Research Article

Bacillus-Derived Probiotics Inhibit Multidrug-Resistant, Virulent *Escherichia coli* in Ducks

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Abstract | Duck farming faces increasing risks posed by multidrug-resistant *E. coli* strains, including those harboring multiple virulence genes. Consequently, the search for antibiotic alternatives such as probiotics or postbiotics has become essential for fostering sustainable livestock development. A total of 273 bacterial strains were isolated from 80 biological samples collected from duck flocks. These strains were screened for antimicrobial activity using the agar diffusion method. Promising strains were identified via 16S rRNA gene sequencing. Antibiotic susceptibility was assessed using the disk diffusion method, while the antimicrobial activity of cell-free supernatants was determined based on minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. Eight *Bacillus* spp. strains demonstrated strong inhibitory activity against *E. coli* harboring five virulence genes (*iss*, *iutA*, *hlyF*, *ompT*, and *iroN*). These strains were identified as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Rossellomorea marisflavi*, and *Bacillus altitudinis*. Among them, *B. amyloliquefaciens* B20 and *B. subtilis* B23-4 exhibited the highest efficacy, characterized by low MIC values and distinct bactericidal activity. The cell-free supernatants of these strains likely contain antimicrobial peptides and cyclic lipopeptides that function by disrupting bacterial cell membranes. Notably, *R. marisflavi* B24-5 also showed considerable activity, suggesting the potential of non-traditional taxa as novel antimicrobial sources. All selected strains were susceptible to most of the antibiotics tested. Cell-free supernatants derived from *Bacillus* spp. represent a promising postbiotic approach for controlling multidrug-resistant *E. coli* in poultry farming. Further studies involving whole-genome sequencing and *in vivo* trials are required to validate the mechanisms of action and confirm practical applicability.

Keywords | Multidrug-resistant, *Escherichia coli*, *Bacillus* spp., Antibacterial activity, Probiotics, Ducks

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INTRODUCTION

Duck farming particularly the "free-range duck" model plays a vital role in the livelihoods of residents in Vinh Long province specifically, and the Mekong Delta region generally, contributing significantly to food security and rural economic development. However, this farming system currently faces numerous challenges stemming from the rise of infectious diseases specifically gastrointestinal infections caused by *E. coli* which have been widely documented both globally and within the Mekong Delta region (Thu *et al.*, 2019). Of even greater concern is the recent emergence of highly virulent *E. coli* strains. Molecular-level analyses have identified the presence of key virulence genes such as *iss*, *iutA*, *hlyF*, *ompT*, and *iroN* which are directly linked to the capacity to cause severe disease, systemic infections,

and increased mortality rates in poultry (Mbanga and Nyararai, 2015; Joseph *et al*., 2024; Monroy *et al*., 2025). These bacterial strains are posing a major threat to duck flock health, particularly within extensive farming systems where biosecurity measures remain limited.

Amidst the pressures of disease outbreaks, the use of antibiotics in poultry farming has become widespread, particularly in the Mekong Delta region and Vinh Long province (Thu *et al*., 2019). However, this pattern of misuse has led to the rapid emergence of multidrug-resistant (MDR) bacteria. Virulence-carrying *E. coli* strains in this region have demonstrated resistance to various classes of antibiotics, thereby significantly diminishing treatment efficacy. The primary underlying cause is that conventional antibiotics typically target a specific site (such as an enzyme or receptor), thereby creating conditions for bacteria to evolve through genetic mutation, leading to the formation of efflux pump systems or drug-degrading enzymes (Nguyen *et al*., 2021; Nasrollahian *et al*., 2024; Kerek *et al*., 2026). Consequently, treatment regimens are gradually losing their effectiveness while simultaneously leaving behind antibiotic residues that compromise biosecurity and public health. Against the backdrop of diminishing antibiotic efficacy, the search for alternative solutions particularly biological preparations such as probiotics and postbiotics has become imperative in the pursuit of sustainable livestock development. Among these options, *Bacillus* spp. is considered promising candidates due to their ability to form highly resistant endospores, enabling them to survive under harsh environmental conditions as well as under antibiotic pressure (Naumova *et al*., 2021; Thuy and Tuu, 2025). Notably, indigenous *Bacillus* strains isolated from the digestive tracts of local duck flocks may offer superior efficacy, having already adapted to the specific ecological conditions of the region.

These bacteria possess numerous biosynthetic gene clusters, enabling the production of a diverse array of antimicrobial compounds, such as antimicrobial peptides (AMPs) and cyclic lipopeptides (surfactin, iturin, fengycin) (Sagar *et al*., 2024). Unlike conventional antibiotics, these compounds act directly on bacterial cell membranes through physicochemical mechanisms, disrupting the phospholipid bilayer structure, inducing pore formation, and ultimately leading to cell lysis. This multi-target mechanism significantly reduces the likelihood of resistance development and facilitates the effective control of multidrug-resistant bacteria (Saiyam *et al*., 2024; Markelova and Chumak, 2025). Against the backdrop of a complex epidemiological landscape characterized by the rapid rise of antibiotic resistance and the growing need to harness indigenous antagonistic microbial resources this study was conducted to isolate and select *Bacillus* spp. strains capable of inhibiting antibiotic-resistant and virulence-gene-carrying *E. coli* strains isolated from ducks. This research not only provides a scientific foundation for understanding the mechanisms of pathogen inhibition but also paves the way for applying probiotic or synbiotic solutions in disease control, thereby contributing to the sustainable development of the local poultry industry.

MATERIALS AND METHODS

Sample collection and bacterial isolation

A total of 80 biological samples were randomly collected from duck flocks in Vinh Long province (Vietnam) for the isolation of *Bacillus* spp. The sample set comprised 40 fecal samples and 40 intestinal fluid samples. All samples were collected under aseptic conditions, stored in sterile containers, and maintained at 4°C during transport. The samples were immediately transported to the laboratory and processed upon arrival for microbial isolation and culture, in order to ensure the viability of the target microorganisms.

