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Microbiological safety of UHT milk treated at 120 °C for 2 s, as estimated from the distribution of high-heat-resistant *Bacillus cereus* in dairy environments

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ABSTRACT

Unlike common ultra-high-temperature (UHT) milk, so-called "UHT milk" in Japan is typically pasteurised at 120–130 °C for 2 s and distributed at 10 °C or less, and there is a potential risk of *Bacillus cereus*. To estimate the microbiological safety of UHT milk, we surveyed the distribution of high-heat-resistant *B. cereus* strains (defined as showing <~3 log reduction after treatment at 120 °C for 2 s) among 200 isolates from dairy environments. Only four strains, which were isolated from the milk plant environment, showed high-heat resistance. All of them were unable to grow at 10 °C but grew at 12 °C. In contrast, heat-labile strains grew well at 10 °C. Therefore, UHT milk pasteurised at 120 °C for 2 s can be microbiologically safe, provided it is kept at 10 °C or less, within a rational shelf-life and avoiding contamination with *B. cereus*, especially of milk-plant-environment origin.

1. Introduction

In the production of market milk, high-temperature short-time (HTST; 72 °C for 15 s) and ultra-high-temperature (UHT) are the principal pasteurisation techniques. Although HTST-pasteurised milk is closest in flavour to raw milk, spore-forming bacteria can survive in the product, so its shelf-life is set at short periods (typically no more than 1 week at 10 °C or less). Among surviving spore-forming bacteria, those able to grow at 10 °C or less are called psychrotrophic spore-forming bacteria; examples are psychrotrophic *Bacillus cereus* and closely related species. *B. cereus* group is a Gram-positive, facultatively anaerobic spore-forming bacterium that is ubiquitous in the environment. Because it is able to grow at even 4–6 °C (Larsen & Jørgensen, 1999; Meer, Baker, Bodyfelt, & Griffiths, 1991), it can grow in stored refrigerated items. Contamination with this bacterium is thus a limiting factor for the shelf-life of HTST milk (Notermans et al., 1997).

In general, each species belonging to *B. cereus* group is very difficult to distinguish, even with 16S rDNA sequencing, so many publications that refer to *B. cereus* actually refer to *B. cereus* group (IDF, 2016). In dairy, because *B. cereus* group is responsible for the deterioration of milk and dairy products, it practically refers to *B. cereus* (sensu lato) without detailed classification. In this study, we also referred to *B. cereus* group as *B. cereus*.

In contrast with HTST pasteurisation, UHT pasteurisation of milk is intended to inactivate these spore-forming bacteria. Although UHT pasteurisation conditions vary from country to country, for the production of UHT milk distributed via the cold chain the typical treatment conditions are 125–138 °C for 2–4 s. This is called "ultra-pasteurisation (UP)" in some countries, but in Japan there is no such designation. For the production of UHT milk distributed at ambient temperatures (so-called "long-life milk"), 130–150 °C for 2–8 s, followed by aseptic filling, is commonly applied (Boor & Murphy, 2002; Heyndrickx et al., 2010).

The legal distribution temperature of market milk also varies from country to country. For example, in the United Kingdom and the United States it is 6 °C (Ranieri, Huck, Sonnen, Barbano, & Boor, 2009), in Germany it is 8 °C (Doll, Scherer, & Wenning, 2017; Mayr, Gutser, Busse, & Seiler, 2004), while in Japan it is 10 °C, which is much higher than other countries.

In Japan, UHT pasteurisation conditions are defined as a heating at 120–150 °C for 1–3 s, as specified by the Ministerial Ordinance on Milk and Milk Products Concerning Compositional Standards, etc (Japanese Ordinance, 1990). UHT pasteurisation conditions intended to inactivate all spore-forming bacteria which can produce an unfavourable cooked flavour in the final product. Therefore, milk that is processed as far as possible under weak UHT pasteurisation conditions (e.g., 120–130 °C for 2 s) and distributed under refrigeration (at 10 °C or less) accounts for the majority of market milk (about 95%) in Japan. In other words, the UHT milk distributed in

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Japan is close to UP milk internationally. Although these pasteurisation conditions do not inactivate all spores in many cases, UHT milk has a long shelf-life when distributed under refrigeration (approximately 2 weeks at 10 °C or less). Under such UHT pasteurisation conditions, however, there remains concern that, if the raw milk is heavily contaminated with spores, some could survive in the UHT milk, even if in small numbers. However, on an empirical basis, it has been shown that such UHT milk has a long history of stable sales in Japanese markets and is a safe product.

