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# Detection and quantification of viable *Bacillus cereus* group species in milk by propidium monoazide quantitative real-time PCR

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## ABSTRACT

The *Bacillus cereus* group includes important spore-forming bacteria that present spoilage capability and may cause foodborne diseases. These microorganisms are traditionally evaluated in food using culturing methods, which can be laborious and time-consuming, and may also fail to detect bacteria in a viable but nonculturable state. The purpose of this study was to develop a quantitative real-time PCR (qPCR) combined with a propidium monoazide (PMA) treatment to analyze the contamination of UHT milk by *B. cereus* group species viable cells. Thirty micrograms per milliliter of PMA was shown to be the most effective concentration for reducing the PCR amplification of extracellular DNA and DNA from dead cells. The quantification limit of the PMA-qPCR assay was $7.5 \times 10^2$ cfu/mL of milk. One hundred thirty-five UHT milk samples were analyzed to evaluate the association of PMA to qPCR to selectively detect viable cells. The PMA-qPCR was able to detect *B. cereus* group species in 44 samples (32.6%), whereas qPCR without PMA detected 78 positive samples (57.8%). Therefore, the PMA probably inhibited the amplification of DNA from cells that were killed during UHT processing, which avoided an overestimation of bacterial cells when using qPCR and, thus, did not overvalue potential health risks. A culture-based method was also used to detect and quantify *B. cereus* sensu stricto in the same samples and showed positive results in 15 (11.1%) samples. The culture method and PMA-qPCR allowed the detection of *B. cereus* sensu stricto in quantities compatible with the infective dose required to cause foodborne disease in 3 samples, indicating that, depending on the storage conditions, even after UHT treatment, infective doses may be reached in ready-to-consume products.

**Key words:** *Bacillus cereus* group, viability, ultra-high temperature milk, real-time PCR

## INTRODUCTION

The *Bacillus cereus* group comprises spore-forming gram-positive bacteria that are ubiquitously found in the environment and has been isolated from a wide variety of foods (Fricker et al., 2007; Ankolekar et al., 2009), including raw, pasteurized, and UHT milk (Christiansson et al., 1999; Bartoszewicz et al., 2008; Banykó and Vyletelová, 2009; Batchoun et al., 2011). These microorganisms are undesired in the food industry due to its spoilage capability and pathogenic potential (Zhou et al., 2008; Ankolekar et al., 2009). The consumption of food contaminated with *B. cereus* sensu stricto has been implicated in outbreaks of emetic and diarrheal syndrome (Ehling-Schulz et al., 2004; EFSA, 2005; Stenfors Arnesen et al., 2008).

Traditionally, enumeration of *B. cereus* group cells from food samples is performed using culture on selective media and biochemical identification to obtain definitive results, which are laborious and time-consuming procedures (AOAC International, 1995; Brazil, 2003; ISO, 2004). Molecular-based techniques have extensively been applied to detect foodborne pathogens (Wang et al., 1997; Fricker et al., 2007; Ankolekar et al., 2009; Martínez-Blanch et al., 2009; Ceuppens et al., 2010; Rantsiou et al., 2010; Fernández-No et al., 2011; Oliwa-Stasiak et al., 2011; Rantsiou et al., 2012), and real-time PCR has been a reliable tool to detect and quantify *B. cereus* from pure culture (Fykse et al., 2003), gastrointestinal matrix (Ceuppens et al., 2010), and food (Martínez-Blanch et al., 2009; Fernández-No et al., 2011; Oliwa-Stasiak et al., 2011). Nevertheless, the major limitation of any PCR-based assay is its inability to distinguish between DNA from viable cells and free DNA, which are either available in the environment or originate from dead cells (Rudi et al., 2005). If DNA from nonviable cells is amplified, bacterial counts will be overestimated. Therefore, end-point and real-time PCR alone may not provide a reliable indication of human health risk levels posed by the presence of viable pathogens. To overcome this limitation, propidium monoazide (**PMA**) has been used as a nucleic acid-intercalating dye to inhibit PCR amplification of

