



## Assessment of the exposure to cytotoxic *Bacillus cereus* group genotypes through high-temperature, short-time milk consumption

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### ABSTRACT

This study addresses the limited tools available for assessing food safety risks from cytotoxic *Bacillus cereus* group strains in contaminated food. We quantified the growth, in skim milk broth, of 17 cytotoxic *B. cereus* strains across 6 phylogenetic groups with various virulence gene profiles. The strains did not grow in HTST milk at 4 or 6°C. At 10°C, 15 strains exhibited growth; at 8°C, one strain grew; and all strains grew at temperatures  $\geq 14^\circ\text{C}$ . Using growth data from 16 strains, we developed linear secondary growth models and an exposure assessment model. This model, simulating a 5-stage HTST milk supply chain and up to 35 d of consumer storage with an initial contamination of 100 cfu/mL, estimated that  $2.81 \pm 0.66\%$  and  $4.13 \pm 2.53\%$  of milk containers would surpass  $10^5$  cfu/mL of *B. cereus* by d 21 and 35, respectively. A sensitivity analysis identified the initial physiological state of cells as the most influential variable affecting predictions for specific isolates. What-if scenarios indicated that increases in mean and variability of consumer storage temperatures significantly affected the predicted *B. cereus* concentrations in milk. This model serves as an initial tool for risk-based food safety decision-making regarding low-level *B. cereus* contamination.

**Key words:** *Bacillus cereus* group, exposure assessment, microbial risk management, precision food safety

### INTRODUCTION

The *Bacillus cereus* group, also referred to as *B. cereus sensu lato* (*s.l.*), is a complex composed of closely related species that can be categorized into 8 phylogenetic

groups (Carroll et al., 2020). This group of bacteria raises concerns within the food industry due to their ability to form endospores that withstand heat treatments, such as HTST pasteurization of milk (Buss da Silva et al., 2022; Tirloni et al., 2022). Certain strains within this group possess the potential to induce foodborne illness through emetic intoxication, a consequence of the production of heat-stable toxin cereulide within a food matrix. In contrast, other strains from this group can cause diarrheal intoxication. Upon ingestion by the human host, these bacteria, while growing in the small intestine, produce pore-forming enterotoxins, leading to cell death (Jessberger et al., 2020). Notably, the estimated annual number of foodborne illnesses caused by *B. cereus* group members in the United States is reported to be 63,623, with a hospitalization rate of 0.4% (CDC, 2018). Moreover, it is important to acknowledge that foodborne illnesses caused by *B. cereus* group members are often underreported due to their short-lived nature and mild symptoms (Stenfors Arnesen et al., 2008). Most foodborne illnesses caused by the *B. cereus* group have been associated with  $10^5$  to  $10^8$  cells/spores per gram of food. However, it has been proposed that any food that contains  $>10^3$  *B. cereus* group cells or spores per gram cannot be considered safe for consumption (EFSA, 2005).

Members of *B. cereus* group are common biological hazards along the dairy production chain, and they often persist in dairy processing environment biofilms (Tirloni et al., 2022). These hazards are particularly relevant to perishable HTST milk, which requires refrigeration along the entire supply chain to ensure product quality and safety. Despite this requirement, HTST milk can be subject to temperature abuses due to an inefficient cold chain. For instance, although efficient temperature control of food products inside refrigerated trucks can usually be achieved, increases in temperature, sometimes to  $>10^\circ\text{C}$ , during ground operations at the beginning and end

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The list of standard abbreviations for JDS is available at [adsa.org/jds-abbreviations-24](https://adsa.org/jds-abbreviations-24). Nonstandard abbreviations are available in the Notes.

of transportation have been frequently reported (Ndraha et al., 2018). Significant temperature abuses have also been observed during consumer transportation from the retail establishment to the home, and inappropriate home storage of food products, which elevate the food safety risk (Mercier et al., 2017). It has been reported that many home refrigerators worldwide are running at higher than recommended temperatures, and this inadequate refrigeration is frequently cited as a factor in incidents of food poisoning (James et al., 2017).

As *B. cereus* group strains can enter the supply chain at multiple entry points and survive heat treatment, occurrence of low levels of this microorganism in pasteurized milk is not unexpected (Svensson et al., 2004; Bartoszewicz et al., 2008; Berthold-Pluta et al., 2019). For instance, Chitov et al. (2008) reported high incidence of diarrheal *B. cereus* strain contamination in food products including pasteurized milk at a level of  $10^2$  to  $10^3$  cfu/mL in Thailand, implying potential risks of foodborne illness caused by the consumption of these food products. Therefore, to facilitate better food safety management decisions about mitigation strategies for the *B. cereus* group, industry needs a tool to quantitatively estimate the dose of *B. cereus* group strains at the point of human consumption. This is possible using an exposure assessment model, which applies predictive modeling techniques to simulate *B. cereus* growth in food products over time when they are responding to different storage conditions (e.g., changing temperatures), given that the food product is contaminated with a specific concentration and *B. cereus* group genotype (Notermans et al., 1997).

In this project, we modeled the growth of selected cytotoxic *B. cereus* group strains from different phylogenetic groups in contaminated HTST milk along a simulated supply chain and used the data to develop an exposure assessment model to predict the *B. cereus* group concentrations in a product upon human consumption. We hypothesized that different *B. cereus* group strains have different growth capabilities in HTST milk. Therefore, the percentage of HTST milk containers that contain *B. cereus* group concentrations above a given threshold (i.e.,  $10^3$  and  $10^5$  cfu/mL) on a given storage day differs by the *B. cereus* group strain that contaminates the product. Although a more realistic HTST milk shelf life is usually 18 to 21 d, we modeled a 5-stage supply chain with up to 35 consumer storage days, as that was the timing when noticeable variations in the percentage of milk containers over  $10^5$  cfu/mL with different *B. cereus* genotypes were observed.

## MATERIALS AND METHODS

### Selection of *Bacillus cereus* Group Isolates

A total of 17 *B. cereus* group strains were included in this study, spanning 6 of the 8 *B. cereus* phylogenetic

groups, from various sources including food, soil, and bioinsecticides that have been characterized genomically and evaluated for cytotoxicity toward human colorectal adenocarcinoma cells (Miller et al., 2018; Carroll et al., 2020). Isolates were selected from a collection of over 300 *B. cereus* group isolates with available genome assemblies to represent (1) diverse virulence gene clusters (i.e., considering the presence and absence of *ces*, *nhe*, *hbl*, *cytk-1*, *cytk-2*, *sph*, *cap*, *has*, *bps*), and (2) phylogenetic groups, which were assigned using BTyper3 v3.3.3 (Carroll et al., 2020). Specifically, BTyper3 was used to calculate average nucleotide identity (ANI) values relative to the species type strain genomes of all validly published and effective *B. cereus* group species published at the time ( $n = 28$ , accessed March 20, 2023; <https://lpsn.dsmz.de/text/approved-lists>). We used 95 ANI as the threshold to delineate bacterial species for the *B. cereus* group strains (Jain et al., 2018) except for one strain (PS00564) that did not share >95 ANI with any *B. cereus* group species type strain genome. The 16 *B. cereus* group strains used in this study most closely resembled the species type strain genomes of *B. pseudomycooides* (group I isolates PS00125 and PS00135), *albus* (group II isolate PS00193), *tropicus* (group II isolate PS00457), *mobilis* (group II isolate PS00518), *pacificus* (group III isolate PS00474), *cereus* (group IV isolates PS00402, PS00407, PS00433, and PS00495), *thuringiensis* (group IV isolates PS00413 and PS00649), *toyonensis* (group V isolates PS00570 and PS00638), and *cytotoxicus* (group VII isolates PS00194 and PS00536). Isolate PS00564, the one exception, belonged to group II (i.e., the phylogenetic group that contains *B. tropicus*) using *panC* group assignment, and shared >94.0 ANI with the *B. tropicus* type strain genome, and was thus grouped with isolate PS00457 for this study. These selected isolates were grouped into 11 clusters containing unique virulence gene profiles, and further subclustered by phylogenetic group. Consequently, we generated 10 subclusters that contained cytotoxic isolates (61 cytotoxic isolates in total). Up to 2 cytotoxic isolates from each virulence-phylogenetic subcluster were selected for inclusion in this study by using a random number generator (Table 1).