Although *B. cereus* spores have relatively low-heat resistance in general, Stadhouders, Hup, and Hassing (1982) stated that heat treatment at 125 °C for 10–20 s was necessary to inactivate all *B. cereus* spores in milk. There have been many studies about the heat resistance of *B. cereus* (Blake, Weimer, McMahon, & Savello, 1995; Faille, Fontaine, & Bénézech, 2001; Janštová & Lukášová, 2001; Mazas, López, Martínez, Bernardo, & Martin, 1999), and in some cases *B. cereus* has been able to survive heat treatment at above 120 °C. If there are higher numbers of spores in raw milk, the spores may survive in UHT milk and accelerate the deterioration of the product. However, to our knowledge, there has been no comprehensive survey of the distribution of high-heat-resistant *B. cereus* strains in dairy environments.

Here, with a focus on pasteurisation treatment at 120 °C for 2 s, we performed a comprehensive survey of the distribution of high-heat-resistant *B. cereus* strains in dairy environments and estimated the microbiological safety of UHT milk.

2. Materials and methods

2.1. Isolation of B. cereus from dairy environments

We attempted to isolate *B. cereus* from raw milk, commercial HTST milk (pasteurisation at 72 °C for 15 s) and UHT milk (pasteurisation at 120 °C for 2 s), and swab samples from milk plant environments (including the milk processing lines after pasteurisation, filling machine, and floors and drains of the milk processing plant). We used 78 samples of raw milk (100 mL each), 90 samples of HTST milk (100 mL each), and 36 samples of UHT milk (1 L each). Because of the low occurrence of *B. cereus*—especially in raw milk, HTST milk, and UHT milk—we used the membrane filtration method of Christiansson, Ekelund, and Ogura (1997) for raw milk and HTST milk, and the streak plate method with mannitol-egg yolk-polymyxin (MYP) agar plates (Merck, KGaA, Darmstadt, Germany) for UHT milk after incubation of the UHT milk at 30 °C for 7 days. The membrane filtration method was carried out as follows. One hundred millilitres of 1% Triton X-100 and 2% 25 mL trypsin solution were added to 100 mL sample, and incubated at 55 °C for 15 min. The mixture was filtered by suction. The filtrate was transferred on blood agar (Oxoid Ltd., Basingstoke, UK) supplemented with defibrinated sheep blood (5%) and polymyxin B sulphate (10 ppm), and incubated at 20 °C for 2 days. Colonies surrounded by a clear zone of haemolysis were streaked onto MYP agar plates. Isolation from swab samples was carried out in the same manner as UHT milk except for incubation. MYP agar plates were used for isolation of *B. cereus*. The ISO 7932 method (ISO, 2004) was used for confirmation of *B. cereus*. After incubation at 30 °C for one day, typical mannitol-negative colonies with egg–yolk reaction on the MYP agar plate were streak onto blood agar plate mentioned above and incubated at 30 °C for one day. Colonies surrounded by a clear zone of haemolysis were identified as *B. cereus*. All strains were stored as glycerol stock (15%, v/v) at −80 °C.