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extracellular DNA and DNA from dead or membrane-compromised cells (Nocker et al., 2006, 2007; Cawthorn and Witthuhn, 2008; Josefsen et al., 2010; Liang et al., 2011; Yang et al., 2011; Mamlouk et al., 2012; Martinon et al., 2012; Barth et al., 2012; Cattani et al., 2013; Zhang et al., 2015). Recently, PMA was associated with an end-point PCR to detect emetic and nonemetic *B. cereus* (Zhang et al., 2014). However, to the best of our knowledge, the effectiveness of PMA combined with quantitative real-time PCR (**qPCR**) has not yet been evaluated for the *B. cereus* group, which would be of value from a food safety perspective. Therefore, we describe qPCR combined with PMA treatment for the detection and quantification of viable *B. cereus* group species, and its evaluation in naturally contaminated UHT milk samples.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Conditions

*Bacillus sporothermodurans* CBMAI 148 was obtained from the Brazilian Collection of Microorganisms from the Environment and Industry UNICAMP-CPQBA. *Bacillus acidicola* (NRRL B-23453), *B. lentus* (NRRL NRS-1262), *B. firmus* (NRRL B-14307), *B. circulans* (NRRL B-378) *B. coagulans* (NRRL NRS-609), *B. mycoides* (NRRL B-14811), *B. pseudomycoides* (NRRL B-617), and *B. thuringiensis* (NRRL B-23440 and BD-515) were provided by the USDA. All strains mentioned above were cultivated in brain heart infusion (**BHI**) broth (Merck, Darmstadt, Germany) at 37°C for 24 h.

### Optimization of PMA Conditions

The optimization of PMA conditions was performed using *B. cereus* ATCC 33019, which was grown overnight in BHI broth (Merck) at 37°C, corresponding to a concentration of $10^8$ cells/mL. Dead cells were obtained from 2 treatments: (i) heat, 500 μL of cell suspension was heated at 100°C in a water bath for 30 min; and (ii) isopropanol, cells were killed by adding 1 mL of isopropanol (F. Maia, São Paulo, Brazil) to 500 μL of a cell suspension followed by incubation for 30 min at room temperature. The isopropanol was removed by harvesting the cells using centrifugation at 5,000 × *g* for 5 min at room temperature and removing the supernatant. Pellets of killed cells were resuspended in 500 μL of BHI broth (Merck). The absence of viable cells from both strategies was confirmed by spread-plating 100-μL aliquots of cells on BHI agar.

The PMA (Biotium Inc., Hayward, CA) was dissolved in 20% dimethyl sulfoxide (Nuclear, São Paulo, Brazil) and added to 500 μL of *B. cereus* cell suspension (viable and dead cells) to achieve final concentrations of 2, 5, 10, 20, and 30 μg/mL. The tubes were then placed in the dark for 10 min to allow PMA to penetrate into dead cells and bind to the DNA. Afterward, tubes were exposed to a 500-W halogen light source (Osram, São Paulo, Brazil) at a 15-cm distance for 10 min. During exposure, the samples were placed on crushed ice to avoid excessive heating. Additionally, to determine the effectiveness of PMA in selectively amplifying DNA from viable cells, mixtures of viable and dead cells were evaluated. Mixtures were prepared so that viable cells corresponded to 100, 75, 50, 25, and 0% of the total bacterial cell concentration, using the same protocol for PMA treatment, before PCR amplification. To assess PMA toxicity and ensure its selectivity, viable cells were submitted to DNA extraction both with and without PMA treatment.

