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Research Note

A Study To Assess the Numbers and Prevalence of *Bacillus cereus* and Its Toxins in Pasteurized Fluid Milk

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ABSTRACT

Bacillus cereus is a pathogenic adulterant of raw milk and can persist as spores and grow in pasteurized milk. The objective of this study was to determine the prevalence of *B. cereus* and its enterotoxins in pasteurized milk at its best-before date when stored at 4, 7, and 10°C. More than 5.5% of moderately temperature-abused products (stored at 7°C) were found to contain $>10^5$ CFU/mL *B. cereus*, and about 4% of them contained enterotoxins at a level that may result in foodborne illness; in addition, more than 31% of the products contained $>10^5$ CFU/mL *B. cereus* and associated enterotoxins when stored at 10°C. Results from a growth kinetic study demonstrated that enterotoxin production by *B. cereus* in pasteurized milk can occur in as short as 7 to 8 days of storage at 7°C. The higher *B. cereus* counts were associated with products containing higher butterfat content or with those produced using the conventional high-temperature, short-time pasteurization process. Traditional indicators, aerobic colony counts and psychrotrophic counts, were found to have no correlation with level of *B. cereus* in milk. The characterization of 17 representative *B. cereus* isolates from pasteurized milk revealed five toxigenic gene patterns, with all the strains carrying genes encoding for diarrheal toxins but not for an emetic toxin, and with one strain containing all four diarrheal enterotoxin genes (*nheA*, *entFM*, *hblC,* and *cytK*). The results of this study demonstrate the risks associated even with moderately temperature-abused pasteurized milk and the necessity of a controlled cold chain throughout the shelf life of fluid milk to enhance product safety and minimize foodborne illness.

Key words: *Bacillus cereus*; *Bacillus cereus* prevalence; *Bacillus cereus* toxins; Enterotoxins; Milk; Pasteurized milk

Bacillus cereus is ubiquitous in nature and is a common contaminant of raw milk, in which its heat-resistant spores often survive pasteurization. *B. cereus* can also be reintroduced into pasteurized milk by inadequately cleaned and sanitized dairy processing pipelines and equipment *(2, 3)*. Previous studies *(2, 3)* indicated that *B. cereus* numbers can reach as high as 10^7 CFU/mL in pasteurized milk before obvious spoilage is observed. This is of concern because published reports *(10, 20)* indicate that *B. cereus* at 10^5 CFU/mL can cause foodborne illness. A risk assessment performed by Notermans et al. *(20)* indicated that up to 7% of milk servings could have *B. cereus* counts (BC) exceeding 10^5 CFU/mL. *B. cereus* can produce emetic and necrotizing enterotoxins, causing diarrhea, nausea, and vomiting *(1, 4, 10, 12, 14)*. The number of foodborne outbreaks attributed to *B. cereus* toxins may be underreported because the duration of the illness is generally short and the disease can be misdiagnosed because its symptoms are similar to those caused by *Staphylococcus aureus* (vomiting) and *Clostridium perfringens* (diarrhea) *(5)*.

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A study *(13)* of Ontario's pasteurized milk conducted during the mid-1990s did not identify any concerns with *B. cereus* in pasteurized milk. However, since that time, the retail shelf life of Ontario's pasteurized dairy products has increased from 12 to 14 days to 21 to 23 days. The effect of this longer retail shelf life on the numbers of *B. cereus* and its production of toxin in pasteurized milk is not known. Data from current laboratory testing of pasteurized milk indicate that microbial numbers greater than 10^6 CFU/mL are often seen in samples at their best-before date, even when these samples are collected, transported, and held at optimal conditions (4°C) (S.L., personal communication, 26 January 2015). Studies *(7, 16, 20, 22)* have indicated that the majority of pasteurized milk is stored at temperatures ranging from less than 5 to 13°C during distribution and in the consumers' refrigerators. A longer retail life may result in a greater potential for the temperature abuse of these products, promoting more microbial growth and toxin production.