All samples were subjected to isolation and identification based on standard microbiological protocols at the Microbiology Laboratory, Tra Vinh University. The sample was homogenized in 50 mL of sterile distilled water; subsequently, 1 mL of the suspension was transferred into 10 mL of Buffered Peptone Water (BPW) medium and incubated at 37°C for 4±1 hours for initial enrichment. To selectively isolate spore-forming *Bacillus* spp. strains, the enriched culture was heat-treated at 80°C for 15 minutes to eliminate vegetative cells (Golnari *et al*., 2024). Following heat treatment, 1 mL of the suspension was inoculated into 10 mL of Tryptic Soy Broth (TSB) medium and incubated at 37°C for 24±1 hours. Next, 100 µL of the culture was spread-plated onto Tryptic Soy Agar (TSA) and incubated at 37°C for 24±1 hours. Well-developed, isolated colonies were selected and subjected to preliminary identification using Gram staining and the catalase test (Al-Azad *et al*., 2020). Strains identified as Gram-positive, catalase-positive, and capable of producing endospores were preserved in glycerol solution at -20°C for subsequent analyses.

Antibacterial activity screening

Escherichia coli was utilized as an indicator strain to assess antagonistic activity. This bacterial strain was isolated from ducks within the ecological zone of Vinh Long province and was selected based on its characteristics of multi-drug resistance and high virulence. The strain demonstrated resistance to 8 out of the 12 antibiotics tested, including ceftiofur, colistin, ampicillin, amoxicillin, gentamicin, streptomycin, tetracycline, and doxycycline. Genotypic analysis via PCR confirmed the presence of five virulence-associated genes (*iss*, *iutA*, *hlyF*, *ompT*, and *iroN*). Given

the combination of a multidrug resistant phenotype and the carriage of multiple virulence factors, this strain serves as a robust pathogenic bacterial model with high practical significance for evaluating antimicrobial efficacy and antagonistic activity. The strain is preserved in a medium supplemented with 20% glycerol at -80°C and is reactivated prior to use in experiments.

The antagonistic activity of *Bacillus* spp. strains against *E. coli* was determined using the agar well diffusion method (Balouiri *et al*., 2016). An *E. coli* suspension was spread evenly onto Mueller-Hinton agar (MHA) medium, after which 6 mm diameter wells were created in the agar under sterile conditions. Each well was filled with 100 µL of a 24-hour *Bacillus* culture, while sterile culture medium was used as a negative control. The plates were incubated at 37°C for 24 hours under aerobic conditions. Antibacterial activity was assessed by measuring the diameter of the inhibition zone (mm). The experiment was repeated three times, and strains exhibiting clear inhibition zones were selected for further studies.

Bacterial strain identification

Bacterial strains exhibiting the strongest antagonistic activity against *E. coli* were selected for molecular identification at the species level. Genomic DNA was extracted and purified according to the manufacturer's instructions using the PHUSA DirectBac Extract kit (PHUSA Biochem, Vietnam). The 16S rRNA gene was amplified via PCR using the primer pair Bsub5F (5′-AAGTCGAGCGGACAGATGG-3′) and Bsub3R (5′-CCAGTTTCCAATGACCCTCCCC-3′), following the method described by Bolivar-Anillo *et al.* (2021). The PCR products were verified via agarose gel electrophoresis, then purified and sequenced using the Sanger method. The resulting nucleotide sequences were processed, assembled, and compared against reference sequences in the NCBI GenBank database using the BLASTn algorithm. Species identification was determined based on the highest percentage of nucleotide sequence similarity relative to reference strains within the database.

Antibiotic susceptibility testing

All *Bacillus* strains demonstrating inhibitory activity against *E. coli* were evaluated for antibiotic susceptibility using the disk diffusion method. The testing procedure and selection of antibiotics were conducted in accordance with the CLSI (2024) guidelines. The antibiotics tested included enrofloxacin, gentamicin, tetracycline, ampicillin, ceftiofur, trimethoprim-sulfamethoxazole, and amoxicillin-clavulanate. Interpretation of the results was based on CLSI zone diameter criteria, categorizing each isolate as susceptible (S), intermediate (I), or resistant (R).

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cell-free supernatant (CFS)

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of cell-free supernatants (CFS) derived from *Bacillus* spp. strains against *E. coli* were determined using the broth microdilution method in 96-well plates, utilizing resazurin as a viability indicator (Bolivar-Anillo *et al*., 2021). Antimicrobial compounds were obtained through a CFS preparation and concentration procedure adapted from the method described by Tran *et al.* (2023). Specifically, *Bacillus* strains were cultured at 37°C for 24 hours with shaking at 300 rpm, followed by sonication for 5 minutes. The culture broth was subsequently centrifuged and filtered through a sterile 0.22 µm membrane to collect the CFS. The resulting supernatant was then lyophilized and reconstituted in sterile water to achieve a stock concentration of 250 µg/mL. To determine the MIC, the CFS was serially diluted twofold in broth medium, starting with a concentration of 125 µg/mL in the first column of the 96-well plate. Each well was inoculated with a density-standardized *E. coli* suspension (approximately 10^5 CFU/mL) and incubated aerobically at 37°C for 18-24 hours. Following the incubation period, 10 µL of a 0.1% resazurin solution was added to each well, and incubation continued for an additional 2-4 hours. Based on the principle that viable bacteria are capable of reducing resazurin (blue) to resorufin (pink), the MIC value was defined as the lowest concentration of CFS that maintained a blue color, indicating complete inhibition of bacterial growth. For the MBC determination, aliquots from wells showing no color change (including the MIC well and those with higher concentrations) were streaked onto the surface of TXB agar and incubated at 37°C for 24 hours. The MBC value was defined as the lowest CFS concentration at which no *E. coli* colonies were observed on the agar surface.

Statistical analysis

Statistical analyses were performed using SPSS software (version 22). Differences in inhibition zone diameters among bacterial strains were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical significance was established at $P<0.05$. Data were presented as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Isolation and morphological characterization of bacterial isolates

A total of 273 bacterial strains were isolated from 80 biological samples collected in Vinh Long province; all exhibited morphological characteristics consistent with the genus *Bacillus* (Table 1 and Figure 1).

