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The detection of viable vegetative cells of *Bacillus sporothermodurans* using propidium monoazide with semi-nested PCR

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ABSTRACT

Bacillus sporothermodurans produces highly heat-resistant spores that can survive ultra-high temperature (UHT) treatment in milk. Therefore, we developed a rapid, specific and sensitive semi-nested touchdown PCR assay combined with propidium monoazide (PMA) treatment for the detection of viable *B. sporothermodurans* vegetative cells. The semi-nested touchdown PCR alone proved to be specific for *B. sporothermodurans*, and the achieved detection limit was 4 CFU/mL from bacterial culture and artificially contaminated UHT milk. This method combined with PMA treatment was shown to amplify DNA specifically from viable cells and presented a detection limit of 10^2 CFU/mL in UHT milk. The developed PMA-PCR assay shows applicability for the specific detection of viable cells of *B. sporothermodurans* from UHT milk. This method is of special significance for applications in the food industry by reducing the time required for the analysis of milk and dairy products for the presence of this microorganism.

1. Introduction

Bacillus sporothermodurans is a Gram positive, aerobic and mesophilic bacterium, which is characterized by the production of spores that are highly heat-resistant and capable of surviving industrial ultra-high temperature (UHT) milk processing (140 °C for 4 s) (Hammer et al., 1995; Pettersson et al., 1996; Scheldeman et al., 2006). Moreover, the spores of *B. sporothermodurans* can germinate and grow up to 10^5 CFU/mL in stored UHT milk, reaching concentrations that are above the maximum allowable thresholds for mesophilic bacteria (Pettersson et al., 1996; Klijn et al., 1997; Herman et al., 1998), which can cause product instability and therefore reduce both shelf life and acceptability to consumers (Tabit and Buys, 2010). However, an increase in temperature and holding time in an attempt to inactivate *B. sporothermodurans* spores can affect the organoleptic and nutritional qualities of UHT products (Claeys et al., 2001).

The high resistance of *B. sporothermodurans* to the heat treatments used in the processing of dairy products underscores the importance of its accurate detection. Phenotypic tests for the identification of this microorganism can be complex and laborious. Additionally, the highly competitive microbiota encountered in milk further increases the difficulties encountered in isolating *B. sporothermodurans* with high sensitivity, specificity and in a short period of time. Therefore, end-point and real time PCR targeting 16S rDNA have been developed to detect *B. sporothermodurans* (Scheldeman et al., 2002; Tabit and Buys, 2011). However, these molecular assays cannot discriminate between DNA from viable and dead *B. sporothermodurans*, which can lead to false-positive results as well as to the overestimation of cell numbers when evaluating food products (Josephson et al., 1993; Nogva et al., 2003). A suggested approach to address this problem is to block the availability of DNA originating from dead cells for PCR amplification, which can be achieved by using DNA-intercalating dyes, such as propidium monoazide (PMA). PMA intercalates into DNA by a covalent linkage induced by light exposure (Nocker et al., 2006). As PMA only penetrates membrane-damaged cells, it has been widely used as an indicator of viability in a variety of bacteria, protozoa, virus and fungi, including pathogenic, environmental and food strains (Nocker et al., 2006, 2007; Cawthorn and Witthuhn, 2008; Vesper et al., 2008; Bae and Wuertz, 2009; Brescia et al., 2009; Josefsen et al., 2010; Taskin et al., 2011; Yáñez et al., 2011; Mamlouk et al., 2012).

In this context, the aim of this study was to develop a specific and sensitive PCR-based method coupled to PMA treatment in order to detect only viable *B. sporothermodurans* vegetative cells in the presence of dead cells from bacterial cultures and milk.

