Effect of Storage Temperatures and Ingredients on Growth of *Bacillus cereus* in Coffee Creamers[1]

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ABSTRACT

Growth of *Bacillus cereus* ATCC 33018 was evaluated in half and half (10.5% fat), whipping cream (30% fat), and nondairy creamer (7.5% fat). Samples were inoculated with approximately 10 vegetative cells/ml or 100 spores/ml and were subsequently stored at 4, 7, 23, and 32°C. Within 9 h at 32°C and 11 h at 23°C, in both half and half and whipping cream, vegetative cells and spores reached population levels that can cause foodborne illness. No growth occurred in any product stored at 4 or 7°C. Sodium stearoyl lactylate, a fatty acid derivative that is used as an emulsifier, inhibited growth of spores and vegetative cells in the nondairy creamers stored at either 32 or 23°C.

(**Key words**: *Bacillus cereus*, storage temperature, coffee creamers)

Abbreviation key: **BHI** = brain-heart infusion, **PCA** = plate count agar, **RCB** = randomized complete blocks, **UP** = ultra-pasteurization.

INTRODUCTION

Since the first description of foodborne illness from *Bacillus cereus* in the 1950s (9), this microorganism has received much attention and has become a possible cause of food poisoning (26). *Bacillus cereus* is an aerobic, rod-shaped bacterium that is widely distributed in the environment and that produces spores, enterotoxins, and lecithinase (2, 30). Approximately 5% of all foodborne illnesses reported to the World Health Organization Surveillance Programme in Europe in 1990 involved *B. cereus* (29). Generally, acceptable epidemiological evidence to incriminate *B. cereus* as the causative agent of foodborne illness requires the presence of greater than 10^5 cells/g of food (5).

Although the significance of *B. cereus* as a dairy foods pathogen has not been fully established, it has been isolated from raw and pasteurized milk (14, 17, 18), infant foods, and dried milk products (3). *Bacillus cereus* has been implicated in a foodborne illness outbreak involving ice cream (2). Unconfirmed cases of *B. cereus* gastroenteritis also may have involved feta cheese and skim milk powder (21). Ahmed et al. (1) isolated *B. cereus* from pasteurized milk, cheese, and ice cream samples, although numbers were less than those normally required for foodborne illness.

Bean and Griffin (2) reported that 94% of outbreaks involving *B. cereus* were attributed to improper holding temperature; therefore, the importance of maintaining adequate refrigerated storage of dairy products is obvious. Fluid coffee creamers are examples of dairy products that are frequently held at ambient temperature in restaurants and food service units. Consequently, temperature abuse is an important consideration for these products.

Most coffee creamers are processed at ultra-pasteurization (**UP**) temperature, and the combination of higher temperature and brief holding time are assumed to destroy all spores of *B. cereus*; however, early work in our laboratory indicated that a few *B. cereus* spores can survive even the most drastic UHT treatments (15, 16). Therefore, the present study was designed to evaluate the growth of *B. cereus* spores and vegetative cells in coffee creamers at several storage temperatures.

MATERIALS AND METHODS

Microorganism

Bacillus cereus ATCC 33018 is an enterotoxin-producing strain that was originally isolated from infant formula based on powdered milk. This strain was obtained freeze-dried from the American Type Culture Collection (Rockville, MD). The culture was

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transferred every 24 h in brain-heart infusion (**BHI**) broth (Difco Laboratories, Detroit, MI) at 32°C until a steady state of growth was reached. The bacteria were subsequently enumerated in plate count agar (**PCA**; Difco Laboratories). For growth experiments in coffee creamers, vegetative cells were standardized to approximately 10 cfu/ml.

Preparation of Spore Suspension

Spore suspensions of *Bacillus cereus* were prepared on fortified nutrient agar as described by Johnson et al. (11). An 18-h culture of *B. cereus* vegetative cells grown in 50 ml of BHI broth in a 125-ml Erlenmeyer flask at 32°C under agitation at 150 rpm was used to surface inoculate fortified nutrient agar (20 ml) previously poured into 100- × 15-mm Petri plates at a rate of 0.2 ml per plate in a laminar flow cabinet. Plates initially were incubated upright for 6 h at 32°C and then were inverted for 5 d at 32°C to optimize sporulation.

