Journal of Science and Technology – Engineering and Technology for Sustainable Development
Volume 36, Issue 2, April 2026, 018-027

Assessment of Cefotaxime Susceptibility and Resistance Gene Transfer Risk between *Bacillus* Strains and *Escherichia coli* Ec457

Bui Hoang Ha[1], Dam Thuy Hang[1], Luu Quynh Huong[2], Ho Phu Ha[1,*]
[1]Hanoi University of Science and Technology, Ha Noi, Vietnam
[2] National Institute of Veterinary Research, Ha Noi, Vietnam
[*]Corresponding author email: ha.hophu@hust.edu.vn

Abstract

Antibiotic resistance in husbandry remains a major global challenge due to the extensive use of antimicrobials in animal production. Probiotics with genetically stable profiles are therefore considered promising alternatives to reduce antibiotic reliance and limit resistance dissemination. This study evaluated the susceptibility of three Bacillus strains: P4QN11, BAD7, and S2.5, and Escherichia coli Ec457 to cefotaxime, and investigated the potential transfer of the bla-CTX-M resistance gene. Disk diffusion assays showed that Bacillus P4QN11, BAD7, and S2.5 had large inhibition zones of 26 to 27 millimeters, indicating high susceptibility, while E. coli Ec457 exhibited a small inhibition zone of 13 ± 1 millimeters, confirming resistance. Cefotaxime resistance of E. coli Ec457 was evidenced by the presence of the blaCTX-M gene detected by PCR. Co-cultivation experiments under selective pressure with LiCl and cefotaxime tested whether Bacillus strains could acquire resistance genes. After repeated co-culture, Bacillus P4QN11, BAD7, and S2.5 remained phenotypically susceptible in disk diffusion tests, and PCR showed no blaCTX-M amplification, confirming gene transfer was not detected. These results demonstrated that the Bacillus strains do not acquire cefotaxime resistance genes from E. coli Ec457, even under selective conditions. This supports their genetic stability and safety for use as probiotics in food and animal feed, help to reduce the spread of antibiotic resistance. Further research should explore longer co-culture periods and genomic analysis to confirm these findings under diverse environmental conditions.

Keywords: Antibiotics, animal feed, *blaCTX-M gene*, *Bacillus* strain.

1. Introduction

Antibiotics continue to be extensively used in animal husbandry worldwide, not only for therapeutic purposes but also for disease prevention and growth promotion. In Vietnam, it has been reported that up to 43.7% of commercial animal feed products contain at least one antibiotic, while more than 21.5% and 5.4% of industrial feed for pigs and poultry, respectively, contain multiple antibiotics [1]. The misuse and overuse of antibiotics in livestock production have accelerated the emergence of antibiotic-resistant bacterial strains, undermining treatment efficacy, complicating disease control, and posing significant risks to public health through the food chain.

In response to these challenges, the search for effective and safe alternatives to antibiotics in animal feed has become increasingly urgent. Among various strategies, the use of probiotics, particularly beneficial bacteria belonging to the genus *Bacillus*, has gained considerable attention. *Bacillus* is Gram-positive, low G+C content, aerobic, and rod-shaped bacteria capable of forming endospores and motile via flagella. Their spores exhibit high resistance to extreme environmental conditions, including low gastric pH, which allows them to survive gastrointestinal transit and reach the small intestine, where they can exert health-promoting effects. Several species, such as *B. amyloliquefaciens*, *B. coagulans*, *B. clausii*, *B. cereus*, *B. subtilis*, and *B. licheniformis*, have been developed and applied as probiotic feed additives in livestock production [2].

However, there is growing concern about the antibiotic resistance of some *Bacillus* strains, especially those exposed to antibiotic-rich environments like feed and commercial probiotic products. Antibiotic resistance in *Bacillus* can be intrinsic or acquired through horizontal gene transfer mediated by mobile genetic elements such as plasmids and transposons [3]. Moreover, certain *Bacillus* species, including *B. subtilis* and *B. licheniformis*, are naturally competent for transformation, allowing them to take up and incorporate exogenous DNA under stress conditions, such as nutrient limitation or the presence of antibiotics [4, 5]. This process increases their genetic diversity and the potential dissemination of resistance genes within microbial communities. Several studies have reported the detection of antibiotic resistance genes in commercial *Bacillus*-based probiotics used for both humans and animals, raising concerns about the possible spread of resistance through the food chain and the environment [6, 7]. The use of antibiotic-resistant *Bacillus* strains may disrupt the gut microbiota and promote the transfer of resistance genes to pathogenic bacteria,

p-ISSN 3093-3242
e-ISSN 3093-3579
https://doi.org/10.51316/jst.189.etsd.2026.36.2.3
Received: Sep 20, 2025; Revised: Dec 1, 2025;
Accepted: Dec 3, 2025; Online: Mar 23, 2026.

In press

ultimately diminishing treatment effectiveness and increasing the risk of antimicrobial resistance spreading to humans via the food chain or direct contact [8]. Therefore, it is essential to carefully evaluate the antibiotic susceptibility and the potential for horizontal gene transfer of *Bacillus* strains intended for probiotic use in animal feed.

Cefotaxime, a third-generation cephalosporin antibiotic with broad-spectrum activity, is still widely used in livestock production. Recent studies have reported the emergence of *Bacillus* strains resistant to cefotaxime [3].

In this study, we aimed to screen and select *Bacillus* strains that exhibit high susceptibility to antibiotics and do not carry, acquire, or transfer cefotaxime resistance genes, such as *bla*-$_{\text{CTX-M}}$, thereby ensuring their safety as potential probiotics for application in livestock production. To achieve this, we cultured the strains and applied molecular biology methods to comprehensively assess the ability of these *Bacillus* strains to act as donors or recipients of resistance genes through horizontal gene transfer. This integrated approach enables the identification of safe and effective probiotic candidates that can help reduce the spread of antibiotic resistance in livestock farming.

