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POST-PASTEURIZATION PERSISTENCE OF ENTEROTOXIGENIC MULTI-DRUG RESISTANT *BACILLUS CEREUS* IN THE DAIRY CHAIN

Passant A. Elhenawy[1], Adel M. Saudi[1], Samah F. Darwish[2], Tarek N. Soliman[3] and Zeinab I. Ali[1]*

Address(es):
[1] Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt.
[2] Biotechnology Research Unit, Animal Reproduction Research Institute (ARRI), Agricultural Research Center (ARC), Giza, 65221, Egypt.
[3] Dairy Department, Food Industries and Nutrition Research Institute, National Research Center, El-Buhouth St., Dokki, Cairo 12622, Egypt.

*Corresponding author: Passantadel259@gmail.com

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ABSTRACT

Milk is a crucial global food source, yet raw milk serves as vehicle for foodborne pathogens, including spore-forming bacteria that challenge thermal processing. The effectiveness of standard batch pasteurization (63°C/30 min) against the public health risk posed by *Bacillus cereus* in raw milk from diverse Egyptian sources remains ambiguous. Fifty raw milk samples from farms, shops, smallholders, and street vendors were analyzed pre- and post-laboratory pasteurization for physicochemical quality and microbial load. Surviving *B. cereus* isolates (N=11) were characterized by five enterotoxin genes and their resistance to ten antibiotics. The findings revealed significant differences in the chemical composition within raw milk samples, where milk from dairy farms and small holdings exhibiting higher fat and protein levels compared to milk from street vendors and dairy shops. Street vendors' milk showed the poorest physicochemical quality, with the highest levels of added water (8.8%) and the highest microbial contamination, including a significantly higher *B. cereus* count (0.8 ± 0.3 $\log_{10}$ CFU/mL). Pasteurization effectively lowered aerobic plate counts by 99.99% and eliminated coliforms and Enterobacteriaceae but was completely ineffective against aerobic spores and *B. cereus* counts, which remained unchanged. Genotypic profiling of the *B. cereus* isolates (N=11) revealed a high prevalence of enterotoxin genes, primarily *nhe* (81.8%) and *bceT* (72.7%). Furthermore, these isolates showed high resistance to penicillin (100%), trimethoprim-sulfamethoxazole (90.9%), and cefoxitin (81.8%). The study concludes that pasteurization alone is insufficient to eliminate the critical public health risk posed by virulent and multi-drug resistant *B. cereus* in raw milk, underscoring a critical need for improved pre-processing hygiene and alternative control strategies.

Keywords: Raw milk, dairy farms, street vendors, *Bacillus cereus*

INTRODUCTION

Throughout human civilization, milk and dairy products have played pivotal role in providing numerous nutrients, including proteins, lipids, carbohydrates, vitamins, and minerals. It is extensively consumed globally in diverse forms and constitutes a crucial part of the human diet. However, because of its high nutritional value and balanced composition, milk is considered an ideal environment for the proliferation of many microbes which may then impact the safety of processed dairy products (**Li *et al.*, 2024 & Shawki et al., 2024**). There are several potential contaminants to milk once it leaves the udder, including hair, waste, bedding, air, water and feed, which may be a source of spoilage and pathogenic microorganisms (**Owusu-Kwarteng *et al.*, 2020**). Thus, the initial microbial load significantly influences the safety and quality of raw milk and reflects the hygienic standards applied during milking and processing (**Evanowski *et al.*, 2020** and **Martin *et al.*, 2023**). Raw milk poses an elevated risk of foodborne illnesses as it can contain harmful pathogens, including *Staphylococcus aureus*, *Bacillus cereus*, *Shiga toxin-producing Escherichia coli* (STEC), *Listeria monocytogenes*, *Salmonella* species, *Yersinia enterocolitica*, *Non-tuberculous mycobacteria* and *Campylobacter* species **(Ali *et al.*, 2020, Hassan & Ali, 2024** and **Elhenawy *et al.*, 2025).** According to the Centers for Disease Control and Prevention, retail sale of unpasteurized milk is legally permitted in a limited number of U.S. states, including California, Pennsylvania, Maine, Washington, Idaho, and New Mexico, which represent the main states allowing direct consumer access to raw milk under regulatory oversight **(CDC, 2019).** Many farm families, workers, and a growing segment of the general population believe raw milk is safe and healthy, claiming it retains beneficial effects that pasteurization may destroy (**O'Callaghan et al., 2019**).