Table 1: Morphological characterization of the isolated *Bacillus* spp. strains.

Parameters	Characteristics	Number of isolates (strains)	Percentage (%)
Colony color	Opaque white or grayish white	224	82.05
	Creamy white or pale yellow	49	17.95
Colony shape	Rhizoid or irregular	158	57.88
	Circular	115	42.12
Colony surface	Dry, rough, and wrinkled	185	67.77
	Smooth, shiny, and mucoid	88	32.23
Gram staining	Gram-positive (+)	273	100
Cell shape	Rod-shaped (bacilli)	273	100
Cell arrangement	Single or short chains	273	100

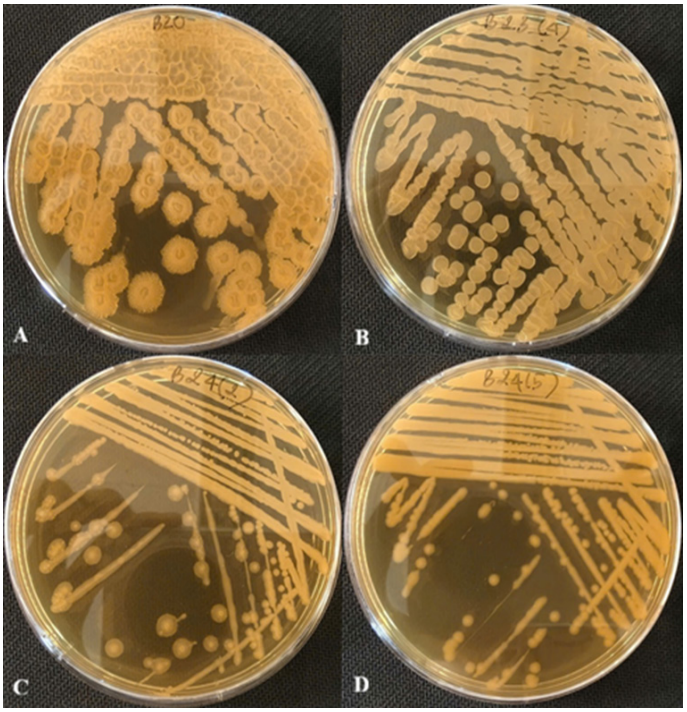

Figure 1: Morphological characteristics of colonies formed by the isolated *Bacillus* spp. on culture media: (A) strain B20 *(Bacillus amyloliquefaciens)*; (B) strain B23-4 *(Bacillus subtilis)*; (C) strain B24-2 *(Bacillus altitudinis)*; and (D) strain B24-5 *(Rossellomorea marisflavi)*.

Specifically, all strains were Gram-positive rods, arranged singly or in short chains (100%), and notably possessed the ability to form endospores following heat treatment at 80 °C for 15 minutes. These characteristics are considered typical criteria for identifying *Bacillus* spp., while also reflecting the high adaptability and survival capacity of this bacterial group under harsh environmental conditions. Colony morphology analysis revealed that opaque white or grayish-white colonies predominated (82.05%), followed by creamy white or pale-yellow colonies (17.95%). Regarding shape, rhizoid or irregular colonies occurred at a higher frequency (57.88%) compared to regular circular colonies (42.12%); this suggests diversity in growth patterns and may be linked to bacterial motility, the secretion of extracellular matrix substances, or surface adhesion strategies. Notably, the majority of strains (67.77%) exhibited dry, rough, and wrinkled colony surfaces, whereas only 32.23% possessed smooth, glossy, and mucoid surfaces. From a functional perspective, the predominance of rough and wrinkled colony phenotypes holds significant biological importance. This phenotype is often closely associated with the capacity for biofilm formation, mediated through the production of extracellular matrix substances (EPS) (Vlamakis *et al*., 2013). Studies published indicate that *Bacillus* strains with robust biofilm-forming capabilities typically exhibit high adhesion efficiency within the gastrointestinal tract; this enhances their colonization potential and contributes to host protection through the mechanism of competitive exclusion against pathogenic bacteria (Lebeer *et al*., 2008). Similarly, publications emphasize that EPS-producing *Bacillus* strains possess superior probiotic potential, owing to their strong resilience against environmental fluctuations and their ability to maintain biological activity under stressful conditions (Cutting, 2011). Mechanistically, the wrinkled colony phenotype is typically linked to the expression of gene clusters regulating biofilm formation (such as *epsA–O* and *tapA-sipW-tasA*), alongside the synthesis of structural components such as exopolysaccharides and amyloid fibers (Vlamakis *et al*., 2013). These structures serve to enhance adhesion capabilities and create a protective microenvironment, thereby increasing resistance to antimicrobial agents and adverse host-derived factors. This is particularly significant within the avian digestive system, an environment characterized by substantial biological pressures and intense microbial competition.

The morphological characteristics recorded in this study align with numerous previous publications; for instance, Thuy and Tuu (2025) noted the predominance of Gram-positive, spore-forming, rod-shaped bacteria exhibiting rough colony morphology within poultry microbiota. Furthermore, studies by Ramlucken *et al*. (2020) indicate that these characteristics are typical markers of superior probiotic *Bacillus* strains particularly those demonstrating robust antimicrobial activity and excellent intestinal colonization capabilities. These traits have been widely

documented in published literature, underscoring their critical role in the selection and functional efficacy assessment of probiotic strains. Notably, the establishment of a diverse *Bacillus* strain bank derived from field conditions in Vinh Long province offers a significant advantage for subsequent selection stages. This biodiversity enhances the likelihood of identifying strains possessing exceptional functional attributes, such as potent antagonistic activity against *E. coli*, robust spore-forming capacity, and high resilience to adverse conditions including antibiotic pressure. From an applied perspective, these findings align with the global trend toward minimizing antibiotic usage in livestock production and replacing it with sustainable biological alternatives (Cutting, 2011; Bahaddad *et al*., 2023). The morphological and physiological characteristics recorded in this study not only confirm the successful isolation of *Bacillus* strains but also demonstrate their distinct potential for probiotic applications. These results establish a solid foundation for subsequent research into their antimicrobial activity, antibiotic resistance, and *in vivo* efficacy, thereby contributing to the development of biological solutions for sustainable duck farming.

