62	9.20	± 0.71	8.54	± 0.34
BD13	10.25	± 0.73	8.95	± 1.28	7.04	± 0.74
BD14	10.76	± 0.50	8.90	± 0.34	7.43	± 1.60
BD15	10.39	± 1.32	0.00	± 0.00	0.00	± 0.00
BD16	10.12	± 0.76	9.08	± 1.74	0.00	± 0.00
BD17	10.10	± 0.54	7.85	± 0.21	5.69	± 0.52
BD18	10.31	± 0.30	7.10	± 0.71	4.15	± 0.21
BD19	10.13	± 0.86	0.00	± 0.00	0.00	± 0.00
BD20	10.50	± 0.48	8.98	± 0.40	3.95	± 0.92
BD21	10.03	± 0.57	9.88	± 0.81	6.97	± 1.15
BD22	10.04	± 0.56	9.23	± 0.09	0.00	± 0.00
BD23	10.04	± 0.68	7.40	± 0.71	0.00	± 0.00
BD24	9.90	± 0.66	8.74	± 2.04	7.53	± 2.39
BD25	9.97	± 0.87	9.74	± 0.91	7.54	± 1.75
BD26	10.07	± 0.66	9.19	± 0.38	3.95	± 1.35
BD27	9.85	± 0.61	8.24	± 0.34	7.32	± 2.08
BD28	10.03	± 0.83	8.64	± 0.08	6.52	± 2.71
BD29	9.99	± 0.67	8.93	± 2.05	7.98	± 0.71
BD30	9.89	± 0.83	7.74	± 1.04	6.35	± 1.31
BD31	10.88	± 0.50	9.97	± 0.78	8.24	± 1.50
BD32	9.95	± 0.78	8.47	± 1.23	7.18	± 1.48
BD33	10.14	± 0.44	8.70	± 0.48	6.81	± 0.64
BD34	9.91	± 0.54	9.73	± 0.62	5.08	± 0.71
BD35	10.05	± 0.88	8.46	± 0.45	4.91	± 0.96
BD36	9.91	± 0.68	9.57	± 0.12	7.15	± 2.89
BD37	9.99	± 0.74	8.38	± 0.14	6.47	± 2.22
BD38	9.96	± 0.67	9.47	± 1.30	5.20	± 1.30
BD39	9.91	± 0.69	9.49	± 0.07	0.00	± 0.00
BD40	9.99	± 0.66	8.96	± 0.39	6.63	± 0.38