Although the range of bacteria in raw milk is highly diverse, certain groups are significantly impacting their hygienic quality and considered a key indicator of poor hygiene in food safety, specially the Enterobacteriaceae family (**Martin *et al.*, 2023**). The Enterobacteriaceae members include *Salmonella* spp. and *Shigella* spp., as well as pathogenic *E. coli* strains, notably *E. coli O157:H7*. The presence and number of Enterobacteriaceae and coliforms/*E. coli* are key indicators of poor hygiene in food safety assessments (**Mladenović *et al.*, 2021& Fahim et al., 2023**). *Listeria monocytogenes* is considered one of the major food-borne pathogens. Invasive listeriosis results in high hospitalization and fatality rates, with vulnerable groups being particularly susceptible (**Bartula *et al.*, 2023 and Hassan & Ali, 2024**). The predominant thermoduric bacteria in raw milk are gram-positive spore-formers as *Bacillus species.* These bacteria are prevalent in nature and frequently found in both raw and processed foods, mostly owing to their sporulation capability (**Qin *et al.*, 2024**). Endospores exhibit resistance to high-temperature processes such as pasteurization and UHT, enabling bacterial germination and proliferation during food storage, in addition to the ability of vegetative cells to produce extra-cellular enzymes causing deterioration of milk and milk products (**Bartula *et al.*, 2023** and **Lee *et al.*, 2024**). The *B. cereus* group include species such as *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus*, *B. anthracis*, and *B. toyonensis*, with *B. thuringiensis* sharing many toxins with *B. cereus*. The pathogenic *Bacillus species* produces enterotoxins (diarrheal toxins) and a non-ribosomal peptide synthetase toxin (emetic toxin). Hemolysin BL (*Hbl*), Non-hemolytic enterotoxin (*Nhe*), Cytotoxin K (*CytK*), and Enterotoxin T (*BcET*) are diarrheal enterotoxins produced by viable vegetative cells of *B. cereus* in the small intestine. While the emetic toxin cereulide, produced in food by specific strains harboring the *ces* gene, exhibits stability to heat and acid, rendering it resistant to food processing and digestion (**Chang, *et al.*, 2021**). The infective dose is estimated from 10^5–10^7 CFU/mL (**Jessberger *et al.*, 2020** and **El-Arabi** and **Griffiths, 2021**). Antibiotic resistance has been developed due to the overuse of antibiotics. Antibiotic resistance in *B. cereus* can be intrinsic or acquired, with acquired resistance genes often located on mobile genetic elements like plasmids or transposons, allowing horizontal gene transfer and spreading resistance to other pathogens (**Hwang *et al.*, 2022** and **Zhai *et al.*, 2023**).

Despite the well-documented presence of *Bacillus cereus* in raw milk, limited studies have comprehensively assessed the combined risk posed by strains that survive pasteurization, particularly in terms of their enterotoxin gene profiles and antimicrobial resistance patterns. In Egypt, available data focusing either on microbial prevalence or milk quality without integrating post-pasteurization survival with virulence and resistance characteristics. Therefore, this study provides an integrated assessment linking physicochemical and microbiological quality of raw milk from both formal (farms) and informal (shops and street vendors) sources with pasteurization efficacy. It further evaluates the survival of

B. cereus spores and characterizes the enterotoxin gene profile (*nhe, hbl, cytK, ces, bceT*) and antimicrobial resistance patterns of the surviving isolates along the dairy chain.

MATERIAL AND METHODS

Samples collection and Laboratory pasteurization

Fifty bulk tank raw milk samples were randomly collected under complete hygienic conditions from dairy farms (N=15), dairy shops (N=15), small holders (N=10) and street vendors (N=10) in Cairo, Giza and Qalyubia governorates. Samples were transported to the laboratory in a cool (4ºC), insulated ice box with minimal delay for immediate examination. Guaiac test was performed to ensure raw milk samples weren't subjected to any heat treatments **(Schonberg, 1956).** Each sample was split into two portions: one kept raw, while the other pasteurized in the laboratory (63 ºC for 30 min) (**Goyal *et al.*, 2024**), simulating the batch pasteurization process, before being analyzed as pasteurized.

Physico-chemical analysis

Measurement of pH value using a digital PH -meter (HI 98/30, Hanna Instruments) according to **AOAC (2003).** Titratable acidity of milk was determined according to **Wehr & Frank (2004).** Chemical composition (fat %, solids non-fat% (S.N.F.), protein%, lactose%, ash%, water added% and freezing point) was examined using the Lactoscan ultrasonic milk analyzer (Bulgaria-25010).

Microbiological analysis

Tenth fold serial dilutions were performed according to **(ISO 6887-5: 2020).** Total aerobic count and aerobic spore formers count were done according to **APHA, (2015**). Enumeration of *B. cereus* was conducted according to **Bennet *et al.*, (2015)** using mannitol egg yolk polymixin B agar (MYP) medium (HiMedia, M636S). Enterobacteriaceae count was performed based on (**ISO, 2004**). Coliforms count (Most probable number) was carried out based on **(BAM, 2020)** using Lauryl Sulphate Tryptose Broth (LSTB) (HiMedia, M1023I). Total yeast and mold were counted on Sabaroud Dextrose Agar (HiMedia, MH063) based on (**ISO 6611, ES, 2012**). The isolation of *L. monocytogenes* and *E. coli* were performed based on (**ISO 11290-1:2017**) and (**ISO 7251:2005**) respectively.
Suspected isolates of Enterobacteriaceae and coliforms were identified according to **Brenner *et al.*, (2007).** All isolated strains of Aerobic spore-formers and *B. cereus* were identified biochemically according to **De Vos *et al.*, (2011).** For more identification, molecular characterization of *B. cereus* sensu lato group, amplification of the gene encoding the flagella motor protein (motB gene) was performed using BCFomp1 and BCRomp1 primers according to **OliwaStasiak et al., (2010).**

The reduction percentage was calculated according to **Fahim et al., (2023)** as:
Log reduction of the inoculated strains = Log_{10} (A/B)
Percent reduction of the inoculated strains = (A–B) × 100/A
A: is the number of initial viable count
B: is the number of viable microorganisms after treatment.

Phenotypic antibiotic susceptibility testing of *B. cereus* isolates using broth dilution method

Resistance/susceptibility of *B. cereus* isolates to ten antibiotics were determined in Mueller-Hinton (MH) (HiMedia, M173) broth using the broth dilution method recommended by the standard criteria of the Clinical and Laboratory Standards Institute (CLSI) guide (**CLSI, 2015**). Penicillin, Cefoxitine, Cefotaxime, Gentamycin, Chloramphenicol, Trimethoprim /sulfumethoxazole, Vancomycin, Clindamycin, Erythromycin and Tetracycline were each diluted in two-fold in the range of 64 to 0.015 mg/L of MH-broth. Overnight culture of *B. cereus* isolates approximately 1×10^5 CFU/mL were used for inoculation. Growth was carried out at 35 ± 2 °C for 18–20 h incubation period and examined in a microplate reader (OD at 610 nm). The breakpoints against *B. cereus* in the CLSI guideline M45-A2E **(CLSI, 2015**) were used for all antimicrobial agents except Cefoxitine and Cefotaxime for which the breakpoints in the CLSI guideline M 100-S25 were used (**Luna *et al.*, 2007** and **CLSI, 2020**).