The objectives of the current study were to (i) estimate the numbers of *B. cereus* in samples of pasteurized milk at their best-before dates when stored at 4, 7, and 10°C; (ii) determine the prevalence of *B. cereus* enterotoxins in pasteurized milk; and (iii) conduct toxigenic characterization of *B. cereus* isolated from milk. The data presented in this

TABLE 1. *Milk samples tested*

Classification and parameter	No. of samples
Packaging type	
Carton	108
Pouch	119
Plastic bottle	4
Glass bottle	23
Butterfat content	
Skim	62
1%	30
2%	92
Homogenized or whole milk	70
No. of days from production date to best-before date	
18–23	206
30–39	40
40–49	8
Pasteurization process[a]	
HTST	206
Microfiltration and HTST	40
UHT	8

[a] HTST, high-temperature, short-time; UHT, ultrahigh temperature.

study can be used to assess the risks associated with *B. cereus* toxins in temperature-abused pasteurized milk.

MATERIALS AND METHODS

Milk samples. Retail-size packages of pasteurized fluid cow milk samples were collected every 2 to 3 weeks from 14 licensed dairy processors located in eastern, central, and southwestern Ontario, Canada, between November 2014 and March 2015. The samples were collected to reflect the variety of container types, process types, and butterfat levels available in Ontario's marketplace (Table 1). The samples were collected on the day of production, kept at 4°C, and shipped to the laboratory within 24 h of collection. Each sample was aseptically divided into three aliquots of approximately 100 mL each. The aliquots were then incubated at 4, 7, or 10°C until the sample's best-before date. At that time, microbial analyses were conducted on the incubated samples.

Microbiological analyses. The total aerobic bacteria were enumerated according to Government of Canada HPB Method MFHPB-33 *(18)*. We obtained the psychotrophic colony counts (PC) following the MFHPB-33 procedure, except that inoculated petrifilms were incubated at 7 ± 1°C for 10 days prior to estimating the cell numbers. The BC were estimated according to Government of Canada Laboratory Procedure MFLP-42 *(8)*. Briefly, we plated known quantities (dilutions) of the food samples onto polymyxin pyruvate egg yolk mannitol bromthymol blue agar. After incubation, we selected presumptive *B. cereus* colonies and subjected them to confirmatory testing, such as motility, rhizoid growth, hemolytic activity, and production of protein toxin crystals *(8)*. We froze the *B. cereus* isolates in 20% glycerol at –80°C until further analysis.

***B. cereus* enterotoxin test.** Samples in which the BC exceeded 10^4 CFU/mL were analyzed for the presence of enterotoxins using the 3M TECRA *Bacillus* diarrheal enterotoxin visual immunoassay kit (Tecra International Pty. Ltd., Frenchs Forest, New South Wales, Australia), as per the manufacturer's protocol. A 200-μL aliquot of each milk sample was tested. The reaction signal was acquired using a spectrophotometer, SPECTRAmax 340PC384 (Molecular Systems, Sunnyvale, CA). The assay detects the *B. cereus* NHE protein complex (related to the *B. cereus nheA* toxin gene) with a detection limit of 1 ng of *Bacillus* diarrheal enterotoxin per mL.

Toxigenic profiling of *B. cereus* isolates using multiplex PCR. We analyzed the *B. cereus* isolates obtained from the milk samples for the presence of the toxin genes *nheA, entFM, hblC, cytK,* and *CER* following the multiplex PCR method of Forghani et al. *(9)*. Briefly, we extracted DNA from the *B. cereus* isolates using the DNeasy tissue kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. The PCR reaction mixture (25 μL) contained 1× HotStarTaq Master Mix (Qiagen), 0.4 μM *cytK* and *nheA* primers, 0.3 μM *CER* and *hblC* primers, 0.2 μM *entFM* primers, and 3 μL of the template DNA. We conducted thermal cycling using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) under following conditions: one cycle at 95°C for 10 min; 35 cycles at 95°C for 15 s, 54°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. We analyzed the PCR products using 2% SYBR Safe (Applied Biosystems) stained agarose gels to determine the presence or absence of the target genes.

***B. cereus* growth analysis.** We selected 17 *B. cereus* isolates, based on the diversity of toxigenic profiles, and subjected them to a bioscreen assay in brain heart infusion broth at 4 and 7°C to determine the growth kinetics. The growth was monitored by measuring the increase in the optical density using a Bioscreen C system at 610 nm (Growth Curves USA, Piscataway, NJ).