### DNA Extraction

Immediately after PMA treatment, bacterial genomic DNA from pure culture or milk was extracted as described by Rademaker and de Bruijn (1997). Briefly, bacterial cells were lysed with 500 μL of 5 *M* guanidine thiocyanate, 0.03 *M N*-lauroyl sarkosine, 0.1 *M* EDTA, at 4°C for 5 min. Afterward, 250 μL cold 7.5 *M* ammonium acetate was added, and tubes were gently shaken and incubated at 4°C for 5 min. An aliquot of 500 μL of chloroform/isoamylalcohol (24:1) was added and the mixture was vortexed vigorously. After centrifugation at 16,000 × *g* for 10 min at room temperature, the pellet was further washed with isopropyl alcohol. The extracted DNA was resuspended in a final volume of 50 μL of milli-Q water.

### End-Point PCR

The optimization of PMA treatment was performed via end-point PCR using oligonucleotides targeting a hemolysin gene (Wang et al., 1997). Polymerase chain reaction was performed in a solution containing 1.5 U of *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany), 1× PCR buffer (Fermentas), 3 m*M* $MgCl_2$, 0.2 m*M* of each deoxynucleoside triphosphate (Fermentas), 0.8 μ*M* of each primer (Invitrogen, São Paulo, Brazil), and 1 μL of genomic DNA, in a final volume of 25 μL. The reactions were run on a Thermocycler (MiniCycler MJ Research, Watertown, MA) at 95°C for 3 min followed by 35 cycles at 95°C for 10 s, 56°C for 15 s, 72°C for 15 s, and 72°C for 3 min. The PCR products were visualized on 1% agarose gel (Invitrogen) in Tris-borate-EDTA buffer with ethidium bromide (0.5 μg/mL; Ludwig Biotecnologia Ltda, Porto Alegre, Brazil) under UV light.

The band intensity was determined using Quantity One 4.6.3 Software (BioRad Laboratories, Hercules, CA). The differences of band intensities between groups were analyzed by Student's *t*-test using IBM SPSS Statistics version 20 (Somers, NY). A $P$-value $< 0.05$ was considered statistically significant.

### Sample Preparation

A total of 135 UHT milk samples from 14 brands and all from different lots were purchased from local retail markets during the period between October 2011 and January 2012. All samples were incubated for 7 d at 36 ± 1°C to favor spore germination and analyzed in parallel using a standard microbiological method and qPCR. For the molecular analysis, two 500-μL aliquots were collected from each milk sample; one aliquot was treated with PMA (30 μg/mL) before DNA extraction, whereas the other was directly submitted to DNA extraction.

### Detection of B. cereus in Milk by Culturing

After incubation for 7 d, 25-mL aliquots of each milk sample were taken aseptically and then diluted 10-fold in 0.1% peptone saline (pH 7.0). Immediately after, all dilutions were spread on *B. cereus* selective agar base mannitol egg yolk polymyxin B (Himedia, Mumbai, India) in duplicate and incubated at 30°C for 24 to 48 h. Colonies surrounded by a precipitate zone were selected, transferred onto the stock agar, and submitted for confirmation by Gram staining and other phenotypic tests, which included motility, nitrate reduction, hemolytic activity on sheep blood agar, tyrosine decomposition, rhizoid growth, and the absence of crystal parasporal inclusion (Brazil, 2003). *Bacillus cereus* ATCC 33019 was used as a reference culture.

### Standard Calibration Curves

A standard curve was generated using 10-fold dilutions of plasmid DNA harboring the target gene by qPCR. The 185-bp fragment of the hemolysin gene was cloned into the pGEM-T Easy vector system (Promega, Madison, WI), according to the manufacturer's instructions. The recombinant vectors were used to transform Top10 *Escherichia coli* strain by heat shock (42°C/45 s) and were then spread on Luria Bertani agar with ampicillin (100 μg/mL) and incubated for 12 h at 37°C. Colonies were selected and inoculated in Luria Bertani broth with ampicillin (100 μg/mL) for 12 h at 37°C with shaking at 200 rpm. Plasmid DNA was purified using a PureYield Plasmid Miniprep kit (Promega), separated by electrophoresis in a 0.6% agarose gel, stained with ethidium bromide, and visualized under UV light. The corresponding band was isolated from the agarose gel and purified using a Quick gel extraction kit (Promega). The concentration of extracted plasmid DNA harboring the target insert was determined using fluorimetry (Qubit, Invitrogen). Subsequently, 10-fold serial dilutions of the extract were prepared, ranging from $10^6$ to $10^0$ plasmid copies per PCR. A standard curve was generated by plotting the DNA amount (mathematically adjusted to the supposed copies/mL) against the threshold cycle (**Ct**) value exported from the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA).