Sporulation was monitored by phase contrast microscopy until fewer than 5% vegetative cells remained. Three milliliters of sterile, deionized water (4°C) were added to the agar surface, and spores were removed with a sterile bent glass rod. The spores contained in the deionized water were then centrifuged and washed 10 times in 30 ml of sterile deionized water (4°C). Between washings, the suspensions were centrifuged at 10,000 × *g* for 30 min.

The concentrated spore suspension was prepared in 30 ml of sterile 10 m*M* phosphate buffer (4°C), pH 7.0, and heated for 12 min at 80°C (16, 18) in a circulating thermostatically controlled water bath to inactivate any remaining vegetative cells. The spore suspension was immediately cooled in an ice-water bath and stored at 4°C. A concentration of approximately 10^{11} cfu/ml of *B. cereus* spores was in the final suspension.

Sample Preparation, Inoculation, and Plating

Half and half (10.5% fat), whipping cream (30% fat), and liquid nondairy coffee creamer (7.5% fat) were obtained commercially. One hundred-milliliter portions of each dairy product were placed into 160-ml, screw-cap milk dilution bottles and sterilized at 121°C for 15 min at 121° for 15 min. Samples were then adjusted to treatment temperatures of 32, 23, 7, or 4°C in water baths. Each sample was then inoculated with 1 ml of a diluted culture of vegetative cells or spore suspension of *B. cereus* to yield final populations of approximately 10 vegetative cells/ml or 100 spores/ml. The inoculated products were subsequently stored at ambient (23°C) and optimum (32°C) temperatures. Samples were removed and pour plated at intervals of 0, 3, 7, 10, 13, 17, 21, and 25 h. Samples were also stored at 7°C, a refrigeration temperature at which psychrotrophic *B. cereus* cells have been reported to grow (25), and at 4°C, the recommended temperature for cold storage of most fluid dairy products; samples were plated after storage for 0, 5, 10, 15, 20, and 25 d. The PCA was used for plating vegetative cells, and products containing spores were pour plated in PCA that had been supplemented with 0.1% soluble starch (10, 16, 17). Dilutions were made in sterile 0.1% peptone solutions, pH 7.2 (Fisher Scientific Co., Pittsburgh, PA). To minimize germination of spores before plating, the complete plating procedure for each sample did not exceed 20 min (24).

Growth Characteristics Calculations

Linear regression equations ($y = a + kx$) were calculated from the linear portion of the growth curves to determine specific growth rates (k) per hour by using the regression procedure of SAS (20). The $\log_{10}$ of colony-forming units per milliliter was expressed as y, and incubation time, in hours, was expressed as x. The slope (k) of the linear portion of the growth curve was the specific growth rate. Generation times (G) were determined by the equation $G = \ln 2/k$.

Predicted Time Required for *B. cereus* Spores and Vegetative Cells to Reach Foodborne Illness Levels

The previously calculated equations for linear regression also were used to determine time required, under various storage conditions, for *B. cereus* to reach levels that were sufficient to cause foodborne illness. To pinpoint this level graphically, horizontal lines were drawn from the y axis (Figures 1 and 2). The intersection between the slope of the growth curves and the respective horizontal lines indicates the predicted times needed for *B. cereus* spores and vegetative cells to reach 100,000 (5 $\log_{10}$), 500,000 (5.7 $\log_{10}$), and 1,000,000 (6 $\log_{10}$) cfu/ml.

Inhibition of Germination and Outgrowth of *B. cereus* Spores in the Nondairy Creamer

The ingredients used in the formulation of fluid nondairy coffee creamer were supplied by the manufacturer. These ingredients, in either dry or liquid form, were individually reconstituted in deionized

water and incorporated into PCA, followed by sterilization at 121°C for 15 min. Hydrogenated vegetable oil was added directly during agar preparation. Agars containing the different ingredients were then inoculated with approximately 10^9 spores/ml. The inoculated medium was incubated at 32°C for 48 h.

