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Seasonal variation in spore levels of *Bacillus cereus* and its psychrotrophic strains in raw milk in Hokkaido, Japan, and evaluation of strain diversity

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ABSTRACT

Bacillus cereus is a spore-forming bacterium that adversely affects the quality of high-temperature short-time (HTST) pasteurised milk. Using a membrane filtration method, we investigated *B. cereus* spore levels in raw milk from dairy plants in two different regions of Hokkaido, Japan, over 1 year. *B. cereus* spore levels were significantly higher in autumn (median, 25 spores L^{-1}) in the Tokachi region and in summer (median, 38 spores L^{-1}) in the Kushiro region than in the other seasons, whereas the bacterium's psychrotrophic spore levels showed no seasonal variation. Multiple-locus variable-number tandem repeat analysis (MLVA) showed that *B. cereus* in raw milk had high strain diversity; MLVA groups consisting of a single strain accounted for 76.6% of the total number of MLVA groups in Tokachi and 68.5% in Kushiro. Three MLVA groups were detected in both regions in three seasons or more; these MLVA groups are therefore widely distributed in Hokkaido.

1. Introduction

Spore-forming bacteria present in raw milk form heat-resistant spores that are not inactivated by high-temperature short-time (HTST; 72 °C for 15 s) pasteurisation, so these bacteria can survive in HTST milk. Among these surviving spore-forming bacteria, *Bacillus cereus* is able to grow at low temperatures; it germinates and grows during cold-chain distribution, causing eventual deterioration of HTST milk. Hence, *B. cereus* is the practical limiting factor in the shelf-life of HTST milk (Notermans et al., 1997). *B. cereus* is a Gram-positive, facultatively anaerobic spore-forming bacterium that is ubiquitous in the environment. It grows at 4–50 °C (IDF, 2016) and is classified either into psychrotrophic strains that can grow at ≤7 °C or mesophilic strains that grow at >7 °C.

The *B. cereus* group is currently composed of 21 closely related species that are very difficult to distinguish from one other. In the dairy industry, the *B. cereus* group is responsible for the deterioration of liquid milk and dairy products, so in most papers the term "*B. cereus*" is used to mean the *B. cereus* group [*B. cereus* (*sensu lato*)] (IDF, 2016). Therefore, in this paper, "*B. cereus*" means *B. cereus* (*sensu lato*).

In Japan, the legal distribution temperature of market milk is ≤ 10 °C; this value is higher than those of other countries (Ohkubo, Uchida, Motoshima, & Katano, 2019). Consequently, HTST milk sold in Japan is more likely to be susceptible to deterioration by *B. cereus*, so its shelf-life in Japan is set at shorter periods (typically no more than 1 week) than those of other countries. In general, *B. cereus* grows well at 10 °C, even in the case of mesophilic strains (Heyndrickx, 2011), and psychrotrophic strains grow faster than mesophilic strains at 10 °C (Ohkubo et al., 2019; te Giffel, Beumer, Slaghuis, & Rombouts, 1995). Therefore, it is important to control the spore levels of both psychrotrophic and mesophilic *B. cereus* strains in the raw milk used for HTST milk production.

The distribution of *B. cereus* in raw milk and HTST milk has been studied in European countries (Bartoszewicz, Hansen, & Swiecicka, 2008; Larsen & Jørgensen, 1997; Svensson, Ekelund, Ogura, & Christiansson, 2004). However, to our knowledge there have been no published surveys of the distribution of *B. cereus* spores in raw milk in Japan, although this information seems important for controlling raw milk hygiene.

Strain typing is used in surveys of contamination sources and evaluations of strain diversity. Many methods of typing *B. cereus* have been reported, such as the RAPD (randomly amplified

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polymorphic DNA) method (Nilsson, Svensson, Ekelund, & Christiansson, 1998), the rep-PCR (repetitive element palindromic PCR) method (Cherif et al., 2003), the AFLP (amplified fragment length polymorphism) method (Ripabelli, McLauchlin, Mithani, & Threlfall, 2000), and the MLVA (multiple-locus variable-number tandem repeat analysis) method (Valjevac et al., 2005). The MLVA method is often used for epidemiological surveys and has the advantages of high reproducibility and an easy-to-construct database; strains with identical MLVA patterns can be regarded as identical strains or genetically very closely related strains (Wolffs, Geelen, & van Alphen, 2017).