2.2. Preparation of spore suspensions

Nutrient broth (Bectone, Dickinson and Company, Sparks, MD) was inoculated with one loopful of glycerol stock culture of *B. cereus* and the inoculated tubes were incubated overnight at 30 °C. The cultures were spread onto tryptone-glucose-yeast extract agar (Oxoid Ltd., Basingstoke, UK) supplemented with 25 mg L^{-1} $MnCl_2.4H_2O$, 250 mg L^{-1} $MgSO_4.7H_2O$, 300 μg L^{-1} $FeSO_4.7H_2O$, 150 mg L^{-1} $CaCl_2.2H_2O$, and the plates were incubated for 7–10 days at 30 °C. Colonies were harvested as spores and washed twice in 1/15 M potassium phosphate buffer (pH 7.0) by centrifugation at 5000×*g* for 10 min at 4 °C. After centrifugation, the spore pellets were suspended in the same buffer. The spore counts in the spore suspension were determined by the pour plate technique on nutrient agar (Bectone, Dickinson and Company, Sparks, MD) at 30 °C for 2 days. All spore suspensions were confirmed to be contained approximately 10^8 spores mL^{-1}. Spore suspensions were stored at 2 °C until use.

2.3. Heat resistance experiments

In the heat resistance experiments, we defined high-heat-resistant *B. cereus* as a strain with less than approximately 3 log reductions after exposure to 120 °C for 2 s. Assuming that the *z*-values of *B. cereus* are 7–9 °C, 3 log reductions after exposure to 120 °C for 2 s is approximately equal to less than 3 log reductions after 100 °C for 5 min. Common heat-labile *B. cereus* strains exhibit more than 5 log reductions under the same conditions. Hence, heat treatment at 100 °C for 5 min was used to pre-screening for high-heat-resistant strains.

Spore suspensions were diluted in 1/15 M potassium phosphate buffer (pH 7.0) to a final concentration of approximately 10^7 spores mL^{-1} and heated at 80 °C for 10 min for spore activation. Two millilitres of diluted spore suspension were dispensed into a glass tube (7 mm inner diameter, 122 mm long), sealed, and heated at 100 °C for 5 min in an oil bath. After being cooled on ice, the spore suspension was poured into nutrient agar and the plates were incubated for 2 days at 30 °C. After incubation, the colonies on the nutrient agar were counted as the number of surviving spores. The pre-screening experiments were performed in duplicate.

Strains that showed no more than approximately 3 log reductions in the pre-screening were carried out further heat resistance experiments to obtain *D*-values at several temperatures and *z*-values.

2.4. Determination of D-values and z-values

Heat-activated spore suspensions were dispensed into glass tubes, sealed, and heated at 90.0–105.0 °C at appropriate intervals. The number of surviving spores was determined in the same manner as described in the pre-screening section. The heat resistant experiments for determination of *D*-values and *z*-values were performed in duplicate. Thermal death curves were plotted at each temperature and the *D*-value was calculated by using linear regression from the straight line portion of the thermal death curve. The *z*-value was calculated by using linear regression from the straight line portion of log *D*-values plotted against their corresponding heating temperatures. We estimated the log reductions at 120 °C for 2 s from calculations using the *D*- and *z*-values obtained in this experiment.

2.5. Growth experiments on high-heat-resistant strains at 10 and 12 °C in UHT milk under pre- and post-pasteurisation contamination conditions

Growth experiments were conducted under pre- and post-pasteurisation contamination conditions using four high-heat-resistant *B. cereus* spores (HR1, HR2, CL6, and CL21) in duplicate (Fig. 1). In both cases, spore counts in the UHT milk at the time of testing were adjusted to approximately 10^3 spores mL^{-1}.

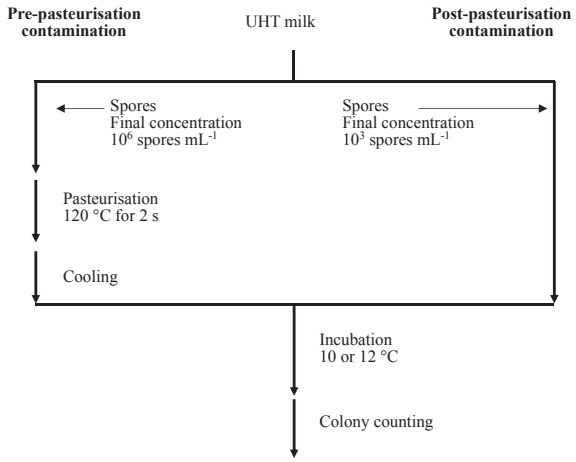

Fig. 1. Schematic flow diagram of pre- and post-pasteurisation contamination experiments.

