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Research Article

A Multidisciplinary Approach to Understanding *Bacillus cereus* in Fresh Meat: Isolation, Biochemical and Molecular Characterization, and Bacteriophages-Mediated Control Strategies for Improved Food Safety and Public Health

Waseem Sajjad[1], Mudassir Ahmad[2]*, Amna Waqar[1], Dalia Mehaisi[2]

[1]Department of Microbiology, Abbottabad University of Science and Technology, Abbottabad Pakistan.

[2]School of Science and Technology, Nottingham Trent University, Nottingham, UK.

***Corresponding Author:** Mudassir Ahmad, School of Science and Technology, Nottingham Trent University, Nottingham, UK.

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Abstract

Bacillus cereus is a major foodborne pathogen linked to contaminated fresh meat that poses major health hazards to the general public since it may generate toxins and heat-resistant spores. Investigating *B. cereus* in fresh meat utilizing a multidisciplinary approach, this work focuses on bacteriophage-mediated biocontrol methods, biochemical and molecular characterization, and isolation. After analyzing fresh meat samples using selective culturing techniques, virulence genes (nhe, hbl, cytK, ces) were identified using molecular techniques (PCR, whole-genome sequencing) and biochemical assays. The effectiveness of lytic bacteriophages in reducing *B. cereus* infection in meat products was also assessed after they were isolated. The findings showed that *B. cereus* was highly prevalent in fresh meat, and molecular characterization confirmed that enterotoxin-producing strains were present. Treatment with bacteriophages showed notable decreases in the bacterial load, indicating that it may be used as a natural biocontrol agent. Phage-based treatments might improve food safety and reduce the need for chemical preservatives by being included into food processing and packaging. In order to reduce the dangers associated with *B. cereus* in the food business, this study emphasizes the significance of integrating cutting-edge genomic and phage treatment techniques with conventional microbiological procedures. In ultimately, the findings protect public health by enhancing food safety procedures and providing lasting solutions for stopping foodborne outbreaks.

Keywords: *Bacillus cereus; Foodborne outbreak; Bacteriophages; Foodborne pathogen*

1. Introduction

Foodborne infections continue to be a major global public health problem, and food safety is seriously threatened by pathogenic microorganisms (Rana et al., 2020). One of these pathogens, *Bacillus cereus*, is a Gram-positive spore-forming bacteria that is commonly linked to food contamination, especially in fresh meat and meat products (Stenfors Arnesen et al., 2008). This bacterium is well-known for producing toxins and heat-resistant spores that cause diarrheal syndromes and emetic (vomiting) foodborne illnesses (Ehling-Schulz et al., 2019). A multidisciplinary approach is needed to understand the prevalence, virulence mechanisms, and control strategies of *B. cereus* due to its persistence in food processing settings and resistance to traditional preservation techniques(Jessberger et al., 2020).

Due to its high water activity and nutrient-rich composition, which create the perfect conditions for bacterial development, fresh beef is extremely vulnerable to microbial contamination (Hussain and Oh, 2018).

A number of processes, such as slaughtering, processing, packing, and storage, might result in contamination. Because *B. cereus* can withstand pasteurization and refrigeration, it poses a serious risk of outbreaks even in meat products that are properly preserved. Innovative approaches to reduce *B. cereus* infection while maintaining food safety and prolonging shelf life are desperately needed, as consumer demand for fresh and minimally processed foods rises (Sornchuer et al., 2024).

However, since *B. cereus* and other members of the *Bacillus cereus* group (such as *B. anthracis* and *B. thuringiensis*) have a close genetic link, these approaches are time-consuming and may lack specificity (Liu et al., 2020). Exoenzyme synthesis is linked to B cereus's pathogenicity, whether it occurs inside or outside the gastrointestinal tract. Four hemolysins, three different phospholipases, and three pore-forming enterotoxins are among the toxins released. Hemolysin BL, nonhemolytic enterotoxin (NHE), and cytotoxin K are the enterotoxins that cause the nod-like receptor protein-3 (NLRP3) to become active. Vegetative cells that are consumed as living cells or spores in the small intestine produce and discharge a protein enterotoxin, which causes diarrheal syndrome (Carroll et al.,2022).

B cereus infection typically resolves on its own without the need for specific treatment. The majority of patients are treated with oral hydration and symptomatic treatment. The majority of individuals get well 24 hours after their symptoms start. It is not recommended to use empirical antibiotic treatment for gastrointestinal disorders brought on by *B cereus* infections. Intravenous fluid hydration may be required in extreme circumstances (Ceuppens et al.,2013). Beta-lactamases are produced by B cereus, which also resists beta-lactam drugs. Vancomycin, gentamicin, chloramphenicol, or carbapenems should be considered when antibiotics are necessary (Liu et al.,2020).

To evaluate the hazards to public health and create focused control strategies, it is essential to comprehend the genetic diversity and virulence factors of *B. cereus* strains isolated from fresh meat. To overcome the infective alternative therapy is needed (Kowalska et al., 2024). Bacteriophage (phage) therapy has drawn interest as a potential substitute for traditional antimicrobial therapies due to its shortcomings (such as antibiotic resistance and customer demand for chemical-free foods) (Kazantseva et al., 2023).

Without harming human cells or the good microbiota, phages are viruses that selectively attack and lyse bacterial cells (García-Cruz et al., 2023).The effectiveness of phages in lowering *B. cereus* contamination in a variety of food matrices, such as meat and dairy products, has been shown in several studies. To improve food safety, phage cocktails that target *B. cereus* spores and vegetative cells, for example, have demonstrated notable decreases in the bacterial burden (Li et al.,2022). Furthermore, to offer ongoing defense against microbial development, phage-based therapies can be included into food packaging systems (such as phage-coated films) (Sornchuer *et al.,* 2024).

Food safety management systems have a significant problem due to the persistence of *B. cereus* in fresh meat (Jovanovic et al., 2021). Emerging strains with increased virulence and resistance require ongoing surveillance, even if regulatory bodies like the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA) have set criteria for microbial limits in food items. The reduction of dangers associated with *B. cereus* can be greatly enhanced by a multidisciplinary strategy that combines phage-mediated biocontrol, genomic analysis, and sophisticated detection techniques (Lomelí-Ortegaet al., 2023).