Statistical Analysis

Data were analyzed as a series of combined randomized complete blocks (**RCB**) over treatment combinations. Eight separate experiments were conducted: 1) vegetative cells at 23°C, 2) spores at 23°C, 3) vegetative cells at 32°C, 4) spores at 32°C, 5) vegetative cells at 4°C, 6) spores at 4°C, 7) vegetative cells at 7°C, and 8) spores at 7°C.

Each experiment was set up as an RCB design with three replications. Treatments consisted of three products: half and half, whipping cream, and nondairy coffee creamer; repeated measurements were taken at 0, 3, 7, 10, 13, 17, 21, and 25 h of incubation for ambient (23°C) and optimum (32°C) temperatures and at 0, 5, 10, 15, 20, and 25 d for refrigeration temperatures (7 and 4°C).

Data were subjected to ANOVA as a series of combined RCB, and means were separated by least significance difference procedure using SAS (20). The following model was used to analyze growth and outgrowth response of vegetative cells and spores at each incubation temperature:

$$Y_{ijkl} = \mu + T_i + e_{il} + P_j + T_i(P_j) + e_{ijk} + H_k + H_k(T_i) + H_k(P_j) + H_k(T_i)\ (P_j) + e_{ijkl}$$

where

μ = overall mean,
T_i = effect of temperature i,
P_j = effect of product j,
H_k = effect of incubation time k,
e_{il} = error (a), assumed normally distributed $(0, \sigma^2)$,
e_{ijk} = error (b), assumed normally distributed $(0, \sigma^2)$, and
e_{ijkl} = error (c), assumed normally distributed $(0, \sigma^2)$.

Significance was declared at $P < 0.05$ unless otherwise stated.

RESULTS

Effect of Storage Temperature on Growth of *B. cereus* Vegetative Cells

The effect of storage temperature on the growth of vegetative cells of *B. cereus* is shown in Figure 1. Growth in half and half and whipping cream at both 32 and 23°C was quite rapid and followed the normal growth curve pattern for bacteria. These data indicate that, at these storage temperatures, both dairy products can support the growth of *B. cereus* until numbers reach levels that can cause foodborne illness, even from small initial numbers of approximately 10 cells/ml.

No growth was observed in the products stored at either refrigeration temperature; therefore, this strain of *B. cereus* was not psychrotrophic and did not grow at 7 or 4°C.

In the nondairy coffee creamer, no growth of vegetative cells occurred during 48 h of incubation at 32 or 23°C (Figure 1). Agar plates were subsequently sealed with parafilm and kept at ambient temperature for an additional 5 d, and no visible growth was evident. Obviously, some inhibitor was present in the nondairy creamer. Therefore, we evaluated the effect of all ingredients in the nondairy coffee creamer on the growth of vegetative cells of *B. cereus*.

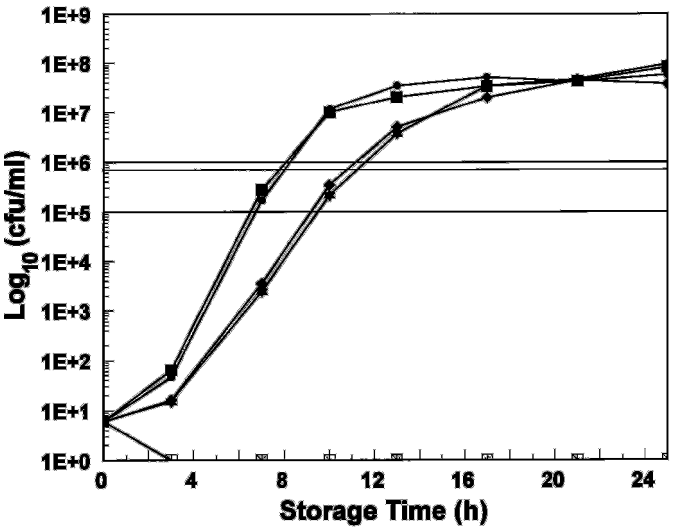

Figure 1. Growth of vegetative cells of *Bacillus cereus* in half and half at 32°C (■), whipping cream at 32°C (●), nondairy coffee creamer at 32°C (∗), half and half at 23°C (♦), whipping cream at 23°C (★), and nondairy coffee creamer at 23°C (□).