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2. Materials and methods

2.1. Bacterial strains, media and culture conditions

B. sporothermodurans CBMAI 148 and CBMAI 155, obtained from the Brazilian Collection of Microorganisms Environment and Industry UNICAMP-CPQBA, and *Bacillus cereus* ATCC 33018, derived from the American Type Culture Collection, were cultivated in BHI broth (Brain Heart Infusion) (Merck, Darmstadt, Germany) at 37 °C for 24 h. *Bacillus acidicola* (NRRL B-23453), *Bacillus lentus* (NRRL NRS-1262), *Bacillus firmus* (NRRL B-14307), *Bacillus circulans* (NRRL B-378), *Bacillus coagulans* (NRRL NRS-609), *Geobacillus stearothermophilus* (NRRL B-11720) and *Geobacillus kaustophilus* (NRRL NRS-81), provided by the United States Department of Agriculture (USDA), were cultivated in TGY broth (5 g tryptone (Himedia, Mumbai, India), 5 g yeast extract (Himedia), 1 g glucose (Vetec, Rio de Janeiro, Brazil) and 1 g K_2HPO_4 (Vetec) in 1 L dH_2O).

2.2. DNA extraction

Bacterial genomic DNA from bacterial culture or milk was extracted as described by Rademaker and de Bruijn (1997). DNA was eluted in a final volume of 50 μL MilliQ water, and its concentration was determined using a fluorometer (Invitrogen, Van Allen Way Carlsbad, USA) according to the manufacturer's specifications.

2.3. Semi-nested PCR assays

2.3.1. DNA amplification

In the first stage, the *B. sporothermodurans* primers BSPO-F2 and BSPO-R2 were used to amplify a 664 bp fragment of the *B. sporothermodurans* 16S rRNA gene by PCR (Scheldeman et al., 2002). PCR was performed in a total volume of 25 μL containing 1.5 U *Taq* DNA polymerase (Invitrogen, São Paulo, Brazil), 1× PCR buffer (Fermentas Life Sciences, Germany), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (Fermentas Life Sciences, Germany), 0.8 μM of each primer (Invitrogen) and 1 μL of genomic DNA. Amplifications were carried out in a Thermocycler (MiniCyclerTM, MJ Research-Watertown, MA, USA) using the following conditions: initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 15 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 8 min. To differentiate *B. sporothermodurans* from *B. acidicola*, semi-nested touchdown PCR was performed using 1 μL of the first amplification product in the same reaction mix. However, BSPO-F2 primer was replaced by the forward internal primer designed in this study (5′AGAAGAGCGGAATTCCAC3′), which shows a C as the last 3′ nucleotide, representing the only nucleotide different from *B. acidicola* in this fragment, according to the sequences deposited in GenBank (ID: EU231617.1; ID: AF329476.1; ID: 49080.1).

The semi-nested touchdown PCR produced a 613 bp fragment using the following conditions: initial denaturation at 95 °C for 5 min, followed by 5 cycles consisting of denaturation at 95 °C for 15 s, annealing at 67 °C for 15 s, and extension at 72 °C for 30 s, 10 cycles carried out under the same conditions (except for the annealing temperature at 66 °C), and 20 cycles with annealing at 65 °C, followed by a final extension at 72 °C for 8 min. Amplification products were checked by agarose gel electrophoresis (1% w/v), 7.5 × 10 cm (*W* × L) gel size, in 0.5 × TBE buffer, at a constant voltage of 100 V for 45 min; stained with 0.5 μg/mL ethidium bromide (Ludwig Biotecnologia) using 2 μL of 100 bp ladder (Ludwig Biotecnologia) as the molecular mass ladder; and visualized under ultraviolet light using a Gel Doc L-Pix image system (Loccus Biotecnologia, Brazil).

The amplification product obtained from the *B. sporothermodurans* CBMAI 148 and CBMAI 155 was purified with PEG 8000 (USB, Cleveland, OH-USA) or with MicroSpin™ S-400 HR Columns (Amershan Biosciences, Piscataway, N.J.), and then submitted to nucleotide sequencing in an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Lincoln Centre Drive Foster City, USA) automated DNA sequencer. All *Bacillus* and *Geobacillus* species described above were used to evaluate the specificity of this method.