Screening of antibacterial activity of isolated bacterial strains

The results presented in Table 2 reveal significant differences in the antagonistic activity of the isolated *Bacillus* strains against a multidrug resistant *E. coli* strain harboring five virulence genes. Among the strains examined, strain B20 demonstrated the strongest antimicrobial activity, exhibiting the largest zone of inhibition (31.83 ± 0.76 mm) a result that was statistically significantly higher ($P < 0.05$) than that of all other strains (Figure 2). Strains B23-4 and B24-5 also demonstrated strong inhibitory capacity, with inhibition zones of 27.50 ± 0.87 mm and 25.33 ± 0.58 mm, respectively. In contrast, the remaining isolates showed moderate activity, with inhibition zones ranging between 19.33 and 20.33 mm. These differences may reflect variations in metabolic capacity, regulatory systems, and the abundance of biosynthetic gene clusters (BGCs) involved in the production of antimicrobial compounds. From a mechanistic perspective, the superior activity of strains B20 and B23-4 may be attributed to their ability to produce various groups of bioactive secondary metabolites particularly cyclic lipopeptides such as surfactin, iturin, and fengycin, as well as antimicrobial peptides (AMPs). These compounds exert their bactericidal effects primarily by targeting the cell membrane; their mechanisms of action include pore formation, disruption of the lipid bilayer's integrity, and the induction of ion leakage, ultimately leading to cell death. In Gram-negative bacteria such as *E. coli*, these effects are further amplified through interactions with the lipopolysaccharide (LPS) layer of the outer membrane, thereby increasing permeability to antimicrobial agents (Latorre *et al*., 2015). Furthermore, recent studies indicate that certain lipopeptides derived from *Bacillus* are capable of inducing intracellular oxidative stress by increasing reactive oxygen species (ROS), thereby amplifying their bactericidal efficacy beyond a mere membrane-disrupting mechanism (Tran *et al*., 2022).

Table 2: Preliminary screening of antibacterial activity of isolated *Bacillus* spp. by agar diffusion technique.

Strain code of the isolated *Bacillus* spp.	Zone of inhibition (mm)
B20	31.83±0.76[a]
B22-6	19.33±1.15[c]
B23-4	27.50±0.87[b]
B24-1	20.17±0.29[c]
B24-2	20.33±0.58[c]
B24-3	20.33±1.15[c]
B24-4	20.17±0.29[c]
B24-5	25.33±0.58[b]

Results are reported as mean ± SD. Means sharing different superscript letters (a, b, or c) in the same column are significantly different ($P < 0.05$).

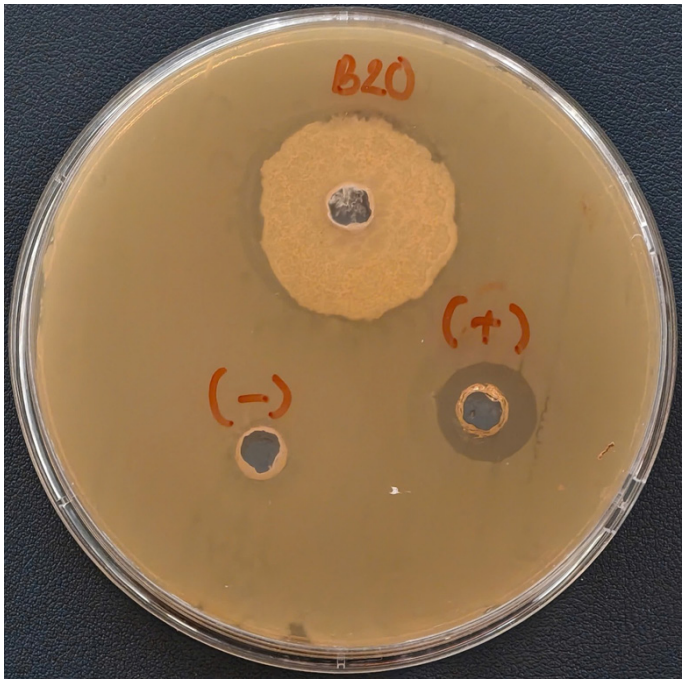

Figure 2: The antimicrobial activity of strain B20 *(Bacillus amyloliquefaciens)* against *E. coli* on agar medium.

In addition to direct antimicrobial effects, *Bacillus* spp. also possesses the ability to interfere with bacterial communication systems. The effective inhibition of *E. coli* strains harboring specific virulence genes (*iss*, *iutA*, *hlyF*, *ompT*, and *iroN*) suggests that these *Bacillus* strains may operate via a mechanism involving the disruption of the quorum sensing system. Specifically, *Bacillus* can produce lactonase enzymes (e.g., AiiA) that degrade

N-acyl homoserine lactone signaling molecules, thereby attenuating the expression of virulence factors and the biofilm-forming capacity of Gram-negative bacteria (Grandclément *et al*., 2016). This "anti-virulence" strategy is particularly valuable because it reduces pathogenicity without imposing strong selective pressures that lead to drug resistance; consequently, it is regarded as a potential alternative to traditional antibiotics.

The markedly larger inhibition zones observed in this study suggest that the isolates from Vinh Long possess enhanced antimicrobial potential. This may be explained by environmental selection pressure in field conditions, which can drive the evolution of strains with greater metabolic versatility and higher expression of antimicrobial compounds. Indeed, genomic analyses have shown that *Bacillus* strains isolated from competitive ecological niches often harbor expanded and diverse BGC repertoires, enabling the synthesis of a wide range of bioactive molecules with synergistic effects (Naser *et al*., 2025).

