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Species-specific real-time PCR assay for enumeration of *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* spores in dairy products

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ABSTRACT

The thermophilic spore-forming bacteria, *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus*, grow readily, especially on milk-powder processing lines, and are important for processing-plant hygiene management. We developed a real-time PCR assay using SYBR Green I to monitor the spores of these species in dairy products. We designed new primer pairs specific for each species from the nucleotide sequences of the stage 0 sporulation gene A (*spo0A*). Ethidium monoazide treatment enabled us to quantify only spores. In dairy products with high protein content, treatment with proteinase K allowed precise quantification of spores. This assay was superior for counting spores, having high linearity ($r^2 = 0.99$) and a wide quantification range (10^1 to 10^6 spores mL^{-1}). This new method can be applied to quantification of spores in samples of milk, skimmed milk, and cream.

1. Introduction

Thermophilic spore-forming bacteria have optimum growth temperatures of approximately 40–70 °C, and can be isolated from dairy products such as milk powder (Burgess, Lindsay, & Flint, 2010; Murphy, Lynch, & Kelly, 1999; Scott, Brooks, Rakonjac, Walker, & Flint, 2007). They can be divided into two groups: the obligate thermophiles and the facultative thermophiles (Burgess et al., 2010). The obligate thermophiles grow only at approximately 40–70 °C and include *Anoxybacillus flavithermus* and *Geobacillus stearothermophilus* (Flint, Ward, & Walker, 2001b; Ronimus et al., 2003; Scott et al., 2007). The facultative thermophiles grow at both mesophilic and thermophilic temperatures, depending on the strains, and include *Bacillus licheniformis*, *Bacillus coagulans* and *Bacillus subtilis* (Flint et al., 2001b; Ronimus et al., 2003).

Although there are usually few thermophilic spore-forming bacteria in raw milk (<10 spores mL^{-1}) (McGuiggan, McCleery, Hannan, & Gilmour, 2002), in the manufacture of milk powder they can grow in the processing line during long-term operation, and may contaminate the finished products in high numbers (>10^4 spores g^{-1}). In the manufacture of milk powder, in particular with a high preheating temperature, the obligate thermophiles grow readily over the approximately 16 h of continuous operation, and consequently, only *A. flavithermus* and *G. stearothermophilus* become major flora. Although the obligate thermophiles are non-pathogenic, they are considered as general indicators of processing-plant hygiene.

In laboratory studies, it was shown that *A. flavithermus* and *G. stearothermophilus* readily form biofilms on stainless-steel surfaces (Burgess, Brooks, Rakonjac, Walker, & Flint, 2009; Flint, Palmer, Bloemen, Brooks, & Crawford, 2001a). They are believed to form biofilms in dairy manufacturing plants at temperatures of 40–65 °C, such as in the cream separator section before preheating, the preheating section, and the evaporator section of a milk powder plant (Burgess et al., 2010). Scott et al. (2007) reported that *A. flavithermus* dominated in the preheating section of the processing line for whole-milk powder whereas there was a mixture of *A. flavithermus* and *Geobacillus* spp. in the evaporator section. However, there is little information about when and where biofilms form or sporulation occurs during the manufacture of dairy products. Burgess et al. (2010) pointed out the need for a greater understanding of biofilm formation on dairy product processing lines, and the link with sporulation, to develop better control measures.

A. flavithermus and *G. stearothermophilus* are known to have different heat resistance and growth temperatures (Zhao et al., 2013). These bacteria therefore tend to form biofilms at different

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places along the processing line, and it is important to distinguish these bacterial species for better control of processing-plant hygiene. However, identification through conventional culture methods is labour-intensive and takes time.

For rapid quantification of common thermophilic spore-forming bacteria, Rueckert, Ronimus, and Morgan (2005a,b; 2006) developed real-time PCR assays to selectively quantify vegetative cells and spores by using DNase I to cleave DNA extracted from vegetative cells. In their assays, they targeted 16S rDNA or *spo0A* (stage 0 sporulation gene A), and these assays can rapidly and selectively quantify vegetative cells and spores of thermophilic spore-forming bacteria in milk powder within 1–1.5 h. Burgess et al. (2010) pointed out that the assay targeting 16S rDNA was less quantitative due to variable copy numbers among the species of thermophilic spore-forming bacteria, and the assay targeting *spo0A* was not sensitive or specific enough because it also amplified DNA from *Bacillus* spp. such as *Bacillus cereus*. Rueckert, Ronimus, and Morgan (2006) used degenerated primer pairs targeting *spo0A* for quantifying all species of thermophilic spore-forming bacteria, but not specifically for quantifying *A. flavithermus* or *G. stearothermophilus* contamination in milk powder. There is currently no literature available about assays for specifically quantifying these two species.