2. Materials and Methods

2.1. Sample Collection

Bacillus strains P4QN11, BAD7, and S2.5, which were previously isolated and screened for probiotic characteristics such as survival in the gastrointestinal tract, production of substrate- degrading enzymes, and antimicrobial activity, along with *Escherichia coli* strain Ec457 (harbouring the *bla*$_{\text{CTX-M}}$ gene conferring resistance to cefotaxime) were obtained from the National Institute of Veterinary Research. The bacterial strains were maintained on LB agar plates at 4 °C for short-term preservation and in LB broth supplemented with 30% (v/v) glycerol at -20 °C for long-term preservation.

2.2. Bacterial Count

The cell density of the *Bacillus* strains was determined by the standard plate count method after serial dilution [9]. *Bacillus* strains and the *E. coli* strain Ec457 were cultured in LB broth using a shaking incubator at 37 °C, 6000 x *g* for 16–20 hours. Following incubation, the cultures were serially diluted until the expected colony count on agar plates ranged ranged from 20 to 200 colonies.

A 100 µL aliquot of each appropriate dilution was spread onto the surface of sterile LB agar plates and incubated at 37 °C for 16–20 hours. Colonies were then counted, and the average cell density (*N*) was calculated using the following formula:

$$N = \frac{\sum C}{(n1+0,1n2).f1.V}\ (CFU/mL) \quad (1)$$

where:

C is total number of colonies counted across all plates

n_1 is number of plates counted at the first selected dilution

n_2 is number of plates counted at the second selected dilution

f_1 is dilution factor corresponding to the first selected dilution

V is volume of inoculum plated on each agar plate (in milliliters)

2.3. Assay of Antibiotic Susceptibility to Cefotaxime

The susceptibility of the bacterial strains to cefotaxime was determined using the Bauer–Kirby disk diffusion method [10]. First, Mueller-Hinton Agar (MHA) plates were prepared. Pure colonies of *Bacillus* strains and the *E. coli* strain Ec457 were inoculated into sterile distilled water and adjusted to a turbidity equivalent to the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL).

Using sterile cotton swabs, the bacterial suspensions were evenly spread over the surface of the MHA plates. Cefotaxime-impregnated paper disks were then placed onto the inoculated plates, which were maintained at room temperature for 30 minutes before incubation at 37 °C for 20–24 hours. After incubation, inhibition zone diameters were measured to determine susceptibility levels. The interpretive used criteria are descirbed in Table 1.

Table 1. Zone diameter breakpoint

Strain	Antibiotic disk content	Susceptible (mm)	Intermediate (mm)	Resistant (mm)
***E. coli* [11]**	30 µg/disk	≥ 23	15 - 22	≤ 14
***Bacillus* spp.* [12]**		≥ 22	-	≤ 21

* Since specific breakpoints for *Bacillus* spp. are not available in CLSI guidelines, and because *Bacillus* are Gram-positive, spore-forming bacteria with growth characteristics similar to *Staphylococcus* spp., the breakpoints established for *Staphylococcus* spp. were used for interpretation.

press

Bacillus strains identified as susceptible were selected for subsequent experiments to ensure that they did not inherently carry resistance genes. This method was also applied to re-evaluate susceptibility after conjugation experiments.

2.4. Co-Cultivation Assay for Cefotaxime Resistance Gene Transfer from E. coli Ec457 to Bacillus Strains

The *Escherichia coli* strain Ec457, known to harbor the cefotaxime resistance gene on a plasmid as confirmed by molecular biology methods, was used as the donor strain. The ability of *Bacillus* strains to acquire the resistance gene was assessed through conjugation experiments.

Three types of agar media were prepared: M1, consisting of Mueller-Hinton Agar (MHA) supplemented with LiCl (10g/L) for selective growth of *Bacillus*; M2, MHA supplemented with antibiotics (AB) for selective growth of *E. coli* Ec457; and M3, MHA supplemented with both LiCl and the antibiotic cefotaxime to suppress both strains and to detect *Bacillus* recipients that acquired the resistance gene. Pure colonies of each strain were suspended in 1 mL sterile distilled water and adjusted to a turbidity equivalent to the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL), followed by further dilution to approximately 10^6 CFU/mL. Then, 100 µL of each *Bacillus* and *E. coli* Ec457 culture was mixed into 9,800 µL of LB (Luria-Bertani) broth, vortexed thoroughly, and incubated at 37 °C with shaking at 6000 x g for 24 hours.

After incubation, the mixture was serially diluted and spread onto M1, M2, and M3 agar plates for colony counting. Two control samples were processed in parallel under the same conditions: one containing only *Bacillus* strains and the other only *E. coli* Ec457, to evaluate individual growth on respective selective media. The conjugation frequency of *Bacillus* strains following incubation (and confirmed gene transfer) was calculated as:

$$Conjugation\ frequency = \frac{Cell\ density\ of\ transconjugants\ (\mathrm{CFU/mL})}{\mathrm{Cell\ density\ of\ recipient\ cells\ (CFU/mL)}} \quad (2)$$

where the cell density of transconjugants corresponds to the number of *Bacillus* colonies growing on M3 medium, and the cell density of recipients (initial *Bacillus*) was determined using the formula:

$$N2 = \frac{\sum C2}{(n1 + 0.1n2).\ f1.\ V}\ (CFU/mL) \quad (3)$$

where $C2$ is the total number of colonies on M1 plates, $n1$ and $n2$ are the numbers of plates counted at the first and second selected dilutions respectively, $f1$ is the dilution factor for the first dilution, and V is the inoculum volume (mL).