Furthermore, their potent inhibitory activity against multidrug-resistant *E. coli* underscores the potential of these strains as next-generation probiotics or biocontrol agents. Spore-forming *Bacillus* species possess numerous distinct advantages including high stability, resilience to adverse environmental conditions, and survivability during processing and storage making them highly suitable for application in animal husbandry (Cutting, 2011; Grant *et al*., 2018). Their dual mechanism of action encompassing both direct antimicrobial activity and modulation of the microbiota aligns with current trends aimed at reducing antibiotic usage while simultaneously ensuring animal health and productivity. Collectively, these results not only validate the robust antagonistic potential of the selected *Bacillus* strains particularly B20 and B23-4 but also provide profound insights into their mechanisms of action. The synergistic interplay of cell membrane disruption, induction of oxidative stress, interference with quorum sensing, and ecological competition likely forms the foundation of their high antimicrobial efficacy. Future research should focus on whole-genome sequencing to identify specific biosynthetic gene clusters, metabolomic analysis to characterize active compounds, and *in vivo* trials to evaluate their protective efficacy in duck flocks. These approaches will play a pivotal role in fully harnessing the potential of these strains as sustainable antibiotic alternatives in modern animal husbandry.

Identification of the isolated bacterial strains

The results of molecular identification based on *16S rRNA* gene sequencing are presented in Table 3 and Figure 3. The analysis revealed distinct species-level diversity among the selected strains exhibiting antagonistic activity, with all sequences achieving 100% nucleotide identity relative to reference strains in the GenBank database. The identified species include *Bacillus amyloliquefaciens* (B20), *Bacillus subtilis* (B23-4), *Rossellomorea marisflavi* (B24-5), and a dominant group belonging to the species *Bacillus altitudinis*. The use of 16S rRNA gene sequencing is considered the gold standard for bacterial identification, enabling reliable

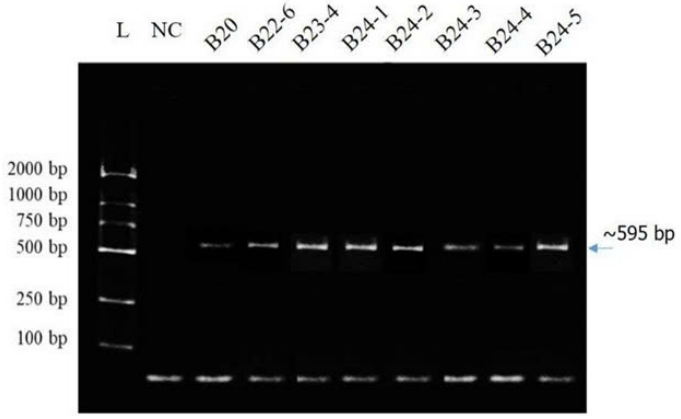

Figure 3: Agarose gel electrophoresis of 16S rRNA gene PCR products from selected *Bacillus* spp. isolates. Lane L: DNA ladder (100–2000 bp); NC: negative control; lanes B20, B22-6, B23-4, B24-1, B24-2, B24-3, B24-4, and B24-5: PCR amplicons of the 16S rRNA gene from corresponding *Bacillus* spp. isolates. A distinct band of approximately ~595 bp was observed in all tested strains.

Table 3: Molecular identification of *Bacillus* spp. using 16S rDNA sequencing.

Strain code of the isolated *Bacillus* spp.	Identified species	NCBI accession number	Nucleotide similarity (%)
B20	*Bacillus amyloliquefaciens*	CP044132.1	100
B22-6	*Bacillus altitudinis*	CP187396.1	100
B23-4	*Bacillus subtilis*	CP017763.1	100
B24-1	*Bacillus altitudinis*	CP099861.1	100
B24-2	*Bacillus altitudinis*	PQ093522.1	100
B24-3	*Bacillus altitudinis*	CP187396.1	100
B24-4	*Bacillus altitudinis*	CP099861.1	100
B24-5	*Rossellomorea marisflavi*	LC875662.1	100

classification down to the species level. The 100% sequence identity achieved in this study confirms the accuracy of the identification results and indicates that the selected strains are representative examples of their respective species. These findings align with numerous previous studies in which 16S rDNA was effectively utilized to characterize both probiotic and antagonistic bacteria (Yarza *et al*., 2014). Notably, the identification of *B. amyloliquefaciens* (B20) and *B. subtilis* (B23-4) is of particular significance, as these species are widely recognized for their potent antagonistic activity against Gram-negative bacteria, including multidrug-resistant *E. coli*. Their antimicrobial potential is primarily attributed to the presence of diverse biosynthetic gene clusters encoding antimicrobial peptides, lipopeptides, and hydrolytic enzymes, which contribute to both the direct inhibition of pathogenic bacteria and the modulation of the intestinal environment (Ishnaiwer *et al*., 2022; Palacios-Rodriguez *et al*., 2024). These characteristics position them as promising probiotic candidates for use in poultry farming. The discovery of *Rossellomorea marisflavi* (B24-5) formerly classified under the genus *Bacillus* alongside the predominance of *Bacillus altitudinis*, reflects the ecological adaptability and diversity of the duck gut microbiota in Vinh Long province. The reclassification of certain species related to *Bacillus* into new genera, such as *Rossellomorea*, highlights the current trend of updating bacterial taxonomy based on genomic data. Notably, species such as *R. marisflavi* typically associated with marine or high-salinity environments are garnering significant interest due to their potential to produce bioactive compounds and enzymes with valuable applications in livestock farming and aquaculture (Ishnaiwer *et al*., 2022; Palacios-Rodriguez *et al*., 2024). The predominance of *B. altitudinis* among the isolated strains also demonstrates this species' high adaptability to specific environmental conditions, including its capacity to withstand stress and compete within complex microbial communities. Previous studies have indicated that *B. altitudinis* possesses various functional attributes such as antimicrobial activity, enzyme production capabilities, and tolerance to adverse conditions thereby reinforcing its potential role as a probiotic or a biocontrol agent. Although 16S rRNA-based identification methods offer high reliability, they remain subject to limitations regarding the differentiation of genetically closely related species and fail to provide detailed information concerning functional genes. Consequently, to fully harness the biological potential and ensure the safety of these elite strains, future studies should employ whole genome sequencing. This approach will enable a comprehensive analysis of biosynthetic gene clusters, as well as genes associated with virulence and antibiotic resistance, thereby facilitating the effective and safe selection and application of these strains within the context of sustainable livestock farming. In summary, the results of molecular identification have confirmed the taxonomic diversity and probiotic potential of the selected bacterial strains. The presence of species exhibiting strong bioactivity such as *B. amyloliquefaciens* and *B. subtilis* alongside potential candidates like *R. marisflavi* and *B. altitudinis*, establishes a solid foundation for subsequent functional studies and for their application in antibiotic-replacement strategies within livestock farming.