Here, we investigated the spore levels of *B. cereus*, with special reference to its psychrotrophic spores, in raw milk from dairy plants in two different regions of Hokkaido, Japan, over 1 year, and elucidated the seasonal variations in these levels. In addition, we used the MLVA method to type *B. cereus* isolates and evaluated them for their strain diversity.

2. Materials and methods

2.1. Sampling of raw milk

Raw milk samples were collected from each silo tank of two dairy plants, one located in the Tokachi region and one in the Kushiro region of Hokkaido. The Tokachi region is located in mid-eastern Hokkaido and the Kushiro region is located in eastern Hokkaido. The two dairy plants were approximately 120 km apart and collected raw milk from their respective regions. The Tokachi silo samples consisted of bulk raw milk from 15 farms and the Kushiro samples consisted of bulk raw milk from 25 farms. Sampling was conducted seasonally four times [spring (April), summer (July), autumn (October), and winter (January)] from April 2017 to January 2018. Seasonal samples were taken for 10 consecutive days in spring and winter, and for 7 consecutive days in summer and autumn. At each sampling, 1 L of raw milk was collected in a sterilised 1-L polypropylene bottle, frozen at −20 °C, and stored frozen until use.

2.2. Enumeration of B. cereus spores in raw milk

For enumeration of *B. cereus* spores in raw milk, we used the membrane filtration method of Christiansson, Ekelund, and Ogura (1997), with slight modification. The procedure is briefly described as follows. One litre of raw milk was dispensed into 10 sterilised 250-mL polypropylene bottles at 100 mL each and then pasteurised at 63 °C for 30 min to eliminate vegetative bacterial cells. One hundred millilitres of polyoxyethylene (10) octylphenyl ether (1% sterilised solution; Wako Pure Chemical Industries Ltd., Osaka, Japan) as a lipid emulsifier, and 25 mL of filter-sterilised trypsin solution [2% trypsin (Nacalai Tesque, Inc. Kyoto, Japan) in 0.1 M Tris-HCl buffer (pH7.8)] and 5 mL of filter-sterilised Protease N solution [2% Protease N (Amano Enzyme Inc., Aichi, Japan) in 0.1 M Tris-HCl buffer (pH7.8)] as proteolytic agents were added to each 100 mL of pasteurised raw milk and mixed well. The mixture was heated at 55 °C for 15 min for proteolytic reaction and then immediately filtered by suction through a Durapore membrane filter (pore size, 0.65 μm; Merck Millipore Ltd., Cork, Ireland). Nalgene reusable filter holders with receiver (Thermo Fisher Scientific, Waltham, MD, USA) were used for filtration. After being rinsed with sterilised water, the filter membrane was placed on a blood agar plate (Oxoid blood agar base No.2; Oxoid Ltd., Basingstoke, UK) supplemented with 5% defibrinated sheep blood (Japan Lamb International Ltd., Hiroshima, Japan) and 10 ppm polymyxin B sulphate. The plates were then incubated at 20 °C for 2 days. All colonies surrounded by a clear zone of haemolysis were purified using the streak plate technique onto a nutrient agar plate (Becton, Dickinson and Company, Sparks, MD, USA) followed by overnight incubation at 30 °C. The ISO 7932 method (ISO, 2004) was used for confirmation of *B. cereus*. The purified colonies were streaked onto a mannitol–egg yolk–polymyxin (MYP) agar plate (Merck Millipore Ltd.). After overnight incubation of the plate at 30 °C, typical mannitol-negative colonies with egg–yolk reaction on the MYP agar plate were streaked onto the blood agar plate mentioned above and incubated overnight at 30 °C. Colonies surrounded by a clear zone of haemolysis were identified as *B. cereus*. All *B. cereus* obtained from raw milk were stored as isolates and used for subsequent experiments.