When *A. flavithermus* or *G. stearothermophilus* grows on the processing line before the pasteurisation section, vegetative cells are killed and only spores remain in the final products. On the other hand, when they grow on the processing line after the pasteurisation section, both vegetative cells and spores are present. It is known that the numbers of vegetative cells of these species gradually decrease when temperatures fall below the growth temperature range (Hashizume, Sekiguchi, & Nosoh, 1976). The presence of large numbers of spores in dairy ingredients such as milk powder threatens the sterility of the final products manufactured using these ingredients. Therefore, for practical hygiene control, it is more important to enumerate only the spores with high sensitivity. Any vegetative cells remaining in the products are easily killed by heat treatment at 80 °C for 10 min. PCR detection methods, however, cannot differentiate the DNA from dead cells and spores. DNA from dead cells can be inactivated using DNA intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) (Pan & Breidt, 2007; Rawsthorne, Dock, & Jaykus, 2009). These can selectively enter dead cells to inactivate DNA. Alternatively, DNase I is used to degrade DNA from dead cells (Rueckert, Ronimus, & Morgan, 2005b).

In this paper, we describe a practical assay for quantifying spores of *A. flavithermus* and *G. stearothermophilus* in dairy products such as milk, skimmed milk, and cream. The real-time PCR assay uses EMA treatment along with SYBR Green I to specifically and sensitively quantify only spores of these two species.

2. Materials and methods

2.1. Bacterial strains

The strains used in this study are listed in Table 1. They were purchased from the American Type Culture Collection (ATCC;

Table 1
Bacterial strains used in this study and the results of primer specificity tests.[a]

Species	Strain	Specificity test results			
		AF-F1/AF-R1 primers for *A. flavithermus*		GS-F2/GS-R2 primers for *G. stearothermophilus*	
		EP-PCR	RT-PCR	EP-PCR	RT-PCR
Thermophilic spore-forming bacteria					
Obligate thermophiles					
Anoxybacillus flavithermus	AF1	+	+	−	−
	AF2	+	+	−	−
	AF3	+	+	−	−
	AF4	+	+	−	−
	AF5	+	+	−	−
Geobacillus stearothermophilus	ATCC 12980	−	−	+	+
	GS1	−	−	+	+
	GS2	−	−	+	+
	GS3	−	−	+	+
	GS4	−	−	+	+
Geobacillus thermoamylovorans	GT1	−	NT	−	NT
Facultative thermophiles					
Bacillus licheniformis	BL1	−	−	−	−
	BL2	−	NT	−	NT
	BL3	−	NT	−	NT
	BL4	−	NT	−	NT
	BL5	−	NT	−	NT
Bacillus subtilis	BS1	−	NT	−	NT
Mesophilic *Bacillus* spp.					
Bacillus cereus	BC1	−	NT	−	NT
Bacillus megaterium	JCM 2506	−	NT	−	NT
Bacillus pumilus	BP1	−	NT	−	NT
Raw-milk flora					
Microbacterium lacticum	JCM 1379	−	−	−	−
Staphylococcus aureus	ATCC 12600	−	−	−	−
Micrococcus luteus	ATCC 4698	−	NT	−	NT
Gram-negative bacteria					
Escherichia coli	IFO 15034	−	−	−	−
Enterobacter cloacae	IFO 13535	−	NT	−	NT
Pseudomonas aeruginosa	ATCC 10145	−	NT	−	NT
Pseudomonas fluorescens	ATCC 13525	−	−	−	−
Acinetobacter baumannii	JCM 6841	−	−	−	−

[a] Abbreviations are: ATCC, American Type Culture Collection; IFO, Institute for Fermentation, Culture Collection of Microorganisms, Osaka; JCM, Japan Collection of Microorganisms (all other strains were isolated from milk products and identified in our laboratory); EP-PCR, end-point PCR; RT-PCR, real-time PCR; +, positive; −, negative; NT, not tested. Template DNA concentration was 2 ng μL^{-1} for target strains and 10 ng μL^{-1} for negative control strains.

Rockville, MD, USA), the Institute for Fermentation (IFO; Culture Collection of Microorganisms, Osaka, Japan), or the Japan Collection of Microorganisms (JCM; Saitama, Japan), or isolated from raw milk and dairy products and identified in our laboratory. They were confirmed to be different strains by the randomly amplified polymorphic DNA (RAPD) method (Ronimus et al., 2003) (data not shown). All strains were stored as glycerol stocks (15%, v/v) at −80 °C.

2.2. Preparation of cultures

One loopful of glycerol stock cultures of thermophilic spore-forming bacteria listed in Table 1 was inoculated in thermophile broth containing (per litre of distilled water): 5 g tryptone (Becton, Dickinson and Company, Sparks, MD), 5 g yeast extract (Bectone, Dickinson and Company), 10 g soluble starch (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1 g K_2HPO_4, 10 mg $FeSO_4.7H_2O$, 20 mg $MgSO_4.7H_2O$, and NaOH to adjust the pH to 7.5. The inoculated tubes were incubated overnight at 55 °C. One loopful of glycerol stock cultures of each mesophilic *Bacillus* spp., raw-milk flora, and Gram-negative bacteria listed in Table 1 was similarly inoculated into tubes containing nutrient broth (Becton, Dickinson and Company) and incubated overnight at 30 °C.