Growth Characteristics of *B. cereus* Vegetative Cells

Table 1 shows the linear regressions and respective coefficients of determination that were obtained during the exponential growth phase in these dairy

TABLE 1. Linear regressions, growth characteristics, and coefficients of determination derived from inoculation of coffee creamers with *Bacillus cereus* vegetative cells or spores.

Product and inoculum	Temperature	Intercept	Slope[1]	SE	Generation time	r^2
	(°C)					
Half and half						
Vegetative, 1×10 cfu/ml	23	–0.417	0.551	0.021	1.258	0.984
	32	–0.383	0.755	0.043	0.918	0.977
Spores, 1×10^2 cfu/ml	23	1.748	0.281	0.020	2.467	0.936
	32	1.811	0.476	0.038	1.456	0.956
Whipping cream						
Vegetative, 1×10 cfu/ml	23	–0.441	0.546	0.015	1.269	0.992
	32	–0.609	0.781	0.036	0.887	0.985
Spores, 1×10^2 cfu/ml	23	1.260	0.241	0.029	2.876	0.839
	32	1.757	0.383	0.019	1.809	0.975

[1]Growth rate, $\log_{10}$ per hour.

products. The high r^2 values obtained suggest that the regression models used were appropriate to explain the growth of *B. cereus* in these products.

Calculated specific growth rates and generation times are also listed in Table 1. Growth rates were higher for half and half containing vegetative cells at 32°C. Growth rate in whipping cream followed a similar trend. Only once at 32°C did growth rate in whipping cream surpass that in half and half.

Predicted Time for *B. cereus* Vegetative Cells to Reach Foodborne Illness Levels

Large numbers of *B. cereus* cells are usually found in foods associated with outbreaks of food poisoning (12). Literature citations (1, 5, 19) indicate that 10^5 to 10^6 *B. cereus* cells/g are required to initiate foodborne illness. However, there is not total agreement on the minimum number of vegetative cells or spores of *B. cereus* required to cause foodborne illness. Calculated linear regressions were used to predict how long an initial inoculum of 10 cells/ml would take to reach 1×10^5, 5×10^5, and 1×10^6 cfu/ml under conditions of temperature abuse (Table 2). Numbers increased to 1×10^5 cells/ml in 7 h, to 5×10^5 cells/ml in 8 h, and to 1×10^6 cells/ml in less than 9 h at 32°C in both half and half and whipping cream.

At ambient temperature (23°C), a concentration of 1×10^5 cells/ml was reached in approximately 10 h; 11 h were required for cell numbers to reach 5×10^5/ml, and almost 12 h were required to reach 1×10^6/ml in both products. Again, at 23°C, there was no significant difference between growth in half and half and whipping cream. After 21 h, numbers of *B. cereus* cells were greater than 3×10^7/ml in both products at both 32 and 23°C.

Effect of Storage Temperature on Germination and Outgrowth of *B. cereus* Spores

The initial work was carried out with vegetative cells of *B. cereus*. Because these cells are more heat sensitive than spores and would not likely survive UP treatment, we investigated the outgrowth of *B. cereus* spores in the same products. During this phase of the

TABLE 2. Predicted hours of temperature abuse for *Bacillus cereus* to reach foodborne illness levels[1] in coffee creamers.