Another standard curve to analyze *B. cereus* contamination in milk was constructed by considering all steps required to perform the PMA-qPCR assay. Then, an overnight culture of *B. cereus* ATCC 33019 in BHI containing $7.5 \times 10^8$ cfu/mL was inoculated in 9 mL of milk. Ten-fold dilutions were prepared in UHT milk and subsequently treated with PMA in duplicate. The DNA from 500-μL aliquots of inoculated milk was extracted as described above. The standard curve was generated by plotting the DNA amount (expressed in the corresponding cell concentration in cfu/mL) against the Ct value exported from the equipment.

The determination of the quantification limit has taken into account that this hemolysin (a putative cereolysin O, a thiol-activated cytolysin from the family of perfringolysin O) gene is present as a single copy in the *B. cereus* strains (*B. cereus* ATCC 14579, NC_004722.1; *B. cereus* ATCC 10987, NC_003909.8; *B. cereus* NC 7401, NC_016771.1; *B. cereus* AH187, NC_011658.1; *B. cereus* AH820, NC_011773.1), as found in the BLAST analysis (http://blast.ncbi.nlm.nhi.gov) of the genomes deposited in the GenBank database (data not shown).

### qPCR

A TaqMan qPCR method able to detect and quantify species of *B. cereus* group based on the amplification of a 185-bp fragment of the hemolysin gene was performed on a StepOne Real-Time PCR System (Applied Biosystems). The primers used were the same as the end-point PCR primers, and the TaqMan MGB probe (5′-FAM-AGCTGTACAACTTGC-3′) was designed using the Primer Express 3.0 software from Applied Biosystems. The qPCR using the primers and the probe was optimized with the Path-ID qPCR Master Mix (Ambion, Life Technologies, Carlsbad, CA) in a final volume of 20 μL that contained 10 μL of 2× Path-ID qPCR Master Mix, 0.6 μ$M$ of each primer, 0.2 μ$M$ of probe, and 2 μL of template DNA. Amplifications were performed with 1 cycle at 95°C for 10 min, 40 cycles

at 95°C for 15 s and 58°C for 45 s. A negative control was always included by using 2 μL of Milli-Q water instead of the DNA template, and the plasmid DNA standard calibration curve was included. Reactions were performed in duplicate.

The specificity of the qPCR assay was tested against different species of *Bacillus*: *B. sporothermodurans*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. acidicola*, *B. lentus*, *B. firmus, B. circulans*, and *B. coagulans*.

### Statistical Analysis

To compare the *B. cereus* positive sample frequencies obtained by the PMA-qPCR and qPCR without PMA, the McNemar test was employed. One-way ANOVA, followed by Tukey's post hoc test, was used to evaluate the significant mean differences among PMA treatments in dead and viable cells. The data were analyzed using IBM SPSS Statistics version 20, and the level of significance ($\alpha$) was set to 0.05 for all tests.