### Selection of Temperatures and Sampling Points

Three temperatures, which represent 3 industrially relevant scenarios, were selected for the growth experiments, which were conducted with 17 cytotoxic *B. cereus* group strains. These include 4°C, the food refrigeration temperature recommended by the Food and Drug Administration (FDA, 2023), 10°C, the refrigeration abuse temperature, and 22°C, the room temperature. For 2 strains (PS00125 and PS00135), which showed growth

**Table 1.** Number of all *Bacillus cereus* group isolates, cytotoxic *B. cereus* group isolates, and *B. cereus* isolates selected for growth experiments in all clusters and subclusters

Cluster <sup>2</sup>	Phylogenetic group <sup>1</sup>								Total
	I	II	III	IV	V	VI	VII	VIII	
Number of all <i>B. cereus</i> group isolates									
1	0	0	0	0	0	0	8	0	8
2	0	0	0	0	1	0	0	0	1
3	0	5	19	1	0	0	0	0	25
4	0	0	0	1	0	0	0	0	1
5	0	5	1	74	3	0	0	0	83
6	0	1	0	2	0	0	0	0	3
7	0	0	20	0	0	0	0	0	20
8	0	16	21	0	0	0	0	0	37
9	0	19	3	4	36	36	0	4	103
10	5	0	0	0	1	3	0	0	9
11	0	0	0	0	1	0	0	0	1
Number of cytotoxic <i>B. cereus</i> group isolates only									
1	0	0	0	0	0	0	1	0	1
2	0	0	0	0	0	0	0	0	0
3	0	3	1	0	0	0	0	0	4
4	0	0	0	0	0	0	0	0	0
5	0	1	0	42	0	0	0	0	43
6	0	0	0	2	0	0	0	0	2
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	1	0	2	4	0	0	0	7
10	4	0	0	0	0	0	0	0	4
11	0	0	0	0	0	0	0	0	0
<i>B. cereus</i> isolates selected for growth experiments only <sup>3</sup>									
1	0	0	0	0	0	0	2	0	2
2	0	0	0	0	0	0	0	0	0
3	0	2	1	0	0	0	0	0	3
4	0	0	0	0	0	0	0	0	0
5	0	1	0	2	0	0	0	0	3
6	0	0	0	2	0	0	0	0	2
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	1	0	2	2	0	0	0	5
10	2	0	0	0	0	0	0	0	2
11	0	0	0	0	0	0	0	0	0

<sup>1</sup>Phylogenetic groups assigned by BTyper3 are used for subclustering.

<sup>2</sup>Isolates are clustered based on unique virulence gene profiles.

<sup>3</sup>Cluster I10 (PS00125 and PS00135), cluster I13 (PS00457 and PS00518), cluster II5 (PS00193), cluster II9 (PS00564), cluster III3 (PS00474), cluster IV5 (PS00407 and PS00495), cluster IV6 (PS00433 and PS00402), cluster IV9 (PS00413 and PS00649), cluster V9 (PS00570 and PS00638), cluster VIII1 (PS00194 and PS00536).

at 22°C but not at 10°C, we also collected growth data at 16°C.

In addition, we also conducted growth/no growth experiments at 6 and 8°C for 15 strains that showed growth at 10°C but not at 4°C to better characterize their growth. Two strains (PS00125 and PS00135) that showed growth at 22°C but not at 10°C were also tested at 14 and 18°C. The sampling points for growth experiments at each temperature were selected based on preliminary growth experiments in skim milk broth (SMB; BD, catalog no. 232100, Franklin Lakes, NJ), which informed the selection of sampling time points to ensure that at least 3 data points were obtained for each growth phase (i.e., lag, exponential growth, and stationary phase).

### Preparation of Spore Suspensions

All strains were streaked from −80°C cryostocks onto brain-heart infusion (BHI) agar (BD, catalog no. 241830, Franklin Lakes, NJ), followed by incubating at 30°C for 24 h. A single isolated colony was then inoculated in 5 mL of BHI broth (BD, catalog no. 237500, Franklin Lakes, NJ) in a 15-mL conical tube (VWR, catalog no. 89039–670, Radnor, PA). Inoculated broths were incubated at 30°C for 24 h without shaking. Two separate AK Agar #2 plates (Sporulating Agar; BD, catalog no. 210912, Franklin Lakes, NJ) were spread-plated with 100 µL of overnight culture and incubated at 30°C for 5 d. To confirm the growth on the AK #2 agar, a wet

mount on a microscopy slide was prepared and examined using a phase contrast microscope. After the presence of spores was confirmed, the plates were flooded with 8 mL of chilled sterile deionized water and biomass was scraped with an L-shaped spreader to suspend the spores. The spores were then transferred into new 15-mL conical tubes, and the suspensions were centrifuged (Avanti Centrifuge J-26 XPI) at  $10,000 \times g$  for 15 min at 4°C. The supernatant was removed, and the spores were resuspended in 3.5 mL of sterile deionized water. Centrifugation was repeated 2 more times for a total of 3 washes. After the final wash, the water was removed, and the spores were resuspended in 5 mL of 50% ethanol and incubated at 4°C in a tube rotator (VWR Mini Tube Rotator) running at 15 rpm for 12 h at 4°C to inactivate remaining vegetative cells. After this incubation, the suspension was centrifuged at  $10,000 \times g$  for 15 min at 4°C. The supernatant was removed, and the spores were resuspended in 3.5 mL of sterile deionized water. Centrifugation was repeated an additional 2 times for 3 total washes. After the last centrifugation, the spores were resuspended in 3 mL of sterile deionized water and stored at 4°C for up to 6 mo.

To determine the spore suspension concentrations, 100  $\mu\text{L}$  of each spore suspension was transferred to a 1.5-mL microcentrifuge tube (VWR, catalog no. 89000–028, Radnor, PA) and incubated in a water bath at 80°C for 12 min, followed by cooling on ice. Using the  $1 \times$  PBS, serial dilutions of the spore suspensions were prepared and spread-plated, in duplicate, onto BHI agar, followed by a 24 h incubation at 30°C. Colonies on each plate were manually counted, and the colony-forming units/milliliter of the heat-treated spore suspension were calculated.

### Growth Experiment in SMB

Growth experiment was initiated by preparing a spore suspension at a concentration of  $10^3$  cfu/mL in 40 mL of SMB followed by heat treatment at 80°C for 12 min. The growth experiments were conducted in 2 independent biological replicates, each with 2 technical replicates, for 17 selected *B. cereus* group strains at 4°C, 10°C, and 22°C. At 4°C, samples were collected at time points 0, 96, 144, 192, 240, 288, 384, and 576 h. At 10°C, samples were collected at time points 0, 6, 24, 48, 96, 192, 240, 288, 384, and 504 h. At 22°C, data were collected in 3 experiments with complementary sampling time points, as well as overlapping time points to ensure reproducibility. Specifically, in one experiment, samples were collected at time points 0, 1, 2, 4, 6, 8, and 24 h. In the second experiment, samples were collected at time points 0, 14, 15, 16, 18, 20, and 22 h. In the third experiment, samples were collected at time points 0, 24, 27, and 30 h, with

a 32 h time point being added for isolates that had not reached the stationary phase at 30 h. Growth experiments conducted at 16°C for 2 strains (PS00125 and PS00135) were also conducted in 2 biological replicates, each with 2 technical replicates, with data collected at time points 0, 1, 2, 4, 6, 8, 14, 18, 24, 48, and 96 h.

At each sampling time point, 1 mL of the sample was serially 10-fold diluted using a  $1 \times$  PBS buffer and 100  $\mu\text{L}$  of dilutions  $10^{-1}$ ,  $10^{-3}$ , and  $10^{-5}$  were plated on BHI agar plates, in duplicates. The BHI agar plates were incubated at 30°C for 12 to 24 h before colony enumeration. Isolates with filamentous colony morphology were pour-plated to prevent colony spreading and were incubated at 30°C for 12 to 24 h. The colonies on countable plates, with 30 to 300 colonies, were manually counted. Plating of 100  $\mu\text{L}$  of SMB and 100  $\mu\text{L}$  of  $1 \times$  PBS were included in each experiment as negative controls. Experiments were not considered valid if the negative controls had observable colony growth.

### Growth/No Growth Experiments

For growth/no growth experiments, spore suspensions were inoculated in SMB at a concentration of  $10^3$  cfu/mL, and incubated at 6°C or 8°C for 504 h. The data were collected at time points 0 and 504 h. Experiments were conducted with 2 independent biological replicates, each with 2 technical replicates. For 2 strains that showed growth at 22°C but not at 10°C, the growth/no growth experiments were also conducted at 14°C and 18°C. Spore suspensions of these strains were inoculated in SMB at a concentration of  $10^3$  cfu/mL and incubated for 384 h. The data were collected at time points 0 and 384 h. Experiments were conducted with 2 independent biological replicates, each with 2 technical replicates. Microbial concentration was determined as described in the previous section. An increase of cell concentration by 1 log cfu/mL during the growth/no growth experiment was considered growth.