The pre-pasteurisation contamination conditions simulated UHT milk manufactured from raw milk that was highly contaminated with high-heat-resistant *B. cereus* spores. Spore suspensions of the high-heat-resistant strains were inoculated into UHT milk (commercially available milk pasteurised at 120 °C for 2 s, Yotsuba Milk Products Co., Ltd., Hokkaido, Japan, 3.7% fat, 8.5% solids-not-fat) to give a concentration of approximately 10^6 spores mL^{-1}, considering that high-heat-resistant strains showed approximately 3 log reductions upon pasteurisation at 120 °C for 2 s. The inoculated milk was then heated at 120 °C for 2 s by using a laboratory-scale UHT plate heat exchanger (STS-100; Hisaka Works, Ltd., Osaka, Japan). The heat-treated milk was collected in sterilised 1 L polypropylene bottles and stored at 10 or 12 °C.

The post-pasteurisation contamination conditions simulated UHT milk highly contaminated with high-heat-resistant *B. cereus* spores after UHT pasteurisation. The same spore suspensions were heated for spore activation at 80 °C for 10 min and then inoculated into UHT milk (as same above) in sterilised 1 L polypropylene bottles to give a concentration of approximately 10^3 spores mL^{-1}. The UHT milk artificially contaminated by high-heat-resistant *B. cereus* spores was stored at 10 or 12 °C.

During the storage periods, samples were taken at appropriate time intervals from the same bottles, and colony counts were conducted by using the pour plate technique on nutrient agar.

As a comparative study, the growth of heat-labile *B. cereus* strains was tested at 10 °C under only post-pasteurisation contamination conditions, because heat-labile *B. cereus* strains from raw milk are inactivated by UHT pasteurisation.

3. Results

3.1. Distribution of high-heat-resistant strains in dairy environments

In our evaluation of the occurrence of *B. cereus* derived from dairy environments and potentially surviving pasteurisation at 120 °C for 2 s, we isolated a total of 200 strains of *B. cereus* from dairy environments. They comprised 105 strains from raw milk, 54 strains from HTST milk, and 41 strains from milk plant environment. In contrast, *B. cereus* was not detected in 36 samples of UHT milk (in 1-L paper cartons), although mesophilic *Bacillus* spp. were detected in 31 samples (data not shown).

In the pre-screening, 18 strains out of a total of 200 showed heat resistance of no more than approximately 3 log reductions after exposure to 100 °C for 5 min; one strain was from HTST milk and the other 17 were from the milk plant environment. One hundred strains out of 105 from raw milk (95.2%), 49 out of 54 from HTST milk (90.7%), and 15 out of 41 from the milk plant environment (36.6%) showed more than 5 log reductions after exposure to 100 °C for 5 min. This indicated that the strains from raw milk and HTST milk were more heat-labile than those from the milk plant environment. The similarity of the percentages observed in raw milk and HTST milk suggests that *B. cereus* strains from raw milk and HTST milk are identical, because the HTST pasteurisation temperature is low (72 °C) and the *B. cereus* spores in the raw milk are likely directly transferred to the HTST milk.

From the *D*-values and *z*-values, we calculated the log reductions after heat treatment at 120 °C for 2 s (Table 1). Only four strains (HR1, HR2, CL6, and CL21) were estimated to be high-heat-resistant strains that showed no more than approximately 3 log reductions at 120 °C for 2 s. All of the isolates were obtained from dairy processing lines after UHT pasteurisation.

Table 1
D-values, *z*-values, and estimated log reductions after heating at 120 °C for 2 s for the 18 strains of *Bacillus cereus* that exhibited less than approximately 3 log reductions in pre-screening at 100 °C for 5 min.