2.3. Discrimination of psychrotrophic strains of B. cereus

Psychrotrophic strains of *B. cereus* isolated from raw milk were discriminated using PCR analysis targeting the cold-shock protein A gene (*cspA*) in accordance with the method described by Francis, Mayr, von Stetten, Stewart, and Scherer (1998). *B. cereus* with *cspA* has the ability to grow at ≤ 7 °C (Francis et al., 1998). The procedure is briefly described as follows. DNA was extracted from the isolates using an InstaGene Matrix Kit (Bio-Rad Laboratories, Hercules, CA, USA). The primers used for PCR were BcAPF1 (5′- GAGGAAATAATTATGACAGTT-3′), BcAPR1 [5′-CTT (C/T) TTGGCCTTCTTCTAA-3′], and BcFF2 (5′-GAGATTTAAATGAGCTGTAA-3′) (Francis et al., 1998). The PCR reaction was performed in 25-μL volumes, each containing 1 U of TaKaRa *Taq* (Takara Co. Ltd., Kyoto, Japan), 2.5 μL of 10 × PCR buffer (Takara Co. Ltd.), 0.2 mM deoxynucleoside triphosphate mixture, 2.0 mM $MgCl_2$, 50 pmol of each primer, and 5 μL of template DNA solution. The PCR protocol was as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 2 min. The amplification products were confirmed by agarose gel electrophoresis. A 284-bp amplification product was obtained from both psychrotrophic and mesophilic strains, whereas a 160-bp product was obtained only from psychrotrophic strains.

2.4. MLVA typing of B. cereus

B. cereus isolated from raw milk was typed using the MLVA method. We used four primer pairs to amplify variable-number tandem repeat (VNTR) regions for MLVA typing [Bcms 08 and Bcms 19 reported by Valjevac et al. (2005) for screening of *B. cereus* group, and Ceb-Bams 13 and Ceb-Bams 22 reported by Le Flèche et al. (2001) for typing of *Bacillus anthracis* and *B. cereus*] (Table 1). We obtained the complete genome sequence of *B. cereus* ATCC 14579^T (accession number AE016877.1) (Ivanova et al., 2003) from GenBank. The sequence and the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) were used to obtain the nucleotide sequences amplified by each primer pair. Using these nucleotide sequences and Tandem Repeats Finder software (http://tandem.bu.edu/trf/trf.submit.options.html), we selected appropriate tandem repeat sequences for MLVA typing.

MLVA typing of the isolates was performed using the DNA described in section 2.3 as a template. The 5′ ends of the forward primers of Bcms 08, Bcms 19, Ceb-Bams 13, and Ceb-Bams 22 were fluorescently labelled with 6-FAM (Applied Biosystems, Foster, CA, USA), NED (Applied Biosystems), VIC (Applied Biosystems), and PET (Applied Biosystems), respectively. The PCR reaction was performed in 10-μL volumes, each containing 0.25 U of Gflex DNA polymerase (Takara Co. Ltd.), 5 μL of 2 × Gflex PCR buffer (Takara Co. Ltd.), 3 pmol of each forward and reverse primer, and 1 μL of

Table 1
Primers used for MLVA typing of *Bacillus cereus*.[a]

Primer name	Sequence	Tandem repeat length (bp)	No. of tandem repeats	Expected PCR product length (bp)
Bcms 08	F: GTGCTGGWGCAAACACAGAC R: TGGTCGCCTGCTTTATAACC	18	20	739
Bcms 19	F: GGAATAGAAGATGAAGAAGAAGTTACG R: TTTCGGTTTTATTGGTGGTTG	23	6	363
Ceb-Bams 13	F: AATTGAGAAATTGCTGTACCAAACT R: CTAGTGCATTTGACCCTAATCTTGT	36	7	553
Ceb-Bams 22	F: ATCAAAAAATTCTTGGCAGACTGA R: ACCGTTAATTCACGTTTAGCAGA	19	22	888

[a] Abbreviations are: F, forward; R, reverse. The number of tandem repeats and expected PCR products length were derived from the sequencing data of *Bacillus cereus* ATCC 14579^T (accession number AE016877.1).