2.3.2. Determination of detection limit

The detection limit of the semi-nested touchdown PCR was determined using a *B. sporothermodurans* strain CBMAI 148 overnight culture of known concentration (4.0×10^7 CFU/mL). Ten-fold dilutions of the original culture were prepared in 0.1% peptone saline and commercial UHT milk. A 1 mL aliquot of each dilution of saline and artificially contaminated milk, in quadruplicate, was subjected to DNA extraction. The CFU/mL number of the dilutions was determined using the standard plate count method.

2.3.3. Detection of B. sporothermodurans in commercial UHT milk

The applicability of the semi-nested touchdown PCR was tested in ten samples of UHT milk from different brands commercialized in the state of Rio Grande do Sul (Southern Brazil). The procedure for the enumeration of total viable microorganisms in liquid UHT dairy products followed the method of the Normative Instruction Nr. 62 of the Ministry of Agriculture of Brazil (MAPA) (Brasil, 2003). Briefly, samples of UHT milk were incubated at 37 °C for 7 days to observe visible changes, such as bloating and casting coagulation. Then, 1 mL aliquots of UHT milk and two decimal dilutions (10^{-1} and 10^{-2}) were spread on brain heart infusion agar and nutrient agar (yeast extract-free) in duplicate. All plates were incubated at 30 °C for 72 h for colony count. Two 1 mL aliquots of each UHT milk sample were submitted to DNA extraction.

2.4. Discrimination of viable and dead B. sporothermodurans

2.4.1. Inactivation treatments

Two strategies were evaluated to kill *B. sporothermodurans* cells: (i) *Heat treatment* – microtubes containing 500 μL of overnight grown cultures ($\sim 10^7$ CFU/mL) were heated at 100 °C in a water bath for 30 min. (ii) *Isopropanol treatment* – cells were killed by adding 1 mL of isopropanol (F. Maia, São Paulo, Brazil) to 500 μL of overnight grown cultures followed by incubation for 30 min at room temperature. The isopropanol was removed by harvesting the cells using centrifugation at 5000 × *g* for 5 min and removing the supernatant. Pellets of killed cells were resuspended in 500 μL of BHI broth (Merck). The viabilities of the cells treated with both strategies were confirmed by plating on BHI agar.

2.4.2. PMA treatment

PMA (Biotium Inc., Hayward, California) was dissolved in 20% dimethyl sulfoxide (DMSO) (Nuclear, São Paulo, Brazil), and added to 500 μL of *B. sporothermodurans* cell suspension (viable and dead cells) at a concentration of approximately 10^7 cells/mL, to achieve final concentrations of 2, 5, 10, 20, and 30 μg/mL. After 10 min of incubation in the dark with occasional mixing, the samples were exposed to light for 10 min at a 15 cm distance using a 500 W halogen light source (Osram, São Paulo, Brazil). After photo-activation, the samples were centrifuged at 6000 × *g* for 10 min prior to DNA extraction.

The effectiveness of the PMA treatment combined with semi-nested touchdown PCR was further evaluated with mixtures containing different concentrations of viable and isopropanol-killed *B. sporothermodurans* cells. The mixtures were prepared at pre-defined ratios of 0, 25, 50, 75 and 100% viable cells. For example, the 100% viable cell mixtures, named 100%, consisted of 500 μL of viable cells (10^7 CFU/mL), while the mixture named 25% was prepared by mixing 125 μL of viable cells with 375 μL of dead cells (isopropanol-killed).

The band intensities were quantified and normalized using the band detection and analysis tools of Quantity One 4.6.3 software (BioRad Laboratories) according to the manufacturer's guidelines. The differences in band intensities between groups were analyzed with Student's *t*-test using IBM® SPSS® Statistics (version 2.0). A *p*-value of less than 0.05 was considered statistically significant.

