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Simple membrane filtration method for estimating numbers of *Paenibacillus* spp. spores in raw milk, using β-galactosidase activity as a selection criterion

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ABSTRACT

Paenibacillus spp. are spore-forming bacteria that adversely affect the quality of dairy products. There is currently no appropriate method for enumerating *Paenibacillus* spp. spores. We developed a simple membrane filtration method to enumerate *Paenibacillus* spp. spores in raw milk, using β-galactosidase activity as a selection criterion. Although *Paenibacillus* spp. spores are relatively small, use of a membrane filter with 0.65-μm pore size allowed us to easily filter raw milk with sufficient recovery. The membrane was put on plates containing X-gal, and detection of β-galactosidase-positive colonies enabled selective enumeration of *Paenibacillus* spp. spores. We investigated *Paenibacillus* spp. spore levels in raw milk from six different areas in the Tokachi region, Hokkaido, Japan over 1 year. There were ≤10 spores 100 mL^{-1} throughout the year, with no significant differences between areas or seasons. *Paenibacillus amylolyticus* and *Paenibacillus odorifer* were the predominant species, accounting for 50.6% and 27.4% of the total spores, respectively.

1. Introduction

Spore-forming bacteria present in raw milk form heat-resistant spores that are not inactivated by HTST (high-temperature short-time) pasteurisation, so these bacteria can survive in HTST milk. In Japan, the legal distribution temperature of market milk is specified to be ≤10 °C, and among surviving spore-forming bacteria that can survive HTST, *Bacillus* spp. and *Paenibacillus* spp., are the main genera of psychrotrophic spore-forming bacteria that can grow at ≤10 °C. Among *Bacillus* spp., *Bacillus cereus* grows well in HTST milk at ≤10 °C, and it germinates and grows during cold distribution, causing eventual deterioration of HTST milk, although in a previous study, we identified some high-heat-resistant strains of *B. cereus* that showed no growth at 10 °C (Ohkubo, Uchida, Motoshima, & Katano, 2019). On the other hand, *Paenibacillus* spp. grow well at temperatures lower than those at which *B. cereus* can grow (Mugadza & Buys, 2018), causing flavour defects in HTST milk (Martin et al., 2011). According to Fromm and Boor (2004) and Ranieri and Boor (2009, 2010), when HTST milk is stored at 6 °C the bacteria detected early during the shelf-life period are predominantly *Bacillus* spp., whereas *Paenibacillus* spp. become predominant later during the shelf-life period. Because *B. cereus* and *Paenibacillus* spp. are practically a limiting factor in the shelf-life of HTST milk, it is important for the manufacture of HTST milk to control these bacteria in raw milk.

B. cereus spores are generally not abundant in raw milk; they have been reported at levels of 10^2 to 10^3 spores L^{-1} (Shaheen, Svensson, Andersson, Christiansson, & Salkinoja-Salonen, 2010). The membrane filtration method reported by Christiansson, Ekelund, and Ogura (1997) has been used to detect *B. cereus* spores in a wide range of studies on *B. cereus*, such as studies of its distribution in raw milk (Svensson, Ekelund, Ogura, & Christiansson, 2004), its seasonal variation (Svensson et al., 2004), and the routes by which it contaminates raw milk (Magnusson, Christiansson, & Svensson, 2007a; Magnusson, Christiansson, Svensson, & Kolstrup, 2006; Magnusson, Svensson, Kolstrup, & Christiansson, 2007b).

Paenibacillus spp. spores are also considered to exist at low levels in raw milk (Huck, Hammond, Murphy, Woodcock, & Boor, 2007; Huck, Sonnen, & Boor, 2008; Martin et al., 2011; Ranieri, Huck, Sonnen, Barbano, & Boor, 2009), and as with *B. cereus* it is difficult to enumerate these spores by conventional plate count methods. Ranieri et al. (2012) reported a real-time PCR method to

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enumerate spores of *Paenibacillus* spp. in raw milk. They stated that *Paenibacillus* spp. spores were not abundant in raw milk, and that an enrichment procedure before analysis was necessary to improve the analytical sensitivity. However, when an enrichment procedure is applied, the numbers of spores originally present in raw milk cannot be determined precisely, and as a consequence this method will not accurately reflect raw milk flora. Therefore, there is currently no accurate method for enumeration of *Paenibacillus* spp. spores in raw milk.