Product and inoculum	Log_{10}	Temperature	Time	SE
		(°C)	(h)	
Half and half				
Vegetative	5	23	9.87	0.16
	5.7		11.10	0.18
	6		11.64	0.19
	5	32	7.13	0.16
	5.7		8.05	0.17
	6		8.45	0.18
Spores	5	23	11.58	0.41
	5.7		14.09	0.50
	6		15.14	0.54
	5	32	6.69	0.33
	5.7		8.16	0.38
	6		8.79	0.41
Whipping cream				
Vegetative	5	23	9.95	0.11
	5.7		11.23	0.12
	6		11.78	0.13
	5	32	7.18	0.13
	5.7		8.07	0.14
	6		8.45	0.15
Spores	5	23	15.48	0.58
	5.7		18.38	0.73
	6		19.62	0.82
	5	32	8.45	0.31
	5.7		10.27	0.36
	6		11.06	0.39

[1]10^5 to 10^6 cfu/ml.

work, the spore inoculum was approximately 1×10^2 per ml of sample. Figure 2 shows the results that were obtained under storage conditions similar to those for vegetative cells. Spores germinated and grew rapidly at both 32 and 23°C. No germination or growth occurred at either 7 or 4°C. These findings again indicated that this particular strain of *B. cereus* did not possess psychrotrophic characteristics, and, therefore, its spores did not germinate and grow under refrigeration temperatures in these dairy products.

Growth of spores was not detected in the nondairy creamer during 25 h of storage at temperatures of 32 or 23°C. After storage, counts corresponded to the original numbers of spores that had been inoculated into the nondairy creamer. Plates were sealed with parafilm and kept at ambient temperature for an additional 5 d. No growth was observed during this time. It was concluded that some inhibitor present in the nondairy creamer prevented germination and subsequent growth of spores in this product.

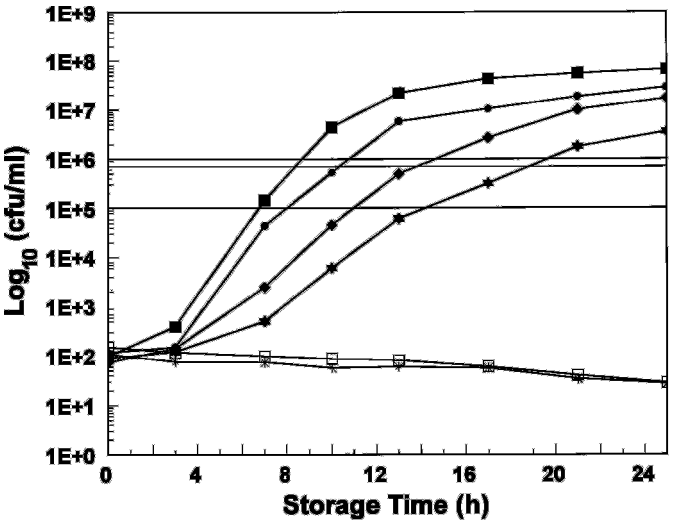

Figure 2. Germination and outgrowth of spores of *Bacillus cereus* in half and half at 32°C (■), whipping cream at 32°C (●), nondairy coffee creamer at 32°C (∗), half and half at 23°C (♦), whipping cream at 23°C (★), and nondairy coffee creamer at 23°C (□).

Outgrowth Characteristics of *B. cereus* Spores

Linear regressions and coefficients of determinations derived from the exponential phase of *B. cereus* growth in half and half and whipping cream are listed in Table 1. As found earlier for vegetative cells, these higher r^2 values suggest that the regression models used were appropriate to explain mathematically the growth of *B. cereus* spores in these products.

Calculated specific growth rates and generation times listed in Table 1 indicate that growth rates were higher at the higher incubation temperature. Generation times were shorter in products stored at 32°C than at 23°C. However, rapid growth occurred in both products at both temperatures.

Predicted Time for *B. cereus* Spores to Germinate, Grow, and Reach Foodborne Illness Levels

Predictions in Table 2 show that high bacteria numbers were reached in less time at 32°C than at 23°C. Counts at 32°C approached 1×10^5 cfu/ml in about 7 and 9 h for both products, 5×10^5 cfu/ml in 8 and 10.30 h, and 1×10^6 cfu/ml at the end of almost 9 and 11 h for half and half and whipping cream, respectively.