### Primary Model Fitting

To estimate the growth parameters, a Baranyi model (Baranyi and Roberts, 1994) was fitted to growth data for 17 selected *B. cereus* group strains collected at 22°C, 16°C, and 10°C using the packages “nlsMicrobio” version 0.0–3 (Baty et al., 2022) and “minpack.lm” version 1.2–3 (Elzhov et al., 2022) in R version 4.1.2 (R Core Team, 2021). The Baranyi model was selected as the primary model because it was the one used for microbial growth prediction under dynamic environmental conditions in R package “biogrowth” version 1.0.2 (Garre et al., 2022). This primary model was used for exposure

assessment model development to facilitate prediction of growth under dynamic temperature conditions. The equation (Equation 1) is presented below:

$$N_t = N_0 + \mu_{max} A(t) - \ln \left[ 1 + \frac{e^{\mu_{max} A(t)} - 1}{e^{(N_{max} - N_0)}} \right], \quad [1]$$

with

$$A(t) = t + \frac{1}{\mu_{max}} \ln \left( e^{-\mu_{max} t} + e^{-\mu_{max} \lambda} - e^{-\mu_{max} t - \mu_{max} \lambda} \right),$$

where  $\mu_{max}$  is the maximum specific growth rate in ln/h,  $\lambda$  is lag time in h,  $N_0$  is the initial population in log<sub>10</sub> cfu/mL,  $N_{max}$  is the maximum population reached in log<sub>10</sub> cfu/mL,  $A(t)$  is the integral function of the adjustment function that describes the lag phase of bacterial growth as the process of adjustment to the new environment,  $t$  is the time, and  $e$  is the base of the natural logarithm.

The last 3 data points of isolate PS00413 replicate 1 at 10°C were excluded when fitting Baranyi model because cells had entered the death phase (Supplemental Figure S1, see Notes).

### Secondary Model Fitting

A reduced Ratkowsky model was selected as the secondary model to calculate theoretical minimum growth temperatures ( $T_{min}$ ) and slope (Ratkowsky et al., 1982), as our available data were confined to growth under sub-optimal temperatures. The unit of  $\mu_{max}$  was converted to log<sub>10</sub>/d before fitting the secondary model in R version 4.1.2 (R Core Team, 2021). The equation (Equation 2) is presented below:

$$\sqrt{\mu_{max}} = b(T - T_{min}), \quad [2]$$

where  $T$  is temperature represented in °C,  $b$  is the slope, and  $T_{min}$  is the theoretical minimum growth temperature represented in °C.

Growth/no growth experiment data at 6°C, 8°C, and 10°C were also used as input data to construct the secondary model. Specifically, for strains that showed no growth at 6°C, 8°C, or 10°C,  $\mu_{max}$  was assumed to be 0 at the given temperature and this  $\mu_{max}$  value was used as an input when fitting the secondary growth model.

### Statistical Analysis

Kruskal-Wallis tests were performed on the  $\lambda$ ,  $\mu_{max}$ , and  $N_{max}$  of all strains whose growth data were obtained from

both 22°C and 10°C growth experiments. A Bonferroni-corrected Dunn's test was used to identify the pairs of strains that showed significant differences in lag time and maximum growth rate. The nonparametric tests were applied because the growth parameters for these strains failed to meet normality and equal variance assumptions.

### Exposure Assessment Model Overview

The exposure assessment model simulates the growth of selected cytotoxic *B. cereus* group strains in one lot of HTST milk transported along a 5-stage supply chain that includes (1) processing facility storage, (2) transportation from the processing facility to retail, (3) retail storage, (4) transportation from retail to the consumer's home, and (5) consumer storage for up to 35 d. We assume standard practices at each stage of the supply chain until the HTST milk products reach the consumer. These include refrigeration storage at the processing facility, retail, and transportation on trucks. Furthermore, we assume that all personnel, including drivers and retail staff, were trained to handle milk products with care, ensuring that they were not exposed to physical damage or extended temperature abuse during transit. Each hypothetical lot of HTST milk was contaminated by one *B. cereus* group strain and had 100 half-gallon milk containers represented by 100 iterations. This model predicts *B. cereus* group concentrations in milk containers on d 14, 21, and 35 of consumer storage. Model outputs were also used to compute the percentage of milk containers with *B. cereus* group concentrations that exceeded 10<sup>3</sup> and 10<sup>5</sup> cfu/mL at d 14, 21, and 35.

### Exposure Assessment Model Parameters

The input parameters for the exposure assessment model include initial contamination levels, growth parameters of selected *B. cereus* group strains, and the temperature profiles for each stage of the supply chain. The initial contamination levels ( $N_0$ ) for 100 iterations were generated from a Poisson distribution in R version 4.1.2 (R Core Team, 2021) with a lambda (i.e., mean) value of 100 cfu/mL. The seed for the initial contamination level generation was set to 42, for consistency.

The initial physiological state of cells ( $Q_0$ ) was calculated as shown in Equations 3 and 4 and described in the Baranyi model (Baranyi and Roberts, 1994):

$$h_0 = \mu_{max} \lambda, \quad [3]$$

where  $\mu_{max}$  is the specific maximum growth rate in ln/h, and  $\lambda$  is the lag time in hours.

Then  $h_0$  was averaged across 2 replicates at 22°C and 2 replicates at 10°C for each strain to obtain  $h_{av}$ , which was used to calculate  $Q_0$  specific to each strain:

$$Q_0 = \frac{1}{\exp(h_{av}) - 1}. \quad [4]$$

The maximum microbial population ( $N_{max}$ ) for each given *B. cereus* group strain was estimated from the Baranyi model and averaged across all replicates from growth experiments at different temperatures for a given *B. cereus* group strain.

The maximum growth rate at the optimum growth temperature ( $\mu_{opt}$ ) was calculated as shown in Equation 5:

$$\mu_{opt} = [b \cdot (T_{opt} - T_{min})]^2, \quad [5]$$

where  $T_{min}$  and  $b$  were calculated from the secondary model.  $T_{opt}$  is the optimum growth temperatures by clade previously reported (Nakamura, 1998; Baranyi et al., 2017). The unit of  $\mu_{opt}$  is in  $\log_{10}/d$ .

A unique time and temperature profile for every milk container in each lot was generated by randomly drawing a time-temperature combination from the corresponding

time and temperature distributions (see Table 2) at each stage of the supply chain as described in a previous study (Qian et al., 2023). The seed was set to 1, for consistency.

### Sensitivity Analysis

A sensitivity analysis was performed to assess the impact of input parameters, specifically  $Q_0$  and  $N_{max}$ , on the model's outcome, which is the percentage of milk containers exceeding *B. cereus* group concentrations of 5 log on consumer storage d 35. In addition to the average  $h_0$ , both the minimum and maximum  $h_0$  were employed to determine the corresponding maximum and minimum  $Q_0$ . The input parameters for running the model on selected strains included the minimum, average, and maximum values for  $Q_0$  (representing the physiological state of the cell, calculated as the product of growth rate and lag time). Similarly, the minimum, average, and maximum values for  $N_{max}$  were used as input parameters for running the model on the selected strains.

### What-If Scenario

Here, we simulated 4 possible scenarios that could occur along an HTST milk supply chain, including (1) a

**Table 2.** Variables used in the time-temperature distributions of the 5-stage supply chain

Symbol	Variable name	Distribution	Unit	Reference
Stage 1. Processing plant storage				
$t_F$	Storage time of milk containers	Uniform (1, 2) <sup>1</sup>	d	Qian et al., 2023
$T_F$	Storage temperature of milk containers	Uniform (3.5, 4.5)	°C	Qian et al., 2023
Stage 2. Transportation from processing plant to retail				
$t_T$	Storage time of milk containers	Triangular (1, 10, 5) <sup>2</sup>	d	FDA and Health Canada, 2015
$T_T$	Storage temperature of milk containers	Triangular (1.7, 10.0, 4.4)	°C	FDA and Health Canada, 2015
Stage 3. Retail storage				
$t_S$	Storage time of milk containers	TruncNormal (0.042, 10.0, 1.821, 3.3) <sup>3</sup>	d	Qian et al., 2023
$T_S$	Storage temperature of milk containers	TruncNormal (-1.4, 5.4, 2.3, 1.8)	°C	FDA and Health Canada, 2015
Stage 4. Transportation from retail to consumer's home				
$t_{T2}$	Storage time of milk containers	TruncNormal (0.01, 0.24, 0.04, 0.02)	d	Expert opinion
$T_{T2}$	Storage temperature of milk containers	TruncNormal (0, 10.0, 8.5, 1.0)	°C	Expert opinion
Stage 5. Consumer storage				
$t_H$	Storage time of milk containers	1–35	d	
$T_H$	Storage temperature of milk containers	TruncLaplace (-1, 15, 4.06, 2.31) <sup>4</sup>	°C	Pouillot et al., 2010

<sup>1</sup>In a Uniform (a, b) distribution, a = minimum, b = maximum.

<sup>2</sup>In a Triangular (a, b, c) distribution, a = minimum, b = maximum, c = mode.

<sup>3</sup>In a TruncNormal (a, b, c, d) distribution, a = minimum, b = maximum, c = mean, and d = SD.

<sup>4</sup>In a TruncLaplace (a, b, c, d) distribution, a = minimum, b = maximum, c = location parameter, and d = dispersion.