2.4.3. Application of PMA associated to PCR in milk

Commercial UHT milk was purchased from a local supermarket. An aliquot of 500 μL of each suspension (viable and dead cells) was diluted in commercial UHT milk to achieve final concentrations ranging from 10^7 to 10^1 CFU/mL. The estimated number of CFU/mL was determined by plating three 100 μL aliquots of the 10^{-5}, 10^{-6} and 10^{-7} dilutions onto BHI agar followed by incubation for 24 h at 37 °C. Two aliquots of each dilution were removed and one was submitted to DNA extraction without prior PMA treatment, and the other was treated with PMA prior to the DNA extraction.

3. Results and discussion

An initial attempt to identify *B. sporothermodurans* cells by PCR amplification of the 16S rDNA was performed based on the method described by Scheldeman et al. (2002). Unfortunately, the tested conditions produced amplification products for five other *Bacillus* species (Fig. 1A). In this context, a semi-nested touchdown PCR method was successfully developed to detect only *B. sporothermodurans* 16S rDNA using the same primers and an additional internal primer, as shown in Fig. 1B. The fragments amplified from *B. sporothermodurans* CBMAI 148 and CBMAI 155 were submitted to automated sequencing, and the sequences were deposited in the GenBank database (GenBank ID: GU238287 and JX569192). Sequence alignment analysis showed 100% identity with the nucleotide sequence of *B. sporothermodurans* strain LMG 17897 (GenBank ID: AJ302941.1), two nucleotide alterations when compared with *B. sporothermodurans* strain LMG 17883 (GenBank ID: AJ302942.1), and one different nucleotide when compared with *B. acidicola* strain TCCC27037 (GenBank ID: EU231617.1) (see Supplementary data). The sequence comparison ensured that specific detection of *B. sporothermodurans* was obtained and, as expected, a high sequence identity was present even when comparing geographically distant strains. However, although the *B. acidicola* sequence also presented a high identity when compared to *B. sporothermodurans* sequences, the semi-nested touchdown PCR design ensured specific detection of *B. sporothermodurans*. The sensitivity of the developed semi-nested touchdown PCR method was evaluated using bacterial culture (Fig. 2A) and artificially contaminated UHT milk (Fig. 2B), and a detection limit of 4.0 CFU/mL of *B. sporothermodurans* was found from both sources. Therefore, the PCR-based method developed here was shown to be highly specific and sensitive to detect *B. sporothermodurans* vegetative cells, even in the presence of milk components, which are usually considered PCR inhibitors (Rossen et al., 1992; Bickley et al., 1996). In addition, this method was shown to be considerably less time-consuming than the classic procedures used for *B. sporothermodurans* detection.

Although PCR-based methods can be sensitive, specific and applicable to food matrices, they do not distinguish between DNA from viable and dead cells. To overcome this limitation, treatment of samples with PMA prior to DNA extraction has been used to evaluate the cellular viability of many different bacteria (Nocker et al., 2006, 2007; Cawthorn and Witthuhn, 2008; Bae and Wuertz, 2009; Josefsen et al., 2010; Taskin et al., 2011; Yáñez et al., 2011; Elizaquível et al., 2012; Mamlouk et al., 2012). Because, to the best of our knowledge, no previous reports have described PMA treatment protocols for *B. sporothermodurans*, experimental conditions were tested that involving bacterial-killing strategies, PMA concentrations, and mixed viable and dead proportions of cells in order to determine the optimal conditions for application to the semi-nested PCR method.

Two killing strategies were tested (heat and isopropanol treatments) and provided similar results (Fig. 3), and isopropanol treatment was used throughout the remaining experiments due to its ease of use. Possible PMA interference in the PCR amplification of DNA from viable cells was initially evaluated and no significant