Table 3 Selected *Bacillus* isolates' bile salt tolerance

Isolates ID	Viable <i>Bacillus</i> bacteria isolates (Log CFU/mL)					
	0% (Control)		0.2%		0.3%	
BD1	10.32	± 0.61	6.78	± 0.53	3.39	± 0.84
BD2	10.51	± 0.79	5.81	± 1.01	0.00	± 0.00
BD3	10.10	± 0.68	5.88	± 1.00	3.21	± 1.38
BD4	10.35	± 0.63	5.99	± 0.17	3.76	± 1.66
BD5	10.30	± 0.77	5.66	± 1.01	3.23	± 0.56
BD6	10.07	± 0.79	5.81	± 0.99	2.92	± 0.82
BD7	10.15	± 0.66	6.15	± 0.65	3.28	± 0.56
BD8	10.08	± 0.68	5.71	± 1.02	3.25	± 0.56
BD9	10.22	± 0.71	6.58	± 1.06	4.75	± 0.54
BD10	10.04	± 0.77	7.58	± 0.69	5.72	± 0.58
BD11	10.03	± 0.69	5.88	± 0.99	3.25	± 0.54
BD12	10.04	± 0.62	5.98	± 1.26	3.31	± 0.56
BD13	10.25	± 0.73	5.87	± 1.24	2.58	± 1.65
BD14	10.76	± 0.50	7.12	± 1.03	3.21	± 0.56
BD15	10.39	± 1.32	7.40	± 1.09	2.63	± 1.64
BD16	10.12	± 0.76	5.62	± 1.24	3.28	± 0.55
BD17	10.10	± 0.54	5.86	± 1.21	0.00	± 0.00
BD18	10.31	± 0.30	5.26	± 0.93	3.19	± 0.53
BD19	10.13	± 0.86	5.86	± 1.25	3.40	± 0.54
BD20	10.50	± 0.48	5.55	± 1.25	2.99	± 1.09
BD21	10.03	± 0.57	5.61	± 1.27	3.17	± 0.55
BD22	10.04	± 0.56	6.04	± 0.40	3.23	± 0.55
BD23	10.04	± 0.68	5.94	± 1.26	3.38	± 0.55
BD24	9.90	± 0.66	5.56	± 1.26	3.22	± 0.56
BD25	9.97	± 0.87	5.58	± 1.15	2.68	± 1.64
BD26	10.07	± 0.66	5.42	± 1.15	4.09	± 1.33
BD27	9.85	± 0.61	5.72	± 0.71	4.23	± 0.61
BD28	10.03	± 0.83	5.35	± 1.14	4.08	± 1.12
BD29	9.99	± 0.67	6.18	± 1.28	0.00	± 0.00
BD30	9.89	± 0.83	5.35	± 1.15	3.26	± 0.56
BD31	10.88	± 0.50	5.64	± 1.14	5.83	± 0.90
BD32	9.95	± 0.78	5.38	± 0.97	3.18	± 0.55
BD33	10.14	± 0.44	5.70	± 1.13	3.20	± 0.55
BD34	9.91	± 0.54	5.71	± 1.00	3.24	± 0.55
BD35	10.05	± 0.88	5.40	± 0.88	3.31	± 0.55
BD36	9.91	± 0.68	5.64	± 0.88	2.77	± 0.74
BD37	9.99	± 0.74	5.40	± 1.15	2.90	± 1.63
BD38	9.96	± 0.67	5.30	± 0.98	3.27	± 0.54
BD39	9.91	± 0.69	5.82	± 0.83	0.00	± 0.00
BD40	9.99	± 0.66	5.53	± 0.96	3.32	± 0.55

Antagonistic activity against key poultry pathogens

The antimicrobial potential of the 40 *Bacillus* isolates was evaluated against three major poultry pathogens. The neutralized, cell-free supernatant (nCFS) from the majority of isolates exhibited inhibitory activity against at least one of the tested pathogens (Table 4).

Table 4 Antimicrobial activity of nCFS from *Bacillus* isolates against pathogenic indicator strains. Inhibition zone diameters are categorized as: + (2–5 mm); ++ (6–9 mm); +++ (≥ 10 mm); – (No inhibition).

Strains ID	<i>E. coli</i> ATCC 25922	<i>S. enterica</i> ATCC 14028	<i>S. aureus</i> ATCC 25923	Strains ID	<i>E. coli</i> ATCC 25922	<i>S. enterica</i> ATCC 14028	<i>S. aureus</i> ATCC 25923
BD1	++	++	++	BD22	++	++	-
BD2	-	++	++	BD23	++	++	++
BD3	++	++	++	BD24	-	++	++
BD4	++	++	++	BD25	++	+++	++
BD5	+++	+++	++	BD26	++	++	++
BD6	++	++	++	BD27	++	++	+++
BD7	-	++	++	BD28	++	++	++
BD8	++	++	+++	BD29	+++	++	-
BD9	++	++	-	BD30	++	++	++
BD10	+++	++	++	BD31	+++	+++	++
BD11	++	++	++	BD32	++	++	++
BD12	++	-	++	BD33	+++	++	++
BD13	++	-	++	BD34	++	++	+
BD14	++	++	++	BD35	-	-	-
BD15	++	++	-	BD36	++	++	++
BD16	++	++	++	BD37	++	+++	++
BD17	++	++	++	BD38	++	++	++
BD18	-	-	-	BD39	++	-	++
BD19	++	++	++	BD40	++	++	++
BD20	++	-	+++	Positive control (ampicilline)	+++	+++	+++
BD21	++	++	-	Negative control	-	-	-
BD1	++	++	++				