In this study, we developed a simple and practical method to enumerate *Paenibacillus* spp. spores with high sensitivity in raw milk. Because it has been reported that most *Paenibacillus* spp. and several *Bacillus* spp. in raw milk show β-galactosidase activity (Ivy et al., 2012), in our method we combined membrane filtration with evaluation of β-galactosidase activity. Using this method, we investigated the distribution of *Paenibacillus* spp. spores in raw milk from six different areas in the Tokachi region of Hokkaido, Japan, over 1 year. This method provides a means to enumerate *Paenibacillus* spp. spores in raw milk and HTST milk and to survey the routes of contamination of raw milk.

2. Materials and methods

2.1. Bacterial strains

The strains used in this study were *Paenibacillus odorifer* PO1 and *Paenibacillus amylolyticus* PA3 isolated from dairy products and identified by 16S rDNA partial sequencing analysis in our laboratory (data not shown). These strains were stored as glycerol (15%, v/v) stocks at −80 °C.

2.2. Preparation of spore suspensions

One loopful of glycerol stock culture of *P. odorifer* PO1 or *P. amylolyticus* PA3 was inoculated into nutrient broth (Becton, Dickinson and Company, Sparks, MD, USA) and the inoculated tubes were incubated overnight at 30 °C. The cultures were spread onto modified tryptone yeast extract agar containing (per litre of distilled water) 10 g tryptone and 2 g yeast extract (both from Becton, Dickinson and Company), 25 mg $MnCl_2 \cdot 4H_2O$, 250 mg $MgSO_4 \cdot 7H_2O$, 300 μg $FeSO_4 \cdot 7H_2O$, 150 mg $CaCl_2 \cdot 2H_2O$, 15 g agar, and NaOH to adjust the pH to 7.0, and the plates were incubated for 7–10 days at 30 °C. The cultures were harvested as spores and washed twice with 0.067 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. Spore counts in the spore suspension were determined by the pour plate technique on brain heart infusion agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), after incubation at 30 °C for 2 days. All spore suspensions were confirmed to contain approximately 10^7 spores mL^{-1}. Spore suspensions were stored at 2 °C until use.

2.3. Membrane filtration method

Membrane filtration was carried out according to the method of Christiansson et al. (1997), with slight modifications. One hundred millilitres of raw milk were 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 filter-sterilised trypsin solution [2% trypsin (Nacalai Tesque, Inc., Kyoto, Japan) in 0.1 M Tris-HCl buffer, pH7.8] and 5 mL 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 the 100 mL of pasteurised raw milk and mixed well. The mixture was heated at 55 °C for 15 min for the proteolytic reaction and 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 put on brain heart infusion agar supplemented with 40 mg L^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and the plates were incubated at 7 °C for 14 days. Colonies coloured blue by the β-galactosidase reaction were counted as *Paenibacillus* spp. When necessary, blue colonies were purified by the streak plate technique on brain heart infusion agar for further identification by MALDI TOF-MS or 16S rDNA sequencing.

2.4. MALDI TOF-MS identification

Isolates were identified by MALDI TOF-MS using a MALDI Biotyper system (Bruker Daltonics GmbH, Leipzig, Germany) according to the manufacturer's instructions, which are, in brief, as follows. A colony of the isolates was spread thinly on the MALDI target plate (Bruker Daltonics GmbH). The microbial film was overlaid with 1 μL 70% formic acid and allowed to dry at room temperature, then overlaid with 1 μL HCCA matrix solution (Bruker Daltonics GmbH) and allowed to dry at room temperature. The assay performed on a Microflex LT instrument with FlexControl (v. 3.4) software (Bruker Daltonics GmbH). Mass spectrum analysis and homology searches were carried out on MALDI Biotyper Compass (v. 4. 1. 80) software (Bruker Daltonics GmbH). According to the criteria proposed by the manufacturer, when the homology score was ≥2.0, the identification was considered to be at the species level. When the homology score was <2.0 and ≥ 1.7, the identification was considered to be at the genus level. In this software, 65 species in the genus *Paenibacillus* are registered in the database.