2.5. Molecular Methods for Detecting the Transfer and Acquisition of Antibiotic Resistance Genes

2.5.1. DNA extraction

Genomic DNA was extracted following the method described by William S. *et al*. [13], with minor modifications. Bacterial cultures grown for 20 hours were centrifuged at 6000 x *g* for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 740 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Then, 20 µL of lysozyme (100 mg/mL) was added, and the mixture was incubated at 37 °C for 1 hour. Subsequently, 40 µL of 10% SDS and 4 µL of proteinase K (20 mg/mL) were added, followed by incubation at 56 °C for 1 hour. Next, 100 µL of 5 M NaCl and 100 µL of CTAB/NaCl solution were added, and the mixture was incubated at 65 °C for 30 minutes. The solution was then extracted with chloroform: isoamyl alcohol (24:1), centrifuged at 6000 x *g* for 10 minutes at 4 °C, and the aqueous phase collected. The extraction step was repeated once more. DNA was precipitated by adding cold isopropanol (−20 °C) and incubating for 2 hours, followed by centrifugation at 6000 x *g* for 15 minutes at 4 °C. The pellet was washed with cold 70% ethanol and centrifuged again for 10 minutes. Finally, the DNA pellet was air dried and dissolved in 25 µL of DNase- free water for storage.

2.5.2. PCR amplification

PCR reactions were carried out in a total volume of 15 µL, containing 7.5 µL of 2x Onetaq PCR Master Mix (New England Biolabs, USA), 4.8 µL of nuclease- free water, 0.6 µL each of forward and reverse primers, and 1.5 µL of template DNA. The primer pair used was KCP 685 (5'-TTTGCGATGTGCAGTACCAGTAA-3') and KCP 686(5'-CGATATCGTTGGTGGTGCCATA-3'), specific for the $bla_{\text{CTX-M}}$ gene, yielding a PCR product of 544 bp [14]. The thermal cycling profile consisted of an initial denaturation at 94 °C for 3 minutes; 35 cycles of denaturation at 94 °C for 15 seconds, annealing at 58 °C for 45 seconds, and extension at 68 °C for 35 seconds; followed by a final extension at 68 °C for 8 minutes.

2.5.3. Agarose gel electrophoresis

PCR products were analyzed by electrophoresis on a 2% agarose gel supplemented with 1% SYBR™ Safe dye. Samples and a 100 bp DNA ladder (Cleaver) were loaded into wells, and electrophoresis was conducted in 1X TAE buffer at 100 V for 30 minutes. Bands were visualized under UV illumination.

2.6. Data Analysis

All experiments were performed in triplicate to ensure accuracy. Data were processed using Microsoft Excel 2019 and presented as mean plus/minus standard deviation.

JST press

3. Results and Discussion

3.1. Evaluation of Cefotaxime Susceptibility in Bacillus spp. and E. coli Ec457 Using the Disk Diffusion Method

Following 24 hours of incubation, the susceptibility of *Bacillus* spp. and *Escherichia coli* Ec457 to cefotaxime was assessed, with the results summarized in Fig. 1 and Table 2. Fig. 1 depicts four Mueller–Hinton Agar (MHA) plates corresponding to the tested strains: *E. coli* Ec457, *Bacillus* P4QN11, BAD7, and S2.5. Each plate was uniformly inoculated with bacterial suspension, and a single cefotaxime disk (30 µg) was placed centrally. The clear zones surrounding the antibiotic disks-termed zones of inhibition-represent areas where bacterial proliferation was effectively suppressed by the antimicrobial agent [15]. Notably, the *E. coli* Ec457 strain exhibited an inhibition zone measuring only 13 ± 1 mm in diameter. This relatively small zone falls below the established clinical breakpoint for cefotaxime susceptibility, thereby indicating a pronounced resistance phenotype [11]. The limited extent of growth inhibition suggests that cefotaxime exerts minimal antibacterial effect against this strain under the tested conditions. This resistance is likely associated with the presence of β lactamase genes, such as *bla*-$_{\text{CTX-M}}$, which is commonly detected among multidrug-resistant *E. coli* isolates [16].

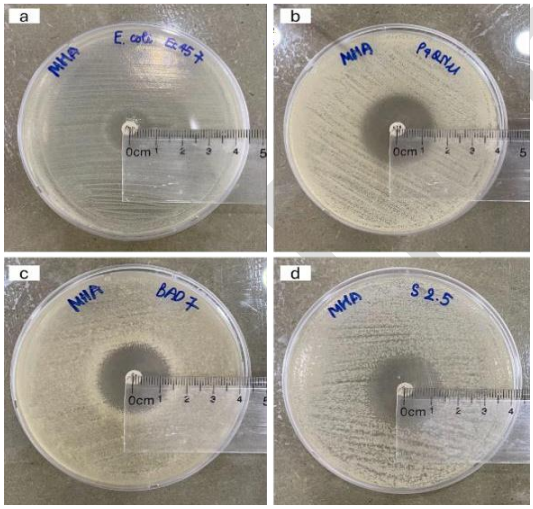

Fig. 1. Antibacterial zone diameter of (a) *E.coli* Ec457 (b) *Bacillus* P4QN11 (c) *Bacillus* BAD7 (d) *Bacillus* S2.5

In contrast, the remaining three *Bacillus* strains exhibited high susceptibility to cefotaxime, as evidenced by the inhibition zone diameters of 26 ± 2 mm for strain P4QN11, 26 ± 2 mm for strain BAD7, and 27 ± 1 mm for strain S2.5 (Table 2). These values exceed the established susceptibility breakpoints, indicating that the antibiotic demonstrated clear antibacterial efficacy against these strains. Nevertheless, to accurately elucidate the relationship between phenotypic resistance and underlying genetic determinants, it is necessary to investigate the presence of cefotaxime resistance genes using molecular biology techniques.

Table 2. Diameter of the Inhibition Zone for cefotaxime of four strains

No.	Strain	Inhibition Zone Diameter (mm)	Interpretation
1	*E. coli* Ec457	13 ± 1	Resistant
2	P4QN11	26 ± 2	Susceptible
3	BAD7	26 ± 2	Susceptible
4	S2.5	27 ± 1	Susceptible

3.2. Detection of Cefotaxime Resistance Genes in Bacillus spp. and E. coli Ec457 Using Molecular Biology Techniques

Following phenotypic susceptibility testing, the *Bacillus* strains and *E. coli* Ec457 were cultured to obtain sufficient biomass. Genomic DNA was then extracted, and polymerase chain reaction (PCR) was performed to detect the presence of antibiotic resistance genes associated with cefotaxime.