Strain	Source	*D*-value (min) at heating temperature (°C)									*z*-value (°C)	Log reduction
		90.0	92.5	95.0	97.5	98.0	100.0	102.0	102.5	105.0		
KH2	HTST			4.8 (1.000)	2.4 (0.998)		1.2 (0.994)				8.3 (1.000)	7.1
HR1	ENV						11.7 (0.993)		6.2 (0.990)	2.2 (0.998)	6.9 (0.981)	2.3
HR2	ENV					13.8 (0.993)	7.1 (1.000)	3.8 (0.993)			7.1 (1.000)	3.1
CL1	ENV		7.7 (1.000)	3.5 (0.999)	1.8 (1.000)						7.9 (0.998)	13.9
CL2	ENV		5.7 (1.000)	2.6 (0.997)	1.2 (1.000)						7.4 (1.000)	30.6
CL6	ENV			8.9 (0.998)	4.7 (1.000)		2.3 (0.998)				8.5 (0.999)	3.3
CL10	ENV			14.3 (1.000)	6.4 (1.000)		2.7 (1.000)				6.9 (1.000)	9.8
CL18	ENV			5.6 (0.999)	2.5 (0.999)		1.2 (0.997)				7.5 (0.999)	12.9
CL21	ENV				9.4 (0.998)		4.7 (1.000)		2.1 (0.999)		7.7 (0.998)	2.8
CL24	ENV			10.3 (0.998)	4.8 (1.000)		2.0 (0.999)				7.0 (0.998)	12.0
CL25	ENV	13.1 (0.998)	5.2 (1.000)	2.9 (1.000)							7.6 (0.983)	22.4
CL26	ENV	11.5 (0.996)	4.6 (0.998)	2.2 (0.998)							7.0 (0.996)	56.5
CL27	ENV	13.2 (1.000)	6.8 (0.999)	3.0 (0.989)							7.8 (0.996)	17.8
CL31	ENV			9.2 (1.000)	4.2 (0.998)		2.1 (0.998)				7.8 (0.999)	5.8
CL32	ENV			5.8 (0.999)	2.7 (1.000)		1.2 (1.000)				7.3 (1.000)	15.3
CL34	ENV		5.7 (1.000)	2.8 (0.998)	1.5 (0.998)						8.6 (0.999)	9.6
CL36	ENV			6.1 (0.990)	2.8 (0.995)		1.3 (0.999)				7.4 (1.000)	12.9
CL37	ENV			6.4 (0.999)	3.0 (0.998)		1.6 (0.996)				8.3 (0.997)	5.4

[a] Abbreviations are: HTST, high-temperature short-time pasteurised milk; ENV, milk plant environment. Correlation coefficients (R^2) are given in parentheses; log reductions are estimated after heating at 120 °C for 2 s.

3.2. Growth of high-heat-resistant strains at 10 and 12 °C in UHT milk under pre- and post-pasteurisation contamination conditions

To investigate the growth of high-heat-resistant *B. cereus* contaminating UHT milk, we conducted growth experiments at 10 and 12 °C after pre- or post-pasteurisation contamination of the milk with the high-heat-resistant strains HR1, HR2, CL6, and CL21 (Fig. 2). After pre- or post-pasteurisation contamination, none of the strains grew at 10 °C for 20 days, whereas all of them grew at 12 °C. However, in the case of pre-pasteurisation contamination conditions, the strains showed growth delay with a long lag phase owing to damage from the heat treatment at 120 °C for 2 s. From the intersection between the initial spore counts and the regression line of exponential phase, lag times of HR1, HR2, CL6, and CL21 under post-pasteurisation contamination conditions were calculated to be 2.3, 4.0, 3.6, and 2.0 days, respectively. On the other hand, under pre-pasteurisation contamination conditions, they were calculated to be 3.8, 8.6, 9.7, and 3.5 days, respectively.

Interestingly, all of the heat-labile strains grew well at 10 °C (Fig. 3). Two strains (BCC1 and BCC2) grew even at 5 °C (data not shown). This suggested that the high-heat-resistant strains have alterations in their growth ranges, making it difficult for them to grow at low temperatures.