Antibiotic susceptibility profile

The results regarding the antibiotic susceptibility of the *Bacillus* strains are presented in Table 4 and Figure 4. Overall, the results indicate that the majority of the strains exhibiting antagonistic activity against pathogenic *E. coli* demonstrated high levels of susceptibility to clinically important antibiotics. Notably, 100% of the strains (8/8) were fully susceptible to ceftiofur (Ce), indicating no signs of resistance to this critical group of cephalosporins. Similarly, very high susceptibility rates were also recorded for amoxicillin–clavulanate (Ac), ampicillin (Am), and gentamicin (Ge), suggesting a favorable safety profile

Table 4: Antibiotic susceptibility testing of *Bacillus* spp. strains capable of inhibiting pathogenic *E. coli.*

Strain code of the isolated *Bacillus* spp.	Zone of inhibition (mm)						
	Ef	Ge	Bt	Te	Am	Ce	Ac
B20	21.33±0.58 (S)	18.67±0.58 (S)	8.83±0.76 (R)	9.67±0.58 (R)	14.67±0.58 (I)	24.67±0.58 (S)	19.67±0.58 (S)
B23-4	16.33±0.58 (I)	18.33±0.58 (S)	13.33±0.58 (I)	13.83±0.29 (I)	20.33±0.58 (S)	24.67±0.58 (S)	19.67±0.58 (S)
B24-5	24.33±0.58 (S)	18.33±0.58 (S)	13.17±0.29 (I)	16.67±0.58 (S)	20.67±0.58 (S)	22.33±0.58 (S)	20.67±0.58 (S)
B24-1	23.33±0.58 (S)	15.67±0.58 (I)	9.67±0.58 (R)	17.33±0.58 (S)	19.67±0.58 (S)	23.33±0.58 (S)	20.67±0.58 (S)
B24-2	23.67±0.58 (S)	17.67±0.58 (S)	11.3±0.58 (I)	10.67±0.58 (R)	20.33±0.58 (S)	24.67±0.58 (S)	16.33±0.58 (I)
B24-3	16.67±0.58 (I)	18.67±0.58 (S)	8.67±0.58 (R)	9.33±0.58 (R)	20.33±0.58 (S)	24.67±0.58 (S)	19.00±1.00 (S)
B24-4	16.33±5.51 (S)	19.00±1.00 (S)	14.67±0.58 (I)	16.67±0.58 (S)	18.67±0.58 (S)	25.67±0.58 (S)	19.67±0.58 (S)
B22-6	13.00±1.00 (R)	18.17±0.29 (S)	11.33±0.58 (R)	10.33±0.58 (R)	19.67±0.58 (S)	23.33±0.58 (S)	18.67±0.58 (S)

Results are reported as mean ± SD. Ef: enrofloxacin; Ge: gentamicin; Bt: trimethoprim-sulfamethoxazole; Te: tetracycline; Am: ampicillin; Ce: ceftiofur; Ac: amoxicillin–clavulanate. S: Susceptible; I: Intermediate; R: Resistant.

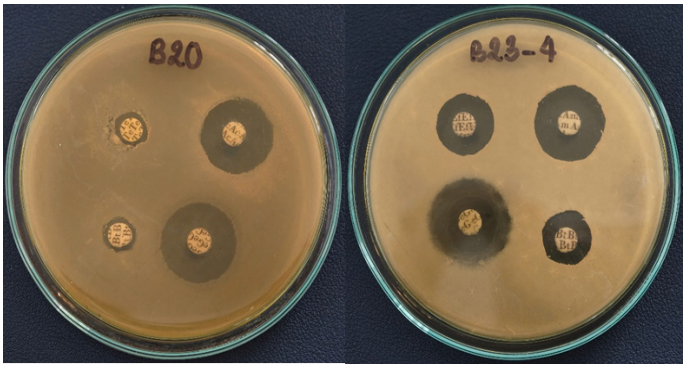

Figure 4: Antibiotic susceptibility profiles of *Bacillus* strains B20 *(Bacillus amyloliquefaciens)* and B23-4 *(Bacillus subtilis)* determined by disk diffusion assay. B20 was resistant to trimethoprim-sulfamethoxazole and tetracycline, but susceptible to amoxicillin-clavulanate and gentamicin. B23-4 was susceptible to ampicillin and gentamicin, with intermediate responses to enrofloxacin and trimethoprim-sulfamethoxazole.

for these strains. Among the strains examined, B24-4 and B24-5 exhibited the broadest susceptibility spectrum, being susceptible to almost all tested antibiotics and showing no distinct signs of multi-drug resistance or cross-resistance. Conversely, certain strains such as B20 and B24-3 demonstrated resistance to trimethoprim-sulfamethoxazole (Bt) and tetracycline (Te); this suggests that some resistance characteristics may still persist in certain strains, potentially linked to environmental conditions or intrinsic resistance mechanisms. From a biosafety perspective, the high level of antibiotic susceptibility observed in the strains within this study constitutes a crucial criterion for potential probiotic candidates. According to guidelines issued by the European Food Safety Authority (EFSA, 2024), microorganisms utilized in animal feed must not harbor antibiotic resistance genes capable of horizontal transfer to antibiotics deemed critical in veterinary and human medicine. Consequently, the antibiotic susceptibility profiles documented in this study reinforce the potential for the safe application of these *Bacillus* strains as probiotics or biocontrol agents. These results align with previous studies, which indicate that *Bacillus* strains isolated from healthy livestock typically exhibit high susceptibility to the majority of antibiotics (Cutting, 2011).