The results of electrophoresis of the PCR products on a 2% agarose gel showed that the positive control, *E. coli* Ec457, produced a distinct DNA band of approximately 544 bp, corresponding to the expected size of the target gene [16], In contrast, no band was observed in the negative control, confirming that the PCR reaction was free from cross-contamination and therefore reliable (Fig. 2).

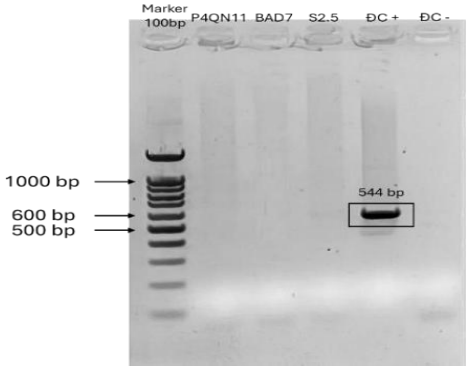

Fig. 2. Detection of *bla*-$_{\text{CTX-M}}$ gene in strains before co-culture

For the tested *Bacillus* strains P4QN11, BAD7, and S2.5, no amplification product was detected. This finding indicates that *E. coli* Ec457 carries the cefotaxime resistance gene, whereas the *Bacillus* strains do not harbor this gene. These results are entirely consistent with those obtained from the disk diffusion method described in Section 3.1, demonstrating high reliability and providing a solid foundation for subsequent experiments.

3.3. Evaluation of Gene Donor and Recipient Potential of Bacillus spp. and E. coli Ec457

In this experiment, *Bacillus* strains P4QN11, BAD7 and S2.5 together with *E. coli* Ec457 (used as the positive control) were cultured in selective media, namely MHA supplemented with LiCl for *Bacillus* strains and MHA supplemented with antibiotics for *E. coli* Ec457 strain to evaluate their growth performance and potential role as gene donors or recipients.

The results showed that all three *Bacillus* strains used as the positive controls exhibited good growth, with cell densities of $(1.86 \pm 0.15) \times 10^7$ CFU/mL for strain P4QN11, $(1 \pm 0.61) \times 10^8$ CFU/mL for strain BAD7, and $(4.8 \pm 0.54) \times 10^7$ CFU/mL for strain S2.5. Among these, BAD7 demonstrated the highest cell density, indicating superior growth performance.

The *E. coli* Ec457 strain also achieved a cell density of $(1.02 \pm 0.1) \times 10^6$ CFU/mL, confirming its robust growth and suitability as a positive control representing *E. coli* Ec457.

Overall, these strains showed stable growth and can be considered reliable standards for subsequent evaluations in this study.

When co-cultivating *Bacillus* P4QN11 with *E. coli* Ec457 on MHA medium supplemented with LiCl exhibited a strong inhibitory effect on *E. coli*. meanwhile, *Bacillus* P4QN11 continued to grow well, achieving a cell density of $(1.26 \pm 0.3) \times 10^7$ CFU/mL (Table 4).

In contrast, on MHA supplemented with antibiotic, *E. coli* exhibited poor growth with a cell density of $(2.7 \pm 0.13) \times 10^5$ CFU/mL, while *Bacillus* growth was inhibited. When LiCl and antibiotic were combined, *E. coli* failed to grow, and only a few small *Bacillus* colonies appeared, indicating a synergistic inhibitory effect of the two agents at a concentration of 10^{-1}.

To evaluate bacterial survival under selective conditions, co-cultures of Escherichia coli Ec457 and Bacillus P4QN11 were plated on selective media supplemented with LiCl and cefotaxime at dilutions ranging from 10^{-1} to 10^{-3}. Fig. 3 illustrates the results after 24 hours of incubation at 37 °C. For each dilution, duplicate plates were prepared to ensure reproducibility. The results showed that a few small colonies appeared at the 10^{-1} dilution, while no colonies were observed at 10^{-2} or 10^{-3}. This indicates a strong inhibitory effect of the LiCl–cefotaxime combination at lower dilutions on both bacterial species. To further identify the small colonies observed on the plates, they were subjected to Gram staining. The results revealed Gram-positive characteristics typical of Bacillus, and no Gram negative cells, which are characteristic of *E. coli*, were detected. This confirms that the small colonies are indeed Bacillus. These colonies were subsequently cultured in liquid medium and re-streaked onto nutrient agar plates. The resulting colonies were uniform and displayed the typical morphology of Bacillus, confirming that no *E. coli* survived on MHA supplemented with LiCl and cefotaxime. The antibiotic susceptibility/resistance phenotype of the observed colonies will be investigated in sections 3.4 and 3.5.

Table 3. Bacterial growth performance

No.	Culture media	Strain	Cell density (CFU/mL)
1	MHA + LiCl + AB	P4QN11	$(1.86 \pm 0.15) \times 10^7$
2		BAD7	$(1 \pm 0.61) \times 10^8$
3		S2.5	$(4.8 \pm 0.54) \times 10^7$
4		Ec457	$(1.02 \pm 0.1) \times 10^6$

Table 4. Cell density of *Bacillus* P4QN11 strain after co-cultivation with *E. coli* Ec457

No.	Culture medium	The microbial strain grows	Cell density (CFU/mL)
1	MHA + LiCl	*Bacillus* P4QN11	$(1.26 \pm 0.3) \times 10^7$
2	MHA + AB	*E. coli* Ec457	$(2.7 \pm 0.13) \times 10^5$
3	MHA + LiCl + AB	*Bacillus* P4QN11	Small colonies appeared