Genotypic analysis (Identification of antibiotic-resistance and enterotoxin genes)

Crude DNA was extracted from bacterial isolates using the Chelex-100 method as reported by (**Elhenawy *et al.*, 2025**) Bacteria grown on the brain heart infusion (BHI) agar plate (HiMedia, M1069) were harvested and dispersed in 200 µl of 5% Chelex-100. The suspension was boiled for 10 min followed by 10 min centrifugation to precipitate bacterial debris. The supernatant was collected and stored at -20˚C to be used as crude template DNA for PCR amplification.
The used primers are presented in Table (1). PCR reactions were established in 20 µl reaction volume containing 5µl of DNA as template, 20 pmol of each primer, and 1X of PCR master mix (Dream Taq Green PCR Master Mix, ThermoFisher). The amplification cycles were carried out in SimpliAmp Thermal Cycler (ThermoFisher Scientific). Reaction conditions were optimized to 94°C for 4 min. as initial denaturation, followed by 35 cycles of 94°C for 30 seconds, specific annealing temperature of each primer pair for 30 seconds and 72 °C for 30 seconds. A final extension step at 72°C for 10 min. was followed. Each run included positive and negative controls. Amplification products were electrophoresed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. A 100bp DNA marker was run simultaneously.

Table 1 Primers used in the genotypic analysis

Primer name	Species &/or gene specific	Primer sequence 5'-3'	Ta ˚c	PCR product size	References
BCFomp1 BCRomp1	*B. cereus* group specific (motB gene)	ATCGCCTCGTTGGATGACGA CTGCATATCCTACCGCAGCTA	54.5	575	OliwaStasiak *et al.* (2010)
mPCR: HD2 F HA4 R	*hbl* gene	GTAAATTGATGAICAATTTC AGAATAGGCATTCATAGATT	49	1091	EhlingSchulz *et al.* (2006)
NA2 F NB 1 R	*nhe* gene	AAGCIGCTCTTCGIATTC ITIGTTGAAATAAGCTGTGG		766	
CK F2 CK R5	*cytK* gene	ACAGATATCGGICAAAATGC CAAGTIACTTGACCIGTTGC		421	
CesF 1 CesR 2	*ces* gene	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA		1271	EhlingSchulz *et al.* (2005)
bceT F bceT R	*bceT* gene	TTACATTACCAGGACGTGCTT TGTTTGTGATTGTAATTCAGG	58	428	Agata *et al.* 1995

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA), version 27 All experiments were performed in duplicate (n = 2 biological replicates), and results were expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA followed by post hoc multiple comparison tests, with $p < 0.05$ considered significant.

RESULTS

Table (2) represented the chemical composition of the examined raw milk samples (N=50), significant differences were observed, especially in fat percentage and added water according to the source. Milk collected from dairy farms and small holders exhibited the highest fat content, with a mean of 6.3% and 6.1%, respectively, while dairy shops (5.3%) and street vendors (4.6%) had considerably lower fat percentages. Similarly, solid not fat (SNF) was highest in dairy farms (9.1%) and small holders (8.7%), compared to dairy shops (8.3%) and street vendors (8.1%).
Milk from dairy farms and small holders had higher protein levels (3.5% and 3.3%) respectively, compared to street vendors (2.8%). The lactose content was also lower in dairy shops and street vendors, with values of 4.5% and 4.4%, respectively. Additionally, moisture content was the highest in street vendors (86.2%) and the lowest in dairy farms (84.1%). After laboratory pasteurization, no change was observed in the chemical composition.

Table 2 Physico-chemical parameters of the examined raw milk samples

Parameters	Dairy farms (N=15)	Small holders (N=10)	Dairy shops (N=15)	Street vendors (N=10)
Parameters	**Mean ±SEM**			
Acidity %	$0.15^{a} \pm 0.1$	$0.14^{a} \pm 0.1$	$0.16^{a} \pm 0.1$	$0.17^{b} \pm 0.1$
pH value	$6.6^{a} \pm 0.4$	$6.7^{b} \pm 0.4$	$6.5^{a} \pm 0.3$	$6.5^{a} \pm 0.5$
Fat%	$6.3^{a} \pm 0.3$	$6.1^{a} \pm 0.4$	$5.3^{b} \pm 0.4$	$4.6^{c} \pm 0.4$
SNF%	$9.1^{a} \pm 0.1$	$8.7^{a} \pm 0.1$	$8.3^{b} \pm 0.1$	$8.1^{b} \pm 0.2$
TS%	$15.8^{a} \pm 0.5$	$13.8^{b} \pm 0.5$	$13.6^{b} \pm 0.6$	$13.7^{b} \pm 0.7$
Moisture%	$84.1^{a} \pm 0.5$	$84.2^{b} \pm 0.5$	$85.4^{b} \pm 0.6$	$86.2^{b} \pm 0.7$
Protein%	$3.5^{a} \pm 0.2$	$3.3^{a} \pm 0.2$	$3.4^{a} \pm 0.2$	$2.8^{b} \pm 0.3$
Lactose%	$4.9^{a} \pm 0.3$	$5.3^{a} \pm 0.3$	$4.5^{b} \pm 0.3$	$4.4^{b} \pm 0.4$
Ash%	$0.7^{a} \pm 0.5$	$0.8^{a} \pm 0.4$	$0.7^{a} \pm 0.5$	$0.6^{a} \pm 0.4$
Added water%	$0^{a} \pm 0$	$0^{a} \pm 0$	$3.8^{b} \pm 1.9$	$8.8^{c} \pm 1.4$
Freezing point °C	$-0.56^{a} \pm 0.5$	$-0.57^{a} \pm 0.4$	$-0.53^{b} \pm 0.4$	$-0.52^{c} \pm 0.5$

N= total number of the examined samples; SEM: Standard error of mean.
Different superscript letters in the same row indicate significant difference ($p<0.05$).
Same superscript letters in the same row indicate non-significant difference ($p>0.05$).