2.3. Preparation of spore suspensions

Spore suspensions of *A. flavithermus* AF1 and *G. stearothermophilus* ATCC 12980 were prepared as follows. The cultures prepared as described in section 2.2 were spread onto modified tryptone yeast extract agar containing (per litre of distilled water): 10 g tryptone, 2 g yeast extract, 25 mg $MnCl_2.4H_2O$, 250 mg $MgSO_4.7H_2O$, 300 μg $FeSO_4.7H_2O$, 150 mg $CaCl_2.2H_2O$, 15 g agar, and NaOH to adjust the pH to 7.0, and the plates were incubated for 14 days at 55 °C. The cultures were harvested as spores and washed twice in 10 mM Tris-HCl buffer (pH 7.8) by centrifugation at 5000× *g* for 10 min at 4 °C. The washed spore pellets were suspended in 9.5 mL of the same buffer; 0.5 mL of 10% sodium dodecyl sulphate (SDS) and 100 μL of proteinase K (>600 mAU mL^{-1}, QIAGEN GmbH, Hilden, Germany) were added to lyse the remaining cells and the suspensions were incubated overnight at 37 °C. Proteinase K was inactivated at 100 °C for 8 min followed by centrifugation. The spore pellets were suspended in 1.8 mL 10 mM Tris-HCl buffer (pH 7.8), then 20 μL DNase I (10 U μL^{-1}; Roche Diagnostics GmbH, Mannheim, Germany) and 200 μL incubation buffer (Roche Diagnostics GmbH) were added to degrade DNA from vegetative cells. Following incubation of the mixtures for 15 min at 37 °C, the DNase I was inactivated at 80 °C for 10 min followed by centrifugation, and then the spore pellets were washed twice in 10 mM Tris-HCl buffer (pH 7.8). The washed spore pellets were suspended in 10 mL of the same buffer. Spore suspensions were stored at −20 °C until use.

2.4. Cell and spore counts

Vegetative cell counts in the cultures and spore counts in the spore suspensions of *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 were determined by the pour plate technique on thermophile agar (thermophile broth containing 15 g L^{-1} agar) at 55 °C for 48 h.

2.5. DNA extraction for specificity tests

DNA extraction from the pure cultures of the target strains or the negative control strains was carried out using the InstaGene Matrix Kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. DNA concentrations were determined using NanoDrop ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA). The extracted DNAs were used for the specificity test.

2.6. Standard DNA extraction from spore suspensions for sensitivity and quantification tests

Standard DNA for quantification of spores was prepared from spore suspensions of 5.9×10^1 to 1.2×10^6 spores mL^{-1} for *A. flavithermus* AF1 and 6.4×10^1 to 1.3×10^6 spores mL^{-1} for *G. stearothermophilus* ATCC12980 using a bead-beating method. The spore pellet after centrifugation of the 1 mL spore suspension was resuspended in 200 μL of TE (Tris-EDTA) buffer. The suspension was transferred to a 2.0 mL tube (Master Tube Hard, Bio Medical Science Co. Ltd., Tokyo, Japan) containing 0.2–0.3 g of zirconium-silica beads (0.2 mm diameter, Bio Medical Science) and vortexed for 5 min using a vortex mixer (Delta Mixer Se-04, TAITEC Co., Saitama, Japan) followed by centrifugation at 13,000× *g* for 3 min. The supernatant was used as the standard DNA for quantification of spores.

2.7. Primer design

We obtained a partial nucleotide sequence for *spo0A* of *A. flavithermus* isolate B (accession number AY672767.1) (Rueckert et al., 2006) and *G. stearothermophilus* BB4419 (accession number U09977.1) (Brown et al., 1994) from GenBank. These nucleotide sequences were used as templates to design primer pairs specific to *A. flavithermus* and *G. stearothermophilus* with the NCBI Primer-BLAST Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) under conditions of an amplification product size of 80–150 bases, primer size of 17–25 bases, and GC content of 40%–60%. The *A. flavithermus*-specific primer pair had the following sequences: AF-F1 (forward primer), 5′-ACATTACAAGCCAAGGAGATATGG-3′; AF-R1 (reverse primer), 5′-CAGCCAAGCCGTCTAAATGC-3′. The *G. stearothermophilus*-specific primer pair had the following sequences: GS-F2 (forward primer), 5′-ACGCCGGTTGTGAGGAAAGC-3′; GS-R2 (reverse primer), 5′-GCCTCGCGCAAATACAAATAGC-3′. The amplification product sizes were 135 bases and 140 bases, respectively.