We subsequently chose five *B. cereus* strains that had diverse toxigenic profiles and were fast growing (identified by the greatest change in optical density) for a growth kinetics study in milk. We placed 250-mL portions of microfiltered milk into sterile screw-cap glass bottles from 2-L cartons and spiked them with each of the five selected strains (ST1, ST2, ST3, ST4, and ST5) to obtain an inoculation level of 10 to 100 cells per 250-mL sample. We then incubated the samples at 4 and 7°C and analyzed them for levels of *B. cereus* and toxin production at 0, 3, 4, 5, 6, 7, 8, and 10 days as previously described. All samples were prepared and analyzed in duplicate.

Statistical analysis. The correlations among the BC, aerobic colony count (ACC), and PC were investigated using Spearman correlation coefficients. We determined whether there was an association between the BC and packaging types using the nonparametric Kruskal-Wallis method. This method was also used for investigating the association between the BC and butterfat level and between the BC and pasteurization process.

RESULTS AND DISCUSSION

Prevalence of *B. cereus* and *B. cereus* enterotoxins. We determined the prevalence of *B. cereus* and *B. cereus* enterotoxins in the samples of pasteurized fluid milk incubated at 4, 7, and 10°C at their best-before dates (Table 2). As the incubation temperature increased, the prevalence of *B. cereus* and its associated enterotoxins also increased. The data indicate that more than 5.5% of the moderately

TABLE 2. *Prevalence of* B. cereus *and diarrheal enterotoxin at three incubation temperatures*[a]

Temp (°C)	Prevalence of *B. cereus* (%)				Prevalence of *B. cereus* enterotoxin (%)
	Overall	$>1 \times 10^4$	$>5 \times 10^{4b}$	$>1 \times 10^{5c}$	
4	0.8	0.4	0.4	0.4	0.0
7	13.4	7.9	5.5	5.5	3.9
10	40.9	34.6	32.3	31.1	31.8

[a] $n = 254$.
[b] Level at which *B. cereus* enterotoxin may be detected.
[c] Level at which *B. cereus* may cause foodborne illness.

temperature-abused products (stored at 7°C) resulted in BC greater than 10^5 CFU/mL and that about 4% contained enterotoxins that may cause foodborne illness. More than 31% of the products stored at 10°C contained BC greater than 10^5 CFU/mL and enterotoxins. Enterotoxins are heat and acid labile (although the emetic toxin is stable under these conditions generally); therefore, in theory the actual toxin presence in milk is secondary to the potential of these bacteria. At ingested densities greater than 10^5 CFU/mL, *B. cereus* would probably result in gastroenteritis via a toxicoinfection.

The correlation analyses revealed little or no correlation between the ACC and BC (at 4°C, $r = 0.048$, $P = 0.56$; at 7°C, $r = 0.060$, $P = 0.46$; and at 10°C, $r = 0.049$, $P = 0.55$) or between the PC and BC (at 4°C, $r = 0.043$, $P = 0.60$; at 7°C, $r = 0.02$, $P = 0.79$; and at 10°C, $r = 0.08$, $P = 0.32$). However, a significant positive correlation did exist between the ACC and PC at each of the incubation temperatures (at 4°C, $r = 0.95$, $P < 0.0001$; at 7°C, $r = 0.82$, $P < 0.0001$; and at 10°C, $r = 0.42$, $P < 0.0001$). The lack of correlation between the ACC and BC or between the PC and BC indicates that these common microbiological indicators are not reliable predictors of *B. cereus* prevalence and numbers. Most important, our results suggest that temperature-abused yet organoleptically acceptable products with ACC or PC levels up to 1 to 2×10^6 CFU/mL could contain sufficient levels of *B. cereus* and enterotoxins to cause foodborne illness.

The associations between the BC and the packaging type, butterfat content, and pasteurization process were also investigated. The results indicated a significant difference in the BC among different packaging types for milk stored at 7°C ($P = 0.0015$) and 10°C ($P = 0.0263$) but not at 4°C ($P = 0.9598$). The highest BC were found in pasteurized milk packed in glass bottles. Although we did not investigate this, the elevated BC could be due to the reuse of glass bottles and the ineffectiveness of washing and sanitizing them. In addition, most of the pasteurized milk samples packed in glass bottles were organically produced. The higher BC could possibly reflect different on-farm practices used in the production of organic milk compared with raw milk produced using conventional dairy practices.