Five isolates BD5, BD10, BD25, BD31, and BD37 were identified as having particularly potent, broad-spectrum antimicrobial activity, producing inhibition zones of ≥ 10 mm against at least two pathogens. Isolate BD31 emerged as the most promising candidate, displaying strong inhibition against the Gram-negative pathogens *E. coli* (12.5 mm zone) and *S. enterica* (11.8 mm zone), and moderate activity against the Gram-positive pathogen *S. aureus* (8.5 mm zone) (Figure 2). Based on its superior and consistent performance across all screening assays (acid tolerance, bile resistance, and antimicrobial efficacy), isolate BD31 was selected for definitive molecular identification.

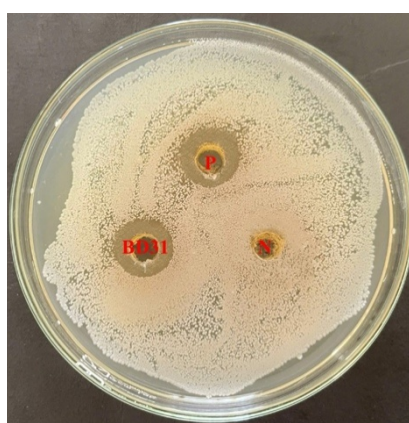


Figure 2. A representative agar well diffusion assay showing the zone of inhibition produced by the nCFS of isolate BD31 against *S. enterica* serovar Typhimurium ATCC 14028. BD31: nCFS from *Bacillus velezensis* BD31; P: Positive control (Ampicillin, 10 μ g); N: Negative control (sterile neutralized LB broth). The diameter of the clear zone indicates the extent of bacterial growth inhibition.

Molecular identification and phylogenetic analysis of isolate BD31

To confirm the species identity of the most promising probiotic candidate, the 16S rRNA gene of isolate BD31 was amplified via PCR. The amplification yielded a single, distinct band of the expected size, approximately 1,500 base pairs (bp), as visualized by agarose gel electrophoresis (Figure 3). The purified PCR product was sequenced, and the resulting 1,489 bp sequence was analyzed using the BLASTn algorithm against the NCBI GenBank database.

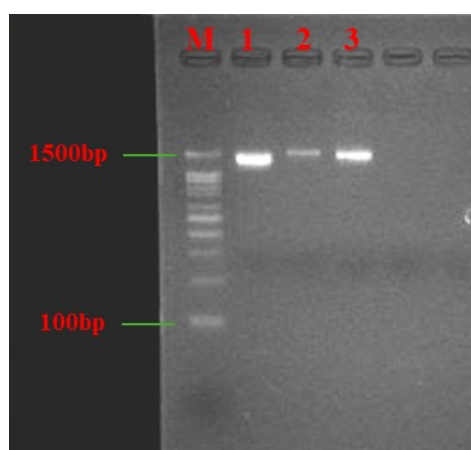


Figure 3. Agarose gel electrophoresis of the 16S rRNA gene amplified from isolate BD31. M: 100 bp DNA ladder; Lane 1: Positive control; Lane 2, 3: PCR product from BD31 showing a clear band at ~1500 bp.

The sequence analysis provided a definitive identification, revealing that the 16S rRNA gene of isolated BD31 shared a 99.76% identity with the reference sequence of *Bacillus velezensis* strain FZB42 (Accession No. NR_075005.2), with 100% query coverage (Table 5). This high degree of sequence similarity unequivocally identifies isolate BD31 as *Bacillus velezensis*. To further elucidate its taxonomic relationship with closely related species, a phylogenetic tree was constructed using the Neighbor-Joining method. The phylogenetic analysis demonstrated that isolate BD31 clustered robustly within the clade formed by other authenticated *Bacillus velezensis* strains, clearly distinguishing it from other species within the *Bacillus subtilis* group, such as *B. amyloliquefaciens* (Figure 4). This molecular confirmation solidifies the identity of BD31 as a native Muscovy duck-derived strain of *Bacillus velezensis* with significant probiotic potential.