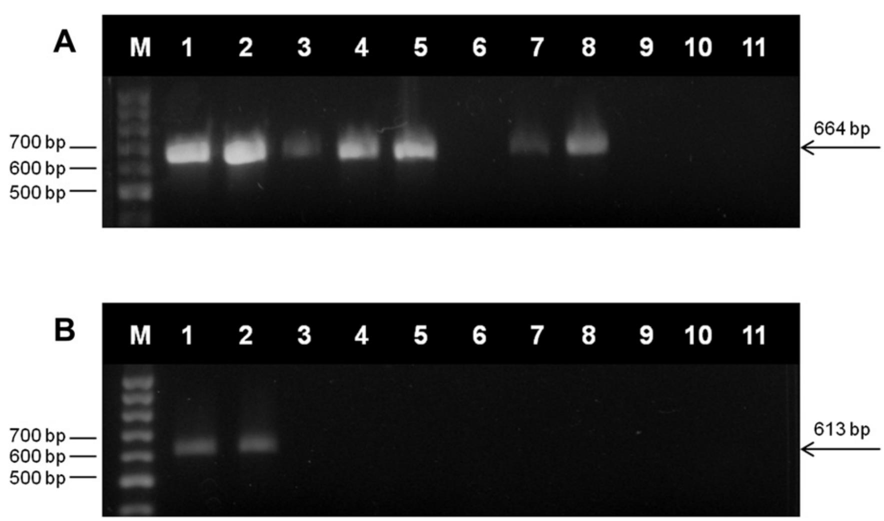


Fig. 1. (A) Detection of *Bacillus sporothermodurans* by PCR. Amplicons originated from genomic DNA of the following: (lane 1) *B. sporothermodurans* CBMAI 148; (lane 2) *B. sporothermodurans* CBMAI 155; (lane 3) *Bacillus cereus*; (lane 4) *Bacillus acidicola*; (lane 5) *Bacillus lentus*; (lane 6) *Bacillus firmus*; (lane 7) *Bacillus circulans*; (lane 8) *Bacillus coagulans*; (lane 9) *Geobacillus stearothermophilus*; (lane 10) *Geobacillus kaustophilus*; and (lane 11) negative control (without template DNA); (M) 100 bp DNA ladder. (B) Detection of *B. sporothermodurans* by semi-nested touchdown PCR. The 613 bp PCR products amplified from genomic DNA of the following: (lane 1) *B. sporothermodurans* CBMAI 148; (lane 2) *B. sporothermodurans* CBMAI 155; (lane 3) *B. cereus*; (lane 4) *B. acidicola*; (lane 5) *B. lentus*; (lane 6) *B. firmus*; (lane 7) *B. circulans*; (lane 8) *B. coagulans*; (lane 9) *G. stearothermophilus*; (lane 10) *G. kaustophilus*; and (lane 11) negative control (without template DNA); (M) 100 bp DNA ladder.