## RESULTS AND DISCUSSION

In the present study, we report a useful PMA-qPCR assay to allow the detection and quantification of viable species of the *B. cereus* group, enabling the DNA-based method to evaluate the microbial load within foods, mainly those that are submitted to stressing conditions during processing and storage. First, end-point PCR was used to evaluate the efficiency of PMA to exclude the DNA from dead cells. The amplifications of DNA from PMA-treated dead cells gradually reduced with increasing concentrations of PMA ($P < 0.05$; Figure 1, lanes 5, 6, 8, 9, 11, 12, 14, 15, 17, and 18). Similar results were obtained using either heat or isopropanol as cell killing methods, and isopropanol treatment was used throughout the remainder of this study because of its ease of use. The PMA at 30 μg/mL was used in the remaining experiments, as this concentration was shown to completely inhibit the PCR amplification from dead cell DNA (Figure 1, lanes 17 and 18). In contrast, we detected specific amplifications from PMA-treated viable cell DNA, nontreated viable cells (Figure 1, lanes 1, 4, 7, 10, 13, and 16), and nontreated dead cells (Figure 1, lanes 2 and 3). Furthermore, we showed that PMA associated with PCR could specifically detect DNA from *B. cereus*-viable cells in the presence of DNA from dead cells, which can be observed from the direct relation between the relative intensity of the DNA bands ($P < 0.05$) and the percentage of viable cells in the range of cell mixtures (Figure 2). Therefore, PMA treatment effectively inhibited the PCR amplification of DNA from dead *B. cereus* cells without having an effect on the amplifications of DNA from viable cells. Viable cells did not interfere with the effect of PMA on dead cells, even in a background containing a high load of bacterial cells. These findings indicate the potential application of the PMA-PCR method to evaluate food exposed to treatments that injure bacterial cells, especially those treatments that interfere with membrane integrity. However, end-point PCR is not an ideal method to evaluate contamination in some foods because certain levels of *B. cereus* sensu stricto may be acceptable, which indicates the need for a quantitative method. We associated the standardized PMA treatment with qPCR to specifically quantify viable *B. cereus* cells. Evaluating the effect of PMA on qPCR-amplifiable DNA from viable or dead cells, the ΔCt (Ct value for PMA-treated cells − Ct values for untreated cells) values found were 0.83 and 14.93, respectively (Table 1), which indicates that PMA showed no significant interference in the detection of viable *B. cereus* using the qPCR assay. Although PMA is extensively more specific for live cells when compared with EMA (Nocker et al., 2006; Cawthorn and Witthuhn,

| | 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) |

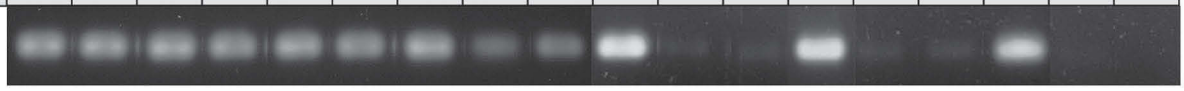

**Figure 1**. The effect of different concentrations of propidium monoazide (PMA) on the detection of viable and dead (heat- or isopropanol-killed) *Bacillus cereus* cells using PCR. Lanes 1 to 3: control samples, without PMA treatment; lanes 4 to 6: 2 μg/mL PMA; lanes 7 to 9: 5 μg/mL PMA; lanes 10 to 12: 10 μg/mL PMA; lanes 13 to 15: 20 μg/mL PMA; lanes 15 to 18: 30 μg/mL PMA; V = viable, H = heat-killed, and I = isopropanol-killed. See Supplemental Figure S1 (http://dx.doi.org/10.3168/jds.2015-10019) for corresponding agarose gels.