template DNA solution. The PCR protocol was as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of 98 °C for 10 s, 56 °C for 15 s and 68 °C for 30 s. Taking into consideration differences in the fluorescence intensity of each fluorescent dye, 1 μL of the amplification products with Bcms 08, 1.5 μL with Bcms 19, 0.5 μL with Ceb-Bams 13, and 2 μL with Ceb-Bams 22 were collected into a single tube, to which 48 μL of sterilised water was added. The solution was then mixed well. One microlitre of the mixture was added 24 μL of Hi-Di Formamide (Applied Biosystems) and 0.5 μL of GeneScan 1200 LIZ dye Size Standard (Applied Biosystems). The mixture was heated at 94 °C for 3 min for denaturation and then used for capillary electrophoresis on an ABI PRISM 310 Genetic Analyser (Applied Biosystems) with POP-4 polymer (Applied Biosystems). Capillary electrophoresis was run at 60 °C for 60 min at a voltage of 15 kV. The size of each VNTR region was determined using GeneMapper (v. 4.1) software (Applied Biosystems). The number of tandem repeats of each VNTR region was calculated from the size of the VNTR region, the offset value (the size of the fragment with no tandem repeat), and the size of the tandem repeat. The MVLA pattern of each isolate was designated as a combination of the number of tandem repeats calculated from the amplification products obtained using the primer pairs of Bcms 08, Bcms 19, Ceb-Bams 13, and Ceb-Bams 22. For example, the MVLA pattern of *B. cereus* ATCC 14579^T was designated as 20-6-7-22 (Table 1). Isolates were classified into MLVA groups according to their MLVA patterns.

2.5. Statistical analysis

To estimate the seasonal variation of *B. cereus* and its psychrotrophic spore levels in raw milk, statistical analyses were performed on the number of spores in each region. Using Mann–Whitney's *U*-test, the number of spores obtained from each sample were compared between seasons. Significant differences were defined as $P < 0.05$.

3. Results and discussion

3.1. Distribution and seasonal variation of B. cereus *spore levels in raw milk*

To estimate the distribution and seasonal variation of *B. cereus* spores in raw milk, we used a membrane filtration method to survey *B. cereus* spore levels in raw milk collected from the silo tanks of two dairy plants over 1 year.

A total of 399 *B. cereus* isolates were obtained from raw milk samples from the Tokachi region, and 606 from the Kushiro region (Table 2). The total number of *B. cereus* spores ranged between 1 and 10^2 L^{-1} through the year (Fig. 1). In general, spore concentrations of 10^2–10^3 L^{-1} have been reported in European countries (Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010); spore levels in raw milk in Hokkaido were therefore relatively low.

In the Tokachi region, total spore levels of *B. cereus* ranged from 1–6 L^{-1} (median, 3 spores L^{-1}) in spring, 9–22 L^{-1} (median, 12 spores L^{-1}) in summer, 20–62 L^{-1} (median, 25 spores L^{-1}) in autumn, and 2–10 L^{-1} (median, 5 spores L^{-1}) in winter (Fig. 1). Spore levels were significantly higher in autumn than in the other seasons ($P < 0.01$) and significantly higher in summer than in spring and winter ($P < 0.01$).

In the Kushiro region, total spore levels of *B. cereus* were 6–17 L^{-1} (median, 9 spores L^{-1}) in spring, 13–88 L^{-1} (median, 38 spores L^{-1}) in summer, 7–22 L^{-1} (median, 16 spores L^{-1}) in autumn, and 3–14 L^{-1} (median, 9 spores L^{-1}) in winter (Fig. 1). Spore levels were significantly higher in summer than in the other seasons ($P < 0.01$) and significantly higher in autumn than in winter ($P < 0.05$).

In European countries, *B. cereus* spore levels in raw milk are generally high in summer and low in winter (Larsen & Jørgensen, 1997; Sutherland & Murdoch, 1994; Svensson et al., 2004); the high summer levels are closely associated with the period of outdoor grazing. Slaghuis, Te Giffel, Beumer, and André (1997) reported that raw milk from cows that were grazed was more likely to be contaminated with *B. cereus* spores than raw milk from cows that were housed. Christiansson, Bertilsson, and Svensson (1999) showed by the RAPD method that *B. cereus* in raw milk was derived from soil; the main contamination route was soil-contamination of cows' teats during grazing. The grazing period in Hokkaido runs from May to October, thus likely explaining the occurrence of high spore levels in raw milk in Hokkaido collected from summer or autumn.