**Table 3.** Implementation of what-if scenarios and outcomes

Scenario	Implementation <sup>1</sup>	Modified time/temperature distribution <sup>1</sup>	Percent milk containers exceeding 5 logs on consumer storage d 35 (mean ± SD)
Baseline	NA	NA	4.13 ± 2.53
Scenario 1 A 3-d delay on HTST milk product delivery from processing facility to retail without temperature abuse	Shift in the distribution of transportation time from processing plant to retail 3 units toward the right	$t_T \sim \text{Triangular}(4, 13, 8)$	4.13 ± 2.53
Scenario 2 A temperature abuse when a consumer transported the HTST milk products from retail back home in a hot summer	Shift in the distribution of transportation temperature from retail to consumer's home 25 units toward the right	$T_{T2} \sim \text{TruncNormal}(25, 35, 33.5, 1.0)$	4.44 ± 3.03
Scenario 3 A slight temperature deviation in consumer's home refrigerator	Shift in the distribution of consumer home storage temperature 1 unit toward the right	$T_H \sim \text{TruncLaplace}(0, 16, 5.06, 2.31)$	8.88 ± 3.84
Scenario 4 An increase in the variability of refrigeration temperatures at consumer's home	Increase in the dispersion parameter of the distribution of consumer home storage temperature by 1 unit	$T_H \sim \text{TruncLaplace}(-1, 15, 4.06, 3.31)$	10.81 ± 3.15

<sup>1</sup>Details of variables used in the time-temperature distributions of the 5-stage supply chain can be found in Table 2. NA = not applicable.

delivery delay by 3 d when HTST milk products were transported from processing facility to retail, with no temperature abuse (e.g., amid a severe snowstorm in New York, milk delivery was delayed by 3 d due to impassable roads, safety concerns, and the time required for road clearing and recovery operations; the products were kept in a well-controlled distribution center during the delay), (2) a temperature abuse simulating a consumer's transportation of HTST milk products from retail to home in a hot summer, which results in an average of 33.5°C (i.e., 25°C higher than the average temperature of the base model), (3) a slight temperature deviation in a consumer's home refrigerator, modeled as a 1°C temperature increase (i.e., an average home storage temperature of 5.45°C), and (4) an increase in the variability of refrigeration temperatures due to unstable power supply (e.g., rolling blackout in California during summer), modeled as a 1°C increase in the dispersion parameter of the distribution of consumer home storage temperature. Percent milk containers that had *B. cereus* group concentrations >10<sup>5</sup> cfu/mL on consumer storage d 21 and 35 were calculated for all 4 scenarios and compared with the base model prediction (Table 3).

### Model Programming and Data Availability

The model was developed in R version 4.1.2 (R Core Team, 2021) with the modified “biogrowth” package version 1.0.2 (Garre et al., 2022). The seed for Monte Carlo simulation was set to 1, for consistency. The raw data used for model construction and R code for the entire

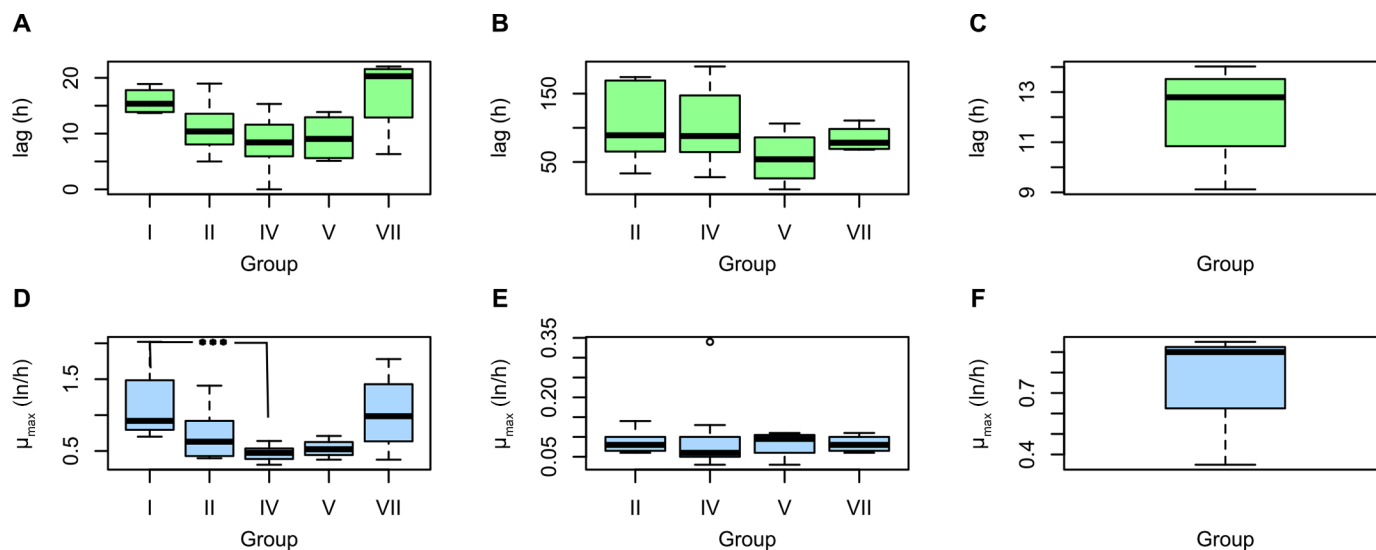
study are available at <https://github.com/FSL-MQIP/Bacillus-cereus-exposure-assessment-model>.

## RESULTS

### *Bacillus cereus* Group Strains Differ in Growth Characteristics, Including the Observed Minimum Growth Temperature

The 17 selected *B. cereus* group strains (representing 6 phylogenetic groups) differed in terms of their minimum growth temperature in SMB. No strain showed detectable growth (i.e., >1 log increase in cfu/mL) at 4°C, 14 out of 17 strains did not grow at 6 or 8°C, and 2 out of 17 strains did not grow at 10°C (Supplemental Table S1, see Notes). The 2 strains that did not show growth at 10°C, showed growth at 14 and 16°C, as determined based on the growth/no growth experiments at these 2 temperatures. Hence, the experimentally obtained growth boundaries for the 17 strains assessed here were (1) 8°C for 1 strain, (2) 10°C for 14 strains, and (3) 14°C for 2 strains, representing the only 2 group I strains characterized.

Due to different minimum growth temperatures associated with the 17 tested strains, the following datasets were available to estimate growth parameters (i.e., lag time, maximum growth rate, maximum microbial population): (1) 22°C for all 17 strains; (2) 10°C for 15 strains, and (3) 16°C for 2 strains. For 22°C, parameter estimates obtained after fitting the growth data with the Baranyi model were (1) lag time ranging from 0 h to 22.04 h, (2) maximum growth rate ranging from 0.31



**Figure 1.** Boxplot showing the within- and between-group variability of growth parameters (i.e., lag and  $\mu_{\max}$  at 22°C) for (A, D) 16 *Bacillus cereus* group strains representing 5 phylogenetic groups (group I [n = 4], group II [n = 8], group IV [n = 12], group V [n = 4], group VII [n = 4]), (B, E) 14 *B. cereus* group strains at 10°C, representing 4 phylogenetic groups, and (C, F) 2 *B. cereus* group strains from group I at 16°C (group I strains did not grow at 10°C). Group III was excluded from this plot because it only had 1 strain. \*\*\* $P < 0.05$  based on post hoc Dunn's test performed following a Kruskal-Wallis test that found  $\mu_{\max}$  to be significantly different between group I and group IV strains when grown at 22°C. The upper and lower edges of boxes are the third and first quartiles. Midlines are medians. The whiskers are the 2 lines outside the box that go from the maximum to the third quartile and then from the first quartile to the minimum. The dots are the outliers.

ln/h to 2.02 ln/h; and (3) maximum microbial population ranging from 5.24 log<sub>10</sub> cfu/mL to 7.61 log<sub>10</sub> cfu/mL. By comparison, for 10°C, parameter estimates were (1) lag times from 10.02 to 194.05 h, (2) maximum growth rates from 0.03 to 0.34 ln/h, and (3) maximum microbial population from 5.01 log<sub>10</sub> cfu/mL to 7.37 log<sub>10</sub> cfu/mL. At 16°C, parameter estimates were (1) lag times from 9.12 to 14.02 h, (2) maximum growth rates from 0.35 to 0.95 ln/h, and (3) maximum microbial populations from 6.17 log<sub>10</sub> cfu/mL to 6.28 log<sub>10</sub> cfu/mL (Supplemental Table S2, see Notes).

### **Bacillus cereus Isolates from Different Phylogenetic Groups Differ in Growth Characteristics, Including the Maximum Growth Rate at 22°C and Hypothetical Minimum Growth Temperature**

To further evaluate *B. cereus* group growth characteristics, the growth parameters obtained for different strains were analyzed based on phylogenetic groups (i.e., group I, II, IV, V, and VII; see Figure 1). Group III was excluded from these analyses because it only contained one strain. The Kruskal-Wallis test for 22°C growth parameters indicated that both lag time and maximum growth rate differed significantly among phylogenetic groups ( $P$ -values of 0.040 and 0.036, respectively), although maximum microbial population did not differ significantly ( $P > 0.1$ ). Group I strains showed the highest median maximum growth rate (0.92 ln/h), which was significantly

( $P = 0.024$ ) higher than that for group IV strains (0.48 ln/h). With regard to the lag time, group VII strains had the longest median lag time (20.30 h), whereas group IV strains had the shortest median lag time (8.43 h), meaning that group VII strains took longest, and group IV strains took shortest time to start growing.