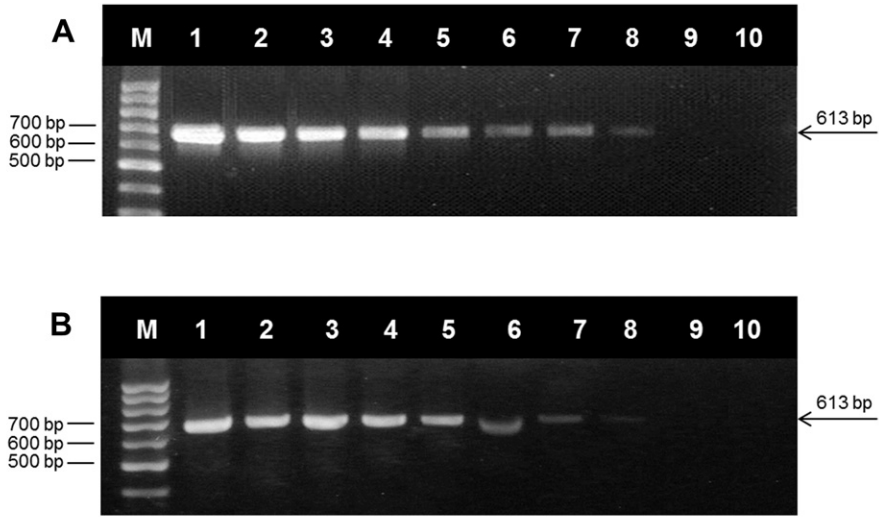


Fig. 2. Detection limit of *Bacillus sporothermodurans* by semi-nested touchdown PCR. Amplifications were performed from genomic DNA of (lane 1) *B. sporothermodurans* CBMAI 148 culture (4.0×10^7 CFU/mL); (lanes 2–9) ten-fold dilutions of a *B. sporothermodurans* CBMAI 148 culture (4.0×10^7 CFU/mL) until 10^0 CFU/mL in 1% peptone saline (A) and UHT milk (B); and (lane 10) negative control (without template DNA); (M) 100 bp DNA ladder.

difference could be found in comparison to the controls at all concentrations used (Fig. 3, lanes 4, 7, 10, 13 and 16; $p > 0.05$). However, the amplification of DNA from PMA-treated dead cells (Fig. 3, lanes 5, 6, 8, 9, 11, 12, 14, 15, 17 and 18) was reduced with increasing PMA concentrations and was completely inhibited at 30 μg/mL. PMA treatment at 30 μg/mL produced the same results when mixing viable and dead *B. sporothermodurans* cells at different ratios prior to DNA extraction (Fig. 4A) because a significant decrease in the band intensity ($p < 0.05$) was found to correlate with the increase in the proportion of dead cells (Fig. 4B), although the total amount of DNA in the reactions remained the same. Additionally, these results ensure that the amplification of DNA from viable cells is not affected by different concentrations of background dead cells in the presence of PMA. This finding is very important for the potential applicability of the method developed here because raw milk may contain up to 10^7 CFU of bacteria per mL (Arenas et al., 2004; Chye et al., 2004; Torkar and Teger, 2008; Tabit and Buys, 2011), and a considerable portion most likely dies during thermal processing. Thus, the optimization of the PMA protocol to this bacterial density makes this method applicable not only to UHT milk analyses but also to other food matrices with high bacterial loads. Regarding the proportion of PMA used, it was expected that a high PMA concentration would be required to inhibit the amplification signal because high concentrations of cells have been suggested to inhibit the crosslinking step when PMA is light activated (Løvdal et al., 2011). However, the optimized PMA concentration was not as high as those reported by other authors who used high bacterial densities in their experiments (Cawthorn and Witthuhn, 2008; Bae and Wuertz, 2009; Chen et al., 2011; Taskin et al., 2011).

Another important point is the use of PMA as viability marker because its use is based on the loss of membrane integrity, which can be considered a conservative viability criterion when analyzing heat treated samples (Contreras et al., 2011). In this regard, Yang et al. (2011) has reported that cells killed by heating to ≤72 °C may not allow PMA penetration, which can limit the use of PMA-PCR for the analysis of some heat treated samples. Therefore, during the design of PMA-PCR based procedures for the analysis of heat treated food, especially when targeting milk contaminants, the fact that the pasteurization temperature may not exceed 72 °C in some instances must be considered. However, PMA can be considered a successful viability marker to detect microorganisms in food treated at high temperatures, such as UHT milk, as well as food exposed to treatments directly targeting membranes. The protocol developed here tested the applicability of this method in milk and it was observed that after addition of PMA in the milk samples, this dye reduced the intensity of false-positive signals (Fig. 5, lanes 6–10), and its effect was not inhibited by milk components. The

	Control			PMA														
Viable: dead cells	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100
Viability[1]	V	H	I	V	H	I	V	H	I	V	H	I	V	H	I	V	H	I
PMA (μg/mL)	0	0	0	2	2	2	5	5	5	10	10	10	20	20	20	30	30	30
Lane	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)