In press

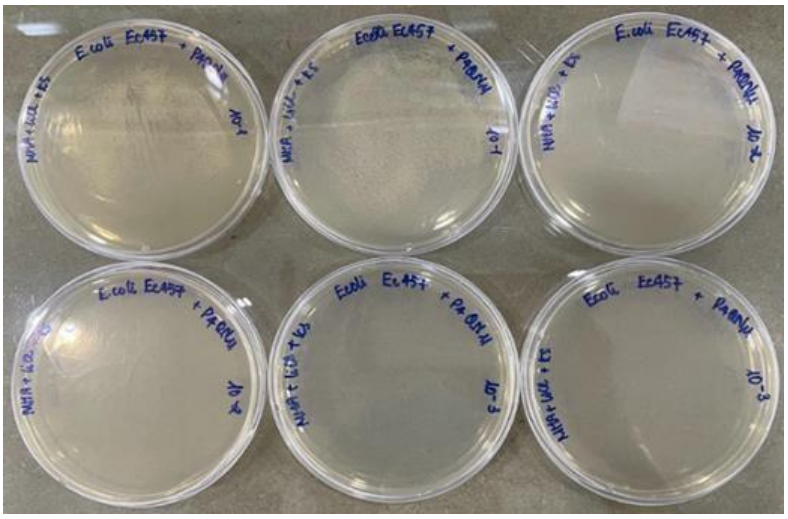

Fig. 3. Results of plating of *Bacillus* P4QN11 strain after co-culture

Table 5. Cell density of *Bacillus* BAD 7 strain after co-cultivation with *E. coli* Ec457

No.	Culture medium	The microbial strain grows	Cell density (CFU/mL)
1	MHA + LiCl	*Bacillus* BAD7	$(1.43 \pm 0.13) \times 10^7$
2	MHA + AB	*E. coli* Ec457	$(1.22 \pm 0.24) \times 10^5$
3	MHA + LiCl + AB	*Bacillus* BAD7	Small colonies appeared

Table 6. Cell density of *Bacillus* S2.5 after co-cultivation with *E. coli* Ec457

No.	Culture medium	The microbial strain grows	Cell density (CFU/mL)
1	MHA + LiCl	*Bacillus* S2.5	$(2.47 \pm 0.38) \times 10^6$
2	MHA + AB	*E. coli* Ec457	$(1.94 \pm 0.22) \times 10^5$
3	MHA + LiCl + AB	*Bacillus* S2.5	Small colonies appeared

Similar to the P4QN11 strain, the *Bacillus* BAD7 strain reached its highest cell density of $(1.43 \pm 0.13) \times 10^7$ CFU/mL when cultured on MHA supplemented with LiCl, whereas *E. coli* growth was inhibited. On the medium containing only antibiotic (AB), *E. coli* still grew at low levels, while BAD7 was completely inhibited. The combination of LiCl and antibiotic completely suppressed *E. coli* growth, and only small colonies of BAD7 remained (Table 5).

Additionally, the BAD7 strain after co-culture was spread on plates with concentrations ranging from 10^{-1} to 10^{-3} and incubated for 24 hours. Only a few small colonies appeared on the plate with a 10^{-1} concentration of LiCl and cefotaxime, while no colonies were observed at concentrations of 10^{-2} and 10^{-3} (Fig. 4). These results indicate a strong inhibitory effect of the LiCl–cefotaxime combination at higher concentrations). Similarly to *Bacillus* P4QN11, these small colonies were verified with Gram staining and morphological characterization on Nutrient Agar plate to confirm *Bacillus* identity; and *E. coli* did not survive on MHA supplemented with LiCl and cefotaxime. The *Bacillus* S2.5 strain exhibited a similar trend. When cultured on MHA supplemented with LiCl, it reached a cell density of $(2.47 \pm 0.38) \times 10^6$ CFU/mL, while *E. coli* was completely inhibited.

When cultured with antibiotic alone, *E. coli* Ec457 exhibited weak growth with a cell density of $(1.94 \pm 0.22) \times 10^5$ CFU/mL, while the Bacillus S2.5 strain was completely inhibited. The combination of both inhibitory agents completely suppressed *E. coli* growth, leaving only a few small colonies of S2.5 (Table 6).