Phage delivery system optimization, long-term effectiveness assessment, and potential regulatory barriers for commercial applications should be the main areas of future study (Kazantseva et al., 2023).In brief, a thorough approach including separation, biochemical and molecular characterization, and cutting-edge control techniques like bacteriophage treatment is needed to address *B. cereus* infection in fresh meat. The food business may improve safety standards, lower spoilage, and safeguard the public's health from foodborne diseases by combining these strategies.

The aim of this study was to isolate *Bacillus cereus* from Fresh Meat, confirm it by biochemical and molecular characterization and find the alternative therapy to antibiotics (bacteriophages) to improve food safety and public health.

2. Materials and Method

2.1 Study area and isolation of samples

Samples of fresh meat were collected from different butcher shops of Abbottabad, Hazara Division, Khyber Pakhtunkhwa, Pakistan. Each sample was properly labeled for diagnostic purposes and collected in compliance with Standard Operating Procedures (SOPs).

2.2. Sample transportation and storage

Samples were collected and immediately transferred to Abbottabad University of Science and Technology's Microbiology Laboratory for the purpose of identifying and isolating bacteria. After being labeled, the samples were kept until further processing could be completed.

2.3. Samples process

Following collection, samples were carefully cleaned to remove surface contaminants and particle debris using distilled water or sterile phosphate-buffered saline (PBS). Using a tissue grinder or sterile mortar and pestle, the cleaned specimens were further physically homogenized to a fine particle fineness in order to ensure equal microbial dispersion. This homogenate was then floated in a pre-measured volume of nutrient-rich liquid culture medium, For optimal growth conditions, the sample-to-media ratio was carefully maintained at 1:10. Then, for a standard 24-hour incubation period, the inoculation tubes were put in a shaking incubator set to 37°C (±1°C) and agitated at 180–200 rpm (David and Daum, 2010).

2.4. Isolation and Growth of Bacteria

Clinical samples were streaked onto selective medium such as Nutrient agar and Mannitol Yolk Polymyxin (MYP) Agar then cultured for 24 to 48 hours at 37°C. Observe the growth characteristics and appearance of the bacterial colonies over a 24-hour incubation period at 37°C (Fernandes Queiroga Moraes et al.,2021).

2.5. Gram staining

To perform the Gram staining, a tiny amount of distilled water was applied to a transparent slide. A sterile needle was used to apply a little amount of pure culture on the slide. The culture was evenly distributed throughout the surface using the needle. Using sterilized water, a drop of crystal violet was added to the slide smear. The crystal violet swirled in and let to dry for around 30 seconds once a uniform dispersion was achieved. Following the application of crystal violet stain, the slide was carefully cleaned using sterile distilled water. The slide was properly washed with pure distilled water, and then a droplet of Lugol's iodine was applied to the smear to remove any leftover crystal violet hue. Lugol's iodine and crystal violet combine to keep the stain in place. After applying Lugol's iodine, the slide was cleaned with acetone. Acetone, a decolorizer, aids in removing excess stains from the slide. To cover up the smudge, a drop of safranin was put on the slide. The counterstained safranin stain gives gram-negative bacteria their distinctive color. After thoroughly cleaning the slide with water to get rid of any remaining traces of safranin, it was eroded clean. Blotting paper was used to gently remove the excess liquid from the slide. The slide was coated with a drop of mounting chemical Canada balsam in order to retain the discolored smear. To observe the slide containing the plated smear, a 100X magnification microscope was utilized (Greenwood et al., 2012).

2.6. Biochemical Characterization

Biochemical assays, including catalase, indole test, urease test, citrate utilization test and motility test were carried out. Briefly described as follow:

2.7. Catalase Test

The test indicates the presence of the catalase enzyme, which causes hydrogen peroxide (H2O2) to release more oxygen. It is employed to distinguish between different bacteria that produce the catalase enzyme. The *Bacillus cereus* strain catalase test was carried out by gently mixing one colony with hydrogen peroxide on a sterile slide. The appearance of gas bubbles on the surface of the culture material indicated that the test was effective (Reiner, 2010).

2.8. Indole Production test

An indole test is used to determine out if an organism can convert tryptophan to indole. A culture of the bacterial strains was injected into a tube filled with tryptophan broth and kept at 37 °C for 24 to 48 hours in order to perform the Indole test. Add 0.5 ml (5 drops) of Kovac's regent and mix gently. Look at the topmost layer of the liquid; if purple or red rings appear there, a favorable result is shown; if yellow rings appear, an unsuccessful outcome is shown (KOMAL, 2019).

2.9. Urease test

Urea was created when amino acids underwent decarboxylation. The breakdown of urea produced CO2 and ammonia. The presence of ammonia, which made the solution more alkaline, was indicated by phenol red, which changed from a light orange hue at a pH of 6.8 to a magenta (pink) tint at a pH of 8.1. Phenol red was employed to detect pH variations in solutions. Urease-positive bacteria colored the medium pink after a day. Because of the generation of acid, negative organisms either produced a yellow color change or no color change at all. A urea agar slant can be inoculated with one or two drops of an overnight brain-heart infusion broth culture, or a piece of a well-isolated colony can be applied to its surface. Additionally, incubate the tube at 35 to 37 degrees for 48 hours to 7 days. After then, watch for the emergence of a pink hue for at least seven days (Brink, 2010).

2.10. Citrate Agar Test

The purpose of this investigation is to ascertain whether an organism can use citrate as an energy source. Inorganic ammonium salts ($NH_4H_2PO_4$) and citrate are the only sources of carbon and nitrogen in the medium. A mild inoculum that was meticulously separated from the colony's core was used to inoculate the slant. At a temperature of 35 to 37 degrees, incubate aerobically for up to 24 hours. A color shift from green to blue was seen along the slope(MacWilliams, 2009).