The results did not indicate a significant difference ($P = 0.898$) in the BC for samples with different butterfat levels stored at 4°C ($P = 0.898$). However, significant differences were noted in products stored at 10°C ($P = 0.0081$), and marginally significant differences were noted in products stored at 7°C ($P = 0.059$); at these temperatures, higher BC were associated with products containing higher butterfat levels.

We observed an influence of the pasteurization process; however, the number of samples included for the comparison of the pasteurization processes was not balanced. Almost all the *B. cereus*–positive samples had been subjected to high-temperature, short-time pasteurization (HTST); in contrast, *B. cereus* was found in only 1 of the 40 samples subjected to microfiltration coupled with HTST pasteurization, and that sample had low numbers (25 CFU/mL) of *B. cereus*.

TABLE 3. *Profiles of enterotoxin genes from selected* B. cereus *isolates*[a]

No. of isolates	*cytK*	*nheA*	*CER*	*hblC*	*entFM*	Toxin profile
9	−	+	−	−	+	ST1
4	−	−	−	+	−	ST2
1	−	+	−	−	−	ST3
2	−	+	−	+	+	ST4
1	+	+	−	+	+	ST5

[a] $n = 17$. The strains were isolated from different milk samples incubated at 7°C (10 isolates) or at 10°C (7 isolates). +, presence of gene; −, absence of gene; ST1 to ST5, each toxigenic profile and the strain chosen to be studied with that profile.

Toxigenic characterization of *B. cereus*. Different toxins of *B. cereus* are responsible for diarrheal and emetic types of food poisoning. The diarrheal food poisoning is associated with heat-labile enterotoxins, especially nonhemolytic enterotoxin, enterotoxin FM, hemolysin BL, and cytotoxin K, whereas emesis is caused by the heat-stable emetic toxin cereulide. These toxins are encoded by single or multiple genes. In this study, we used the gene sequences for the toxigenic characterization of *B. cereus*. Table 3 shows the results from selected strains of *B. cereus* isolated from different milk samples incubated at 7°C (10 isolates) or at 10°C (7 isolates). All the strains tested carried genes encoding for diarrheal toxins but not for an emetic toxin.

We observed five toxigenic gene patterns (ST1 to ST5, each toxigenic profile and the strain chosen to be studied with that profile), with the nonhemolytic enterotoxin gene (*nheA*) and enterotoxin FM gene (*entFM*) both present in the majority (>70%) of the *B. cereus* strains tested, followed by the hemolysin BL gene (*hblC*, 41%). One of the strains

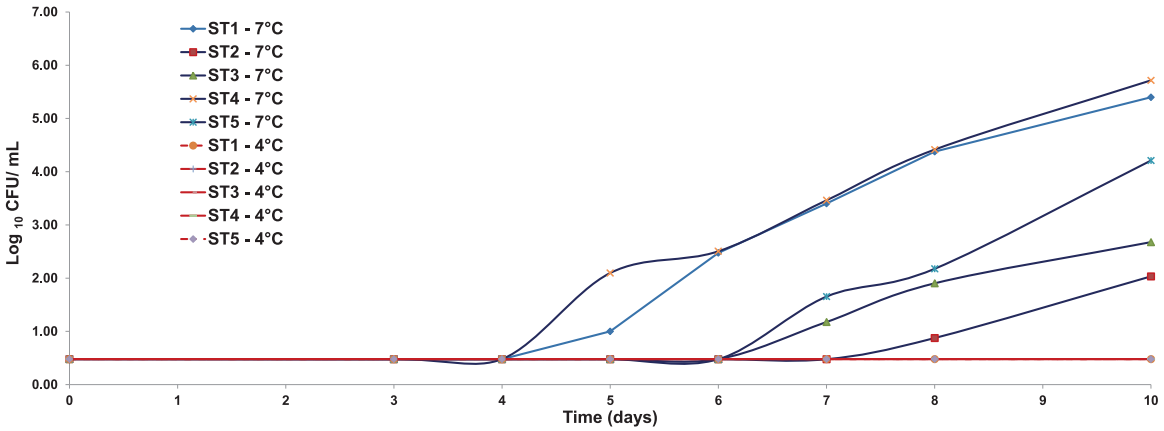

FIGURE 1. *Growth kinetics of five representative* B. cereus *strains (ST1 to ST5) in microfiltered milk incubated at 4°C (red lines) and 7°C (blue lines). ST1 to ST5 represent each toxigenic profile and the strain chosen to be studied with that profile.*

carried all of the four diarrheal enterotoxin genes. These observations are consistent with published data *(17, 21),* in which the diarrheal toxins are commonly associated with proteinaceous foods, sauces, meat products, and dairy products while the emetic enterotoxins are mostly found in cooked rice, pasta, pastry, and noodles.