In press

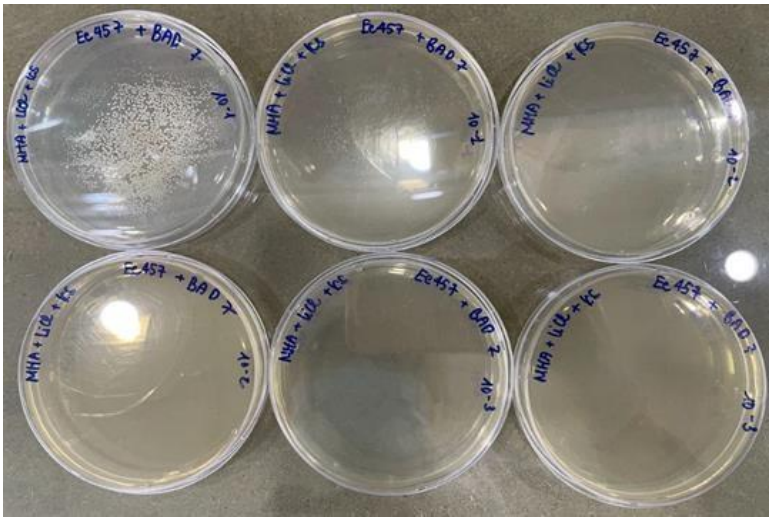

Fig. 4. Results of streak plating of *Bacillus* BAD7 strain after co-culture

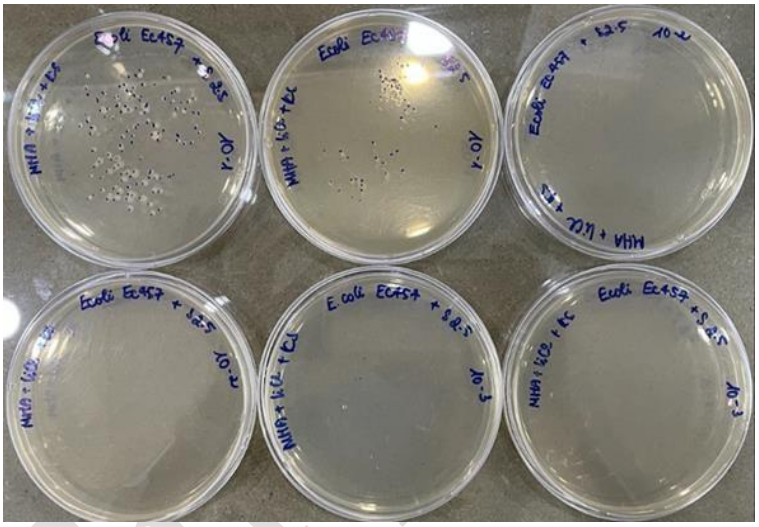

Fig. 5. Results of streak plating of *Bacillus* S2.5 strain after co-culture

After co-cultivation and plating at dilution levels of 10^{-1}, 10^{-2}, and 10^{-3}, exhibited only a few small colonies at the 10^{-1} dilution on medium containing LiCl and cefotaxime, while no colonies were observed at the 10^{-2} and 10^{-3} dilutions (Fig. 5). In the same manner as strains P4QN11 and BAD7, the small colonies were subjected to Gram staining and examined for their colony morphology on Nutrient Agar to verify their *Bacillus* identity

Compared to monoculture controls, the cell densities of both *Bacillus* and *E. coli* in co-culture samples were reduced by 10–20 fold, indicating competitive or interactive effects within the environment that negatively impacted growth. Among the three *Bacillus* strains, BAD7 exhibited the highest cell density, followed by P4QN11, with S2.5 showing the lowest.

The presence of small colonies on selective media containing both LiCl and cefotaxime antibiotics may suggest the acquisition of antibiotic resistance genes from *E. coli* via horizontal gene transfer or uptake of extracellular DNA. Alternatively, it could reflect sub-inhibitory concentrations of antibiotics insufficient to completely suppress growth [4, 17-18]. Therefore, the colonies of *Bacillus* strains (P4QN11, BAD7, S2.5) grown on plates containing LiCl and cefotaxime were subjected to following investigation on antibiotic susceptibility in section 3.4 and verification of no $bla_{\text{CTX-M}}$ gene transfer in section 3.5.

3.4. Assessment of Antibiotic Resistance Gene Transfer between Bacillus spp. and E. coli Using the Disk Diffusion Method

The evaluation of antibiotic resistance gene transfer between *Bacillus* spp. and *E. coli* via the disk diffusion method on MHA supplemented with LiCl and LiCl combined with antibiotic revealed that all three *Bacillus* strains (P4QN11, BAD7, and S2.5) after co- culture with *E. coli* Ec457 produced clear inhibition zones when tested with cefotaxime disks (Fig. 6).

Specifically, on MHA + LiCl, the inhibition zone diameters were as follows: P4QN11, 26 ± 1 mm; BAD7, 26 ± 2 mm; and S2.5, 25 ± 1 mm. On MHA supplemented with both LiCl and cefotaxime, the inhibition zones slightly decreased to 24 ± 1 mm for P4QN11, 23 ± 1 mm for BAD7, and 24 ± 1 mm for S2.5 (Table 7). All measured values remained within the susceptibility thresholds according to the Bauer-Kirby standards, indicating that the *Bacillus* strains-maintained susceptibility to cefotaxime even after co-cultivation with *E. coli* harbouring the *bla*-$_{\text{CTX-M}}$ resistance gene [12].

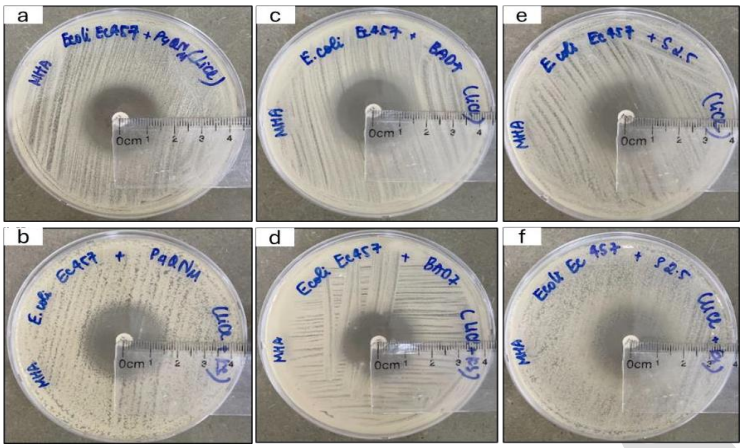

Fig. 6. Antibacterial zone diameter of the *Bacillus* strains after co-culture with *E. coli* Ec457 (a) P4QN11 strain in LiCl (b) P4QN11 strain in LiCl + cefotaxime antibiotic (AB) (c) BAD7 strain in LiCl (d) BAD7 strain in LiCl + AB (e) S2.5 strain in LiCl (f) S2.5 strain in LiCl + AB

Table 7. Diameter of the Inhibition Zone for cefotaxime of three *Bacillus strains* after co-culture in different media

No.	Culture medium	Strain	Antibacterial zone diameter (mm)	Result
1	MHA + LiCl	P4QN11	26 ± 1	Sensitive
2		BAD7	26 ± 2	Sensitive
3		S2.5	25 ± 1	Sensitive
4	MHA + LiCl + AB	P4QN11	24 ± 1	Sensitive
5		BAD7	23 ± 1	Sensitive
6		S2.5	24 ± 1	Sensitive