2.8. Real-time PCR assay

Real-time PCR was carried out using a LightCycler DX-400 (Roche Diagnostics GmbH). The PCR reaction mixture was as follows: 4 μL FastStart DNA Master PLUS SYBR Green I Master Mix (Roche Diagnostics GmbH), 1 μL of each forward and reverse primers (10 μM), 5 μL template DNA solution, and 9 μL water (PCR grade).

The real-time PCR protocol was as follows: initial denaturation of 95 °C for 10 min followed by 50 cycles of 95 °C for 10 s, 65 °C for 10 s and 72 °C for 6 s. Melting-curve analysis was performed after amplification to assess whether the real-time PCR assay produced a single, specific product. The amplification product was cooled to 70 °C and then heated to 97 °C at a rate of 0.1 °C s^{-1}. The fluorescence signals were continuously monitored during the analysis to confirm specificity from the obtained melting temperature (T_m).

2.9. Specificity test

Specificity of the designed primer pairs was confirmed by endpoint PCR/real-time PCR assay using DNA extracted from the pure cultures of the target strains or the negative control strains. The concentration of the template DNA solution was 2 ng μL^{-1} for target strains in the real-time PCR assay and 10 ng μL^{-1} for the negative control strains.

2.10. Sensitivity and quantification test

The sensitivity and quantification capability of real-time PCR was tested using the standard DNAs extracted from the spore suspension of *A. flavithermus* AF1 and *G. stearothermophilus* ATCC 12980. The standard curve was generated from the threshold cycles (C_t values), which were obtained from the real-time PCR assay for the standard DNAs in triplicate, plotted against the logarithm of spore counts per millilitre. The amplification efficiency (E) was calculated from equation $E = 10^{(-1/\text{slope})} - 1$.

E values from 0.8 to 1.2 were considered to be appropriate.

2.11. Pretreatment before DNA extraction from milk, 10% reconstituted skimmed milk, and cream

Dairy products contain various amplification-inhibiting substances (McKillip & Drake, 2004; Straub, Hertel, & Hammes, 1999; Wilson, 1997), and these interfere with PCR-based quantification, especially in cream. We partially modified the method of Rueckert et al. (2005a) for pretreatment before DNA extraction from milk, skimmed milk, and cream. The procedure can be briefly described as follows. Two hundred microliters of 1.2 M trisodium citrate as protein solubilising agent was added to 1 mL sample and mixed well. The pellet after centrifugation at 20,000× *g* for 5 min was resuspended in 1 mL sterilised water, to which was added 200 μL 1.2 M trisodium citrate and 200 μL n-decane as lipid solubilising agent. The suspension was mixed well and centrifuged at 20,000× *g* for 5 min. The pellet obtained from milk and skimmed milk was resuspended in 200 μL sterilised water and centrifuged at 20,000× *g* for 5 min. For cream, the pellet obtained after the treatment of trisodium citrate and n-decane as described above was resuspended in 200 μL sterilised water, to which was added 20 μL proteinase K to lyse the remaining amplification-inhibiting substances. The suspension was incubated at 56 °C for 5 min, inactivated at 95 °C for 5 min, and centrifuged at 20,000× *g* for 5 min to obtain pellets containing both spores and dead cells.

2.12. Ethidium monoazide treatment

DNA extraction applied to dairy products extracts DNA from both spores and vegetative cells. Therefore, to practically enumerate only spores by real-time PCR, it is necessary to inactivate the DNA from vegetative cells before DNA extraction. We used EMA to inactivate DNA from vegetative cells. EMA penetrates vegetative cells via damaged membranes, then covalently binds to DNA by photoactivation. The DNA bound EMA cannot be amplified. The pellet obtained from 1 mL of sample after pretreatment of milk, skimmed milk, or cream as described in section 2.11, was suspended in 45 μL sterilised water. To the suspension was added 5 μL EMA (1 mg mL^{-1}, Sigma–Aldrich Co. Ltd., St Louis, MO, USA); it was placed on ice and incubated in the dark for 5 min, then irradiated for 5 min by LED light (LDA7LA1, 450 lm, 6.9W; Panasonic, Osaka, Japan) at a height of 2 cm, followed by centrifugation at 20,000× *g* for 5 min to collect the pellet. This process was repeated three times. The final irradiation by LED light lasted 15 min. After centrifugation at 20,000× *g* for 5 min, the pellet was washed once and suspended in 200 μL TE buffer.

2.13. DNA extraction from spores in pretreated or EMA-treated samples

DNA extraction from spores in pretreated or EMA-treated samples was carried out by the bead-beating method as described in section 2.6. The extracted DNAs were subjected to the real-time PCR assay.