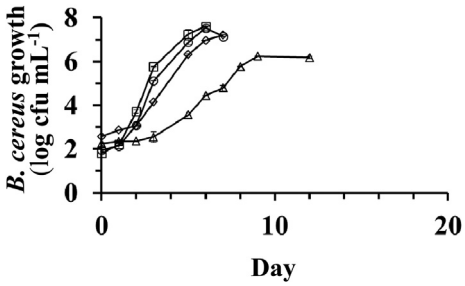

Fig. 3. Growth curves of heat-labile *Bacillus cereus* BCC1, BCC2, BCM4, and BCM5 at 10 °C in UHT milk under post-pasteurisation contamination conditions. Spores were heated for spore activation at 80 °C for 10 min and inoculated into UHT milk. All strains were isolated from raw milk and showed more than 5 log reductions at 100 °C for 5 min. Strains: BCC1 (○), BCC2 (□), BCM4 (△), and BCM5 (◇).

4. Discussion

To our knowledge, this is the first comprehensive report to investigate the distribution of high-heat-resistant *B. cereus* strains in dairy environments. We showed that high-heat-resistant *B. cereus* strains were not detectable in raw milk, HTST milk, or UHT milk, but they were found in the milk plant environment.

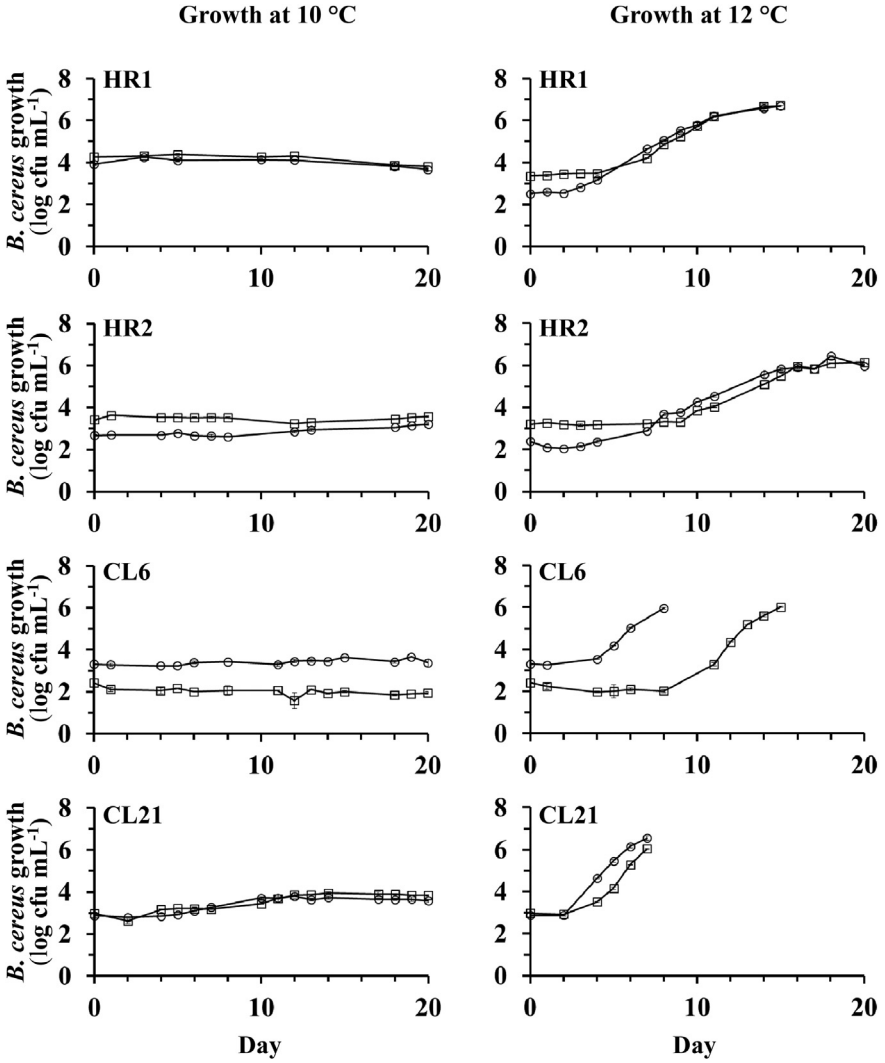

Fig. 2. Growth curves of high-heat-resistant *Bacillus cereus* HR1, HR2, CL6, and CL21 at 10 °C (left column) and 12 °C (right column) in UHT milk under pre- and post-pasteurisation contamination conditions. For pre-pasteurisation contamination, spores were inoculated into UHT milk, and the inoculated milk was pasteurised at 120 °C for 2 s (□). For post-pasteurisation contamination, spores were heated for spore activation at 80 °C for 10 min and then inoculated into UHT milk (○).