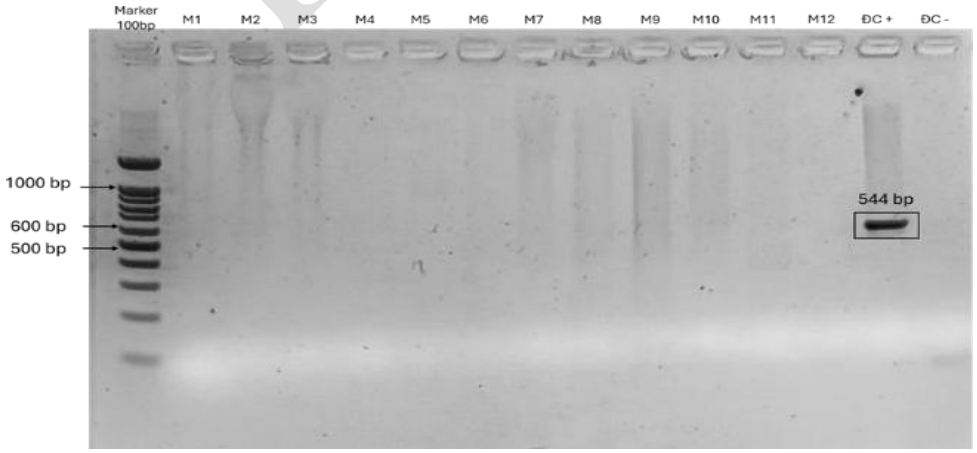

Fig. 7. Detection of *bla*-$_{\text{CTX-M}}$ gene in strains after co-culture with M1: P4QN11 (LiCl – first time) M2: BAD7 (LiCl – first time) M3: S2.5 (LiCl – first time) M4: P4QN11 (LiCl + AB – first time) M5: BAD7 (LiCl + AB – first time) M6: S2.5 (LiCl + AB – first time) M7: P4QN11 (LiCl – second time) M8: BAD7 (LiCl – second time) M9: S2.5 (LiCl – second time) M10: P4QN11 (LiCl + AB – second time) M11: BAD7 (LiCl + AB – second time) M12: S2.5 (LiCl + AB – second time) DC +: Positive control DC -: Negative control.

In press

The slight variation in inhibition zone diameters upon the addition of selective antibiotics may be attributed to interactions between the culture medium and the biological characteristics of the bacteria. Antibiotics can slow the growth of resistant *E. coli* strains or affect *Bacillus* spp. through feedback regulation and substrate competition [19]. LiCl contributes to the inhibition of Gram-negative bacteria by targeting enzymes and cellular structures [20], while exerting minimal effects on Gram-positive bacteria. However, given the fluctuation in inhibition zone diameters of only approximately ±1 mm, no significant decrease in antibiotic susceptibility was observed.

The results indicate that the *Bacillus* strains under investigation lack the ability to acquire antibiotic resistance genes from *E. coli* Ec457 harbouring the *bla*-$_{\text{CTX-M}}$ gene, even under selective conditions involving antibiotics and LiCl. The inhibitory capacity of the *Bacillus* strains remained stable, reflecting genetic stability and strong biological adaptability.

The absence of observed resistance gene transfer further supports the genetic safety of these *Bacillus* strains, reinforcing their potential as safe probiotics for use in food and animal feed applications. This also contributes to minimizing the risk of antibiotic resistance gene dissemination in the environment.

3.5. Assessment of Antibiotic Resistance Gene Transfer between Bacillus spp. and E. coli Using Molecular Biology Techniques

Genomic DNA from the three Bacillus strains (P4QN11, BAD7, and S2.5) after co-culture with *E.coli* Ec457 was extracted from samples cultured on two different media (MHA + LiCl and MHA + LiCl combined with antibiotic), with experiments performed in duplicate at two independent time points. PCR products were analyzed by electrophoresis on a 2% agarose gel. The positive control (*E. coli* Ec457) exhibited a specific band of approximately 544 bp, corresponding to the *bla*-$_{\text{CTX-M}}$ gene [16], whereas no band was detected in the negative control (Fig.7), confirming the absence of cross-contamination and validating the reliability of the PCR assay. Notably, none of the three Bacillus strains showed amplification at the 544 bp position under any tested condition, indicating that they did not acquire the *bla*-$_{\text{CTX-M}}$ gene from *E. coli* Ec457, even under selective pressure. These results confirm that the Bacillus strains do not undergo antibiotic resistance gene acquisition, fulfilling genetic safety criteria and supporting their potential as safe probiotics for use in animal husbandry.

4. Conclusions

This study demonstrated that three *Bacillus* spp. strains P4QN11, BAD7, and S2.5 did not acquire cefotaxime resistance genes from *E. coli* Ec457 carrying *bla*-$_{\text{CTX-M}}$ when co-cultured and spread on selective media containing LiCl and antibiotics. Disk diffusion assays showed that *Bacillus* strains consistently remained susceptible to cefotaxime after co-culture, while PCR analyses confirmed the absence of *bla*-$_{\text{CTX-M}}$ gene transfer. The results indicate that the transfer of the cefotaxime-resistance gene from *E. coli* to the three *Bacillus* strains was not detected using bulk culture based analytical methods This finding reinforces their genetic safety and supports their application as probiotics in food and animal feed, contributing to efforts to limit the spread of antibiotic resistance.

Acknowledgments

This work was partially supported by the Ministry of Science and Technology (Grant number: ĐTĐL.CN-71/19), Viet Nam.

References

[1] K. N. Di, D. T. Pham, T. S. Tee, Q. A. Binh, and T. C. Nguyen, Antibiotic usage and resistance in animal production in Vietnam: a review of existing literature, Tropical Animal Health and Production, vol. 53, Jun. 2021, Art. no. 340. https://doi.org/10.1007/s11250-021-02780-6

[2] S. Mingmongkolchai and W. Panbangred, *Bacillus* probiotics: an alternative to antibiotics for livestock production, Journal of Applied Microbiology, vol. 124, iss. 6, pp. 1334–1346, Jun. 2018. https://doi.org/10.1111/jam.13690

[3] Z. Zhai, C. Cui, X. Li, J. Yan, E. Sun, C. Wang, H. Guo, and Y. Hao, Prevalence, antimicrobial susceptibility, and antibiotic resistance gene transfer of *Bacillus* strains isolated from pasteurized milk, Journal of Dairy Science, vol. 106, iss. 1, pp. 75–83, Jan. 2023. https://doi.org/10.3168/jds.2022-22199