2.5. 16S rDNA identification

For the strains that could not be identified to species level by MALDI TOF-MS identification, 16S rDNA identification was performed according to the method of Johnson (1994). The procedure is briefly described as follows. DNA from the isolates was extracted using an InstaGene Matrix Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Using the DNA as a template, 16S rDNA was amplified. Approximately 300 bases from the 5′ end of the 16S rDNA were sequenced using an ABI PRISM 310 Genetic Analyzer and an ABI PRISM Big Dye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Homology searches were carried out on NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). When the sequence identity was ≥99%, the identification was considered to be at the species level. When the sequence identity was <99% and ≥97%, the identification was considered to be at the genus level.

2.6. Spore recovery tests using membrane filters with pore sizes of 0.45 μm and 0.65 μm

To investigate the effect of membrane filter pore size on the recovery of spores, we compared the recovery of *P. odorifer* PO1 and *P. amylolyticus* PA3 spores using membrane filters with pore sizes of 0.45 μm and 0.65 μm. Spore suspensions of *P. odorifer* PO1 and *P. amylolyticus* PA3 were diluted in sterilised water to a final concentration of approximately 50 spores mL^{-1}. Then, the spores in 1 mL of the diluted spore suspension were enumerated using the pour plate method and the membrane filtration method. In the pour plate method, 1 mL of diluted spore suspension was poured into brain heart infusion agar and the plates were incubated at 30 °C for 2 days. In the membrane filtration method, 1 mL of diluted spore suspension was dispersed in 100 mL of sterilised water and directly filtered by suction through a Durapore membrane filter with a pore size of 0.45 μm or 0.65 μm. The filter membrane was put on brain heart infusion agar, and the plates were incubated at 30 °C for 2 days. The numbers of spores obtained from the two methods were compared five times.

2.7. Effect of culture temperatures on ability to differentially detect spores of Paenibacillus *spp. from among those of other psychrotrophic spore-formers*

In addition to *Paenibacillus* spp., psychrotrophic spore-forming bacteria such as *B. cereus* are present in raw milk. We investigated the incubation conditions that would allow us to differentially enumerate spores of *Paenibacillus* spp. from among other psychrotrophic spore-forming bacteria. One hundred millilitres of raw milk from two different areas (A and B) in the Tokachi region of Hokkaido, Japan, was subjected to the membrane filtration method described in section 2.3. The filter membrane was put on brain heart infusion agar supplemented with 40 mg L^{-1} of X-gal, and the plates were incubated at 7 °C for 14 days or at 10 °C for 10 days. After the incubation, the numbers of blue colonies and white colonies obtained under each incubation condition were compared. All the blue colonies that formed under both incubation conditions, all the white colonies from 7 °C, and 10 arbitrarily chosen white colonies from 10 °C were purified in the same manner as mentioned in section 2.3 and identified using MALDI TOF-MS or 16S rDNA sequencing as described in sections 2.4 and 2.5.

2.8. Survey of the distribution of Paenibacillus *spp. spores in raw milk*

To estimate the distribution of *Paenibacillus* spp. spores in raw milk, we surveyed spore levels in raw milk from six different areas (A to F) in the Tokachi region over 1 year. 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 on 3 consecutive days. Two hundred millilitres of raw milk were taken in a 250 mL sterilised polypropylene bottle, frozen at −20 °C, and stored frozen until use. The raw milk sample was thawed in a water bath at 10 °C, and immediately 100 mL of the samples was subjected to membrane filtration as mentioned in section 2.3. The blue colonies thus obtained were purified in the same manner as mentioned in section 2.3 and identified using MALDI TOF-MS or 16S rDNA sequencing as described in sections 2.4 and 2.5.