2.11. Motility test

To determine if an organism can move by utilizing its flagella, this test is conducted. The kind of bacteria determines where the flagella are located. After making the semisolid agar, put it into test tubes to conduct the motility test for *B. cereus* strains. After a culture has developed on nutrient agar medium for 18 to 24 hours, affix a straight needle to the colony. Only puncture 1/3 to ½ inch deep once you are at the center of the tube. As the needle leaves the medium, make sure it does so in the same direction. To determine whether a diffuse growth zone has emerged from the inoculation line, incubate for up to seven days at 35° to 37°C (Shields and Cathcart, 2011).

2.12. Disk Diffusion Susceptibility Testing

Mueller-Hinton agar coated with different antibacterial filter paper disks is used to cultivate facultative anaerobic and pathogenic aerobic bacteria. By figuring out how sensitive or resistant these bacteria are to various antibiotic drugs, the disk diffusion susceptibility test assists clinicians in selecting alternatives to therapy for their patients. The ability of that drug to inhibit that organism can be inferred indirectly from the proliferation surrounding the disks (Hudzicki, 2009).Bacterial suspensions were prepared using the 0.5 McFarland standard. Antibiotic disks were placed on the surface of Mueller-Hinton agar plates after the suspension was applied. The plates were incubated at 37°C for 16–18 hours in order to determine their sensitivity to antibiotics. Next, the inhibition zones were measured in mm(Hudzicki, 2009).

2.13. DNA Extraction

The Qiagen RTU kit was used to extract the whole genomic DNA of the tested bacterial culture. To determine the spore concentration needed for extraction, 1 mL of the culture containing 108 cfu/mL was centrifuged. The extraction tube (2.5 mL) was filled with 250 µL of proteinase K to remove any potential proteins and lysis buffer AL. After centrifuging the suspension, the supernatant was disposed of. To get rid of the particulates. 95% ethanol was added to the lysate.

After passing the cleaned lysate through a purification micro spin column, AE buffer was used to elute the column. AW1 and AW2 were the washing buffers that were utilized. Quantification of the isolated DNA was done with the Nanodrop spectrophotometer NS1020. For subsequent downstream analysis, the isolated DNA was kept at -20 °C. 1% agarose gel electrophoresis was used to evaluate the isolated DNA's purity(Vilain et al., 2009).

2.14. PCR Amplification and Sanger Sequencing

The F/R primers specific to 16S were used to amplify the isolated DNA. 27F 5' (AGA GTT TGA TCM TGG CTC AG) and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) are the sequences. The PCR product was predicted to be between 1.4 and 1.6 kb. Exonuclease I and SAP enzymes were used for enzymatic digestion in order to sequence the PCR product. The PCR product from agarose gel electrophoresis was then run through a purification column and elution buffer. Sanger sequencing was performed on the cleaned PCR product using primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT)(Chen and Tsen, 2002).

Stage	PCR Protocol	Temperature (°C)	Time (min.)
1st	Initial Denaturation	94	5.0
2nd	Denaturing	94	0.5
	Annealing	52.7	0.5
(35 Cycles)	Extension	72	2.0
3rd	Final Extension	72	5.0
4th	Hold	4	∞

2.15. Bioinformatics Analysis

Chromas and BioEdit version 7.7.1 programs were employed to examine the sequence in order to determine the bacterial strain's evolutionary connection. The sequence has been altered for low-quality and superfluous amplifications, and the peaks were adjusted. The highly matched sequences from the databank were obtained using the NCBI's basic local alignment search tool (BLASTn). The Clustal Omega bioinformatics program was used to perform multiple sequence alignment of the chosen BLASTn returned sequences before the phylogenetic tree building process. The sequenced bacterial strain was used using MEGAX software to create the phylogenetic tree for the evolutionary connection with other species. For the creation of phylogenetic trees, the Fast Minimum Evolution Method and Max Sequence Difference 0.75 were employed.

2.16. Isolation of bacteriophages from sewage

Samples of soil from rice fields and sewage have been collected in the Abbottabad district. The samples were collected in a sterilized pot and sent to Abbottabad University's Microbiology Lab for bacteriophage isolation. After two minutes of stirring, the samples were centrifuged for ten minutes at 15,000 rpm. 40 mL of soil and rice field water sample, 10 mL of sterile 5 X nutritional broth, and one microliter of an overnight culture of *Bacillus cereus* were added to the flask as an inoculant in order to process the sample. The flask was incubated at 37°C for the whole night in a shaking incubator fixed at 120 rpm. After incubation, the contents of the flask were centrifuged for 10 minutes at 10,000 rpm. A new, sterile falcon tube was used to collect the clear supernatant, which was then kept at 4°C. The presence or absence of bacteriophages in the filtrate was determined using a spot test (Liu et al., 2021).

2.17. Detection of bacteriophages in the filtrate

Following bacteriophage enrichment, a spot test revealed the presence of a *Bacillus cereus*phage as follows: For the spot test, 100 μL of a *Bacillus cereus*culture that has been cultivated overnight is spread out on a nutrient agar plate. The plates were left to dry for about ten minutes after seven milliliters of the filtrate containing the suspicious bacteriophage were applied. After that, the plates were incubated at 37°C for the whole night. The plates were then inspected to see whether bacteriophages had created a distinct lysis zone. The presence of the clear zone, or plaque, indicates the presence of a certain bacteriophage (Liu *et al.,* 2021).

2.18. Purification of bacteriophages using a double layer agar assay

A two-layer agar overlay approach was used to quantify and extract the bacteriophage from the lysate. A conical flask containing 100 mL of semisolid nutrition agar was autoclaved and cooked to 48°C in a water bath before the experiment began. Initially, the lysate was serially diluted (1:9) in microtubes containing 900 μL of nutritional broth. After that, the selected dilutions were mixed with 100 μL of a Bacillus cereus culture. To allow the phages to attach to the bacterial cells, the phage lysate and bacterial culture were incubated for five minutes.

After the selected dilutions have been poured onto nutrient agar plates. After applying 3 mL of semisolid agar to the nutritional agar plate's surface, it was incubated for the whole night at 37°C. The mixture was dispersed over the dish by swirling the semisolid agar. We looked at the countable plaques (30 pfu–200 pfu) on the plates (Alvi et al., 2021).Certain plates with plaque were chosen for phage purification. The plaque-containing tip was put in a test tube with 10 mL of nutritional broth and 1 mL of Bacillus cereus bacteria in order to propagate the phage.