3.2. Distribution and seasonal variation of B. cereus *psychrotrophic spore levels in raw milk*

To estimate the distribution and seasonal variation of *B. cereus* psychrotrophic spore levels in raw milk, we discriminated the psychrotrophic strains from among a total of 399 isolates from raw milk in the Tokachi region and 606 in the Kushiro region (Table 2). In our preliminary discrimination experiment in which we applied PCR to 42 strains of *B. cereus* in our culture collections, including 13 psychrotrophic strains, we found that only psychrotrophic strains possessed *cspA* (data not shown). This was in agreement with the results of the PCR analysis by Francis et al. (1998). We therefore employed a PCR method for the discrimination of psychrotrophic strains.

The PCR analysis showed that, in the Tokachi region, 22 strains out of 399 isolates (5.5%) were psychrotrophic (Table 2). Seasonal

Table 2
Total numbers of *Bacillus cereus* isolates and numbers of psychrotrophic isolates.[a]

Season	Number of *B. cereus* isolates	
	Total	Psychrotrophic
Tokachi region		
Spring	28	2 (7.1)
Summer	90	6 (6.7)
Autumn	233	9 (3.9)
Winter	48	5 (10.4)
All seasons	399	22 (5.5)
Kushiro region		
Spring	97	30 (30.9)
Summer	321	22 (6.9)
Autumn	103	18 (17.5)
Winter	85	28 (32.9)
All seasons	606	98 (16.2)

[a] The percentage of psychrotrophic isolates (relative to total isolates) is given in parentheses.

psychrotrophic spore levels were 0–1 L^{-1} (median, 0 spores L^{-1}) in spring, 0–3 L^{-1} (median, 0 spores L^{-1}) in summer, 0–7 L^{-1} (median, 0 spores L^{-1}) in autumn, and 0 to 2 spores L^{-1} (median, 0 spores L^{-1}) in winter (Fig. 1). There were no significant differences in psychrotrophic spore levels between seasons ($P > 0.05$).

In the Kushiro region, 98 strains out of 606 isolates (16.2%) were psychrotrophic (Table 2). Seasonal psychrotrophic spore levels were 0–5 L^{-1} (median, 4 spores L^{-1}) in spring, 1–5 L^{-1} (median, 3 spores L^{-1}) in summer, 1–6 L^{-1} (median, 2 spores L^{-1}) in autumn, and 0–7 L^{-1} (median, 2 spores L^{-1}) in winter (Fig. 1). There were no significant differences in psychrotrophic spore levels between seasons ($P > 0.05$).

Svensson et al. (2004) investigated raw milk from eight dairy plants in Sweden; *B. cereus* psychrotrophic spore levels were high in summer, as were total *B. cereus* spore levels. In their study, 48% of isolates in summer and 35% of isolates in winter were