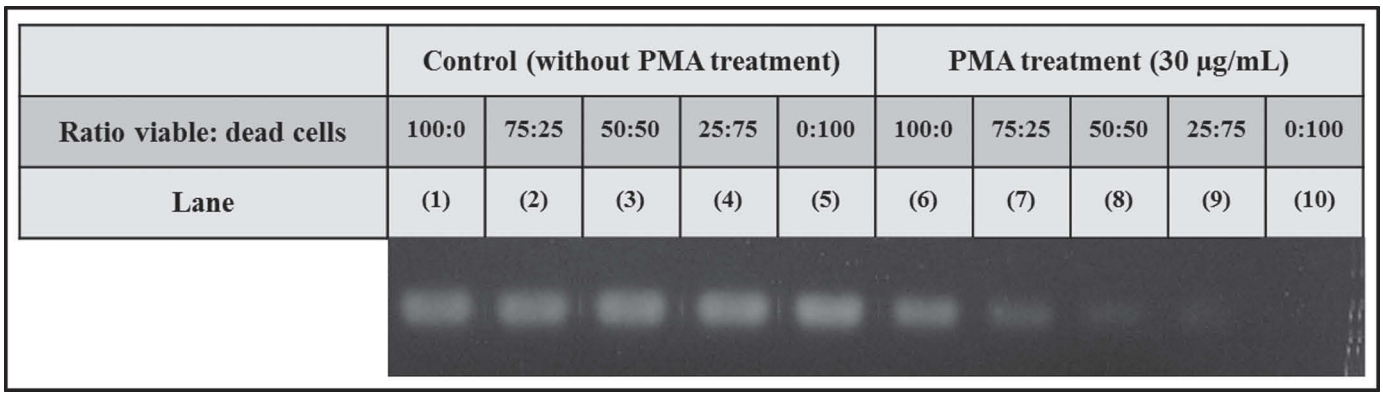


**Figure 2**. The effect of propidium monoazide (PMA) treatment on the PCR-based detection of viable and isopropanol-killed *Bacillus cereus* cells mixed at different ratios. See Supplemental Figure S2 (http://dx.doi.org/10.3168/jds.2015-10019) for corresponding agarose gels.

2008), a slight increase in Ct value was observed in viable cells treated with PMA, which possibly indicates the presence of some viable cells with compromised membrane due to a physiological status that can imply changes in membrane permeability, or can even be assigned to a small proportion of dead cells present in the viable cell culture (Yañez et al., 2011; Fittipaldi et al., 2012). Conversely, amplification signal was not completely suppressed (Ct value of 36.45) when dead cells were treated with 30 µg/mL PMA, indicating that PMA did not penetrate into some dead cells, which enables the generation of false-positive results. This has been pointed out as a disadvantage of the use of PMA combined with PCR for the detection of viable microorganisms, because not all nonviable cells have compromised membranes (Nocker and Camper, 2009; Fittipaldi et al., 2011; Løvdal et al., 2011; Fittipaldi et al., 2012; Cangelosi and Meschke, 2014).

Regarding the specificity of the qPCR method employed, the primers selected had previously presented a high specificity when tested against a broad panel of bacterial species (Wang et al., 1997). Furthermore, the TaqMan probe used was shown to restrict the detection of 3 genetically close species of the *B. cereus* group (*B. cereus* sensu stricto, *B. mycoides*, and *B. thuringiensis*), and accordingly, these species were detected by the PMA-qPCR method developed here, whereas the DNA of 6 other species of *Bacillus* was not amplified. In this sense, although the main concern has focused on the pathogenicity of *B. cereus* sensu stricto, *B. mycoides* and *B. thuringiensis* were also isolated from different milk samples (Bartoszewicz et al., 2008; Zhou et al., 2008). Indeed, it has been described that some *B. thuringiensis* strains can produce the same diarrheal enterotoxin as *B. cereus* sensu stricto, indicating a potential effect on food safety (Hansen and Hendriksen, 2001); it was shown that *B. mycoides* might present enterotoxin genes and affect the quality of milk during storage (Prüss et al., 1999).