Microbiological analysis of raw milk samples was demonstrated in table (3) Aerobic plate count (APC) was observed to be nearly similar in dairy farms (6.8 ± 0.2 $\log_{10}$ CFU/mL), small holders (7.3 ± 0.3), dairy shops (7.6 ± 0.2), with no significant differences between these groups ($P > 0.05$). However, a significant increase in APC was found in street vendors, with a $\log_{10}$ CFU count of 8.0 ± 0.3 ($P < 0.05$). Additionally, the Aerobic Spore Formers Count (ASF) was lowest in dairy farms (2.6 ± 0.1) and higher in small holders (3.8 ± 0.3), dairy shops (3.9 ± 0.1), and street vendors (4.7 ± 0.4) ($P< 0.05$). *B. cereus* was detected with mean counts of 0.3 ± 0.1, 0.4 ± 0.2, 0.5 ± 0.3 and 0.8 ± 0.3 $\log_{10}$ CFU/mL in dairy farms, dairy shops, small holders and street vendors, respectively with a significant increase in street vendors ($P < 0.05$). Enterobacteriaceae, counts were highest in street vendors (5.6 ± 0.3), followed by dairy shops (5.4 ± 0.5) and small holders (4.8 ± 0.7), while dairy farms recorded the lowest count (3.3 ± 0.7) ($P < 0.05$). Coliform counts were highest in street vendors (4.7 ± 0.6) and dairy shops (4.5 ± 0.4), with small holders (4.0 ± 0.4) and dairy farms (3.3 ± 0.8) showing lower levels, though these differences were not significant ($P > 0.05$). Lastly, yeast and mold count was highest in street vendors (6.8 ± 0.3) and dairy shops (6.6 ± 0.2), followed by small holders (6.3 ± 0.6), with dairy farms showing the lowest count (4.9 ± 0.3) ($P < 0.05$).

Table 3 Microbiological analysis of the examined raw milk samples ($\log_{10}$ CFU/mL)

Parameters	Dairy farms (N=15)	Small holders (N=10)	Dairy shops (N=15)	Street vendors (N=10)
	Mean ±SEM			
Aerobic plate count (APC)	$6.8^{a} \pm 0.2$	$7.3^{a} \pm 0.3$	$7.6^{a} \pm 0.2$	$8^{b} \pm 0.3$
Aerobic spore formers count (ASF)	$2.6^{a} \pm 0.1$	$3.8^{b} \pm 0.3$	$3.9^{b} \pm 0.1$	$4.7^{c} \pm 0.4$
***B. cereus* count**	$0.3^{a} \pm 0.1$	$0.5^{a} \pm 0.3$	$0.4^{a} \pm 0.2$	$0.8^{b} \pm 0.3$
Enterobacteriaceae count	$3.3^{a} \pm 0.7$	$4.8^{ab} \pm 0.7$	$5.4^{ab} \pm 0.5$	$5.6^{bc} \pm 0.3$
Coliform count ($\log_{10}$ MPN/ml)	$3.3^{a} \pm 0.8$	$4^{a} \pm 0.4$	$4.5^{a} \pm 0.4$	$4.7^{a} \pm 0.6$
Yeast and mold count	$4.9^{a} \pm 0.3$	$6.3^{a} \pm 0.6$	$6.6^{ba} \pm 0.2$	$6.8^{ba} \pm 0.3$

N= total number of the examined samples; SEM: Standard error of mean.
Different superscript letters in the same row indicate significant difference ($P<0.05$).
Same superscript letters in the same row indicate non-significant difference ($P>0.05$).

Table (4) presented the effect of laboratory pasteurization on the microbial quality of milk. A significant reduction ($P < 0.05$) was observed in the aerobic plate count (APC) and yeast and mold count following pasteurization. The mean APC decreased from 8.8 ± 0.1 $\log_{10}$ CFU/mL in raw milk to 4± 0.1 in pasteurized milk (99.99% reduction). A complete elimination (below detectable limits) of yeast and mold counts, Enterobacteriaceae and coliform bacteria was recorded in pasteurized milk (99.99% reduction).

Table 4 Microbial reduction after laboratory pasteurization of raw milk samples

Parameters	Raw milk (N=50)	Pasteurized milk (N=50)	Log reduction	Reduction %
	Mean ±SEM			
Aerobic plate count (APC)	8.8 ± 0.1^{a}	4 ± 0.1^{b}	4.8	**99.99%**
Aerobic spore formers count (ASF)	3.7 ± 0.1^{a}	3.7 ± 0.1^{a}	0	0%
***B. cereus* count**	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0	0%
Enterobacteriaceae count	4.9 ± 0.3^{a}	ND^{b}	≥6.8	**99.99%**
Coliform count ($\log_{10}$ MPN/ml)	4.1 ± 0.3^{a}	ND^{b}	≥5.6	**99.99%**
Yeast and mold count	6.2 ± 0.2^{a}	ND^{b}	≥7.3	**99.99%**

N= total number of the examined samples; SEM: Standard error of mean. Different superscript letters in the same row indicate significant difference ($P<0.05$). Same superscript letters in the same row indicate non-significant difference ($P>0.05$). ND: Not Detected.