***B. cereus* growth kinetics in pasteurized milk.** We conducted a further study of the growth characteristics of the toxigenic *B. cereus* strains to determine the likelihood of *B. cereus* growth and toxin production in pasteurized milk stored at 4 and 7°C because milk stored under these conditions may still be acceptable to consumers at its best-before date. A bioscreen analysis of the 17 isolates (used in the toxigenic analysis in Table 3) grown in brain heart infusion broth indicated little growth occurred at 4°C; however, at 7°C, the optical density increased dramatically after a lag of about 6 days for 6 of the 17 *B. cereus* isolates (data not shown), indicating the potential for these strains to grow in milk to levels that may cause foodborne illness.

We selected five strains exhibiting varying toxigenic profiles, as well as accelerated growth rates, for further growth kinetics studies at 4 and 7°C. We used microfiltered milk as the growth substrate because previous results indicated very low levels of *B. cereus* contamination (data not shown). At 4°C, none of the five strains demonstrated substantial growth (<3 CFU/mL) or toxin production during the 10-day incubation period (Fig. 1). In contrast, at 7°C after a lag period of 4 to 7 days, significant growth (Fig. 1) was observed for all five strains. In particular, after 7 to 8 days of growth at 7°C, the *B. cereus* strains ST1 and ST4 reached levels greater than 10^4 CFU/mL, a level at which enterotoxins may be detected in pasteurized fluid milk *(11, 19)*; this also correlated with the enterotoxin detection in these samples on day 10.

We conducted an additional study involving 102 of the 254 pasteurized fluid milk samples to gain some insight into the rate at which *B. cereus* numbers could become problematic in temperature-abused milk. When the milk samples were stored at 10°C, the *B. cereus* numbers reached 10^5 CFU/mL in 36 (35%) of the 102 samples by their best-before date (<21 days). For example, four of the products that were held for only 5 to 6 days, and which therefore could still be organoleptically acceptable, already contained a BC of 10^5 CFU/mL.

These results suggest that keeping milk at 4°C supports little growth of *B. cereus* in milk; that keeping milk at 7°C, which occurs in household fridges, can result in substantial increases in the numbers of *B. cereus* after 7 to 8 days; and that keeping milk at 10°C can increase *B. cereus* levels to 10^5 CFU/mL in 5 to 6 days. Therefore, storing milk above 4°C for several days may lead to *B. cereus* in sufficient concentrations to be of concern. The results also reinforce previous observations that little to no *B. cereus* growth occurred in milk stored at 4°C *(23)* and that psychrotrophic and mesophilic *B. cereus* strains can grow to 10^5 to 10^6 CFU/g and produce toxins in food within their shelf life when stored between 5 and 10°C *(6, 15)*.

We can conclude from this study that the integrity of the cold chain throughout the life of fluid milk is crucial for product safety. When milk is stored at 4°C, the growth of *B. cereus* and the production of its enterotoxins are minimized; however, when milk is subjected to moderate temperature abuse, which may still be organoleptically acceptable, *B. cereus* could pose a health risk to consumers. Moreover, we found that ACC and PC did not reflect the dynamics of *B. cereus* and cannot be used to predict the risk. Higher BC were more associated with higher butterfat levels and the traditional HTST pasteurization process.

We recommend that regulatory agencies reinforce the importance of maintaining the temperature of pasteurized milk at or below 4°C via the enforcement of current regulations and stakeholder education. The finding of higher BC in pasteurized organic milk packed in glass bottles should be further investigated. Specifically, the prevalence and numbers of *B. cereus* in raw organic milk compared with raw conventionally produced milk, as well as the efficacy of the equipment and process used to wash and sanitize glass bottles should be investigated.

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