The enumeration of species of *B. cereus* group by qPCR was performed using cloned DNA- and cell-based standard curves. The plasmid DNA standard curve showed an amplification efficiency of 96.15%, with good quantitative accuracy ($R^2 = 0.9995$), and allowed us to determine the quantification limit of the designed qPCR in $1.6 \times 10^2$ DNA copies/mL (Figure 3A). However, to determine the actual sensitivity, which takes into account the possible losses that can occur when processing the samples, we constructed another standard curve from milk that was artificially contaminated with a known number of *B. cereus*. The contaminated milk was submitted to PMA treatment and DNA extraction, and the curve generated from these cells showed an amplification efficiency of 97.41% and a high coefficient of determination ($R^2 = 0.9971$), providing a quantification limit of $7.5 \times 10^2$ cfu/mL (Figure 3A). Because

**Table 1**. Effect of propidium monoazide (PMA) treatment on cycle threshold (Ct) values obtained in quantitative PCR assay from viable or dead *Bacillus cereus* derived from a culture with cell density of $10^8$ cfu/mL

| | Ct value | | | | |
|---|---|---|---|---|---|
| Cells | Not treated with PMA | SD | PMA treated | SD | ΔCt |
| Viable | 21.88[a] | 0.35 | 22.48[a] | 0.57 | 0.83 |
| Dead | 21.52[a] | 0.48 | 36.45[b] | 0.7 | 14.93 |

[a,b]Numbers with different letters differ significantly at $P < 0.05$.

both standard curves had very similar efficiencies, we used the linear equation resulting from the cell-derived curve and compared it to each point of the plasmid DNA curve, thus estimating the differences in the final outcome for both curves (Figure 3B). The comparison showed a reduction of approximately 38% of genetic material in the cell-derived curve, indicating the proportion of DNA loss during the process. This difference reinforces the need to estimate the possible bias between plasmid and cell-based standard curves and correct the quantification error in the final result, as described by Pérez et al. (2013). Therefore, the loss of 38% of DNA, as demonstrated by the comparison of both curves, was then considered when determining the quantity of viable cells in UHT milk samples. The developed method was compared with the traditional culturing technique by analyzing 135 UHT milk samples. The qPCR without PMA treatment detected *B. cereus* group species in 78 samples (57.8%), and the PMA-qPCR method detected 44 positive samples (32.6%; Ct ranging from 26.9 to 37.0), presenting a significant difference ($P < 0.001$; Table 2). The PMA treatment is believed to have inhibited the amplification of a large amount of DNA from cells that were killed during the UHT processing, which prevented an overestimation of bacterial cells when using qPCR and, thus, did not overvalue potential health risks. Importantly, *B. cereus* sensu stricto was detected by culture-based method in 15 samples, and also detected by PMA-qPCR; here, 29 additional samples were positive only by PMA-qPCR. The detection of more positive samples by PMA-qPCR can be due to the presence of *B. mycoides*, *B. thuringiensis*, or both, or the presence of *B. cereus* sensu stricto at quantities below the culture-based method detection limit. Moreover, another difference among these methods is that the cultural assay can detect only viable and replicating bacteria, whereas the molecular-based assays associated with PMA can detect DNA from viable and viable but nonculturable microorganisms. The viable but nonculturable state has previously been described in *B. stratosphericus* (Cooper et al., 2010), which enables us to consider that it can also occur in other *Bacillus* species, especially when submitted to stress conditions, such as high temperature. This cellular condition is of special concern because of the maintenance of infection potential, despite the inability of cells to grow in culture media (McDougald et al., 1998; Wéry et al., 2008). It should also be considered that not all nonviable cells have compromised membranes, including spores that can remain structurally intact after heat inactivation, despite the loss of viability (Rawsthorne et al., 2009; Tabit and Buys, 2010; Probst et al., 2012; Cangelosi and Meschke, 2014), which prevents PMA penetration and, consequently, might be detected by PMA-qPCR.