Table 5 Top BLASTn hit for the 16S rRNA gene sequence of isolate BD31.

Subject description	Max score	Query cover	E-value	Percent identity	Accession number
<i>Bacillus velezensis</i> strain FZB42 16S ribosomal RNA, 1515 complete sequence		100 %	0.0	99.76 %	NR_075005.2

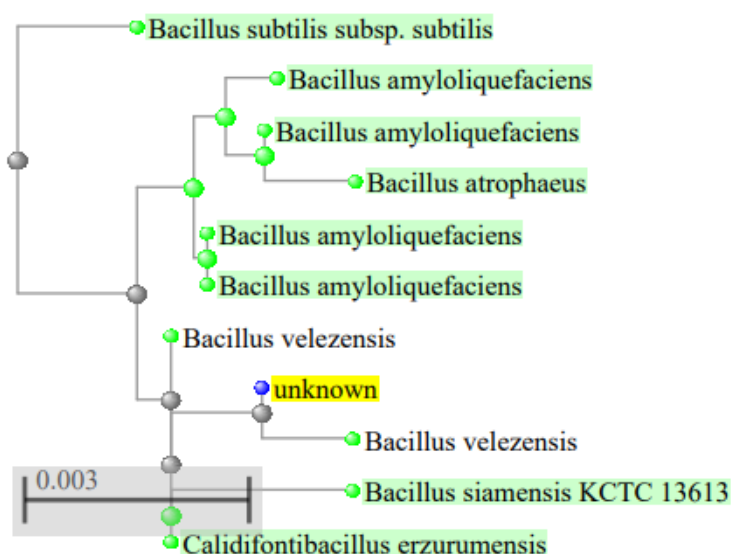


Figure 4. Phylogenetic tree constructed using the Neighbor-Joining method, illustrating the evolutionary relationship of isolate BD31. The analysis, based on 16S rRNA gene sequences, shows BD31 clustering with reference strains of *Bacillus velezensis*. Bootstrap values (from 1000 replicates) are indicated at the nodes.

DISCUSSION

The Muscovy duck gut: A strategic frontier for probiotic bioprospecting

This study ventured into the relatively unexplored microbial ecosystem of the indigenous Muscovy duck (*Cairina moschata*) gastrointestinal tract (GIT). This strategic choice for bioprospecting proved highly fruitful. Our central hypothesis was that the gut microbiota of autochthonous animals, which are thoroughly adapted to their local environment, constitutes a rich and untapped reservoir of beneficial microorganisms. The successful isolation of forty distinct bacterial strains, including the highly promising candidate *Bacillus velezensis* BD31, validates this approach. Unlike commercially standardized poultry breeds raised in controlled environments, indigenous animals like the Muscovy duck co-evolve with regional diets, unique environmental pressures, and endemic pathogen challenges. This long-term evolutionary selection process likely cultivates a gut microbiome uniquely tailored to enhance host digestive efficiency, immunological resilience, and overall health. Our work aligns with a substantial body of research that has successfully identified beneficial *Bacillus* species from the gut microbiota of diverse animal hosts, including broiler chickens (Mazanko et al., 2022), cattle (Naeem et al., 2018), and fish (Penaloza-Vazquez et al., 2019), collectively reinforcing the genus's ubiquitous and symbiotic presence in animal microbiomes. By focusing on a previously under-explored avian host within the key biodiversity hotspot of Vietnam, this research adds a crucial data point to the global effort of discovering novel probiotic candidates.