By comparison, the Kruskal-Wallis test for 10°C growth parameters showed no significant differences ( $P > 0.1$ ) for any of the 3 parameters (i.e., lag time, maximum growth rate, maximum microbial population) among phylogenetic groups. Numerically, group V strains had the highest median maximum growth rate (0.10 ln/h), whereas group IV strains had the lowest median maximum growth rate (0.06 ln/h). Group II strains had the longest median lag time of 89.11 h, whereas group V strains had the shortest median lag time of 53.94 h (Figure 1). This suggested that this temperature was high enough to not significantly affect growth dynamics of any of the isolates that were able to grow at this temperature.

To further assess differences in secondary growth parameters between groups, we successfully fitted maximum growth rates for 16 strains with a secondary growth model (i.e., reduced Ratkowsky model). This model converged when using data from all strains except isolate PS00135 (group I), which had a nonlinear relationship between maximum growth rate and temperature under suboptimal growth conditions (Supplemental Figure S2, see Notes). We therefore were not able

**Table 4.** Slope of secondary model (b) and theoretical minimum growth temperature ( $T_{\min}$ ) for 16 isolates

Isolate	Phylogenetic group	b	$T_{\min}$ (°C)
PS00125	I	0.30	9.22
PS00193	II	0.17	6.79
PS00457	II	0.14	5.88
PS00564	II	0.20	6.28
PS00518	II	0.20	6.86
PS00474	III	0.15	6.41
PS00413	IV	0.13	4.88
PS00433	IV	0.12	5.83
PS00495	IV	0.17	6.79
PS00407	IV	0.15	6.71
PS00402	IV	0.13	4.26
PS00649	IV	0.15	6.46
PS00570	V	0.14	5.42
PS00638	V	0.15	5.52
PS00194	VII	0.21	6.89
PS00536	VII	0.21	7.06

to calculate  $T_{\min}$  (i.e., hypothetical minimum growth temperature) for this strain. For the rest of the strains,  $T_{\min}$  ranged from 4.26°C to 9.22°C. The average  $T_{\min}$  of strains from groups II, III, IV, V, and VII were similar, ranging from 5.47°C to 6.98°C. The only strain from group I (PS00125) showed a substantially higher  $T_{\min}$  (9.22°C) compared with the strains from other groups (Table 4). This suggests different ecological adaptation of strains from group I, which require a higher minimum temperature for growth.

#### **The Base Model Predicts a Higher Percent of Containers Exceeding *B. cereus* Group Concentrations of $10^5$ and $10^3$ cfu/mL for One Group IV Strain and One Group V Strain**

The growth data for the 16 *B. cereus* group strains that showed linear secondary models were used for the development of an exposure assessment model. We assessed the growth predictions of the base model for 16 *B. cereus* group strains in HTST milk products stored at 3°C for 90 d, with an initial contamination level of 100 cfu/mL. This temperature was selected because it was 1°C below the lowest  $T_{\min}$  of all strains calculated from the secondary model. Hence, we expected that the model would predict no growth for any of the 16 strains at 3°C. Indeed, the model predicted no growth for any of the 16 strains at 3°C, which aligned with the experimental outcomes that none of the strains had detectable growth at 4°C.

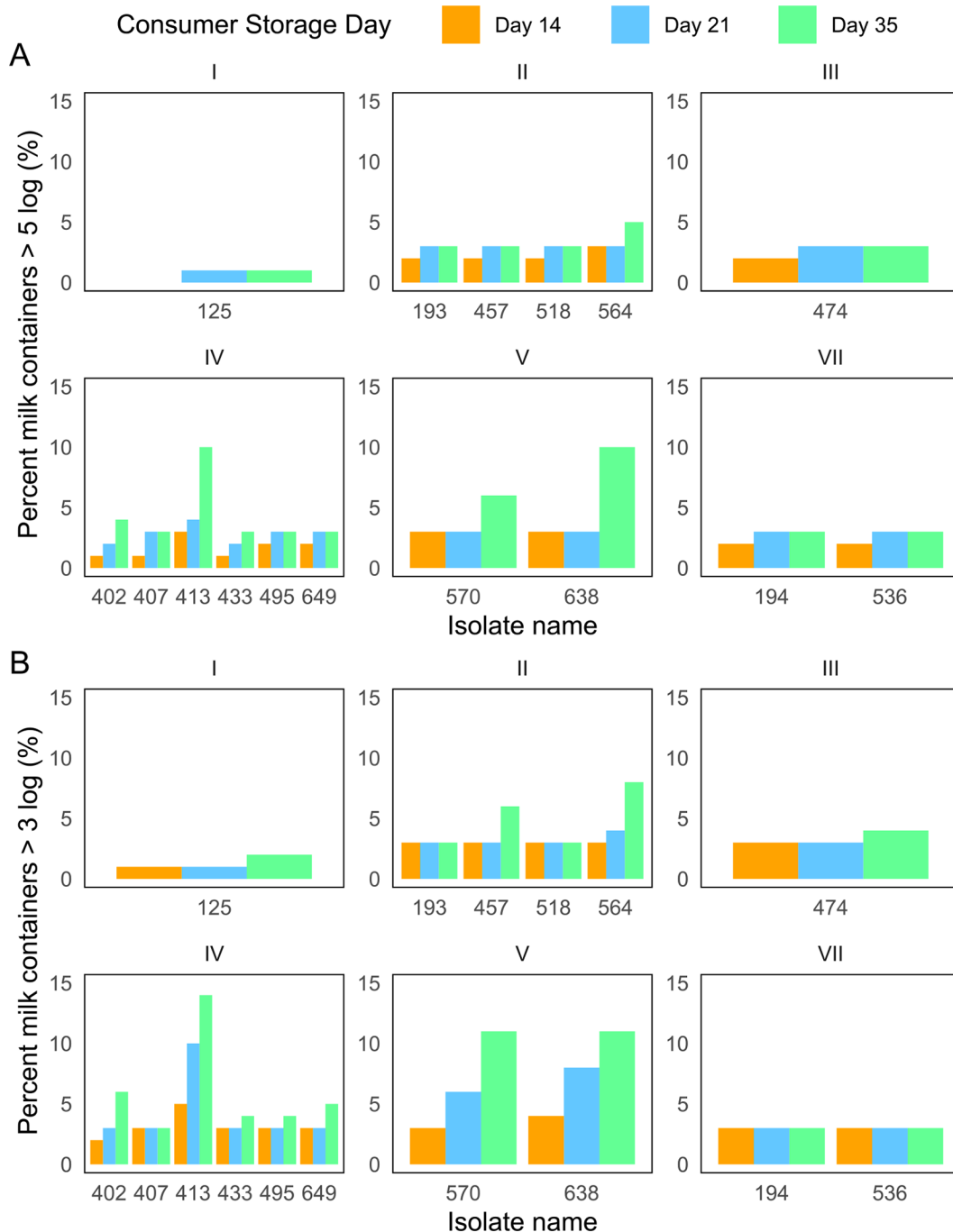
The exposure assessment model was subsequently used to predict growth in HTST milk for each of the 16 included strains up to d 35 of consumer storage. Exposure assessment model used temperature profiles that include different time and temperature distributions for 5 stages of the supply chain (Table 2). The growth predictions were used to compute the percentage of simulated milk

containers that had *B. cereus* group concentrations  $>10^5$  cfu/mL and  $10^3$  cfu/mL on consumer home storage d 14, 21, and 35, respectively. The percentage of milk containers exceeding  $10^5$  cfu/mL on d 14, 21, and 35 were  $1.94 \pm 0.85$  (mean  $\pm$  SD),  $2.81 \pm 0.66$ , and  $4.13 \pm 2.53$ , respectively, whereas the percentage of milk containers exceeding  $10^3$  cfu/mL on the same days were  $3.00 \pm 0.82$ ,  $3.88 \pm 2.25$ , and  $5.63 \pm 3.67$ .

Our analyses identified 5 strains for which the percentage of containers above  $10^3$  cfu/mL (i.e., 3%) was the same at consumer storage d 14, 21, and 35 (i.e., isolates PS00193 and PS00518 from group II, isolate PS00407 from group IV, and isolates PS00194 and PS00536 from group VII; Figure 2b). These 5 strains, as well as 4 additional strains (i.e., isolate PS00457 from group II, isolate PS00474 from group III, isolates PS00495 and PS00649 from group IV) also showed the same percentage (i.e., 3%) of containers above  $10^5$  cfu/mL at consumer storage d 21 and 35 (Figure 2a). We further evaluated the conditions for strains that had the same model predictions at different consumer storage times to verify our model was performing reasonably. An analysis of the 100 model simulations for these 9 strains (which represented 3 group II strains, 1 group III strain, 3 group IV strains, and 2 group VII strains) showed that for each of these strains, only 3 out of 100 simulated temperature profiles had a consumer home storage temperature  $>10^\circ\text{C}$ . For the other 97 simulations, the consumer storage temperature was either (1) below  $T_{\min}$  of these 9 strains (and thus resulted in no predicted growth) or (2) above  $T_{\min}$  but below  $10^\circ\text{C}$ , a temperature at which our model predicts that these isolates grew so slowly that they would not exceed  $10^5$  cfu/mL by consumer storage d 35.