A 24-hour incubation period at 37°C was followed by plaque visualization and purification. Up to ten iterations of the purifying procedure were performed. The titer of the lysate solutions was calculated using the following formula: Titter (*pfu*/mL) = plaque (*pfu*) dilution number x phage (mL) volume added to plate.

3. Results

3.1. Meat sample preparation

After undertaking microbiological analysis, the meat samples were aseptically washed with sterile physiological saline (0.85% NaCl) to remove loosely attached bacteria and surface debris while preserving the adherent microbial flora. After being cleaned, the samples were transferred to sterile Petri plates and carefully cut with sterile surgical scissors to ensure consistent uniformity. This physical split made it easy to release bacteria from the meat matrix for subsequent culture and analysis. Throughout the procedure, laminar airflow was employed to maintain sterility and prevent cross-contamination. While ensuring a representative sample of bacteria that are both surface-associated and tissue-entrapped, this preparation method reduces external contamination (Figure 1).

3.2. Morphological characterization

Bacterial isolates were then characterized by morphology, by using Nutrient Agar. On nutrient agar all B. cereus strains produced white colonies (Figure 2).

Figure . *Meat sample processing.*

Figure 2. *Morphological characterization of bacterial isolates.*

3.3. Gram Staining Results

Using an isolated strain of *B. cereus* cultured for a whole night, Gram staining identified the organism are Gram-positive rod shape. Prior to accomplishing confirmation biochemical testing, the Gram reaction and cellular organization provided first proof that these isolates were bacillus (Figure 3).

3.4. Catalase test results

The isolated bacteria generated gas bubbles on a glass slide after being treated with a few drops of 3% H2O2, indicating that the catalase test was positive. The catalase test result showed that all the *B. cereus* bacterial strain were positive (Figure 4).

3.5. Indole test

A reddish-colored ring appeared on the glass tube surface as soon as the kovac's reaction was injected, signifying a successful indole test. Indole negativity is indicated by yellow or no color. The bacterium *B. cereus*gave a negative indole test by adding 5-6 drops of kovac's reagent (Figure 5).

3.6. Urease Test

The production of urea is confirmed by the appearance of bright pink (fuchsia) colour on the slant. Formation of pink color indicates positive result. The hydrolysis of the media proteins during prolonged incubation could result in a false-positive test. The findings of the urease test for bacterial isolates were negative (Figure 6).

3.7. Citrate Agar Test

The citrate test came back with a negative result. The Citrate Agar test showed bacterial isolates of *B. cereus* were negative (Figure 7).

3.8. Motility test

In this test semisolid agar substrate was used to determine bacterial motility. A diffusive zone of growth from the inoculation line indicates bacterial motility. All strains of *B.cereus* are motile by showing diffusing from the line of inoculation (Figure 8).

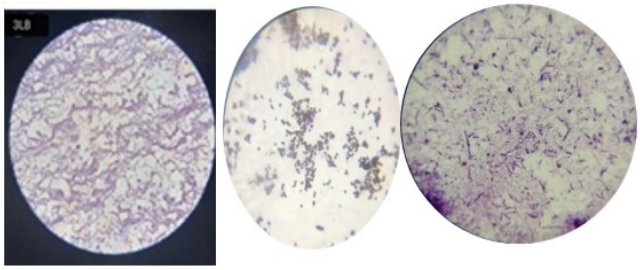


Figure 3. *Microscopy of bacterial isolates.*

Figure 4. *Catalase test results of bacterial isolate*

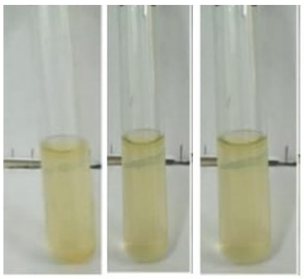


Figure 5. *Indole test results for all bacterial isolates.*

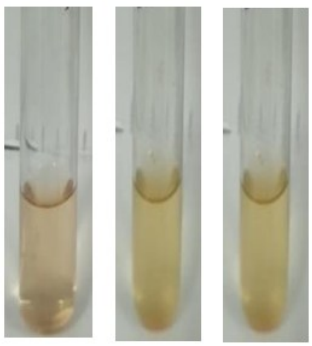


Figure 6. *Urease test results for all bacterial isolates*

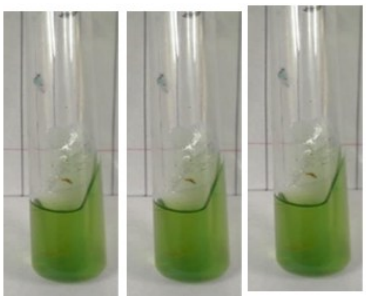


Figure 7. *Citrate agar test results for all bacterial isolates.*

Figure 8. *Motility test results for all bacterial isolates*

3.9. Antimicrobial susceptibility testing

To determine the antibiotic sensitivity pattern of isolated strains, sensitivity testing was performed (Figure 9, Table 1).

The BLASTn analysis of the 16S rRNA gene sequence showed 99.75% similarity with *Bacillus cereus* strain (Accession No. MG561368.1). The phylogenetic tree constructed using MEGA X software showed the evolutionary relationship between the isolate and other *Bacilli* spp. The tree revealed that the isolate clustered with *Bacilli* strains, confirming the BLAST analysis results. The distance-based tree showed a clear separation between the isolate and other *Bacilli* species, indicating a distinct phylogenetic position Table 2.