A pivotal element of our isolation methodology was the deliberate application of a heat-shock treatment. This technique served as a powerful and practical preliminary screen, selectively enriching for endospore-forming bacteria. This is not merely a biological curiosity; it represents a paramount commercial advantage. The

genus *Bacillus* is renowned for its ability to form dormant endospores, which are extraordinarily resilient to environmental stressors such as extreme temperatures, desiccation, and acidity (Truong et al., 2021). This inherent thermostability ensures that the probiotic can withstand the high temperatures and pressures of the commercial feed pelleting process, a significant hurdle that often compromises the viability of non-spore-forming probiotics like many lactic acid bacteria (Ramlucken et al., 2020). The subsequent morphological and biochemical analyses further corroborated the initial classification, with all isolates exhibiting classical *Bacillus* traits: Gram-positive, rod-shaped morphology and the presence of spores. The consistent positive catalase test across isolates is particularly noteworthy, as this enzyme is vital for protecting the bacteria from oxidative stress within the host gut, a critical survival mechanism for successful colonization and function (Golnari et al., 2024). This strategic combination of a targeted isolation source and a functionally relevant screening method proved highly effective in identifying robust probiotic candidates.

Navigating the gastrointestinal gauntlet: A prerequisite for efficacy

For any orally administered probiotic to confer a health benefit, it must first survive the formidable chemical and enzymatic challenge of the host's upper GIT. In poultry, this journey involves passage through the highly acidic proventriculus and gizzard, where pH can drop to 2.0, followed by exposure to the detergent-like action of bile salts in the small intestine. These harsh conditions can readily lyse unprotected bacterial cells, rendering a probiotic supplement ineffective before it can reach its target site in the lower intestine. Consequently, our multi-stage screening process designated acid and bile salt tolerance as a non-negotiable prerequisite for any potential probiotic candidate.

Our in vitro assays revealed significant strain-dependent variations in survival, underscoring the critical importance of this functional screening step. The lead candidate, *B. velezensis* BD31, clearly distinguished itself by demonstrating exceptional resilience. It maintained high viability after a four-hour exposure to a highly challenging acidic pH of 2.0 and a physiologically relevant bile salt concentration of 0.3%, conditions that were lethal to many other isolates. This robust tolerance is a hallmark of superior probiotic *Bacillus* strains and is consistent with numerous studies where the most effective candidates exhibit the highest tolerance to simulate gastrointestinal stresses. For example, (Zhu La et al., 2024) reported that their top-performing *B. velezensis* strain, isolated from chickens, showed the greatest resilience to both acid and bile among all candidates tested. Similarly, studies on *B. velezensis* strains intended for aquaculture (Arya et al., 2025) and human applications (Brutscher et al., 2024) have consistently identified superior survival in simulated GIT conditions as a key selection criterion. The demonstrated ability of BD31 to withstand these harsh conditions strongly indicates that it possesses the intrinsic durability required to successfully transit the avian GIT, germinate from spore to viable vegetative cell in the intestine, and subsequently colonize and exert its beneficial effects. This resilience is likely conferred by a combination of robust cell wall architecture and sophisticated physiological stress-response mechanisms, such as the activation of proton pumps and the expression of bile salt hydrolases, which are known to be active in tolerant probiotic strains (Su et al., 2024).

Potent, broad-spectrum antimicrobial activity: A primary mechanism for pathogen control

One of the most significant functional attributes of a probiotic is its ability to directly inhibit the growth of pathogenic microorganisms, thereby protecting the host from infection. In this study, the neutralized cell-free supernatant of *B. velezensis* BD31 displayed potent and remarkably broad-spectrum antimicrobial

activity. It effectively inhibited the growth of key Gram-negative enteric pathogens, including *Escherichia coli* and *Salmonella enterica*, as well as the formidable Gram-positive pathogen *Staphylococcus aureus*. The capacity to antagonize such a phylogenetically diverse range of pathogens is a highly desirable trait for any poultry probiotic. It suggests the production of a sophisticated arsenal of antimicrobial compounds with multiple modes of action, which not only provides comprehensive protection but may also reduce the likelihood of pathogens developing resistance. This broad-spectrum efficacy is particularly valuable in commercial poultry operations, where birds are often challenged by co-infections from multiple pathogens, creating complex disease scenarios that are difficult to manage with conventional, narrow-spectrum antibiotics (Hamed et al., 2024).