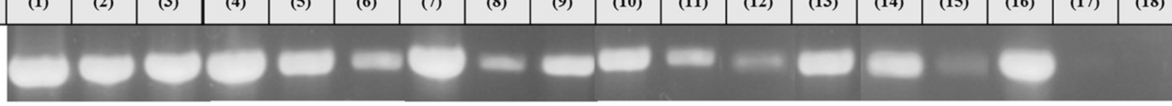
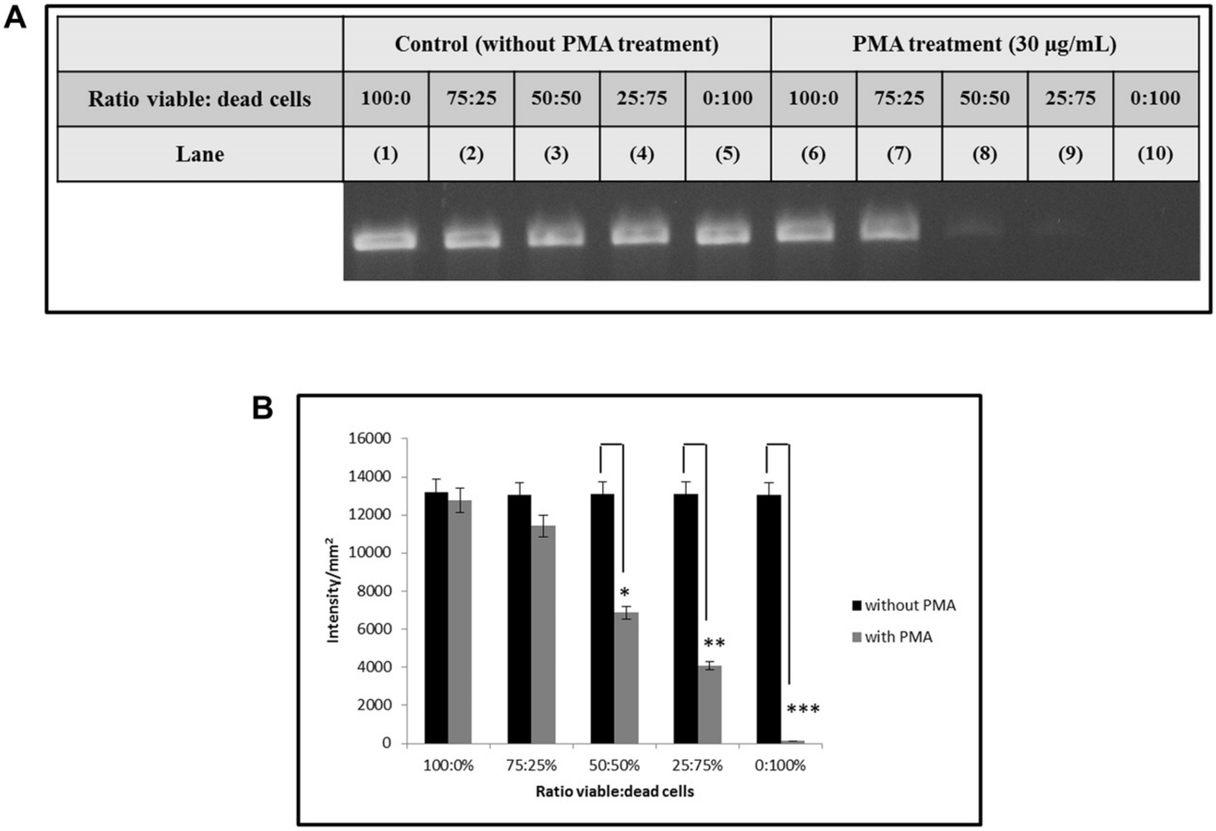


Fig. 3. The effect of different concentrations of propidium monoazide (PMA) for the detection of viable and dead (heat- or isopropanol-killed) *Bacillus sporothermodurans* CBMAI 148 cells by PCR. Lanes 1–3: control samples, without PMA treatment; lanes 4–6: 2 μg/mL PMA; lanes 7–9: 5 μg/mL PMA; lanes 10–12: 10 μg/mL PMA; lanes 13–15: 20 μg/mL PMA; and lanes 15–18: 30 μg/mL PMA; [1]V = viable, H = heat-killed and I = isopropanol-killed.