2.14. Influence of proteinase K treatment on C_t values from cream

To examine the influence of proteinase K treatment on C_t values from cream, duplicate samples of milk and cream were inoculated with *A. flavithermus* AF1 culture to a concentration of 2.0×10^2 cells mL^{-1}. The cream sample was subjected to the pretreatment described in section 2.11 with and without proteinase K. The milk sample was subjected to pretreatment without proteinase K. Pretreatment was followed by DNA extraction using the InstaGene Matrix Kit and real-time PCR assay. The duplicate C_t values obtained were compared. To investigate the influence of pretreatment on the quantification of spores, triplicate samples of milk, skimmed milk, and cream were inoculated with spores of either *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 at concentrations of 10^2 to 10^4 spores mL^{-1}, subjected to pretreatment and DNA extraction, and quantified by real-time PCR.

2.15. Effect of EMA treatment on spore-specific quantification

To investigate the inhibitory effect of EMA on dead cells, *A. flavithermus* AF1 and *G. stearothermophilus* ATCC 12980 cultures prepared as described in section 2.2 were heated at 80 °C for 10 min to prepare dead cells and added to sterilised water to a concentration of 10^4 to 10^6 dead cells mL^{-1}. One millilitre of these inoculated mixtures was subjected to EMA treatment as described in section 2.12, followed by DNA extraction and real-time PCR assay.

2.16. Quantification of spores from samples spiked with both spores and dead cells

Triplicate samples of milk, skimmed milk, and cream were inoculated with both dead cells and spores of *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 to a concentration of approximately 10^4 dead cells mL^{-1} and 10^2 to 10^4 spores mL^{-1}. Each 1 mL sample was subjected to pretreatment, EMA treatment, DNA extraction, and quantification by real-time PCR.

3. Results

3.1. Primer specificity

The specificity of the newly designed AF-F1/AF-R1 and GS-F2/GS-R2 primer pairs was confirmed by endpoint PCR and real-time PCR assay. The results are shown in Table 1. In the endpoint PCR assay using the AF-F1/AF-R1 primer pair with 17 strains of thermophilic spore-forming bacteria, 3 strains of mesophilic *Bacillus* spp., 3 strains of raw-milk flora, and 5 strains of Gram-negative bacteria, all 5 strains of *A. flavithermus* were exclusively amplified, with a product size of 135 bases. Similarly, in the assay using the GS-F2/GS-R2 primer pair, all 5 strains of *G. stearothermophilus* were exclusively amplified, with a product size of 140 bases.

In the real-time PCR assay using the AF-F1/AF-R1 primer pair with 11 strains of thermophilic spore-forming bacteria, 2 strains of raw-milk flora, and 3 strains of gram-negative bacteria, all 5 strains of *A. flavithermus* were exclusively amplified, with mean (±SD) C_t values and T_m of 16.89 ± 0.77 and 83.95 ± 0.01 °C, respectively. Similarly, in the assay using the GS-F2/GS-R2 primer pair, all 5 strains of *G. stearothermophilus* were exclusively amplified, with mean C_t values and T_m of 16.06 ± 0.43 and 87.63 ± 0.02 °C, respectively. The primer pairs AF-F1/AF-R1 and GS-F2/GS-R2 were therefore shown to be specific to *A. flavithermus* and *G. stearothermophilus*, respectively.

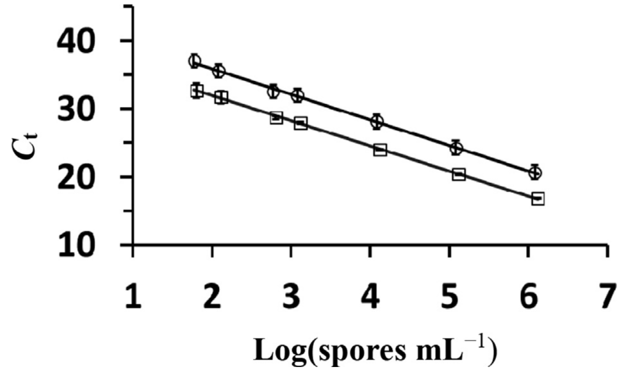

Fig. 1. Standard curves for quantifying *Anoxybacillus flavithermus* AF1 (◯) and *Geobacillus stearothermophilus* ATCC 12980 (□) spores by real-time PCR assay. Spore suspensions containing 10^1–10^6 spores mL^{-1} were subjected to DNA extraction using zirconium-silica beads, and the PCR reactions were performed in triplicate. Error bars indicate standard deviations for triplicate tests.

3.2. *Sensitivity and quantification capability of real-time PCR*

To confirm the sensitivity and quantification capability of real-time PCR, we performed the real-time PCR assay for spore suspensions of *A. flavithermus* AF1 and *G. stearothermophilus* ATCC 12980, and plotted the standard curves from the C_t values obtained against the logarithm of spore counts per millilitre (Fig. 1). The equation for the standard curve for *A. flavithermus* AF1 spores was $C_t = -3.75 \log(\text{spores } mL^{-1}) + 43.39$, with a linear relationship from 5.9×10^1 to 1.2×10^6 spores mL^{-1} ($r^2 = 0.99$, $E = 0.85$). Similarly, the equation for the standard curve for *G. stearothermophilus* ATCC 12980 spores was $C_t = -3.70 \log(\text{spores } mL^{-1}) + 39.34$, with linear relationship from 6.4×10^1 to 1.3×10^6 spores mL^{-1} ($r^2 = 0.99$, $E = 0.86$).