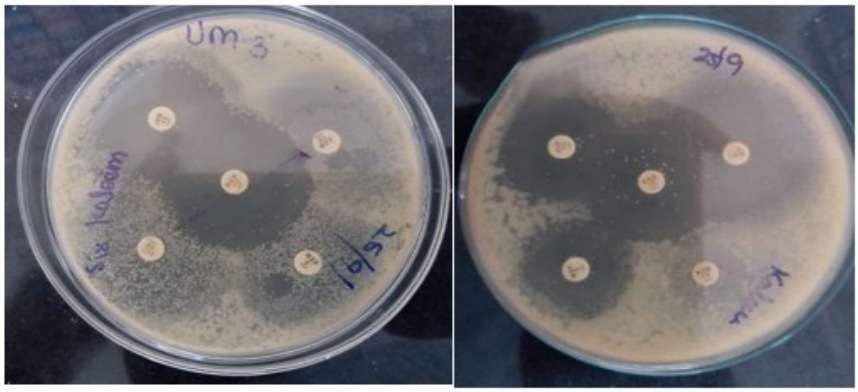


Figure 9. *Antibiotic susceptibility results for all bacterial isolates.*

Table 1. Antibiotic Susceptibility Profile of *Bacillus Cereus* Isolates from Fresh Meat.

Antibiotic Class	Antibiotic (Disk Concentration)	Resistance (%)	Intermediate (%)	Sensitive (%)	Interpretive Criteria (CLSI, 2023)
β-lactams	Penicillin G (10 IU)	92.3	5.4	2.3	R ≥ 28 mm
	Ampicillin (10 µg)	87.6	8.2	4.2	R ≥ 28 mm
Cephalosporins	Cefotaxime (30 µg)	45.2	32.1	22.7	R ≥ 23 mm
	Ceftazidime (30 µg)	38.9	41.3	19.8	R ≥ 18 mm
Aminoglycosides	Gentamicin (10 µg)	12.4	15.6	72.0	R ≥ 15 mm
	Streptomycin (10 µg)	28.7	24.3	47.0	R ≥ 15 mm
Tetracyclines	Tetracycline (30 µg)	23.5	18.9	57.6	R ≥ 15 mm
Macrolides	Erythromycin (15 µg)	34.2	25.7	40.1	R ≥ 14 mm
Quinolones	Ciprofloxacin (5 µg)	8.9	10.2	80.9	R ≥ 21 mm
Others	Vancomycin (30 µg)	5.1	7.3	87.6	R ≥ 15 mm
	Chloramphenicol (30 µg)	14.7	12.5	72.8	

Table 2. Microbial information extracted from BLASTn results.

Subject	Accession No.	MG561368.1
	Description	*Bacillus cereus*
	Length (b)	1476
	Start	12
	End	1216
	Query Cover (%)	97
Score	Bit	2183
	E-value	0.0
Identities	Match/Total	1199/1207
	Percentage (%)	99.34

3.10. Nucleotide Sequence

CCTTTAAGCGATATCTGTATCGTGCACGGCTGAGCGCATGAGATTAAGAGCTT-
GCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTA
ATACCGGATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTT
TCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTT
CTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGG
CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGC
CCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATA
GGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGGGCTACAAGGGACGGGTCAAAAAAGGTGCA

3.11. Taxonomic Hierarchy

The *Bacillus cereus* bacterium belongs to the following taxonomic hierarchyFigure 10, Table 3 and 4:

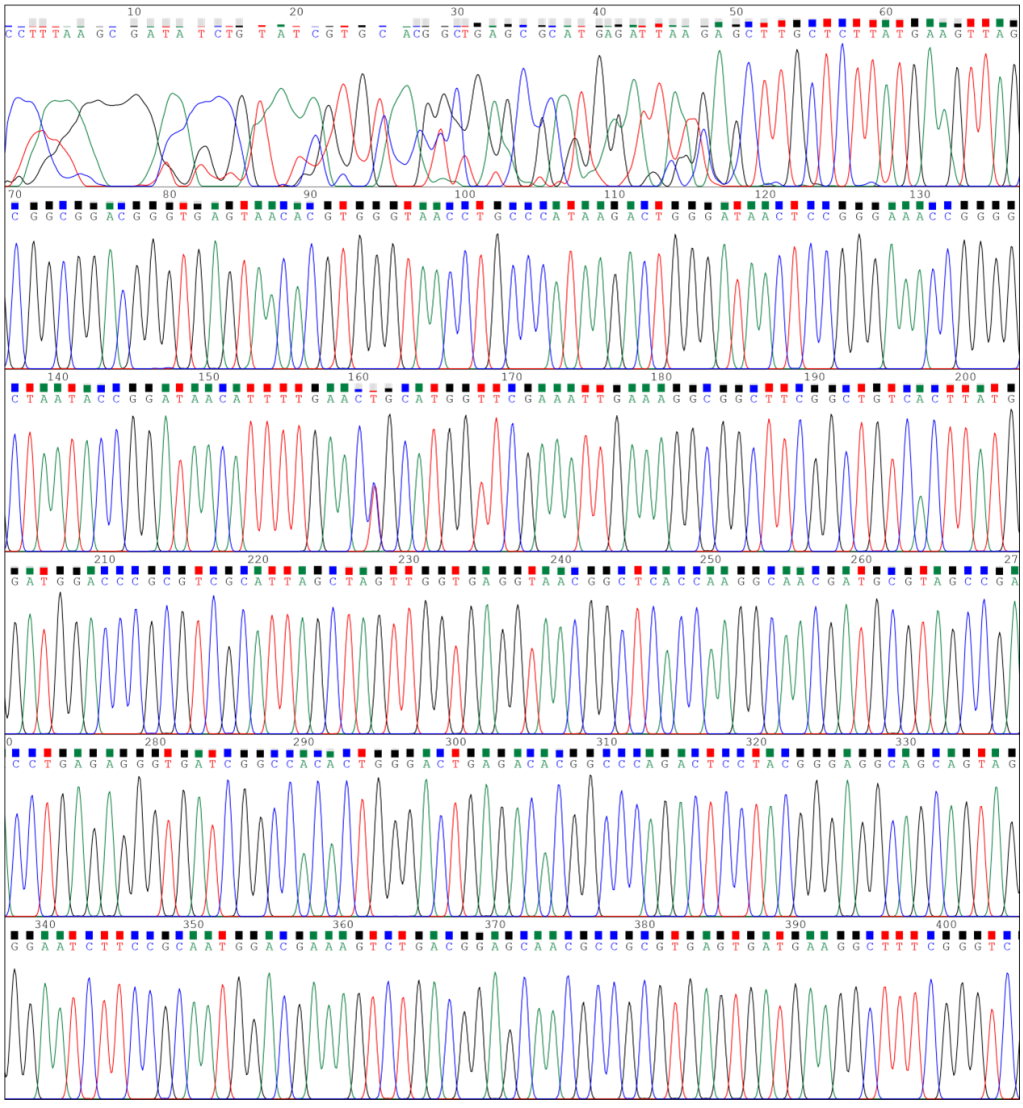
Based on the 16S rRNA gene sequence analysis and phylogenetic tree construction, the isolate was identified as a strain of *Bacillus cereus*. The results suggest a close evolutionary relationship between the isolate and other *B. cereus* strains. These findings provide valuable insights into the genetic diversity and phylogenetic relationships among *Bacilli* species.