When products were stored at 23°C, longer times were predicted for *B. cereus* to achieve numbers of 10^5 to 10^6 cfu/ml. Approximately 12 to 16 h were required to reach 1×10^5 cfu/ml, and 14 to almost 16 h were necessary for numbers to increase to 5×10^5 cfu/ml in half and half and whipping cream. Counts approached 1×10^6 cfu/ml after 15 and 19 h in both products and in the same order, respectively.

Inhibition of Germination and Growth of *B. cereus* Spores by Nondairy Ingredients

Because germination and growth were totally inhibited in the nondairy coffee creamers, we examined specific ingredients for their individual contribution to this inhibition.

Table 3 shows the results of the growth of *B. cereus* spores in PCA containing the various ingredients. Spores failed to germinate and grow in the presence of sodium stearoyl lactylate, but germinated and grew in the presence of the other ingredients, which included corn syrup, hydrogenated vegetable oil, sodium caseinate (the major dairy ingredient), potassium phosphate, polysorbate-60, carrageenan, and β-carotene.

DISCUSSION

In this study, growth characteristics of vegetative cells and spores of *B. cereus* were studied in three different products at four storage temperatures. Generation times ranged from 0.887 to 2.876 h for spores and vegetative cells in half and half and whip-

ping cream stored at 23 or 32°C. Shehata and Collins (22) also studied generation times of three species of *Bacillus* in milk. Calculated doubling times ranged from 5 to 7 h at 7.2°C. Counts were between 1×10^6 and 1×10^7 cfu/ml at the time of off-flavor detection from an initial number of 2×10^4 to 3×10^4 cfu/ml. Larkin and Stokes (13) studied sporeformers isolated from mud and determined their generation times to be 8.5 and 11.5 h at 5°C in BHI broth. Benedict et al. (4) observed generation times of *B. cereus* of 0.23 h from initial levels of 10^3 cells/ml when grown in BHI broth at 37°C. Total numbers ranged from 10^6 to 10^9 cfu/ml in 3.5 and 6.1 h, respectively. Thus, our data agreed with those other growth studies. Obviously, initial inoculum level and temperature were the major determinants in the growth dynamics of *B. cereus*.

Also, Sutherland (23) studied the outgrowth of *B. cereus* spores in whipping cream (39% fat). From an initial inoculum of 1×10^3 spores/ml, numbers reached approximately 2×10^7 cfu/ml after 24 h at 21°C, and 2×10^2 cfu/ml after 14 d at 6°C. Although we used a slightly higher incubation temperature (23°C), our results were obtained from an inoculum of 1 log unit smaller (1×10^2 spores/ml). Because our final counts for whipping cream were also 1 log unit smaller, our results agree with those of Sutherland (23).

Becker et al. (3) found that, in reconstituted infant food that had been naturally contaminated with approximately 1×10^2 spores of *B. cereus*/g, 1×10^5 cfu/ml was reached at 27°C after 7 and 8 h of incubation for two strains and after 9 h for three others. Perhaps the observed differences in incubation periods that were required to reach 1×10^5 cfu/ml could be attributed to slight differences in storage temperatures: 23°C in our study and 27°C in the study by Becker et al. (3). Also, the higher fat content of coffee creamers (10.5% in half and half and 30% in whipping cream) than in infant food might be a factor. Nevertheless, these data show that storage temperature is critical to maintain low numbers of *B. cereus* in coffee creamers, and prolonged storage at ambient temperatures can certainly allow sufficient growth for cell numbers to be in the range that can cause foodborne illness within 9 h.