#### **Sensitivity Analysis Revealed that Variation in the Input Parameter $Q_0$ Significantly Influenced the Model Predictions for Certain Strains**

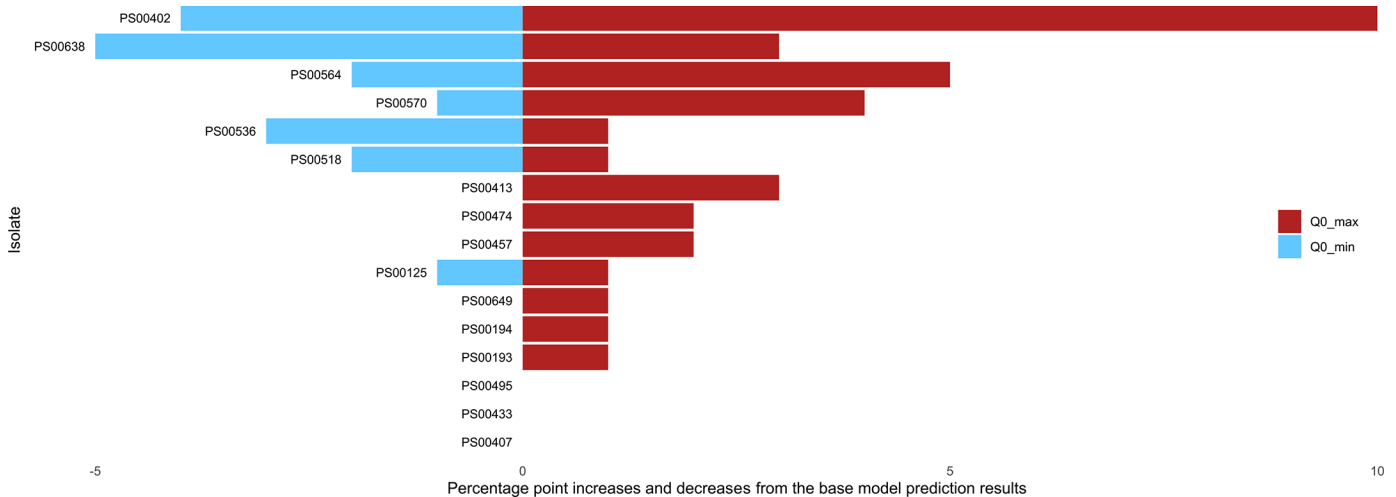
In the sensitivity analysis, we evaluated the effect of both  $N_{\max}$  and  $Q_0$  on the percentage of milk containers exceeding *B. cereus* group concentrations of  $10^5$  cfu/mL on consumer storage d 35 for 16 strains. This combination of consumer storage day and concentration cut-off was used because  $10^5$  cfu/mL represents the *B. cereus* regulatory threshold in several countries and d 35 showed some of the largest variations in model predictions between strains. The sensitivity analysis for  $N_{\max}$  predicted an average of  $4.06 \pm 2.54$  (mean  $\pm$  SD) and  $4.13 \pm 2.53\%$  milk containers exceeding *B. cereus* group concentrations of  $10^5$  cfu/mL on consumer storage d 35 when using the minimum and maximum  $N_{\max}$  as the input parameters, respectively, as compared with  $4.13 \pm 2.53\%$  when using the average  $N_{\max}$ . Only 1 out of the 16 strains (PS00402; group IV) showed detectable changes



**Figure 2.** Base model prediction results. The percentage of simulated milk containers contaminated with a given *Bacillus cereus* group (i.e., group I, II, III, IV, V, VII) that exceeded 5 log<sub>10</sub> cfu/mL (A) and 3 log<sub>10</sub> cfu/mL (B) on consumer home storage d 14, 21, or 35.

in the percentage of milk containers over 10<sup>5</sup> cfu/mL. For this strain, the percentage of milk containers over 5 logs dropped by 1 percentage point when using the minimum  $N_{\max}$  as the input as compared with using the average  $N_{\max}$  (Supplemental Table S3, see Notes). Our data thus indicate that model outcomes showed limited sensitivity to changes in  $N_{\max}$ .

In contrast, the model outcomes were more sensitive to the physiological state parameter,  $Q_0$ . Using the minimum and maximum  $Q_0$  as the input parameters in the sensitivity analysis resulted in an average of 3.00 ± 2.42 (mean ± SD) and 6.31 ± 4.14% milk containers exceeding *B. cereus* group concentrations of 10<sup>5</sup> cfu/mL on consumer storage d 35, respectively, as compared with 4.13 ± 2.53%



**Figure 3.** Tornado diagram for sensitivity analysis of  $Q_0$  showing the percentage point increases (when using the maximum  $Q_0$  as the input) and decreases (when using the minimum  $Q_0$  as the input) from the base model prediction results (i.e., the percent of milk containers over  $5 \log_{10}$  cfu/mL on consumer storage d 35 when using the average  $Q_0$  as the input parameter) for 16 *Bacillus cereus* group strains.

when using the average  $Q_0$ . This analysis also revealed that the sensitivity of model outcomes to variation in  $Q_0$  differed substantially by strain. The 4 strains where model outcomes were most sensitive to changes in  $Q_0$  were isolates PS00402 (group IV), PS00638 (group V), PS00564 (group II), and PS00570 (group V). The model outcomes were not sensitive to the change in  $Q_0$  for 3 strains from group IV and the same predictions were generated with varying  $Q_0$  values (Figure 3). This is because (1) one strain (i.e., isolate PS00433 from group IV) had lower variability in the parameter  $Q_0$  compared with other strains, and (2) our model predicted that the other 2 strains (i.e., isolates PS00407 and PS00495 from group IV) grew very slowly and would not reach  $>10^5$  cfu/mL on consumer storage d 35 under storage temperature of  $<10^\circ\text{C}$  even in the worst-case situation (i.e., highest experimentally determined  $Q_0$ ).

#### **Small Temperature Increases and Increased Temperature Fluctuations in Consumer Refrigerators Can Substantially Increase the Percentage of Milk Containers Predicted to Contain *B. cereus* Group Concentrations $>10^5$ cfu/mL**

What-if scenario analyses were used to evaluate the impact of 4 different practical scenarios on possible consumer exposure to milk with *B. cereus* group concentrations  $>10^5$  cfu/mL at consumer storage d 21 (representing a more realistic HTST milk shelf life) and 35 (the timing when we start to observe distinguishable variations in the percentage of milk containers containing over  $10^5$  cfu/mL among *B. cereus* group strains). Our results show that a 3-d delay on HTST milk product delivery from pro-

cessing facility to retail without temperature abuse (scenario 1) did not alter the percentage of milk containers exceeding  $10^5$  cfu/mL of *B. cereus* group concentrations on consumer home storage d 21 and 35 (i.e., 2.81% and 4.13%, identical to the base model prediction). Similarly, a temperature abuse simulation of consumer transportation of HTST milk products from retail to home in a hot summer (average milk temperature of  $33.5^\circ\text{C}$  with an average time of 1 h; scenario 2) led to 3.06% and 4.44% milk containers exceeding  $10^5$  cfu/mL on consumer home storage d 21 and 35, which are slightly higher than the base model predictions (2.81% and 4.13%). Under scenario 2, the model also predicted that 3 strains had higher percentages of milk containers containing *B. cereus* group concentrations  $>10^5$  cfu/mL compared with the baseline on consumer storage d 21. Specifically, 5% and 3% of the milk containers contaminated with isolates PS00413 and PS00433 from group IV and 5% of the milk containers contaminated with isolate PS00638 from group V had *B. cereus* group concentrations  $>10^5$  cfu/mL, which is higher compared with 4%, 2%, and 3% predicted by the base model, respectively. In contrast, 2 strains (PS00413 from group IV and PS00570 from group V) were predicted to exceed  $10^5$  cfu/mL in a higher percentage (11% and 10%, respectively) of milk containers on consumer storage d 35, compared with the prediction by the baseline model (i.e., 10% and 6%, respectively).