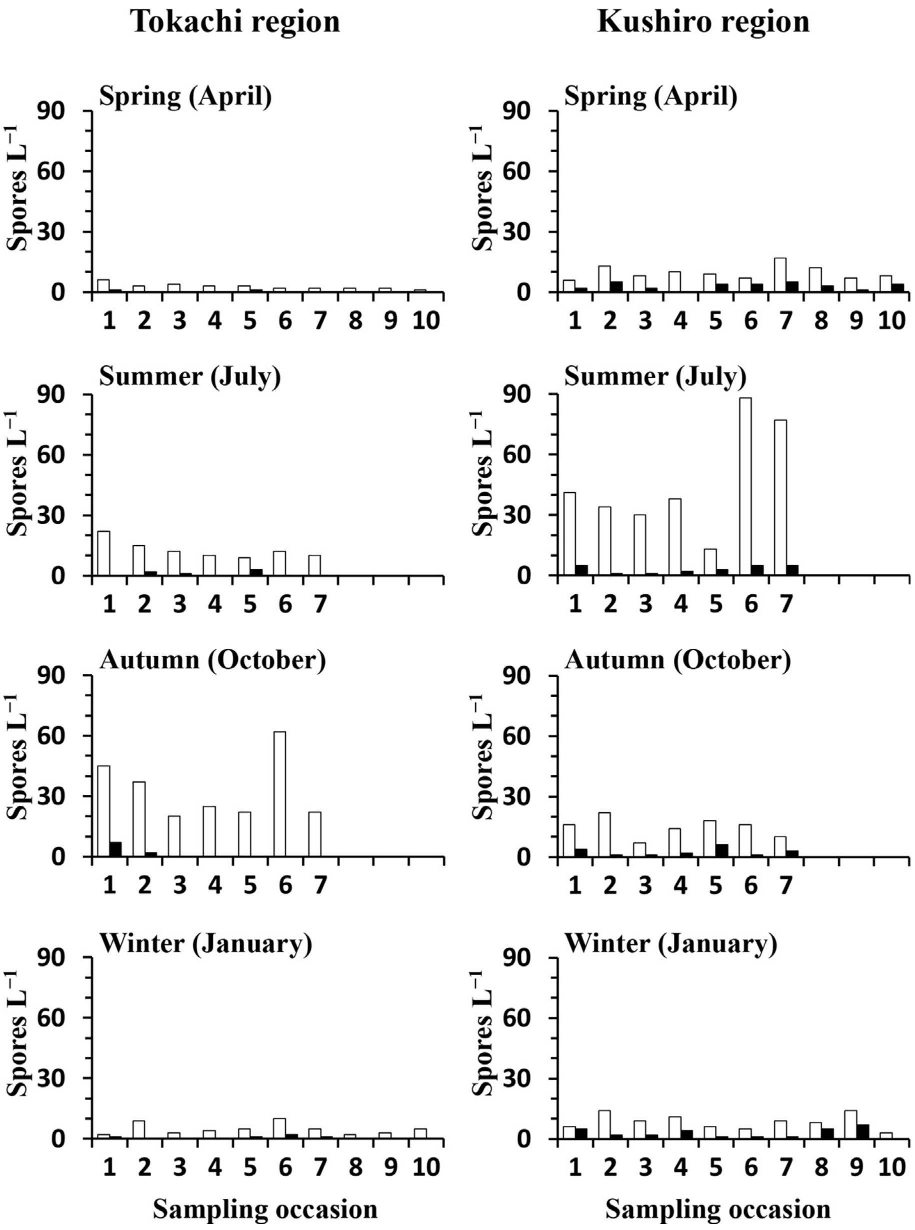

Fig. 1. Numbers of *Bacillus cereus* spores and numbers of psychrotrophic spores in 1-L samples of raw milk taken from dairy plants in two different regions over 1 year. Seasonal samples were taken for 10 consecutive days in spring and winter, and 7 consecutive days in summer and autumn. White column, total number of *B. cereus* spores; black column, number of *B. cereus* psychrotrophic spores.

psychrotrophic strains. In contrast, here we found no seasonal variation in psychrotrophic spore levels; moreover, psychrotrophic strains accounted for only 5.5% of total isolates in the Tokachi region and 16.2% in the Kushiro region. Therefore, psychrotrophic spore levels in raw milk in Hokkaido were also relatively low.

In the Netherlands, te Giffel et al. (1995) found the highest levels of psychrotrophic *B. cereus* in soil (66% of total *B. cereus*); soil was the main source of contamination with psychrotrophic *B. cereus*. von Stetten, Mayr, and Scherer (1999) investigated the influence of climate on the proportions of psychrotrophic *B. cereus* in soils; the proportions they found in tropical, temperate, and alpine soils were 0%, 45%, and 98%, respectively. Although Sweden, the Netherlands, and Hokkaido are located in cold climates, the proportion of psychrotrophic strains in soils likely varies considerably from region to region.

In Japan, the quality of HTST milk is likely strongly affected by *B. cereus* owing to the higher distribution temperature (≤10 °C) than in other countries. The microbiological safety of HTST milk can, in practice, be secured using raw milk with low levels of spores from both mesophilic and psychrotrophic *B. cereus* strains as well as by setting an appropriate shelf-life and controlling temperatures at ≤ 10 °C.

3.3. *Strain diversity of* B. cereus *in raw milk in each season*

To estimate the strain diversity of *B. cereus* in raw milk in each season, we performed MLVA typing of *B. cereus* isolated seasonally. In our preliminary experiment comparing the RAPD method of Nilsson et al. (1998) with the MLVA method used here, 118 strains of *B. cereus* in our culture collections were classified into 41 groups by the RAPD method, whereas they were classified into 48 groups by the MLVA method (data not shown). The MLVA method thus had more discriminatory power than the RAPD method, and the MLVA patterns obtained were easily compared. Therefore, we decided to use the MLVA method for typing of *B. cereus*.