Mechanistically, the observed antibiotic susceptibility phenotype may reflect the genomic characteristics of these strains. Unlike many pathogenic bacteria, probiotic *Bacillus* strains typically do not harbor mobile genetic elements such as plasmids or transposons that carry antibiotic resistance genes; this effectively reduces the risk of resistance gene dissemination via horizontal gene transfer (Sorokulova, 2008). However, the resistance observed in certain strains against specific antibiotics such as tetracycline and sulfonamides may be attributed to intrinsic resistance mechanisms (including efflux pumps, target modification, or enzymatic inactivation), rather than the acquisition of resistance genes from external sources (EFSA, 2012).

A noteworthy observation is the combination of potent antimicrobial activity and high antibiotic susceptibility exhibited by the strains under investigation. This characteristic suggests that their ability to inhibit *E. coli* is not linked to conventional antibiotic resistance mechanisms, but is primarily mediated through the production of bioactive compounds, such as lipopeptides and antimicrobial peptides. This finding holds significant importance, as it helps mitigate the risk of co-selection and the subsequent dissemination of antibiotic resistance genes within the environment (Martinez, 2009). Furthermore, the absence of multidrug resistance in the majority of the strains further reinforces their potential for application in livestock production systems that aim to reduce or eliminate antibiotic usage. In the context of antibiotic resistance emerging as a global crisis, the identification of probiotic strains that possess both potent antimicrobial activity and a robust safety profile is of paramount importance. Spore-forming *Bacillus* species possess inherent advantages regarding stability and resilience, making them a suitable choice for inclusion in animal feed formulations (Bahaddad *et al*., 2023).

However, it is important to note that assessments based solely on phenotypic antibiotic susceptibility are insufficient to draw definitive conclusions regarding biosafety. Subsequent studies should employ whole-genome sequencing to detect potential resistance genes, virulence factors, and mobile genetic elements. Genomic-level analysis will provide a more comprehensive insight into the safety and functional potential of these strains, while simultaneously ensuring compliance with international standards. The antibiotic susceptibility profiles of the selected *Bacillus* strains demonstrate favorable biosafety characteristics and reinforce their potential for application as probiotics. The combination of strong antagonistic activity and high antibiotic susceptibility establishes a solid foundation for the development of sustainable antibiotic alternatives in poultry farming.

Antibacterial activity of cell-free supernatants

The antibacterial activity of cell-free supernatants (CFS) derived from various *Bacillus* spp. strains against multidrug-resistant *E. coli* is presented in Table 5 and Figure 5. The results reveal distinct differences in inhibitory potential among the strains examined. Notably, *Bacillus amyloliquefaciens* B20 demonstrated the strongest antibacterial activity, exhibiting the lowest MIC and MBC

values of 0.98 µg/mL and 1.95 µg/mL, respectively. Similarly, *Bacillus subtilis* B23-4 also exhibited high antibacterial efficacy, with MIC and MBC values of 1.95 µg/mL and 3.91 µg/mL, respectively. Conversely, strains belonging to the species *Bacillus altitudinis* (B22-6, B24-1, B24-2, B24-3, and B24-4) exhibited significantly higher MIC values, ranging from 15.63 to 31.25 µg/mL, indicating lower antibacterial efficacy. Notably, *Rossellomorea marisflavi* B24-5 also demonstrated significant antibacterial activity, with an MIC of 3.91 µg/mL and an MBC of 7.81 µg/mL, indicating relatively strong inhibitory potential despite belonging to a less-studied genus.

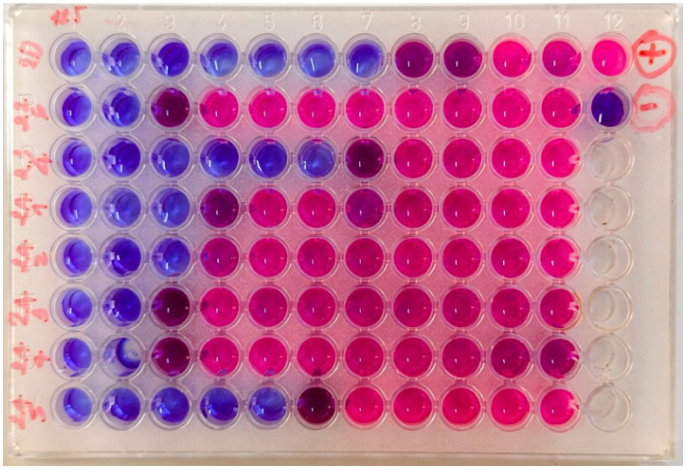

Figure 5: Minimum inhibitory concentration (MIC) of cell-free supernatants (CFS) from *Bacillus* spp. against *E. coli* determined by a resazurin-based broth microdilution assay in a 96-well plate. CFS were twofold serially diluted and incubated with *E. coli*. Blue indicates inhibition, while pink indicates growth. The MIC was defined as the lowest concentration maintaining the blue color.

Table 5: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of cell-free supernatants (CFS) from *Bacillus* spp. against *E. coli.*

Stain code	Identified species	MIC (µg/mL)	MBC (µg/mL)	MBC/ MIC ratio
B20	*Bacillus amyloliquefaciens*	0.98	1.95	2
B22-6	*Bacillus altitudinis*	31.25	62.50	2
B23-4	*Bacillus subtilis*	1.95	3.91	2
B24-1	*Bacillus altitudinis*	15.63	62.50	4
B24-2	*Bacillus altitudinis*	15.63	62.50	4
B24-3	*Bacillus altitudinis*	31.25	62.50	2
B24-4	*Bacillus altitudinis*	31.25	62.50	2
B24-5	*Rossellomorea marisflavi*	3.91	7.81	2

The MBC/MIC ratio for most strains particularly B20, B23-4, and B24-5 was equal to 2, suggesting that the antibacterial compounds within the CFS exert a bactericidal effect (killing bacteria) rather than merely inhibiting growth (a bacteriostatic effect). In contrast, strains B24-1 and B24-2 exhibited a higher ratio (4), indicating weaker bactericidal activity.