In contrast, no significant difference ($p > 0.05$) was detected in aerobic spore formers (ASF), or *B. cereus* counts, (5.2 ± 0.1, 3.7 ± 0.1, and 0.5 ± 0.1, respectively).

Data presented in table (5) showed the acceptability of examined samples to the Egyptian standards. For raw milk samples, only 6% (3/50) of the samples met the legal limit for APC (i.e., less than 500,000 cells/ml). In terms of *B. cereus*, 88% (44/50) of raw milk samples were acceptable (not exceeding one cell/ml). All raw milk samples (100%) were free of *L. monocytogenes* and met the legal requirement, while for *E. coli*, 80% were acceptable. For APC, 66% of pasteurized milk samples were within the acceptable range (less than 30,000 cells/ml). *E. coli* and *L. monocytogens* were absent in all pasteurized milk samples (100% met the legal standards). Similarly, Coliforms were within acceptable limits in all pasteurized samples (100%), with counts of less than 10 cells/ml.

The biochemical identification of aerobic spore formers isolates was presented in fig. (1). A total of 196 isolates were identified. The most prevalent species were *Bacillus subtilis* (26.67%, 52 isolates), followed by *Bacillus pumilus* (22.56%, 44 isolates) and *Bacillus licheniformis* (14.36%, 28 isolates). Other notable isolates included *Bacillus megaterium* (12.82%, 25 isolates), *Bacillus badius* (7.69%, 15 isolates), and *Bacillus cereus* (5.6%, 11 isolates).

Fig. (2) showed biochemical identification of Enterobacteriaceae isolates. A total of 205 isolates were identified, with the most prevalent species being *Hafnia alvei* (17.07%, 35 isolates), followed by *Enterobacter aerogens* (16.58%, 34 isolates) and *Enterobacter dissolvens* (14.63%, 30 isolates). Other common isolates

included *Klebsiella pneumoniae* subsp. *pneumoniae* (14.15%, 29 isolates), *Providencia rettgeri* (6.83%, 14 isolates), and *Escherichia coli* (5.85%, 12 isolates). *Serratia fonticola* (5.36%, 11 isolates) and *Serratia liquefaciens* (4.88%, 10 isolates) were also frequently isolated.

Table 5 Degree of acceptability of the examined raw and pasteurized milk samples based on Egyptian standards

Parameters	Aerobic plate count	*B. cereus* count	Coliform count	*E. coli*	*L. monocytogens*
	Raw milk samples (N=50)				
Legal values	*Less than 500000 cells/ml	*One cell/ml	N/A	*Free	*Free
No. of acceptable samples (%)	3 (6%)	44 (88%)	N/A	40 (80%)	50 (100%)
	Pasteurized milk samples (N=50)				
Legal values	*Less than 30000 cell/ml	*Free	*Less than 10 cell/ml	*Free	*Free
No. of acceptable samples (%)	33 (66%)	44 (88%)	50 (100%)	50 (100%)	50 (100%)

N: total number of the examined samples; Min: Minimum; Max: Maximum; SEM: Standard error of mean. N/A: Not available.
*Egyptian Standards (ES: 154-1/2005) and (ES: 1616/2005)

The antibiotic resistance profile of *B. cereus* isolates presented in table (6) revealed a striking resistance to β-lactam antibiotics. All isolates (100%) were resistant to penicillin, and the majority were resistant to cefoxitin (81.8%). Resistance to the third-generation cephalosporin, cefotaxime, was observed in 27.3% of isolates while Tetracycline (Tetracyclines) demonstrated moderate resistance (18.2%). Clindamycin (Lincosamide) and Erythromycin (Macrolide) also showed lower resistance (9%). In contrast, all tested *B. cereus* isolates were susceptible to Chloramphenicol (Amphenicol) and Vancomycin (Glycopeptide) while 90.9% were susceptible to Gentamicin (Aminoglycoside).

Table 6 Phenotypic antimicrobial resistance profile of *B. cereus* strains (N=11)

Antimicrobial Class	Antimicrobial agent	R	I	S
		N (%)		
β-lactams	Penicillin	11(100)	0(0)	0(0)
	Cefoxitin	9(81.8)	2(18.2)	0(0)
	Cefotaxime	3(27.3)	4(36.4)	4(36.4)
Aminoglycoside	Gentamicin	0(0)	1(9)	10(90.9)
Amphenicol	Chloramphenicol	0(0)	0(0)	11(100)
Folic acid inhibitor	Trimethoprim-sulfamethoxazole	10(90.9)	0(0)	1(9)
Glycopeptide	Vancomycin	0(0)	0(0)	11(100)
Lincosadmide	Clindamycin	1(9)	1(9)	9(81.8)
Macrolides	Erythromycin	1(9)	3(27.3)	7(63.6)
Tetracyclines	Tetracycline	2(18.2)	1(9)	8(72.7)

R: Resistance; I: Intermediate; S: Sensitive; N: Number of isolates

Fig. (3) illustrated the genotypic profiling of various toxin genes among *B. cereus* isolates, 81.8% of *B. cereus* isolates harbored *nhe*. Followed by *bceT* (72.7%), *cytK* (45.4%), *hbl* (18.2%) and *ces* (9%). The co-occurrence of virulence factors was also observed, with *nhe* and *ces* present in 9% of the isolates, *nhe*, *cytK*, and *bceT* in 36.3%, and *nhe*, *hbl*, and *bceT* in 18.2%.