The numbers of MLVA groups into which *B. cereus* isolates were classified in each season are shown in Table 3. In the Tokachi region, 28 isolates in spring, 90 in summer, 233 in autumn, and 48 in winter (Table 2) were classified into 25, 23, 59, and 26 MLVA groups, respectively. There were two MLVA groups in summer, two in autumn, and one in winter to which 10 or more isolates belonged. The proportion of unique MLVA groups consisting of only a single isolate was 88.0% in spring, 65.2% in summer, 72.9% in autumn, and 80.8% in winter (Table 3); the next most common were MLVA groups consisting of a few isolates.

In the Kushiro region, 97 isolates in spring, 321 in summer, 103 in autumn, and 85 in winter (Table 2) were classified into 72, 116, 74, and 75 MLVA groups, respectively. There were five MLVA groups in summer to which 10 or more isolates belonged. The proportion of unique MLVA groups consisting of only a single isolate was 79.2% in spring, 68.1% in summer, 79.7% in autumn, and 90.7% in winter (Table 3).

The MLVA analysis revealed that *B. cereus* in raw milk had very high strain diversity (Table 3). However, MLVA groups to which 10 or more isolates belonged were often found in summer and autumn—during the grazing period. Notably, in autumn in the Tokachi region, 139 isolates belonged to the MLVA group with the MLVA pattern 19-5-6-19 (Table 4), accounting for 59.7% of the total number of isolates in autumn (139 out of 233, Table 2). Detection of a large number of isolates belonging to a single MLVA group strongly suggests the presence of a specific contamination source. To optimise the quality of raw milk it is important to identify the sources of *B. cereus* contamination and improve hygiene. Because we collected raw milk samples from silo tanks at dairy plants, there may have been contamination of the silo tanks or of specific farmers' raw milk. However, we could not identify the contamination source from the results of this survey. To identify the source, further investigations, such as surveys of the hygiene of the silo tanks and analysis of *B. cereus* spore levels in farmers' raw milk, would be necessary.

3.4. *Occurrence of* B. cereus *detected throughout the year*

To estimate the occurrence of *B. cereus* detected throughout the year, we compared the MLVA patterns of all *B. cereus* isolates. A total of 399 isolates in the Tokachi region and 606 in the Kushiro region were classified into 107 and 260 MLVA groups, respectively (Table 3). The proportions of unique MLVA groups consisting of only a single isolate were 76.6% and 68.5%, respectively (Table 3).

The numbers of *B. cereus* isolates with identical MLVA patterns detected in three or more seasons are shown in Table 4. In the Tokachi region, one MLVA group, represented by the MLVA pattern 19-6-7-17, was detected throughout the year, and eight MLVA groups were detected in three seasons. In the Kushiro region, four MLVA groups, represented by MLVA patterns 19-6-6-15, 20-5-5-19, 20-5-6-17, and 20-5-6-20, were detected throughout the year, and 15 MLVA groups were detected in three seasons. Three MLVA groups, represented by MLVA patterns 19-6-7-17, 19-6-6-15, and 20-5-5-19, were detected in both regions in three or more seasons. The two dairy plants were geographically far away from each other

Table 3
Numbers of MLVA groups classified according to the number of *Bacillus cereus* isolates within each MLVA group.[a]

Season	Numbers of MLVA groups					Total MLVA groups
	1	2–9	10–49	50–99	≥100	
Tokachi region						
Spring	22 (88.0)	3 (12.0)				25
Summer	15 (65.2)	6 (26.1)	2 (8.7)			23
Autumn	43 (72.9)	14 (23.7)	1 (1.7)		1 (1.7)	59
Winter	21 (80.8)	4 (15.4)	1 (3.8)			26
All seasons	82 (76.6)	20 (18.7)	3 (2.8)	1 (0.9)	1 (0.9)	107
Kushiro region						
Spring	57 (79.2)	15 (20.8)				72
Summer	79 (68.1)	32 (20.3)	4 (3.4)	1 (0.9)		116
Autumn	59 (79.7)	15 (20.3)				74
Winter	68 (90.7)	7 (9.3)				75
All seasons	178 (68.5)	73 (28.1)	8 (3.1)	1 (0.4)		260

[a] *Bacillus cereus* isolates were classified into MLVA groups by MLVA typing in each season or through the year. Because several MLVA groups were detected in multiple seasons, the total number of MLVA groups through the year was less than the sum of MLVA groups in each season. The percentage of a MLVA group classified according to the number of isolates within each MLVA group per season is given in parentheses.