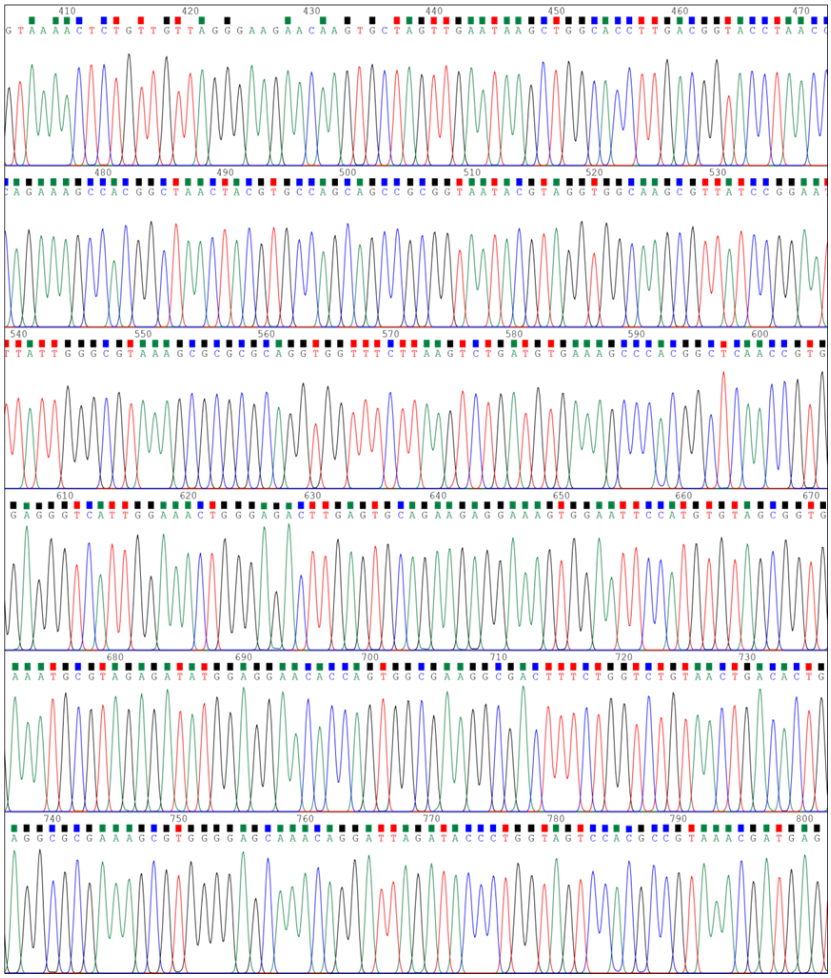
Table 3: Taxonomic hierarchy of the identified strain

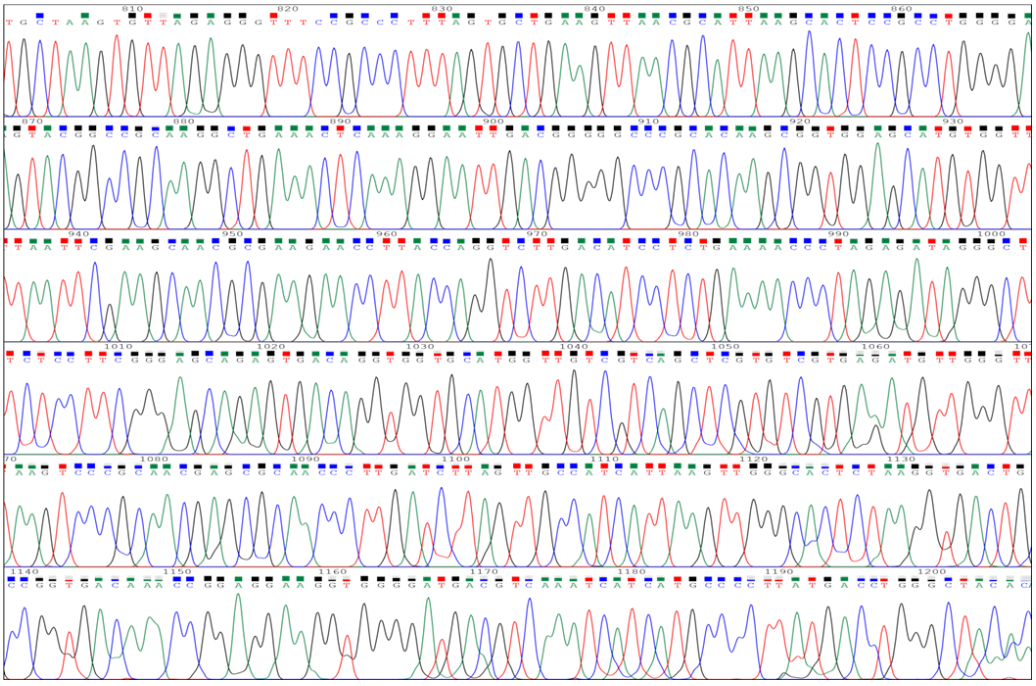
Taxon	Description
Domain	*Bacteria*
Phylum	*Bacillota*
Class	*Bacilli*
Order	*Bacillales*
Family	*Bacillaceae*
Genus	*Bacillus*
Species	*Bacillus cereus*