3.3. *Influence of proteinase K treatment on C_t values from cream*

The pretreatment before DNA extraction from whole- and skimmed-milk powder described by Rueckert et al. (2005a) was rapid and easy, but it could not be applied to cream, which contains large amounts of amplification-inhibiting substances. We therefore tested a modification to their method.

Milk and cream inoculated with *A. flavithermus* AF1 culture were pretreated with trisodium citrate and n-decane, and then DNA was extracted and quantified by real-time PCR assay. In comparison with milk, the DNA solution from cream had a large amount of precipitates and its C_t values were delayed by approximately 4 cycles in the real-time PCR assay (Table 2). Because we considered the precipitates to be mainly proteins, we applied proteinase K treatment in cream after the treatment with trisodium citrate and n-decane. After this treatment, most of the precipitates disappeared and the C_t values of cream matched those of milk (Table 2).

To investigate the effect of this pretreatment on the quantification of spores, we inoculated *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 spores into milk, skimmed milk, and cream, and quantified the spores by real-time PCR assay. The difference between the number of spores inoculated and the numbers from real-time PCR ranged from −0.33 to 0.25 log (spores mL^{-1}) (Table 3), indicating that the assay had sufficient quantification capability.

Table 2
Influence of proteinase K treatment on C_t values from milk and cream.[a]

Milk (−)	Cream (−)	Cream (+)
35.74	39.99	35.09

[a] All samples were spiked with *Anoxybacillus flavithermus* AF1 cells at log (cells mL^{-1}) = 2.30. Values are the mean of duplicate C_t values from real-time PCR assay; (+), with proteinase K treatment; (−), without proteinase K treatment.

Table 3
Quantification of spores of *Anoxybacillus flavithermus* AF1 and *Geobacillus stearothermophilus* ATCC 12980 in artificially spiked milk, skimmed milk, or cream using real-time PCR assay.[a]

Inoculated conc. [log (spores mL^{-1})]	Spore numbers ± SD [log (spores mL^{-1})]		
	Milk	Skimmed milk	Cream
A. flavithermus AF1			
4.38	4.39 ± 0.08	4.16 ± 0.11	4.05 ± 0.08
3.38	3.44 ± 0.05	3.40 ± 0.06	3.29 ± 0.04
2.38	2.35 ± 0.22	2.48 ± 0.07	2.32 ± 0.10
G. stearothermophilus ATCC 12980			
4.41	4.62 ± 0.03	4.66 ± 0.01	4.33 ± 0.28
3.41	3.64 ± 0.02	3.64 ± 0.03	3.41 ± 0.03
2.41	2.26 ± 0.06	2.40 ± 0.16	2.19 ± 0.19

[a] Milk and skimmed milk were pretreated with trisodium citrate and n-decane. Cream received additional pretreatment with proteinase K. Values are the mean of triplicate spore numbers ± standard deviation.

3.4. *Effect of EMA treatment on quantification capability of spore-specific count*

In our test to see if we could inactivate DNA from dead cells using EMA, we subjected sterilised water containing dead cells of *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 at concentrations of 10^4 to 10^6 dead cells mL^{-1} to EMA treatment, DNA extraction, and real-time PCR assay. In the tests with *A. flavithermus* AF1, no amplification was observed in the DNA from the cell suspension containing 10^4–10^5 dead cells mL^{-1}, but there was amplification evident at 10^6 dead cells mL^{-1}. In contrast, the tests with *G. stearothermophilus* ATCC 12980 showed no amplification of DNA from the cell suspension containing 10^4–10^6 dead cells mL^{-1}. These tests therefore showed that EMA treatment could inactivate the DNA from at least 10^5 dead cells mL^{-1} of *A. flavithermus* AF1 and 10^6 dead cells mL^{-1} of *G. stearothermophilus* ATCC 12980.

3.5. *Spore quantification in samples spiked with spores and dead cells*

Under the assumption that actual dairy products contain spores and dead cells, we added approximately 10^4 dead cells mL^{-1} and

Table 4
Quantification of spores of *Anoxybacillus flavithermus* AF1 and *Geobacillus stearothermophilus* ATCC 12980 in artificially spiked milk, skimmed milk, or cream using real-time PCR assay with ethidium monoazide (EMA) treatment.[a]

Inoculated conc. [log (spores mL^{-1})]	Spore numbers ± SD [log (spores mL^{-1})]		
	Milk	Skimmed milk	Cream
A. flavithermus AF1			
4.59	4.26 ± 0.19	4.49 ± 0.27	4.26 ± 0.09
3.59	3.36 ± 0.26	3.54 ± 0.05	3.32 ± 0.25
2.59	2.35 ± 0.00	2.55 ± 0.08	2.80 ± 0.20
G. stearothermophilus ATCC 12980			
4.59	4.55 ± 0.24	4.66 ± 0.05	4.18 ± 0.18
3.59	3.68 ± 0.04	3.82 ± 0.03	3.13 ± 0.06
2.59	2.42 ± 0.17	2.65 ± 0.20	2.13 ± 0.05