Table 4
MLVA groups detected in three or more seasons, and number of isolates belonging to each MLVA group.[a]

MLVA group	Seasons (number of isolates detected)	
	Tokachi region	Kushiro region
19-6-7-17	SPR (1), SMR (4), AUT (4), WTR (1)	SPR (2), SMR (3), WTR (3)
19-6-6-15	SMR (2), AUT (5), WTR (2)	SPR (2), SMR (6), AUT (6), WTR (3)
20-5-5-19	SMR (3), AUT (2), WTR (1)	SPR (3), SMR (6), AUT (1), WTR (1)
20-5-6-17		SPR (2), SMR (4), AUT (1), WTR (1)
20-5-6-20		SPR (1), SMR (4), AUT (1), WTR (3)
19-5-6-19	SMR (2), AUT (139), WTR (13)	
19-5-7-19	SMR (3), AUT (1), WTR (6)	
20-5-6-18	SPR (1), AUT (1), WTR (1)	
20-5-6-19	SPR (1), AUT (2), WTR (1)	
20-5-6-21	SPR (1), SMR (18), AUT (3)	
20-5-7-20	SMR (37), AUT (12), WTR (4)	
18-3-7-19		SPR (1), AUT (2), WTR (2)
19-3-7-19		SPR (1), SMR (3), AUT (2)
19-3-7-21		SPR (1), SMR (6), WTR (1)
19-5-5-20		SPR (1), SMR (9), AUT (1)
20-5-4-19		SPR (3), SMR (1), AUT (1)
21-3-5-0		SPR (1), SMR (3), AUT (1)
21-4-5-27		SMR (3), AUT (3), WTR (1)
21-4-6-20		SPR (3), SMR (3), AUT (5)
21-4-7-18		SMR (1), AUT (1), WTR (1)
21-5-6-23		SPR (1), AUT (1), WTR (1)
21-5-7-23		SPR (1), AUT (4), WTR (1)
22-4-7-21		SPR (1), SMR (1), WTR (1)
22-5-0-26		SPR (1), SMR (1), AUT (1)
23-4-5-17		SPR (1), SMR (1), WTR (1)

[a] Each MLVA group is represented by an MVLA pattern designated as a combination of the number of tandem repeats calculated from amplification products obtained using Bcms 08, Bcms 19, Ceb-Bams 13, and Ceb-Bams 22 as primers. Abbreviations are: SPR, spring; SMR, summer; AUT, autumn; WTR, winter.

(approximately 120 km), so identical raw milk never entered the silo tanks of both dairy plants. Therefore, these three MLVA groups are likely to be widely distributed in raw milk in Hokkaido.

4. Conclusions

The microbiological quality of raw milk as a material is important for the manufacture of HTST milk. Using a membrane filtration method, we investigated *B. cereus* spore levels in raw milk from dairy plants in two different regions of Hokkaido, Japan, over 1 year. *B. cereus* spore levels were significantly higher in summer or autumn than in other seasons, in agreement with the findings in other countries. However, there were no significant differences in psychrotrophic spore levels between the four seasons, and spore levels of *B. cereus* and its psychrotrophic strains in raw milk in Hokkaido were relatively low. We conclude that the microbiological safety of HTST milk can, in practice, be secured using raw milk with low *B. cereus* spore levels for the manufacture of HTST milk, as well as by setting an appropriate shelf-life. MLVA analysis revealed high strain diversity of *B. cereus* in raw milk in Hokkaido, but only a few of these strains were widely distributed. MLVA groups to which at least 10 isolates belonged were often found in summer and autumn, suggesting the presence of specific contamination sources. Our findings provide fundamental information for the control of *B. cereus* spores in raw milk and should help to improve the quality of the raw milk used for manufacturing HTST milk in Japan.

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