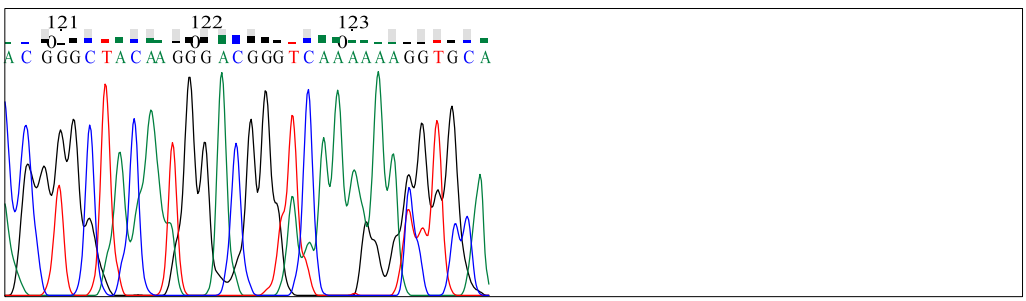
Table 4. Top 10 BLASTn Results.

Scientific Name	Max Score	Total Score	Query Cover	E-value	Per. Ident (%)	Acc. Len (b)	NCBI Accession N0.
Brevibacillus brevis	2187	2187	96%	0	99.75	1486	JX460822.1
Bacillus cereus	2183	2183	97%	0	99.34	1476	MG561368.1
Bacillus arachidis	2183	2183	97%	0	99.34	1442	OR835690.1
Bacillus sp. (in: firmicutes)	2183	2183	97%	0	99.34	1476	MN007096.1
Bacillus sp. HXG-C6	2183	2183	97%	0	99.34	1436	GU257957.1
Bacillus sp. enrichment culture clone SYW2	2183	2183	97%	0	99.34	1475	FJ601632.1
Bacillus sp. enrichment culture clone SYW19	2182	2182	97%	0	99.34	1469	FJ601649.1
Bacillusthuringiensis	2182	2182	96%	0	99.66	1402	OQ975943.1
Bacillus sp. hb83	2182	2182	97%	0	99.34	1451	KF863872.1
Bacilluscereus	2182	2182	97%	0	99.34	1451	MZ433237.1








121
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A C GGGC TACAA GGG ACGGGTCAAAAAAGGTGCA

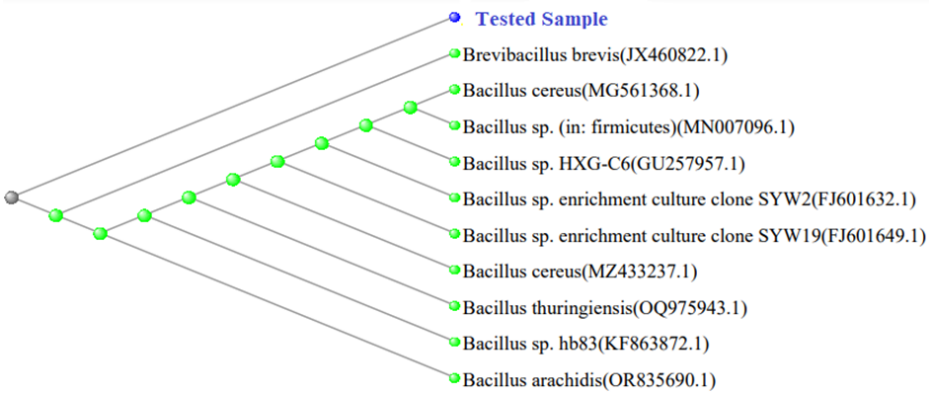

Figure 10. *Phylonetic tree results*

3.12. Spot test for the detection of B. cereus bacteriophages

The presence of bacteriophages against *B. cereus*was detected sewage samples. Spot tests revealed that sewage samples included infective bacteriophage (Figure 11).

3.13. Purification of bacteriophages produced clear transparent plaques

The isolated phage produced a clear, round plaque against *B. cereus* on the double-layer agar plate. The plaque was formed up of two circles: the inner circle was completely transparent, and the outer circle surrounded it. Bacteriophages are generating the depolymerase enzyme when there is a fuzzy layer like this one around the plaque. The halo that surrounds the plaque shows that the phage is producing soluble enzymes, such as depolymerase, that cause the bacterial host cell to break free from its capsule Figure 12.