2.9. Statistical analysis

Mann–Whitney's *U*-test was used for statistical analysis of the data.

3. Results

3.1. Selection of appropriate pore size of membrane filter for collection of Paenibacillus *spp. spores*

We compared the numbers of spores recovered from spore suspensions containing approximately 50 spores mL^{-1} of *P. odorifer* PO1 or *P. amylolyticus* PA3 using membrane filters with pore size of 0.45 μm or 0.65 μm. For *P. odorifer* PO1, the average number of spores (±SD) recovered using the pour plate method was 44.8 ± 6.8; the average number recovered using the membrane filtration method was 50.0 ± 5.0 with a 0.45 μm pore size filter and 45.0 ± 8.4 with a 0.65 μm pore size filter. For *P. amylolyticus* PA3, the average numbers of spores recovered were 74.8 ± 7.9, 60.4 ± 16.0, and 68.6 ± 10.9, respectively. For both *P. odorifer* PO1 and *P. amylolyticus* PA3, there were no significant differences in spore recovery between any of the three methods. However, in practice, the larger pores can filter larger amounts of raw milk more easily, so we decided to use a membrane filter with a pore size of 0.65 μm for the membrane filtration method.

Table 1
Influence of incubation conditions on the enumeration of *Paenibacillus* spp. spores using the membrane filtration method.[a]

Colony colour	Spore numbers (spores 100 mL^{-1})	
	A	B
Incubation at 7 °C for 14 days		
Blue	8	6
White	5	3
Incubation at 10 °C for 10 days		
Blue	9	5
White	60	158

[a] Raw milk samples were collected from two different areas (A and B) in the Tokachi region of Hokkaido, Japan. Blue colonies are β-galactosidase-positive; white colonies are β-galactosidase-negative.

3.2. Appropriate culture conditions for effective enumeration of Paenibacillus *spp. spores*

We applied the membrane filtration method to raw milk sampled from two different areas, and we compared the numbers of blue and white colonies obtained from incubation at 7 °C for 14 days and at 10 °C for 10 days. In each raw milk sample, no difference in the number of blue colonies was observed between incubation conditions, whereas white colonies tended to be detected more frequently with incubation at 10 °C for 10 days (Table 1). Under both incubation conditions, the blue colonies were 1–3 mm in diameter.

Table 2
Identification of isolates purified from white colonies that formed at 7 °C for 14 days or at 10 °C for 10 days.[a]

Incubation conditions	Strains
7 °C for 14 days (38 isolates)	*Psychrobacillus psychrodurans* (8)
	Psychrobacillus spp. (8)
	Solibacillus silvestris (8)
	Sporosarcina globispora (5)
	Sporosarcina spp. (3)
	Solibacillus spp. (2)
	Bacilus cereus (1)
	Bacillus plakortidis (1)
	Bacillus simplex (1)
	Bacillus spp. (1)
10 °C for 10 days (73 isolates)	*Bacillus pumilus* (29)
	Solibacillus silvestris (8)
	Solibacillus spp. (7)
	Bacilus cereus (5)
	Bacillus megaterium (5)
	Bacillus spp. (4)
	Sporosarcina spp. (3)
	Bacillus gibsonii (2)
	Lysinibacillus spp. (2)
	Psychrobacillus psychrodurans (2)
	Psychrobacillus spp. (2)
	Bacillus murimartini (1)
	Bacillus plakortidis (1)
	Bacillus simplex (1)
	Lysinibacillus fusiformis (1)

[a] Strains were identified using MALDI TOF-MS or 16S rDNA sequencing; number of isolates in each species is shown in parentheses.