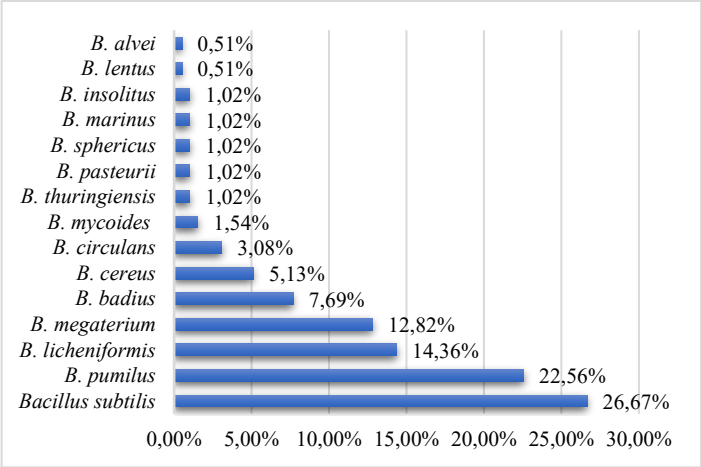

Figure 1 Identification of Aerobic Spore Former isolates

DISCUSSION

Effect of milk source on the physicochemical attributes of raw milk

The physicochemical analysis presented in table (2) revealed the relation between milk source and quality. pH and titratable acidity are milk quality indicators that reflects the freshness, bacterial activity, and flavor of the milk (**Kandeel *et al.*, 2019** and **Bangieva *et al.*, 2020**).Milk from street vendors exhibiting the worst quality profile based on the Egyptian regulatory standards **(ES, 2005).** Particularly, the highest levels of added water (8.8%) and the lowest levels of fat and protein. The obtained findings match with previous reports in Egypt and other developing nations, where informal milk supply chains are often associated with economic adulteration to increase volume **(Wafy, 2019; Ali *et al.*, 2021 and Nyokabi *et al.*, 2021).** The presence of added water not only compromises the nutritional value but also suggests a lack of regulatory oversight and potentially introduces secondary contamination.

The pH value and titratable acidity of milk samples from dairy farms were 6.6 ± 0.4 and 0.15 ± 0.1 lactic acid, respectively. Comparable findings were reported by **Tawfik *et al.*, (2022),** who observed slightly lower pH values and similar titratable acidity levels in milk from dairy farms. In contrast, **Ismail *et al.*, (2024)** reported lower pH values and higher acidity levels in milk samples from supermarkets in the New Valley Governorate.

Estimation of the microbial load of examined raw milk samples

The aerobic plate count (APC) reflects the total viable bacterial population and provides insight into milking hygiene, udder health, and mastitis management. Lower values (below 10,000 CFU/mL) are generally associated with higher milk quality (**Böhnlein *et al.*, 2021, Martin *et al.*, 2023 and Ahmed et al., 2025**). Street vendor milk showed the highest aerobic plate counts (APC) (8.0 ± 0.3 $\log_{10}$ CFU/mL) and significantly higher counts of Enterobacteriaceae and coliforms (4.7 ± 0.6 $\log_{10}$ MPN/ml). These organisms serve as key indicators of fecal contamination and overall poor hygiene during milking, handling, and storage **(Mladenović et al., 2021).** The elevated microbial load, especially in the informal supply chain, highlights the urgent need for stricter hygiene standards and enforcement at the point of sale to protect consumer health **(Meshref et al., 2021).** These elevated levels are indicative of substandard hygiene and handling practices, consistent with findings from previous studies (**Galaby *et al.*, 2021** and **Meshref *et al.*, 2021**). Additionally, the high levels of Enterobacteriaceae and coliforms in informal milk sources further support linking such contamination to poor hygiene and handling practices across the dairy chain (**Sobeih *et al.*, 2020; El-Prince *et al.*, 2023; Fathi *et al.*, 2019**). These findings underscore the microbial threats associated with milk from informal street vendors and highlight the urgent need to improve hygiene practices within the informal milk distribution sector to mitigate potential health risks. Additionally, the present study found that all milk samples were free from *L. monocytogenes*, contrasting with the findings of **Saleh *et al.*, (2021)** and **Ahmed *et al.*, (2022),** who reported a prevalence of *L. monocytogenes* in 13.3% of raw milk samples.

Pasteurization Efficacy and the Persistence of *B. cereus*

The results in table (4) indicated that aerobic spore formers (ASF) and *B. cereus* counts were the lowest in dairy farms, **Owusu-Kwarteng *et al.*, (2017)** and

Yacoub *et al.*, (2017) observed lower ASF counts in dairy farms, while **Khater *et al.*, (2017)** noted comparable ASF contamination levels in dairy shops. On the other hand, higher *B. cereus* counts were reported by **Alnakip *et al.*, (2023)** and **Mohamed *et al.*, (2016)** in raw milk from dairy shops. Interestingly, standard laboratory batch pasteurization (63°C/30 min) proved to be highly effective against non-spore-forming bacteria, achieving a 99.99% reduction in APC (Table 4) and complete elimination of coliforms and Enterobacteriaceae. This result confirms the critical role of pasteurization in neutralizing the immediate threat posed by common vegetative pathogens, such as *E. coli* and *L. monocytogenes*, which were successfully eliminated **(El-Prince et al., 2023).** On the other hand, ineffectiveness of pasteurization against aerobic spore formers and *Bacillus cereus* was recorded. From a dairy processing perspective, this suggests that pasteurization cannot be considered a control step for *B. cereus*, but rather a risk-modifying step under certain conditions. Similar observations have been reported by **Dash *et al.*, (2022)** and **Medjahdi *et al.*, (2025),** who similarly observed that spore-forming bacteria are more resistant to pasteurization, highlighting the limitations of pasteurization in fully eliminating these types of microorganisms.