B. cereus spores are not plentiful in raw milk; they have been reported at concentrations of 10^2 to 10^3 spores L^{-1} (Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010), and we found as few as 10^1 to 10^2 spores L^{-1} (data not shown). Because of its low-heat resistance, most *B. cereus* in raw milk is fully inactivated to safe levels by pasteurisation at 120 °C for 2 s; this is why we did not detect *B. cereus* in commercially available UHT milk.

Nevertheless, high-heat-resistant strains tended to be present in the milk plant environment, and our experimental findings show that it is possible for them to survive even in UHT milk that is contaminated before pasteurisation. This potential for low levels of *B. cereus* to be present in market milk does not necessarily cast doubt on the safety of market milk as a chilled product (Langeveld, van Spronsen, van Beresteijn, & Notermans, 1996; Notermans et al., 1997). In UHT milk for chilled distribution, even if low levels of *B. cereus* are present, milk safety can be sufficiently secured unless *B. cereus* grows during the expiration date under the given distribution conditions; this policy is aimed at securing both the freshness and safety of milk as food. We found here that high-heat-resistant *B. cereus* strains lost their growth ability at 10 °C (Fig. 2). Therefore, even if they survive in UHT milk, they will not grow under distribution at 10 °C or less, and the microbiological safety of the UHT milk will thus be secured.

Generally, most *B. cereus* strains grow well at 10 °C (Guinebretière et al., 2008). Here, we found that four heat-labile strains isolated from raw milk also grew well at 10 °C (Fig. 3). The reason why the high-heat-resistant strains in our study did not grow at 10 °C—unlike common strains—is likely that either they belong to a new species of *B. cereus* group that does not have the ability to grow at 10 °C, or their growth ability at 10 °C is suppressed by their acquisition of high-heat resistance in response to environmental stress. Further investigations are needed to elucidate this issue; high-heat-resistant mutants need to be derived and their temperature ranges for growth compared with that of the parent strain.

Mayr et al. (2004) investigated extended-shelf-life (ESL) milk pasteurised at 127 °C for 5 s; *B. cereus* was not detected during a 23-week period at 10 °C, but it was isolated after subsequent storage at 30 °C for 10 days. They stated that the reason for the lack of detection during storage at 10 °C was that heat damage affected the ability of *B. cereus* to grow at low temperatures. Heat damage to growth was also apparent after heat treatment at 120 °C for 2 s in our study: it took several days at 12 °C to recover (Fig. 2). Although UHT pasteurisation at 120 °C for 2 s does not inactivate all high-heat-resistant *B. cereus*, bacterial growth ability at low temperature is negatively affected by the heat damage. Even if high-heat-resistant *B. cereus* can grow in UHT milk, its growth will be delayed, and this has advantageous for the microbiological safety of UHT milk.

The contamination conditions used in our growth experiments were severe and unrealistic. In practice, it is unlikely that contamination like this would occur in UHT milk. We therefore conclude that the microbiological safety of UHT milk pasteurised at 120 °C for 2 s can, in practice, be secured by eliminating *B. cereus* contamination of the milk plant environment, controlling temperatures at 10 °C or less, and setting an appropriate shelf-life.

5. Conclusions

In this study, we investigated the distribution of high-heat-resistant *B. cereus* in dairy environments and estimated the microbiological safety of UHT milk pasteurised at 120 °C for 2 s. High-heat-resistant strains were only found in the milk plant environment, but they lost their growth ability at 10 °C, which is upper limit of the Japanese legal distribution temperature. Therefore, UHT milk pasteurised at 120 °C for 2 s can be microbiologically safe, as long as it is kept at 10 °C or less, within a rational shelf-life and avoiding contamination with *B. cereus*, especially of milk-plant-environment origin.

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