[a] Milk, skimmed milk, and cream were spiked with both dead cells and spores of *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 to a concentration of approximately 10^4 dead cells mL^{-1} and 10^2 to 10^4 spores mL^{-1}, respectively. Values are the mean of triplicate spore numbers ± standard deviation.

spores of *A. flavithermus* AF1 or *G. stearothermophilus* ATCC 12980 to milk, skimmed milk, and cream at different concentrations from 10^2 to 10^4 spores mL^{-1}. We then quantified only the spores by real-time PCR assay. The difference between the numbers of inoculated spores and those from real-time PCR ranged from −0.46 to 0.23 log (spores mL^{-1}) (Table 4), indicating that our assay was sufficient to quantify only spores in these samples.

4. Discussion

In this study, we established a practical method for enumeration of only spores of *A. flavithermus* and *G. stearothermophilus* separately in dairy products. We summarised the method in a flow diagram (Fig. 2).

We designed primer pairs specific to *A. flavithermus* and *G. stearothermophilus*, respectively, from the nucleotide sequences of *spo0A*, and developed a real-time PCR assay using SYBR Green I. By applying EMA treatment, this assay can be used to quantify only spores in dairy products with high sensitivity; the quantification of spores in dairy products can be accomplished within 2.5 h.

Although in our preliminary experiments we designed primer pairs targeting 16S rDNA for a real-time PCR assay, they were not sufficiently specific (data not shown). We therefore designed new primer pairs targeting *spo0A*, which is widely distributed in spore-forming bacteria. There are well-conserved regions and variable regions in *spo0A* (Wunderlin, Junier, Roussel-Delif, Jeanneret, & Junier, 2013). Kuisiene, Raugalas, and Chitavichius (2009) evaluated the variability of *spo0A* in the genus *Geobacillus* and the applicability of this gene for taxonomy within this genus. They reported that *G. stearothermophilus* could be distinguished within the genus *Geobacillus* by interspecific sequence analysis of *spo0A*. This led us to expect that *spo0A* could be used as a marker capable of specifically and separately quantifying *A. flavithermus* and *G. stearothermophilus*.

The annealing sites of our primer pairs are at positions 22–28 (forward primer) and 60–65 (reverse primer) in the primer pair AF-F1/AF-R1, and positions 125–130 (forward primer) and 165–170 (reverse primer) in GS-F2/GS-R2, referring to the numbering of the amino acid sequence of *spo0A* of *G. stearothermophilus* isolate A (GenBank: accession number AY672766.1) (Rueckert et al., 2006). Unlike the method of Rueckert et al. (2006), which amplifies DNA from *Bacillus* spp., our primer pairs can specifically amplify DNA from *A. flavithermus* or *G. stearothermophilus*, respectively, thereby making it possible to separately quantify these major thermophilic spore-forming bacteria in dairy products.

Regarding DNA extraction from cream, there are reported extraction methods using SDS or CTAB (cetyltrimethylammonium bromide) (Pirondini et al., 2010; Poms, Glössl, & Foissy, 2001). However, both methods include phenol/chloroform treatment and ethanol precipitation to obtain high-purity DNA, which complicate the operation and increase the time required. For our method, we found that proteinase K treatment was simple, and it efficiently removed amplification-inhibiting substances.

Large amounts of DNA from dead cells are expected in dairy products. Hence, we used EMA to inactivate DNA from dead cells so as to quantify only spores. Although inactivation by EMA was sufficient, it took some time for the treatment procedure. There is, however, a method other than EMA that use DNase I or PMA. In our preliminary experiments, we found that inactivation by DNase I was not sufficient. Further investigations are needed to shorten the time required for this assay and to optimise the conditions for the use of these reagents.

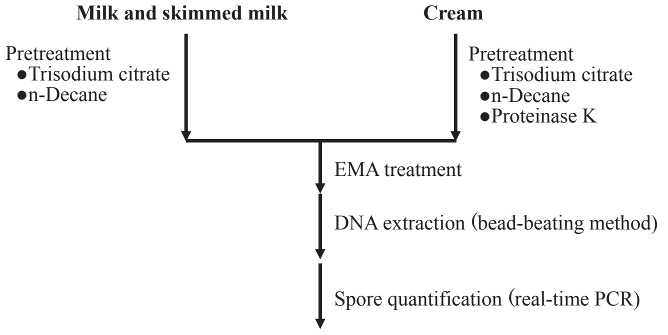

Fig. 2. Flow diagram of our method for the practical quantification of only spores in milk, skimmed milk, and cream. EMA, ethidium monoazide.