[4] L. Deng, C. Wang, X. Zhang, H. Tang, X. Chen, S. Du, and X. Chen, Cell-to-cell natural transformation in *Bacillus subtilis* facilitates large scale of genomic exchanges and the transfer of long continuous DNA regions, Nucleic Acids Research, vol. 51, iss. 8, pp. 3820–3835, May 2023. https://doi.org/10.1093/nar/gkad138

[5] X. Zhang, T. Jin, L. Deng, C. Wang, Y. Zhang, and X. Chen, Stress-induced, highly efficient, donor cell-dependent cell-to-cell natural transformation in *Bacillus subtilis*, Journal of Bacteriology, vol. 200, no. 17, Aug. 2018. https://doi.org/10.1128/JB.00267-18

[6] F. M. F. Elshaghabee, N. Rokana, R. D. Gulhane, C. Sharma, and H. Panwar, *Bacillus* as potential probiotics: status, concerns, and future perspectives, Frontiers in Microbiology, vol. 8, Aug. 2017. https://doi.org/10.3389/fmicb.2017.01490

[7] European Food Safety Authority, EFSA statement on the risk posed to humans by a vitamin B_2 produced by a genetically modified strain of *Bacillus subtilis* used as a feed additive, EFSA Journal, vol. 17, no. 4, Apr. 2019. https://doi.org/10.2903/j.efsa.2019.5615

[8] Y. Agersø, K. Bjerre, E. Brockmann, E. Johansen, B. Nielsen, R. Siezen, B. S-Lauridsen, M. Wels, and A. A. Zeidan, Putative antibiotic resistance genes present in extant *Bacillus licheniformis* and *Bacillus*

In press

paralicheniformis strains are probably intrinsic and part of the ancient resistome, PLoS One, vol. 14, no. 1, Jan. 2019. https://doi.org/10.1371/journal.pone.0210363

[9] A. Raheem, L. Liang, G. Zhang, and S. Cui, Modulatory effects of probiotics during pathogenic infections with emphasis on immune regulation, Frontiers in Immunology, vol. 12, Apr. 2021. https://doi.org/10.3389/fimmu.2021.616713

[10] A. W. Bauer, W. M. Kirby, J. C. Sherris, and M. Turck, Antibiotic susceptibility testing by a standardized single disk method, American Journal of Clinical Pathology, vol. 45, iss. 4, pp. 493–496, Apr. 1966. https://doi.org/10.1093/ajcp/45.4_ts.493

[11] M. P. Weinstein, Performance standards for antimicrobial susceptibility testing, Clinical and Laboratory Standards Institute (CLSI), 2019.

[12] G. M. N. Uddin, M. H. Larsen, H. Christensen, F. M. Aarestrup, T. M. Phu, and A. Dalsgaard, Identification and Antimicrobial Resistance of Bacteria Isolated from Probiotic Products Used in Shrimp Culture, PLoS One, vol. 10, no. 7, Jul. 2015. https://doi.org/10.1371/journal.pone.0132338

[13] M. Dieser, H. J. Smith, and C. M. Foreman, Seven genome sequences of airborne, bacterial isolates from Antarctica, Microbiology Resource Announcements, vol. 13, no. 6, May 2024. https://doi.org/10.1128/mra.01129-23

[14] R. A. Mir, T. A. Weppelmann, L. Teng, A. Kirpich, M. A. Elzo, J. D. Driver, and K. C. Jeong, Colonization dynamics of cefotaxime resistant bacteria in beef cattle raised without cephalosporin antibiotics, Frontiers in Microbiology, vol. 9, Mar. 2018. https://doi.org/10.3389/fmicb.2018.00500

[15] A. A. Adegoke, C. E. Madu, O. A. Aiyegoro, T. A. Stenström, and A. I. Okoh, Antibiogram and beta-lactamase genes among cefotaxime resistant *E. coli* from wastewater treatment plant, Antimicrobial Resistance and Infection Control, vol. 9, no. 1, Mar. 2020, Art. no. 46. https://doi.org/10.1186/s13756-020-0702-4

[16] R. S. P. Sah, B. Dhungel, B. K. Yadav, N. Adhikari, U. T. Shrestha, B. Lekhak, M. R. Banjara, B. Adhikari, P. Ghimire, and K. R. Rijal, Detection of *TEM* and *CTX-M* genes in *Escherichia coli* isolated from clinical specimens at Tertiary Care Heart Hospital, Kathmandu, Nepal, *Diseases*, vol. 9, no. 1, Feb. 2021, Art. no. 15. https://doi.org/10.3390/diseases9010015

[17] I. L. Brito, Examining horizontal gene transfer in microbial communities, Nature Reviews Microbiology, vol. 19, pp. 442–453, Apr. 2021. https://doi.org/10.1038/s41579-021-00534-7

[18] J. Davies and D. Davies, Origins and evolution of antibiotic resistance, Microbiologia, vol. 12, no. 1, pp. 9–16, Mar. 1996. https://doi.org/10.1128/mmbr.00016-10

[19] H. Takada, Z. F. Mandell, H. Yakhnin, A. Glazyrina, S. Chiba, T. Kurata, K. J. Y. Wu, B. I. C. Tresco, A. G. Myers, G. C. Aktinson, P. Babizke, and V. Hauryliuk, Expression of *Bacillus subtilis* ABCF antibiotic resistance factor VmlR is regulated by RNA polymerase pausing, transcription attenuation, translation attenuation and (p)ppGpp, Nucleic Acids Research, vol. 50, iss. 11, pp. 6174–6189, Jun. 2022. https://doi.org/10.1093/nar/gkac497

[20] M. Bonnet, J. C. Lagier, D. Raoult, and S. Khelaifia, Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology, New Microbes and New Infections, vol. 34, Mar. 2020, Art. no. 100622. https://doi.org/10.1016/j.nmni.2019.100

In press