The first 2 scenarios, which represent practical events during transportation, did not change model predictions for most, if not all, strains. However, the last 2 scenarios, which represent consumer behavior or ability to manage refrigeration temperature, resulted in a more profound effect. A slight temperature deviation at prolonged con-

**Table 5.** Percentage of milk containers with over 5 log<sub>10</sub> cfu/mL at consumer storage d 35 and 21 predicted by base model compared with what-if scenarios

Isolate	Group	Base model		Scenario 1 <sup>1</sup>		Scenario 2 <sup>2</sup>		Scenario 3 <sup>3</sup>		Scenario 4 <sup>4</sup>	
		d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21	d 35	d 21
Average of all isolates <sup>5</sup>		4.13	2.81	4.13	2.81	4.44	3.06	8.88	4.56	10.81	7.56
PS00125	I	1	1	1	1	1	1	3	2	2	2
PS00193	II	3	3	3	3	3	3	6	3	10	5
PS00457	II	3	3	3	3	3	3	10	3	12	9
PS00518	II	3	3	3	3	3	3	7	3	10	9
PS00564	II	5	3	5	3	5	3	12	7	13	10
PS00474	III	3	3	3	3	3	3	10	3	10	7
PS00402	IV	4	2	4	2	4	2	9	3	11	5
PS00407	IV	3	3	3	3	3	3	5	3	10	5
PS00413	IV	10	4	10	4	11	5	17	11	17	14
PS00433	IV	3	2	3	2	3	3	6	3	10	4
PS00495	IV	3	3	3	3	3	3	7	3	10	9
PS00649	IV	3	3	3	3	3	3	10	3	10	9
PS00570	V	6	3	6	3	10	3	14	10	14	10
PS00638	V	10	3	10	3	10	5	14	10	14	12
PS00194	VII	3	3	3	3	3	3	6	3	10	6
PS00536	VII	3	3	3	3	3	3	6	3	10	5

<sup>1</sup>Scenario 1, a 3-d delay on HTST milk product delivery from processing facility to retail without temperature abuse.

<sup>2</sup>Scenario 2, a temperature abuse when a consumer transported the HTST milk products from retail back home in a hot summer.

<sup>3</sup>Scenario 3, a slight temperature deviation in consumer's home refrigerator.

<sup>4</sup>Scenario 4, an increase in the variability of refrigeration temperatures at consumer's home.

<sup>5</sup>Percent milk containers (%) over 5 log<sub>10</sub> cfu/mL at consumer storage d 35 and d 21 averaged across all 16 isolates.

sumer home storage (scenario 3) had a much higher predicted effect on the percentage of milk containers containing >10<sup>5</sup> cfu/mL than the 2 scenarios detailed above. The model predicted 4.56 ± 3.05 (mean ± SD) and 8.88 ± 3.84% milk containers with *B. cereus* group concentrations >10<sup>5</sup> cfu/mL on consumer storage d 21 and 35, respectively (as compared with the base model prediction of 2.81% and 4.13%). Under scenario 3, 1 of 6 group IV strains and both group V strains were predicted to exceed 10<sup>5</sup> cfu/mL on consumer storage d 21 in more than 10% milk containers, whereas the base model predicted that none of the strains would exceed 10<sup>5</sup> cfu/mL in more than 10% milk containers. In contrast, 2 of 4 group II strains, 1 of 1 group III strain, 2 of 6 group IV strains and 2 of 2 group V strains were predicted to exceed 10<sup>5</sup> cfu/mL in more than 10% milk containers on consumer storage d 35 (compared with 1 of 6 group IV strains and 1 of 2 group V strains predicted by the base model). Increasing the variability in consumer storage temperature has an even more substantial effect on the percentage of milk containers exceeding *B. cereus* group concentrations of 10<sup>5</sup> cfu/mL than a consistently higher average storage temperature (scenario 3). The model predicts that 7.56 ± 3.20% and 10.81 ± 3.15% milk containers had *B. cereus* group concentrations over 10<sup>5</sup> cfu/mL on consumer storage d 21 and 35. Under this scenario, 1 of 4 group II strains, 1 of 6 group IV strains and both group V strains were predicted to have more than 10% of milk containers exceeding 10<sup>5</sup> cfu/mL on consumer storage d 21, while

15 of 16 strains were predicted to have more than 10% of milk containers exceeding 10<sup>5</sup> cfu/mL on consumer storage d 35 (compared with 0 and 2 strains predicted to have more than 10% of milk containers with over 10<sup>5</sup> cfu/mL on consumer storage d 21 and 35 by the base model; Table 5). The more profound effect from scenarios 3 and 4 can be explained by the fact that (1) consumer storage is the longest time in the entire supply chain, and therefore any temperature change would have a more extended effect, and (2) changing deviation and variability increases the number of abnormal temperature conditions that exceed the growth boundaries of more strains.

## DISCUSSION

The exposure assessment model we developed using a genomically diverse set of *B. cereus* group strains presents a tool to facilitate industry risk assessments for diarrheal *B. cereus* group members in HTST milk. Our experimental data suggest that *B. cereus* group strains show a range of growth characteristics, including differences in minimum growth temperature, which ultimately affect the exposure through consumption of contaminated HTST milk. Specifically, our research reveals 2 significant insights (1) *B. cereus* group strains from different phylogenetic groups differ in key cardinal values (i.e., minimum growth temperatures) and (2) there are differences in growth parameters within a given phylogenetic group. Consequently, our data support that the

accuracy of *B. cereus* group exposure assessments can be improved by accounting for genetic diversity within the *B. cereus* group. Notably, what-if scenarios run with the initial exposure assessment yield crucial insights into *B. cereus* group risk assessment and risk management. These analyses highlight the substantial effect of both absolute temperature and temperature variation during consumer storage on predicted *B. cereus* group exposure. Although our model proves beneficial for industry risk assessments concerning diarrheal disease-causing *B. cereus* group members, advances in the understanding of *B. cereus* group virulence potential are needed to develop full risk assessment models that include public health metrics.

### ***Bacillus cereus* Group Members Show a Range of Growth Characteristics, Including Differences in Minimum Growth Temperatures and Maximum Growth Rates, Which Affect the Risk Associated with the Contamination of HTST Milk**

We found that the 16 tested *B. cereus* group strains showed practically relevant variations in both experimental and theoretical minimum growth temperatures. The experimentally obtained growth boundaries of these 16 strains are consistent with the findings of Lott et al. (2023) and Saleh-Lakha et al. (2017) that some cytotoxic sporeformers in the *B. cereus* group may grow at abuse temperatures (e.g., 10°C), but not at lower temperatures (e.g., 4°C). By comparison, one study reported that experimentally observed minimal growth temperatures of 30 diarrheal enterotoxin producing *B. cereus* group strains ranged from ≤5°C to 11°C (Dufrenne et al., 1994). Similarly, a growth study of 11 psychrotolerant *B. cereus* strains (Andersen Borge et al., 2001) reported that the lowest temperatures with observed growth (in BHI medium and commercial 1.5% UHT milk) were 4°C for one strain, 6°C for 2 strains, and 7°C for 7 strains. Guinebrière et al. (2008) reported phylogenetic group-specific minimum growth temperatures: (1) 10°C for group I (28 strains tested), (2) 7°C for group II (33 strains tested), (3) 15°C for group III (96 strains tested), 10°C for group IV (101 strains tested), 8°C for group V (17 strains tested), and 20°C for group VII (2 strains tested). This is generally consistent with our findings except that the group III and group VII isolates in our study had lower experimental minimum growth temperatures (i.e., 10°C) as compared with data reported by Guinebrière et al. (2008). This finding is novel and significant, because these 2 phylogenetic groups are known to contain strains with disease-causing potential. Specifically, group III isolates contain cereulide-producing strains that can cause emetic illness, and group VII isolates contain cytotoxinK-1-producing

strains that are known to cause diarrheal illness (Dietrich et al., 2021; Jovanovic et al., 2021; Cairo et al., 2022).

The experimental minimum growth temperatures for all strains in our study were consistent with their theoretical  $T_{\min}$  predicted by the secondary model. Specifically, the one group I strain with a minimum growth temperature of 14°C also had a substantially higher theoretical  $T_{\min}$  of 9.2°C compared with the other 15 strains that showed detectable growth at 10°C ( $T_{\min}$  ranging from 4.26–7.06°C). Notably, Carlin et al. (2013) reported  $T_{\min}$  for strains from groups II to V are comparable to our study; however, 2 group VII strains in their study were reported to have substantially higher  $T_{\min}$  (i.e., 12.6°C and 19.1°C) than the  $T_{\min}$  in our study (i.e., 6.89°C and 7.06°C). Our growth experiment also showed that *B. cereus* group members have a wide range of estimated maximum growth rates at 22°C and 10°C. This is comparable to the reported range of *B. cereus* group maximum growth rates (12 strains, growth experiment conducted in BHI supplemented with yeast extract at 2 g/L and glucose at 3 g/L) of 0.27/h to 1.17/h at 22°C and 0.02/h to 0.17/h at 10°C (Carlin et al., 2013). Other studies such as Ellouze et al. (2021) also investigated *B. cereus* growth in a dairy matrix (i.e., reconstituted milk) and collected kinetic data across a temperature range of 9 to 45°C. However, they examined only one reference emetic *B. cereus* strain, F4810/72, which was not included in our study. Overall, the high variability of growth characteristics of *B. cereus* group members, including minimum growth temperatures and maximum growth rates, suggests that it is crucial to evaluate a diverse set of strains when predicting *B. cereus* group growth. This is particularly important, as accurate predictions of the growth of different *B. cereus* strains are essential to correctly predict the exposure risk if milk is contaminated with a specific *B. cereus* group strain.