This result is a direct consequence of the intrinsic heat resistance of *B. cereus* endospores. The thermal processing conditions of 63°C for 30 min are insufficient to inactivate these spores, which are known to survive temperatures up to 100°C **(Chang et al., 2021).** The survival of *B. cereus* spores indicates that, although pasteurization reduces the initial microbial load in raw milk, these heat-resistant spores may subsequently germinate and develop into toxin-producing vegetative cells during refrigerated storage of the pasteurized product. This highlights a critical limitation in current milk safety protocols, as the surviving *B. cereus* population represents a latent public health threat **(Medjahdi et al., 2025).**

Biochemical identification of aerobic spore former isolates presented in fig. 1 revealed that the most prevalent isolates were *B. subtilis* with incidence rate of 26.67%, while *B. cereus* was found at a percentage of 5.6%. Similar findings were reported by **Ali *et al.*, (2013**), who identified *B. subtilis* as the most common strain, occurring at an incidence rate of 24.08%, while *B. cereus* was detected at a rate of 8.38%. As illustrated in fig. 2, the predominant strain was *Hafnia alvei*, accounting for 17.07% of the isolates, while *E. coli* was present at a frequency of 5.85%. These findings align with those reported by **Sobeih *et al.*, (2020)**. In contrast, **El-Mokadem *et al.*, (2020)** identified *Serratia marcescens* as the most prevalent isolate, with *E. coli* being detected at a higher incidence than observed in this study. The degree of acceptability of raw and pasteurized milk samples was assessed based on Egyptian standards in table (5). Raw milk samples showed lower microbiological quality, with only 6% meeting the legal limit for APC. In contrast, after pasteurization, milk showed significantly better compliance, with 66% meeting the APC standards and 100% complying with limits for *E. coli* and *L. monocytogenes*.

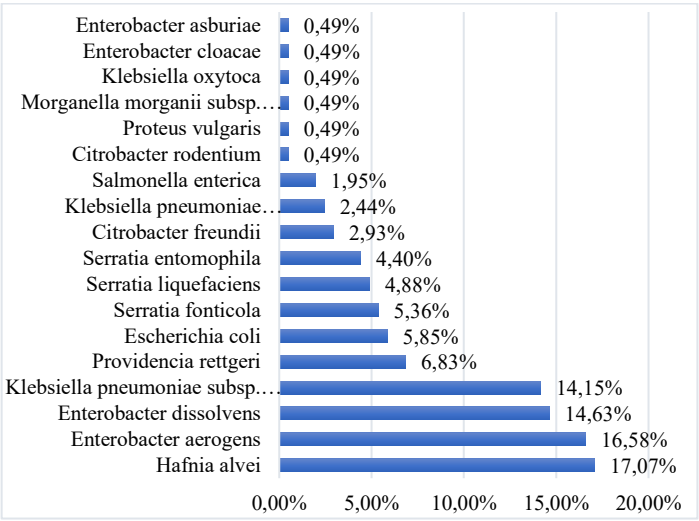

Figure 2 Identification of Enterobacteriaceae isolates

Virulence potential and antimicrobial resistance profile

The phenotypic antimicrobial susceptibility testing further compounded the public health concern. The post-pasteurization *B. cereus* isolates exhibited a high level of resistance to several clinically important antibiotics. The Phenotypic characterization demonstrated in table (6) of the 11 *B. cereus* isolates that survived pasteurization provides the crucial evidence for the severity of this latent threat with high resistance to Penicillin (100%), Trimethoprim-sulfamethoxazole (90.9%) and Cefoxitin (81.8%), these results were similarly consistent with the findings of **Amer *et al.*, (2019), Abouelhag *et al.*, (2021)** and **Abubaker *et al.*, (2023)** who observed high resistance to β-lactams. Moreover, **Abdel-Hak, *et al.* (2024)** reported 73% susceptibility to Trimethoprim-sulfamethoxazole. On the other hand, *B. cereus* isolates exhibited low resistance to erythromycin (9%), whereas **Abubaker *et al.* (2023)** reported complete resistance (100%) to erythromycin. The findings showed that 18.2% of the isolates exhibited resistance to Tetracycline which matched with those of **Osama *et al.*, (2020)** who observed 22.6% resistance to Tetracycline. On the other hand, isolates demonstrated full susceptibility to Chloramphenicol and Vancomycin, with 90.9% susceptibility to Gentamicin. Similar results obtained by (**Dowidar *et al.*, 2023**), while **Elhaw *et al.*, (2024)** detected 50% resistance to Chloramphenicol. This suggests a pressing need for effective antibiotic stewardship in agriculture to reduce the emergence of multidrug-resistant bacteria in the food chain.

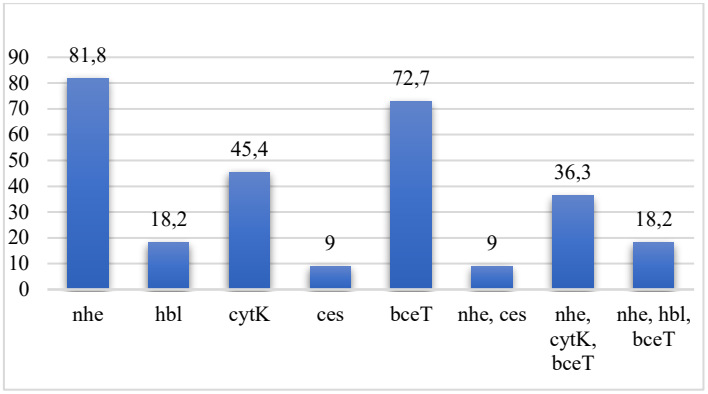

Figure 3 Genotypic profiling of the toxin genes (*nhe, hbl, cytK, ces, bceT*) in *B. cereus* isolates, illustrating their presence and co-occurrence through PCR analysis.