Table 3
Numbers of *Paenibacillus* spp. spores in 100 mL of raw milk sampled from six different areas over 1 year.[a]

Sampling occasion	Spore numbers (spores 100 mL^{-1})					
	A	B	C	D	E	F
Spring (April)						
Day 1	0	1	1	0	1	7
Day 2	0	0	0	3	1	1
Day 3	0	2	1	1	0	0
Summer (July)						
Day 1	0	2	0	0	5	0
Day 2	1	4	1	3	7	2
Day 3	5	5	0	4	7	0
Autumn (October)						
Day 1	2	1	1	1	2	1
Day 2	9	3	0	0	3	2
Day 3	10	4	0	8	1	3
Winter (January)						
Day 1	2	0	2	8	1	0
Day 2	8	1	2	10	0	0
Day 3	6	0	0	10	1	1

[a] Raw milk samples were collected from six different areas (A to F) in the Tokachi region of Hokkaido, Japan.

We purified all colonies that formed at 7 °C and all blue colonies and 10 arbitrarily chosen white colonies that formed at 10 °C, and we identified them using MALDI TOF-MS or 16S rDNA sequencing. In raw milk from area A, isolates from the blue colonies were identified as *P. amylolyticus* (7 strains) and *Paenibacillus pabuli* (1 strain) at 7 °C, and *P. amylolyticus* (8 strains) and *P. pabuli* (1 strain) at 10 °C. In raw milk from area B, they were identified as *P. amylolyticus* (5 strains) and *P. odorifer* (1 strain) at 7 °C, and *P. amylolyticus* (3 strains) and *P. odorifer* (2 strains) at 10 °C. Under both incubation conditions, all isolates from blue colonies were composed of *Paenibacillus* spp. and did not contain *Bacillus* spp.

In contrast, isolates from the white colonies were composed of spore-forming bacteria other than *Paenibacillus* spp. In raw milk from area A, they were identified as *Psychrobacillus psychrodurans* (2 strains), *Sporosarcina globispora* (2 strains), and *B. cereus* (1 strain) at 7 °C, and *B. cereus* (3 strains), *Bacillus megaterium* (3 strains), *Ps. psychrodurans* (2 strains), *Solibacillus silvestris* (1 strain), and *Sp. globispora* (1 strain) at 10 °C. In raw milk from area B, they were identified as *S. silvestris* (2 strains) and *Bacillus simplex* (1 strain) at 7 °C, and *Bacillus pumilus* (3 strains), *B. cereus* (2 strains), *S. silvestris* (2 strains), *B. megaterium* (1 strain), *B. simplex* (1 strain), and *Lysinibacillus fusiformis* (1 strain) at 10 °C.

In our preliminary experiment, we identified 38 white colonies at 7 °C and 73 white colonies at 10 °C; all were identified as spore-forming bacteria other than *Paenibacillus* spp. (Table 2). Therefore, under both incubation conditions, white colonies did not contain *Paenibacillus* spp.

In comparison with incubation at 10 °C for 10 days, incubation at 7 °C for 14 days suppressed white-colony-forming spore-forming bacteria and enabled the more efficient enumeration of *Paenibacillus* spp. that formed the blue colonies. In addition, there was no difference in the numbers of *Paenibacillus* spp. spores detected under the two incubation conditions. Therefore, we decided to use the incubation conditions of 7 °C for 14 days for the membrane filtration method.

3.3. *Distribution of* Paenibacillus *spp. spores in raw milk*

Using the membrane filtration method, we investigated the distribution of *Paenibacillus* spp. spores in raw milk from six different areas over 1 year. The spore levels of *Paenibacillus* spp. in raw milk ranged from 0 to 10 spores 100 mL^{-1} throughout the year (Table 3); there was no significant difference in the number of spores between areas or between seasons ($p > 0.05$).

In this survey, we isolated and identified 168 strains of *Paenibacillus* spp. (Table 4). In MALDI TOF-MS identification, 148 strains out of 168 belonged to 5 different species: *P. amylolyticus* (85 strains), *P. odorifer* (46 strains), *P. pabuli* (14 strains), *Paenibacillus anaericanus* (2 strains), and *Paenibacillus barcinonensis* (1 strain). The remaining 20 strains were not identified, even to the genus level, with MALDI TOF-MS, so we applied 16S rDNA identification to them. Although they were identified as *Paenibacillus* spp. by this method, they could not be identified to the species level. Highly frequently detected species were *P. amylolyticus* and *P. odorifer*, which accounted for 50.6% and 27.4% of all isolates, respectively.