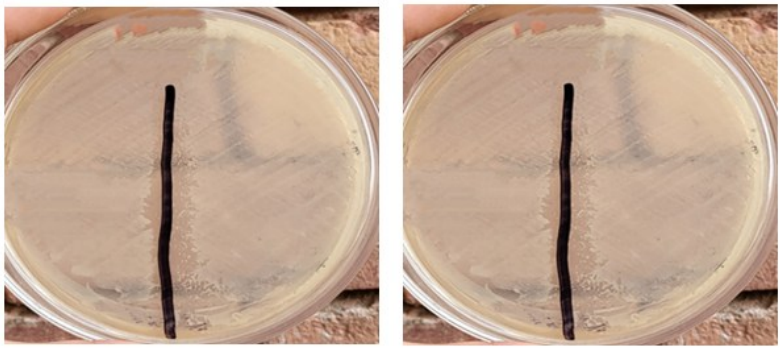

Figure 11. *Detection of bacteriophages through spot test.*

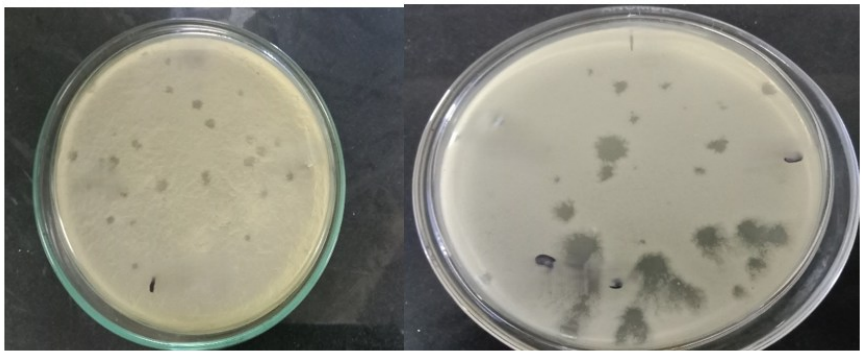

Figure 12. *Purified bacteriophages against B. cereus*

4. Discussion

The persistence of Bacillus cereus as a foodborne pathogen in fresh meat presents significant challenges to food safety systems worldwide. Our findings demonstrate that this spore-forming bacterium exhibits remarkable resilience in meat processing environments, with contamination occurring at multiple points from slaughter to retail (Fiedleret al., 2019). The ecological success of *B. cereus* can be attributed to its ability to form heat-resistant endospores that withstand common food processing treatments, including pasteurization and refrigeration (Jonsmoen et al., 2024).The current study was carried out to isolate and characterize*Bacillus cereus* from fresh meat as well as to isolates bacteriophages against *B. cereus* strains.The bacterial isolates were characterized by morphological, by using Nutrient Agar (NA) medium. The bacterial isolates produces grew whitish colonies. Additionally, this was noted by Isaka (2015) which shows *B. cereus* colonies are grew whitish convex, mucoid and shiny in texture. After applying the Gram-staining method, microscopic examination demonstrated that every bacterial isolate consisted of gram–positive rods bacteria distributed irregularly. A similar study was conducted by Isaka (2015) which shows Bacillus cereus isolated from meat samples were gram positive rod shaped.The biochemical analysis of the *B. cereus* isolates was another aspect of this work. The results of this experiment showed that the *B. cereus* isolates were positive for catalase, negative for indole, negative for urease, negative for citrate agar and positive for motility test. Similar, study was conducted by Rehman et al., (2018) which shows *B. cereus* isolates were positive for catalase, negative for indole and methyl red test, negative for urease, negative for citrate agar and positive for motility test.

A concerning prevalence of enterotoxin genes (nhe, hbl, and cytK) among meat-derived strains was found by molecular characterization of isolates, which is consistent with clinical evidence that links these virulence factors to outbreaks of foodborne disease(Ehling-Schulz et al., 2019). These findings support epidemiological research that indicates meat products are the source of around 20% of documented *B. cereus* outbreaks. The genetic diversity seen in *B. cereus* isolates points to intricate adaptation strategies for settings used in meat processing.

Some meat isolates have closer genetic ties to clinical strains than to environmental isolates, according to different phylogenetic groups found by whole genome sequencing (Schmid et al., 2021). This discovery has significant ramifications for risk assessment as it suggests that some lineages that have evolved to survive meat processing may have increased pathogenic potential. The discovery that several isolates included plasmid-borne toxin genes, which might promote horizontal gene transfer across bacterial communities in meat ecosystems, was especially worrisome (Liu et al., 2020)

Clinical management of such infections is made more difficult by the fact that certain isolates include antimicrobial resistance determinants against tetracyclines and β-lactams. Selected phage cocktails achieved >3 log decreases in *B. cereus* counts on meat surfaces, which is encouraging for our bacteriophage-based intervention investigations (Zhang et al., 2020). Phage selectivity for distinct *B. cereus* sequence types indicates that customized phage combinations would be required for efficient biocontrol in various meat processing plants. The necessity for dynamic phage rotation strategies or combination approaches with other antimicrobials is highlighted by the establishment of phage-resistant mutants upon repeated exposure (Kazantseva et al., 2023). Application procedures need to be tailored for certain product types, as evidenced by the considerable variation in the stability of phage activity under different meat storage conditions (aerobic, vacuum-packed, and frozen). A number of real-world issues need to be taken into account while creating phage-based therapies. Many nations still lack adequate regulatory frameworks for phage uses in food, necessitating precise standards for assessing safety and efficacy (Kazantseva et al., 2023).

Phage technology is seen differently by consumers, according to research on consumer acceptability, which highlights the necessity of open and honest communication on its advantages and safety. Phage incorporation into active packaging materials or edible coatings holds promise for long-lasting antimicrobial action over the course of a product's shelf life from a technical standpoint. Scale-up issues, however, include preserving phage viability throughout industrial processing and storage, which would call for creative stabilizing methods such microencapsulation (Tan et al., 2024). Our results have greater implications for food safety management systems. The current microbiological standards for *B. cereus* in food sometimes ignore strain virulence potential in favor of enumeration alone (Haque et al., 2021). Our findings indicate the need for more advanced risk assessment techniques that take isolate molecular characterization into account (Stefanič et al., 2024). More accurate monitoring of high-risk strains in meat processing settings may be made possible by the development of quick detection techniques that target virulence genes. Additionally, given that distinct *B. cereus* lineages exhibit differing levels of disinfectant resistance, the observed strain variety raises the possibility that sanitation techniques may require facility-specific adjustment (Stefanič et al., 2024).

Studies monitoring *B. cereus* population dynamics across the meat supply chain should be the main focus of future research objectives. Important ecological relationships between *B. cereus* and other meat microbiota that affect its persistence and virulence expression may be revealed by metagenomic techniques (Yuan et al.,2023). For improved pathogen control, research should be done on the possible synergies between phage treatment and other cutting-edge technologies as cold plasma or high-pressure processing. Furthermore, in industrial contexts, economic assessments are required to assess the cost-effectiveness of phage-based therapies in comparison to traditional preservation techniques (Kambová, 2021).

5. Conclusion

The results of the study show that fresh meat frequently contains *Bacillus cereus*, which is very resistant to antibiotics. Our isolation and biochemical analysis confirmed the presence of *B. cereus* strains. A lot of research has been done to identify biomarkers and characteristics that set the closely related members of the *B. cereus* group apart. Finding the pathogenic strains of *B. cereus* that cause food poisoning and diarrheal symptoms is a major concern. These syndromes seem to be caused by the different cytotoxin and degradative enzymes generated by bacteria under the direction of the virulence regulator. Therefore, in order tackle this problem, new approaches are required. One of the most effective ways to address the issue of antibiotic resistance is to treat patients using bacteriophages.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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