This multi-drug resistance profile is critical, as Penicillin and its derivatives are often the first-line treatment for *Bacillus* infections. The observed resistance is likely due to the intrinsic production of β-lactamase by *B. cereus* **(Owusu-Kwarteng *et al.*, 2017).** The high resistance to Trimethoprim-sulfamethoxazole and Cefoxitin suggests potential acquired resistance mechanisms, which are often found on mobile genetic elements, raising the possibility of horizontal gene transfer to other foodborne pathogens in the dairy environment **(Hwang *et al.*, 2022; Zhai *et al.*, 2023).**

Genotypic analysis of virulent genes associated with *B. cereus*

Genotypic analysis revealed a high prevalence of enterotoxin genes, with the non-hemolytic enterotoxin (*nhe*) gene present in 81.8% and the enterotoxin (*bceT*) gene present in 72.7% of the surviving isolates. The *nhe* gene, in particular, is considered one of the major virulence factors responsible for the diarrheal type of *B. cereus* food poisoning **(Jessberger *et al.*, 2020).** These results are consistent with other studies that have identified *nhe* as a predominant virulence factor in *B. cereus* **(Hefny *et al.*, 2020; Hammad *et al.*, 2021; Mostafa *et al.*, 2022; Dowidar *et al.*, 2023** and **Abdel-Hak *et al.*, 2024**). However, **Asfour *et al.*, (2024)** detected *cytK* and *bceT* toxin genes at higher frequencies, and they were observed as the predominant toxin genes in their study. In the present study, the detection rate of the emetic *ces* gene was 9%, whereas **Abubaker *et al.*, (2023)** reported a higher detection rate of 20%. Furthermore, **Hefny *et al.*, (2020)** reported that none of the *B. cereus* strains they examined carried the *ces* gene. The co-occurrence of multiple virulence factors (*nhe*, *cytK*, and *bceT*) in 36.3% of isolates suggests that the surviving population is highly virulent and capable of causing severe gastrointestinal illness if allowed to proliferate. This finding is particularly concerning because it demonstrates that the thermal process is selectively enriching for the most robust and potentially the most pathogenic strains, a phenomenon previously reported in other food matrices **(Hammad *et al.*, 2021)** and indicates the potential pathogenicity of *B. cereus* in milk, particularly in milk from street vendors.

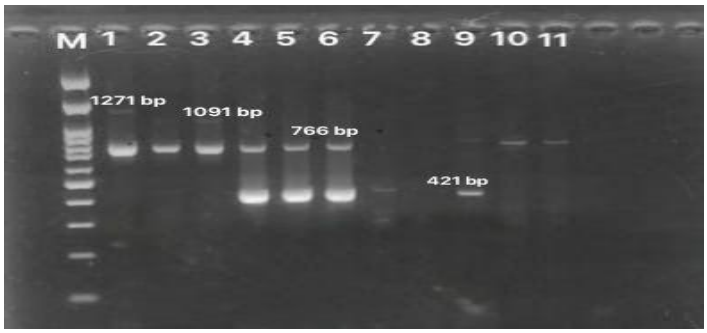

Figure 4 Agarose gel electrophoresis of the multiplex PCR assay showed that the *ces* gene (1271 bp) was positive in lane 1, while the *hbl* gene (1091 bp) was positive in lane 3. The *nhe* gene (766 bp) was detected in lanes 1, 2, 3, 4, 5, 6, 9, 10, and 11, whereas the *cytK* gene (421 bp) was positive in lanes 4, 5, 6, 7, and 9. Lane M contained the 100 bp DNA ladder.

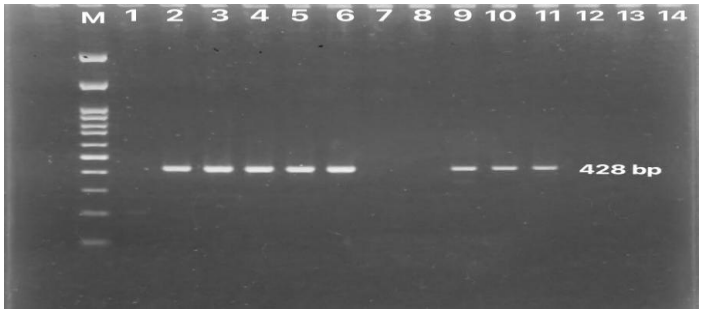

Figure 5 PCR assay for *bceT* gene (428bp). Lane M 100 bp DNA ladder, lane 1 (Negative control), lane 2 (positive *bceT*), lanes 3,4,5,6,9,10,11 are positive samples, lanes 7,8,12,13,14 are negative samples.

CONCLUSION

This study demonstrates that *Bacillus cereus* persists after standard batch pasteurization due to spore resistance, with no significant reduction in its counts. The surviving isolates exhibited a high prevalence of enterotoxin genes, particularly *nhe* and *bceT*, along with notable resistance to β-lactam antibiotics and trimethoprim-sulfamethoxazole. These findings highlight that milk from informal sources represents a significant risk factor for the introduction of virulent and multi-drug resistant *B. cereus* into the dairy chain. Therefore, improving pre-processing hygiene and implementing additional control measures beyond pasteurization are essential to ensure milk safety.

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