The capability of *B. cereus* to grow in pasteurized milk and whipping cream (40% fat) at refrigeration temperatures has been reported (6). Although our strain did not grow at refrigeration temperatures, some investigators have reported that *B. cereus* survives, grows, and even produces toxins at low temperatures (8, 23, 27). Griffiths (8), using a reverse passive latex agglutination assay, demonstrated that some psychrotrophic *Bacillus* growing in milk at temperatures ranging from 6 to 21°C could produce toxin. Sutherland (23), working with a known toxigenic isolate of *B. cereus*, reported failure of that strain to grow and produce diarrhoeagenic toxin in creams and dairy-based products at 6°C. However, growth and toxin were readily detected in the same products stored at 21°C. The inability of other strains of *B. cereus* to grow at 5, 6, and 7°C also has been reported (4), indicating that storage temperature is critical to control growth of *B. cereus* in dairy products.

According to the International Dairy Federation, as cited by te Giffel et al. (25), to be considered psychrotrophic, a microorganism should be able to grow at 7°C or less, regardless of its optimum growth temperature. With constant refrigeration at 4°C or even at

TABLE 3. Effect of nondairy coffee creamer ingredients on germination and outgrowth of *Bacillus cereus* spores.[1]

	Incorporated in the agar matrix[2]						
Ingredient	0.1%	1%	2%	2.5%	3%	5%	10%
	(cfu × 10^8/ml)						
Corn syrup	ND[3]	5.0	ND	ND	ND	4.9	4.2
Hydrogenated oil	ND	6.4	ND	ND	ND	5.1	3.4
Sodium caseinate	ND	4.6	ND	ND	ND	3.5	2.9
K_4HPO_4	ND	4.4	ND	ND	ND	2.7	1.6
Polysorbate-60	ND	6.7	ND	ND	ND	3.8	3.8
Carrageenan	ND	4.7	4.3	ND	4.7	ND	ND
β-Carotene	4.8	4.9	ND	5.1	ND	5.9	ND
S-S-Lactylate[4]	<1	<1	ND	ND	<1	ND	ND

[1]Inoculum level: 1×10^9 spores/ml.

[2]Combined with plate count agar and incubated at 32°C for 48 h.

[3]Not determined.

[4]Sodium stearoyl lactylate.

7°C, no growth occurred in the coffee creamers in this study, confirming that our strain is not psychrotrophic. However, in situations in which temperature abuse took place, numbers sufficient to cause foodborne illness (10^5 cells/ml and above) were reached quickly from a very small inoculum. This fact could be critical to prevent outbreaks of foodborne illness because, in early studies of UHT treatment of milk containing *B. cereus* spores, Martin and Blackwood (15) and Martin et al. (16) found that, even at temperatures of 180°C for a few seconds, a small number of *B. cereus* spores survived. Survival of *B. cereus* in fluid milk after UHT treatment also has been observed by others (7, 28). These *B. cereus* spores possibly could subsequently germinate, grow, and produce toxin in dairy-based creamers if storage temperature is abused.

No germination and growth occurred in the presence of sodium stearoyl lactylate at the concentrations that are normally used in nondairy creamers. These data indicate that this ingredient inhibits germination of *B. cereus* spores in a liquid environment, which raises the possibility of controlling germination and growth of *B. cereus* spores in any fluid product in which sodium stearoyl lactylate, a fatty acid derivative, can be incorporated. Studies are underway to evaluate this possibility and to determine a minimum inhibitory concentration for this compound for *B. cereus*.

The importance of the findings in this study is that non-cultured dairy products, because of their favorable pH and lack of high concentrations of sodium chloride or other inhibitors, may support rapid growth of *B. cereus*. These results agree with findings of Benedict et al. (4). The data indicate that, if storage temperature is abused, less than 24 h are required for very low initial levels of *B. cereus* spores or vegetative cells to increase to levels that are sufficient to cause foodborne illness. Proper refrigeration temperatures (7 or 4°C) were effective in controlling the growth of this particular strain of *B. cereus*. However, it is important to note that some heat-resistant strains of *B. cereus* exhibiting psychrotrophic characteristics have been reported to grow and produce toxins in milk and dairy products at refrigeration temperatures.

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