5. Conclusions

In this study, we successfully developed a practical real-time PCR assay to quantify only the spores of *A. flavithermus* and *G. stearothermophilus*, separately, in dairy products by the application of EMA treatment. In dairy products with high protein content, treatment with proteinase K enabled precise quantification of spores. This assay provides a means to investigate sporulation of *A. flavithermus* and *G. stearothermophilus* on dairy processing lines and is expected to be applied for hygiene management in dairy processing plants.

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References

Brown, D. P., Ganova-Raeva, L., Green, B. D., Wilkinson, S. R., Young, M., & Youngman, P. (1994). Characterization of spo0A homologues in diverse *Bacillus* and *Clostridium* species identifies a probable DNA-binding domain. *Molecular Microbiology, 14*, 411–426.

Burgess, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M., & Flint, S. H. (2009). The formation of spores in biofilms of *Anoxybacillus flavithermus*. *Journal of Applied Microbiology, 107*, 1012–1018.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology, 144*, 215–225.

Flint, S., Palmer, J., Bloemen, K., Brooks, J., & Crawford, R. (2001a). The growth of *Bacillus stearothermophilus* on stainless steel. *Journal of Applied Microbiology, 90*, 151–157.

Flint, S. H., Ward, L. J. H., & Walker, K. M. R. (2001b). Functional grouping of thermophilic *Bacillus* strains using amplification profiles of the 16S–23S internal spacer region. *Systematic & Applied Microbiology, 24*, 539–548.

Hashizume, S., Sekiguchi, T., & Nosoh, Y. (1976). Effect of temperature on the viability of *Bacillus stearothermophilus*. *Archives of Microbiology, 107*, 75–80.

Kuisiene, N., Raugalas, J., & Chitavichius, D. (2009). Phylogenetic, inter, and intraspecific sequence analysis of *spo0A* gene of the genus *Geobacillus*. *Current Microbiology, 58*, 547–553.

McGuiggan, J. T. M., McCleery, D. R., Hannan, A., & Gilmour, A. (2002). Aerobic spore-forming bacteria in bulk raw milk: Factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. *International Journal of Dairy Technology, 55*, 100–107.

McKillip, J. L., & Drake, M. (2004). Real-time nucleic acid-based detection methods for pathogenic bacteria in food. *Journal of Food Protection, 67*, 823–832.

Murphy, P. M., Lynch, D., & Kelly, P. M. (1999). Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. *International Journal of Dairy Technology, 52*, 45–50.

Pan, Y., & Breidt, F., Jr. (2007). Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. *Applied and Environmental Microbiology, 73*, 8028–8031.

Pirondini, A., Bonas, U., Maestri, E., Visioli, G., Marmiroli, M., & Marmiroli, N. (2010). Yield and amplificability of different DNA extraction procedures for traceability in the dairy food chain. *Food Control, 21*, 663–668.

Poms, R. E., Glössl, J., & Foissy, H. (2001). Increased sensitivity for detection of specific target DNA in milk by concentration in milk fat. *European Food Research and Technology, 213*, 361–365.

Rawsthorne, H., Dock, C. N., & Jaykus, L. A. (2009). PCR-based method using propidium monoazide to distinguish viable from nonviable *Bacillus subtilis* spores. *Applied and Environmental Microbiology, 75*, 2936–2939.

Ronimus, R. S., Parker, L. E., Turner, N., Poudel, S., Rückert, A., & Morgan, H. W. (2003). A RAPD-based comparison of thermophilic bacilli from milk powders. *International Journal of Food Microbiology, 85*, 45–61.

Rueckert, A., Ronimus, R. S., & Morgan, H. W. (2005a). Development of a rapid detection and enumeration method for thermophilic bacilli in milk powders. *Journal of Microbiological Methods, 60*, 155–167.

Rueckert, A., Ronimus, R. S., & Morgan, H. W. (2005b). Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus*. *Journal of Applied Microbiology, 99*, 1246–1255.

Rueckert, A., Ronimus, R. S., & Morgan, H. W. (2006). Development of a real-time PCR assay targeting the sporulation gene, *spo0A*, for the enumeration of thermophilic bacilli in milk powder. *Food Microbiology, 23*, 220–230.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology, 60*, 109–117.

Straub, J. A., Hertel, C., & Hammes, W. P. (1999). A 23S rDNA-targeted polymerase chain reaction–based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. *Journal of Food Protection, 62*, 1150–1156.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology, 63*, 3741–3751.

Wunderlin, T., Junier, T., Roussel-Delif, L., Jeanneret, N., & Junier, P. (2013). Stage 0 sporulation gene A as a molecular marker to study diversity of endospore-forming Firmicutes. *Environmental Microbiology Reports, 5*, 911–924.

Zhao, Y., Caspers, M. P. M., Metselaar, K. I., de Boer, P., Roeselers, G., Moezelaar, R., et al. (2013). Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. *Applied and Environmental Microbiology, 79*, 5652–5660.