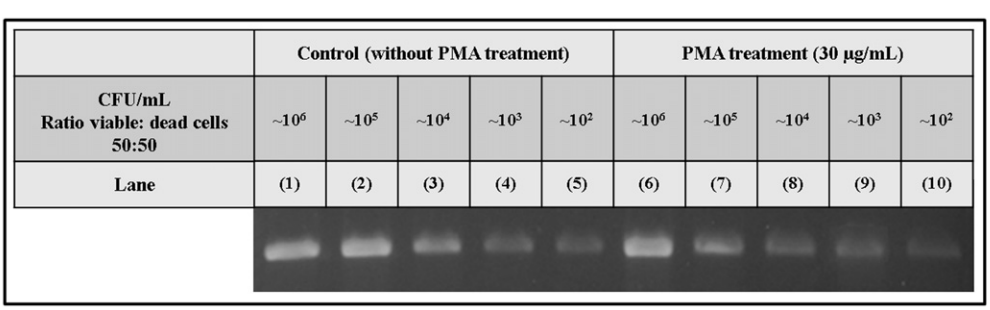


	Control (without PMA treatment)					PMA treatment (30 µg/mL)				
Ratio viable: dead cells	100:0	75:25	50:50	25:75	0:100	100:0	75:25	50:50	25:75	0:100
Lane	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)

Fig. 4. The effect of propidium monoazide (PMA) treatment for the detection by PCR of viable and isopropanol-killed *Bacillus sporothermodurans* CBMAI 148 cells mixed at different ratios. (A) PCR products visualized on agarose gel stained with 0.5 µg/mL ethidium bromide under UV light. (B) Diagram representing the band intensities from amplicons generated with or without PMA treatment using different ratios of viable:dead cells. $^{*}p < 0.001$; $^{**}p < 0.005$; $^{***}p < 0.001$.

limit of detection of PMA treatment associated with semi-nested touchdown PCR method in UHT milk was determined to be 10^2 CFU/mL of viable cells, which is lower than or similar to those described in other studies that have applied PMA-PCR assays to food analysis. For example, a detection limit of 10^2 CFU/mL for *Campylobacter jejuni* (Josefsen et al., 2010) and *Brochothrix thermosphacta* (Mamlouk et al., 2012) was found in chicken carcass rinse and fresh salmon, respectively, while 10^3 CFU/g was described as the limit for *Salmonella* Typhimurium in lettuce (Liang et al., 2011). The difference in sensibility of the PCR method with or without the PMA pre-treatment can result from the loss of cells during the additional PMA step and/or to the possible presence of *B. sporothermodurans* DNA in the late exponential phase cultures used to determine the PCR detection limit, possibly originating from cells that died during the bacterial growth phase. Additionally, the PMA-PCR method detection limit of 10^2 CFU/mL for *B. sporothermodurans* alone meets the criteria of the European Union (EU) and Brazilian legislation for the maximum count of mesophilic microorganisms in UHT milk (Anonymous, 1992; Brasil, 1997).

In conclusion, the new molecular assay developed here for the rapid, sensitive and specific detection of viable *B. sporothermodurans* cells could be a very useful tool for the early identification of undesirable *B. sporothermodurans* vegetative cells in milk, dairy products and additional food matrices. Thus, the application of this method could be of great value for the quality control of food products by monitoring the level of viable *B. sporothermodurans* during manufacture or storage and significantly reducing economic losses to the industry.



	Control (without PMA treatment)					PMA treatment (30 µg/mL)				
CFU/mL Ratio viable: dead cells 50:50	~10^6	~10^5	~10^4	~10^3	~10^2	~10^6	~10^5	~10^4	~10^3	~10^2
Lane	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)

Fig. 5. Detection limit of the PMA-PCR to detect viable and isopropanol-killed *Bacillus sporothermodurans* cells in artificially contaminated milk. The ratio between viable and dead cells is 50:50.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.fm.2012.12.007.

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