4. Discussion

In this study, we developed a practical method for the simple enumeration of *Paenibacillus* spp. spores in raw milk with high sensitivity by means of a membrane filtration method using β-galactosidase activity as a selection criterion. This method enables the analysis of populations of *Paenibacillus* spores in raw milk.

Membrane filtration as a method to enumerate bacterial spores in raw milk has been reported for *B. cereus* (Christiansson et al., 1997) and for *Clostridium tyrobutyricum* (Bourgeois, Le Parc, Abgrall, & Cleret, 1984). The spores of the target bacteria in those two studies were large, so a membrane filter with a pore size of 0.8 μm was used. In contrast, the spores of *Paenibacillus* spp. are not as large as those of *B. cereus* or *C. tyrobutyricum*, so we tested filters with a smaller pore size (0.45 and 0.65 μm). We found that both pore sizes gave sufficient recovery of *Paenibacillus* spp. spores. For practical reasons, we selected filters with a pore size of 0.65 μm, as these allowed us to easily filter 100 mL of raw milk as well as HTST milk.

Ivy et al. (2012) investigated the β-galactosidase activity of *Paenibacillus* spp. isolated from raw milk, pasteurised milk, and dairy farm environments. They stated that although most *Paenibacillus* spp. were positive for β-galactosidase activity, some β-galactosidase-negative *Paenibacillus* spp. existed. In our investigation, all β-galactosidase-positive bacteria isolated from raw milk

Table 4
Distribution of *Paenibacillus* species isolated from raw milk from six different areas over 1 year.[a]

Strains	Spore numbers						Number of isolates	Percentage of isolates
	A	B	C	D	E	F		
Paenibacillus amylolyticus	24	12	4	24	15	6	85	50.6
Paenibacillus odorifer	11	8	4	14	7	2	46	27.4
Paenibacillus pabuli	4	2		6	1	1	14	8.3
Paenibacillus anaericanus		1		1			2	1.2
Paenibacillus barcinonensis				1			1	0.6
Paenibacillus spp.	4			2	6	8	20	11.9
Total	43	23	8	48	29	17	168	100.0

[a] Raw milk samples were collected from six different areas (A to F) in the Tokachi region of Hokkaido, Japan. Strains were identified using MALDI TOF-MS or 16S rDNA sequencing. Spore numbers are total numbers from the 1-year investigation.

were *Paenibacillus* spp., whereas all of the β-galactosidase-negative bacteria were spore-forming bacteria other than *Paenibacillus* spp. Therefore, our method is sufficiently practical to enumerate *Paenibacillus* spp. spores in raw milk.

We used low-temperature incubation conditions for selecting *Paenibacillus* spp. However, under low-temperature conditions, it took time for visible colonies to form on the plates. Rapidity is required for a method to be of practical use in surveying sources of contamination in HTST milk manufacture; hence, it will be necessary to develop a selective medium that can more rapidly detect *Paenibacillus* spp.

In our investigation of the levels of *B. cereus* spores in raw milk, we found <10 spores 100 mL^{-1} (data not shown). In the current study, *Paenibacillus* spp. spores were present in raw milk at the same levels as *B. cereus* spores (Table 3). To our knowledge, this study is the first to clarify the levels of *Paenibacillus* spp. spores present in raw milk.

5. Conclusions

We successfully developed a practical membrane filtration method to enumerate *Paenibacillus* spp. spores in raw milk. Combination of β-galactosidase activity with low-temperature incubation enabled effective enumeration of *Paenibacillus* spp. spores. By this method, we found that the levels of *Paenibacillus* spp. spores in raw milk were $\leq$10 spores 100 mL^{-1}. The method provides a means to investigate the numbers of *Paenibacillus* spp. spores in raw milk and HTST milk. It should be applicable to the selection of high-quality raw milk and to the management of hygiene with respect to *Paenibacillus* spp. in HTST milk manufacturing plants.

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