The genus *Bacillus*, and *B. velezensis* in particular, is renowned as a prolific producer of a vast array of bioactive secondary metabolites. The potent inhibition observed in our assays is almost certainly attributable to the secretion of these compounds into the supernatant. Genomic and metabolomic studies have confirmed that *B. velezensis* possesses genetic machinery to synthesize several families of powerful antimicrobial lipopeptides, most notably surfactins, fengycins, and iturins (Liu et al., 2017; Rabbee et al., 2019). These molecules act through diverse and complementary mechanisms: surfactins are powerful biosurfactants that disrupt the structural integrity of the pathogen's cell membrane, while iturins and fengycins form pores in the membrane, leading to leakage of cellular contents and ultimately, cell lysis (Perini et al., 2024). The strong inhibitory activity of BD31 against both Gram-negative and Gram-positive bacteria strongly suggests it is a proficient producer of one or more of these compound classes. This finding is in robust agreement with numerous studies that have directly linked the probiotic effects of *B. velezensis* to its production of these specific metabolites. Strains have demonstrated potent inhibitory effects against *S. aureus*, *E. coli*, and *Salmonella Typhimurium* in both in vitro and in vivo models, with this activity directly correlating with the presence of lipopeptide biosynthetic gene clusters (Afroj et al., 2021; Husna et al., 2023; Wang et al., 2024). Beyond direct antagonism, this antimicrobial activity also facilitates competitive exclusion, whereby the probiotic outcompetes pathogens for nutrients and adhesion sites on the intestinal epithelium, further preventing pathogen colonization and mitigating the risk of infection (Barzegar et al., 2021).

Identity and multifaceted potential of *Bacillus velezensis* BD31

Accurate taxonomic identification is a cornerstone for both the efficacy and safety assessment of any probiotic candidate. Through 16S rRNA gene sequencing, the gold standard for bacterial identification, coupled with BLASTn analysis, we definitively identified isolate BD31 as *Bacillus velezensis* with 99.76% sequence identity to the well-characterized type of strain FZB42. A subsequent phylogenetic analysis further solidified this classification, showing that BD31 robustly clusters within the *B. velezensis* clade, clearly demarcated from other closely related species in the *Bacillus subtilis* group. This confident molecular identification provides a strong foundation for its continued development as a specialized feed additive.

Bacillus velezensis is increasingly recognized as a "star species" in the world of probiotics and agricultural biocontrol agents. Originally classified as a subspecies of *Bacillus amyloliquefaciens*, it was elevated to a distinct species based on compelling genomic evidence (Liu et al., 2017). Its advantages as a feed additive are multifaceted and extend far beyond its antimicrobial capabilities. As a spore-former, it offers exceptional stability during feed manufacturing and storage as previously discussed (Ramlucken et al., 2020). Upon germination in the gut, it effectively functions as a microbial factory. In addition to antimicrobial compounds, *B. velezensis* secretes a wide range of beneficial extracellular enzymes, such as

amylases, proteases, and cellulases. These enzymes can significantly improve nutrient digestion and absorption from feed, leading to enhanced conversion efficiency and better growth performance in the host animal (Latorre et al., 2016; Hosseini et al., 2018). Furthermore, *B. velezensis* is known to modulate the gut microbiota beneficially, promoting the growth of other symbiotic bacteria while suppressing pathogens. It can also enhance intestinal barrier function by strengthening tight junctions between epithelial cells, thereby preventing the translocation of pathogens and toxins into the bloodstream and reducing gut inflammation (Su et al., 2024). The isolation of a native, highly adapted strain like BD31 from the local Muscovy duck population represents a significant step toward developing a next-generation, regionally tailored feed additive for the Vietnamese poultry industry.