### ***Quantitative B. cereus* Group Exposure Assessments Can Be Improved by Accounting for Genetic Diversity of *B. cereus* Group Members and Common HTST Milk Microbiota**

Very few of the previous quantitative exposure assessments conducted in pasteurized fluid milk have addressed how the genetic diversity of *B. cereus* group members might affect human exposure. For instance, Notermans et al. (1997), Aćai et al. (2014), and Lewin et al. (2019) omitted reporting the phylogenetic grouping of *B. cereus* when presenting their risk assessment findings. Carlin et al. (2013) reported the variation of cardinal values for *B. cereus* group members with respect to their phylogenetic affiliation but were lacking data for isolates from phylogenetic group I. Our study further emphasizes the need to account for genetic variability when conducting

*B. cereus* group quantitative exposure assessments. This is specifically supported by the fact that group I isolate PS00125, which has the highest  $T_{\min}$  (9.22°C) in our study, was indeed predicted to have the lowest percentage of milk containers exceeding  $10^5$  cfu/mL on consumer storage d 35. Importantly, model input parameters other than  $T_{\min}$  (e.g., the initial physiological state of cells,  $Q_0$ ) may also affect exposure risk. This is supported by our exposure assessment model predictions that the risks of *B. cereus* group exposure on a given consumer storage day (especially consumer storage d 35) varies among strains and phylogenetic groups that show similar  $T_{\min}$ . For instance, isolates PS00413 and PS00402, both from group IV, had similar  $T_{\min}$  (i.e., 4.88°C and 4.26°C); however, they showed substantial variations in another model input parameter,  $Q_0$  (i.e., 1.26E-02 and 1.91E-09). As a result, the model predicted that on consumer storage d 35, as high as 10% of the milk containers with isolate PS00413 exceeded  $10^5$  cfu/mL, whereas only 4% of the milk containers with isolate PS00402 exceeded the same threshold. This finding also suggests that, as the sensitivity analysis indicated, additional growth data collection for strains with great uncertainty in  $Q_0$  is necessary to improve the prediction precision.

Additionally, HTST milk is known to have a rich microbiota that consists of thermophilic bacteria (e.g., *Microbacterium* spp.) and gram-negative bacteria associated with postpasteurization contamination (e.g., *Pseudomonas*; Lott, 2023). This suggests the need to account for the effect of these competing microorganisms on the growth of *B. cereus* group strains in HTST milk. Although our current research scope limits the depth of investigation into factors such as competing microbiota, pH, and secondary metabolites such as organic acids produced by lactic acid bacteria, we recognize their relevance. Incorporating these variables in future studies could provide a more comprehensive understanding of microbial interactions within HTST milk and shed further light on the complexities of microbial behavior, enhancing the accuracy of our analysis of *B. cereus* growth dynamics in milk. Future studies might be conducted to quantify the microbial interactions between *B. cereus* group strains and other microbial contaminants in the HTST milk, similar to previous studies that modeled the interactions between 2 microorganisms in the same system (Martens et al., 1999; Courtin and Rul, 2004).

#### **What-If Scenarios Suggest That Absolute Temperature and Temperature Variation During Consumer Storage Have a Large Effect on the Predicted *B. cereus* Group Exposure**

The exposure assessment reported here was used to run selected what-if scenarios to assess the importance

of selected supply chain disruptions and changes, as well as different consumer storage temperature scenarios that may mimic practices in different locations, seasons, or infrastructure conditions. The 2 what-if scenarios that modeled disruption to distribution included scenarios that mimicked (1) extended storage in a distribution center (without temperature abuse) and (2) temperature abuse during consumer transport from retail to home. Both what-if scenarios showed limited effects on risk metrics, which is not surprising, as scenario (2) was modeled as a relatively short time period of temperature abuse (median of 1 h; range of 14 min to 6 h). Our findings indicate short-term temperature deviations do not substantially increase bacterial growth. This reinforces the importance of adhering to recommended consumer time-temperature controls, commonly referred to as the 2-h rule and 1-h rule. This rule suggests that foods should be kept outside the danger zone (4.4–60.0°C) for less than 2 h (for temperature between 4.4 and 32.2°C) or 1 h (for temperatures >32.2°C; Cho et al., 2020). Importantly, these what-if scenarios also illustrate how this model could be used to predict *B. cereus* group exposures if supply chains might experience certain “worst case” disruptions (e.g., lack of refrigeration for extended time periods).

The 2 what-if scenarios assessing the effect of varying temperature conditions during home storage comprised (1) a slight deviation in temperature within a consumer’s home refrigerator, and (2) an increase in the variability of refrigeration temperatures in a consumer’s home. These scenarios demonstrated that even small differences in refrigeration conditions (e.g., a 1°C shift in mean temperature) can substantially affect likely *B. cereus* group exposure. This finding aligns with our existing knowledge that prolonged temperature abuse during consumer home storage accelerates the growth of many cytotoxic *B. cereus* group isolates (Ceuppens et al., 2011). It leads to a notable occurrence, with more than 8% and 4% of milk containers exceeding  $10^5$  cfu/mL on consumer home storage d 35 and d 21, respectively. Changes in the variability of home refrigeration temperatures also yielded substantial effects. A higher variability may arise from the use of older refrigerators, more commonly prevalent in households with lower income, or in regions or seasons (e.g., hot summers) where rolling blackouts (i.e., planned short-term power outages), are common. Our findings are consistent with previous studies (Jofré et al., 2019) that refrigeration temperature fluctuation increases the risk of exposure to foodborne pathogens, thus negatively affecting the safety of food products.

Although our current assessment used a modeled temperature profile for a 5-stage supply chain, accommodating static storage temperatures at each stage, we acknowledge the potential for future model improvements to incorporate a comprehensive dynamic tempera-

ture profile, which would better simulate temperature fluctuations within each stage. However, implementing such improvements would require more precise supply chain temperature data.

**While Our Model Will Facilitate Industry Risk Assessments for Diarrheal *B. cereus* Group Strains, Advances in the Understanding of *B. cereus* Group Virulence Are Needed to Develop Full Risk Assessments That Include Public Health Metrics**

The model reported here will provide industry with an initial tool that can facilitate risk-based food safety decision-making for products that are contaminated with low *B. cereus* group levels. For example, if an HTST fluid milk product is found to contain low levels of a specific *B. cereus* group strain, our model can be used to predict, at different time points, which proportion of products are expected to exceed a certain concentration threshold (e.g., 3 or 5 log<sub>10</sub> cfu/mL). Risk managers could then use this information to inform their decision-making. In addition, this model could also be adopted to assess regulatory risk, such as the likelihood that a lot with low *B. cereus* group levels may yield a test result that exceeds a regulatory threshold, using approaches such as those described previously (Chen et al., 2022). We, however, do appreciate that many users may be hesitant to use this initial model for risk management related decisions because we currently lack the ability to predict the number of human cases that may be caused by a given lot. This is due to the lack of a universally applicable dose-response relationship that will allow us to predict the likelihood of *B. cereus* toxicoinfection per serving and estimate the number of cases within the population (Notermans et al., 1997; Ačai et al., 2014; Lewin et al., 2019). Nevertheless, our model could be easily adapted into a full risk assessment if *B. cereus* group dose-response data become available.

### CONCLUSIONS

This study comprehensively analyzed the growth characteristics of 17 *Bacillus cereus* group strains under various temperature conditions. Key findings include that no strains grew at 4°C, only one grew at temperatures ≤10°C, and all demonstrated growth at ≥14°C. Growth parameters varied significantly with temperature; for instance, at 22°C, lag times ranged from 0 to 22.04 h, whereas at 10°C they extended up to 194.05 h. The exposure assessment model predicted that 1.94 ± 0.85%, 2.81 ± 0.66%, and 4.13 ± 2.53% of milk containers would exceed 10<sup>5</sup> cfu/mL on d 14, 21, and 35, respectively. What-if scenario analyses suggested that small increases in temperature or fluctuations within consumer refrigerators could significantly raise the risk, with predicted concentrations of *B. cereus* over 10<sup>5</sup> cfu/mL in up to 10.81% of milk contain-

ers by d 35 under varying storage conditions. Sensitivity analyses highlighted the physiological state parameter ( $Q_0$ ) as having a significant effect on model predictions, emphasizing its importance in risk assessments. Overall, the results underscore the necessity of maintaining strict temperature controls in consumer settings to manage risks associated with *B. cereus* in milk.

### NOTES

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**Nonstandard abbreviations used:** ANI = average nucleotide identity; BHI = brain-heart infusion; N<sub>0</sub> = initial contamination levels; N<sub>max</sub> = maximum microbial population; Q<sub>0</sub> = initial physiological state of cells; SMB = skim milk broth; T<sub>min</sub> = theoretical minimum growth temperature.







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