This study demonstrates that CFS derived from *Bacillus* spp. strains particularly *B. amyloliquefaciens* B20 and *B. subtilis* B23-4 exhibit potent antimicrobial activity against multidrug-resistant *E. coli*. The exceptionally low MIC values recorded indicate the presence of highly active bioactive compounds, thereby validating the potential of these strains for application as alternative antimicrobial agents. A key finding of this study is the observed MBC/MIC ratio of 2 in the elite strains, which substantiates a bactericidal mechanism of action. This is particularly significant in the context of controlling multidrug-resistant bacteria, as bactericidal agents are generally more effective at completely eradicating highly virulent pathogens (Pankey and Sabath, 2004). In this study, the *E. coli* strain utilized harbored multiple virulence genes (*iss*, *iutA*, *hlyF*, *ompT*, *iroN*) and exhibited resistance to various antibiotics; nevertheless, it remained highly susceptible to the CFS, thereby highlighting the superior antimicrobial efficacy of the metabolites produced by these *Bacillus* strains.

These findings align with the research conducted by Tran *et al.* (2023), in which *Bacillus subtilis* strains were demonstrated to possess a broad antimicrobial spectrum and low MIC values against a wide range of pathogenic bacteria. This further reinforces the role of *Bacillus* species as promising biocontrol agents within the field of veterinary medicine.

The potent antimicrobial mechanism of CFS particularly in strain B20 can be attributed to the presence of a complex mixture of bioactive compounds, including antimicrobial peptides (AMPs) and cyclic lipopeptides such as surfactin, iturin, and fengycin. These compounds exert a multi-target mechanism of action that is fundamentally distinct from that of traditional antibiotics (Caulier *et al.*, 2019). Unlike conventional antibiotics, which target specific enzymes or metabolic pathways, these lipopeptides interact directly with the phospholipid bilayer of bacterial cell membranes. This interaction induces pore formation, leading to the leakage of ions and intracellular components, and ultimately resulting in cell lysis. This physicochemical mechanism significantly mitigates the potential for resistance development, as bacteria find it exceedingly difficult to alter their membrane structure without compromising their viability (Markelova and Chumak, 2025). This mechanism explains why CFS remains highly effective against *E. coli* strains resistant to eight different antibiotics. Common resistance mechanisms such as efflux pumps, enzymatic degradation, or target modification are rendered virtually ineffective against compounds that function by disrupting the cell membrane. Another

noteworthy finding concerns the activity of *Rossellomorea marisflavi* B24-5. Although its MIC was higher than that of B20, this strain nonetheless demonstrated potent inhibitory activity. This is particularly significant given that species belonging to the genus *Rossellomorea* typically inhabit marine or saline environments habitats where microorganisms tend to produce unique, highly active bioactive compounds. These results suggest the existence of novel, untapped biosynthetic pathways, thereby opening up promising avenues for future research (Zhang *et al*., 2025). The observed variations in activity among different *Bacillus altitudinis* strains also indicate that their antimicrobial potential is strain-specific rather than species-specific. This phenomenon may be attributed to differences in secondary metabolite biosynthetic gene clusters, underscoring the critical importance of strain-level selection in research involving probiotics and antimicrobial agents.

From an applied perspective, the study results suggest that CFS derived from *Bacillus* strains could be developed as a novel postbiotic strategy for controlling multidrug-resistant bacteria in livestock production. Given their potent bactericidal activity and low propensity for inducing drug resistance, these compounds are particularly well-suited for use in poultry and duck farming sectors where the issue of antibiotic resistance is becoming increasingly acute. Overall, *Bacillus* spp. strains particularly *B. amyloliquefaciens* B20 and *B. subtilis* B23-4 produce extracellular metabolites exhibiting potent bactericidal activity against multidrug-resistant *E. coli*. The low MIC values, coupled with favorable MBC/MIC ratios, suggest a high potential for practical application. Concurrently, the activity displayed by *R. marisflavi* opens up new avenues for research into novel bioactive compounds.

CONCLUSIONS

This study demonstrated that selected *Bacillus* spp. from duck production systems exhibit strong antibacterial activity against multidrug-resistant *E. coli*. Notably, *Bacillus amyloliquefaciens* B20 and *Bacillus subtilis* B23-4 showed the highest efficacy, with low MIC values and bactericidal effects. Their cell-free supernatants contain potent bioactive compounds capable of eliminating virulent *E. coli*. Additionally, *Rossellomorea marisflavi* B24-5 highlights the potential of non-traditional taxa as novel antimicrobial sources. Overall, *Bacillus*-derived cell-free supernatants represents a promising postbiotic strategy for controlling multidrug-resistant pathogens in poultry systems. Further studies, including whole-genome sequencing and in vivo validation, are recommended to confirm their mechanisms and practical applications.

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NOVELTY STATEMENT

This study is the first to identify indigenous *Bacillus* spp. from duck production systems with strong bactericidal activity against multidrug-resistant *Escherichia coli* carrying multiple virulence genes (*iss*, *iutA*, *hlyF*, *ompT*, and *iroN*). Notably, *B. amyloliquefaciens* B20 and *B. subtilis* B23-4 showed the highest efficacy, with low MIC values and clear bactericidal effects. Their cell-free supernatants likely contain antimicrobial peptides and cyclic lipopeptides that disrupt bacterial membranes. In addition, *R. marisflavi* B24-5 demonstrated considerable activity, highlighting the potential of non-traditional taxa as novel antimicrobial sources. These findings support a new postbiotic strategy for controlling multidrug-resistant pathogens in poultry.

AUTHOR'S CONSTRIBUTION

NVV was responsible for study conception and experimental design. NVV and HMH carried out the experiments and analyzed the data. NVV, NTL, and HMH prepared the manuscript. All authors critically reviewed and approved the final manuscript.

Generative AI and AI-assisted technology statement

The authors declare that ChatGPT was utilized solely for language refinement. All scientific content was critically reviewed, revised, and validated by the authors to ensure accuracy and originality.

Conflict of interest

The authors have declared no conflict of interest.

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