Limitations and future research directions

While the *in vitro* results presented in this study are highly promising, it is essential to acknowledge its limitations and outline a clear, rigorous roadmap for future research. *In vitro* screening assays, though crucial for initial characterization and candidate selection, cannot fully replicate the complex, dynamic, and largely anaerobic environment of the avian gut. This environment involves intricate, three-way interactions between the host's physiology, the complex resident microbiota, and various dietary components. Therefore, the promising traits observed in a controlled laboratory setting must be validated in a live animal model to confirm their physiological relevance and practical efficacy.

The immediate and most critical next step is to perform whole-genome sequencing of *B. velezensis* BD31. This advanced analysis will serve several vital purposes. First, it will provide the highest possible resolution for taxonomic confirmation, definitively resolving any ambiguity that may exist due to the high genetic similarity among species within the *B. subtilis* group (Yilmaz et al., 2021). Second, and most importantly from a regulatory and safety standpoint, it will facilitate a comprehensive *in silico* safety assessment to confirm its non-pathogenic status and absence of undesirable genes. The genome can be screened for any potential virulence-associated genes (those encoding toxins or hemolysins) and transferable antibiotic resistance markers, a mandatory safety check for all new probiotic candidates (Brutscher et al., 2024; da Rosa et al., 2025). Third, a complete genome sequence will enable the identification of the specific biosynthetic gene clusters responsible for the synthesis of the observed antimicrobial compounds (surfactin, fengycin, and iturin synthetase genes), providing a clear genetic basis for its potent antagonistic activity and opening avenues for strain improvement.

Following this essential genomic characterization, research must progress to controlled *in vivo* poultry trials. These studies are indispensable for evaluating the true potential of *B. velezensis* BD31 as a commercial feed additive. The trials should be meticulously designed to assess the strain's impact on key production parameters, including growth performance (body weight gain), feed conversion ratio, and overall flock health and mortality. Concurrently, its physiological effects must be investigated by measuring its ability to beneficially modulate the gut microbiota composition (via 16S rRNA amplicon sequencing), enhance intestinal morphology (e.g., villus height and crypt depth), strengthen intestinal barrier function, and stimulate the host's mucosal immune system. Critically, a pathogen challenge model (with an endemic strain of *Salmonella* or *E. coli*) should be employed to definitively determine if the potent *in vitro* antimicrobial activity translates into a tangible, protective effect against infection *in vivo*. Successful completion of these validation steps is imperative to translate this promising laboratory candidate into a scientifically validated, safe, and effective probiotic for the poultry industry.

CONCLUSIONS

In conclusion, this study successfully demonstrates that the gastrointestinal tract of the indigenous Vietnamese Muscovy duck is a valuable and strategic reservoir for discovering novel *Bacillus* strains with significant probiotic potential. Through a systematic, function-driven screening process, we identified and characterized *Bacillus velezensis* BD31, a strain that exhibited exceptional tolerance to simulated gastrointestinal conditions and displayed potent, broad-spectrum antimicrobial activity against critical poultry pathogens. These compelling in vitro findings strongly suggest that *B. velezensis* BD31 is a superior candidate for development as a direct-fed microbial aimed at enhancing poultry health, reducing reliance on antibiotics, and improving productivity. This work represents a crucial first step in harnessing local microbial biodiversity to create sustainable and effective solutions for agriculture. However, to realize this potential, a rigorous path forward is required. Further investigation, beginning with whole-genome sequencing for comprehensive safety and functional gene analysis, followed by comprehensive in vivo efficacy trials, is essential before this promising isolate can be commercialized as a validated probiotic solution for promoting a healthier and more sustainable poultry industry in Vietnam and beyond.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Nguyen Phuong Thuy: Conceptualization, methodology, investigation, validation, formal analysis, writing – original draft, writing – review and editing.

Nguyen Thanh Tuu: Investigation, resources, data curation.

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