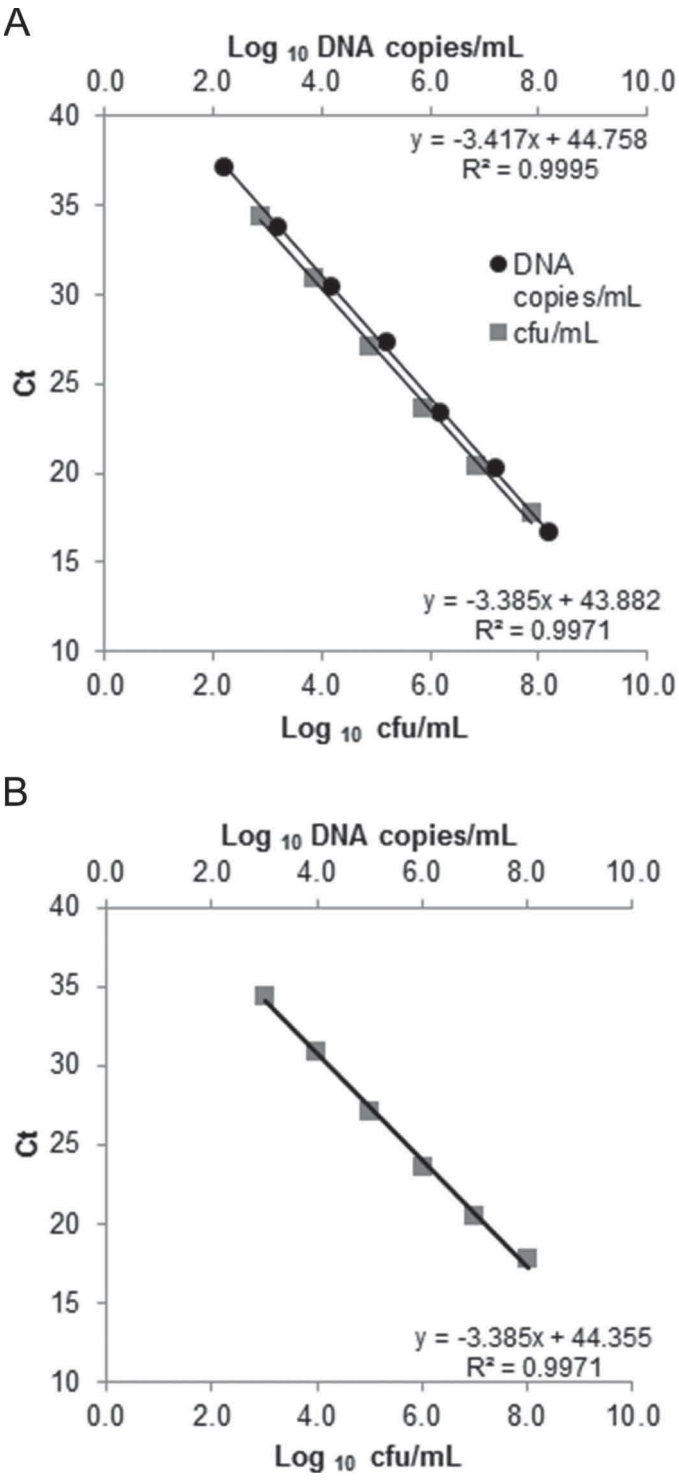


**Figure 3**. Standard curves used to calibrate the propidium monoazide (PMA)-quantitative real-time PCR (qPCR) method. (A) Plasmid DNA and cell-derived standard calibration curves for the *Bacillus cereus* PMA-qPCR assay. A standard curve of 10-fold serial dilutions of (●) plasmid DNA (from $1.6 \times 10^8$ to $1.6 \times 10^2$ of DNA copies/mL) and (■) 10-fold serial dilutions of *B. cereus* in artificially inoculated milk (from $7.5 \times 10^7$ to $7.5 \times 10^2$ cfu/mL). (B) The standard curve generated by the interpolation of the resulting linear equations for the cell-derived curve and the plasmid DNA curve.

**Table 2**. Quantification of viable *Bacillus cereus* group species in UHT milk by quantitative real-time PCR (qPCR) associated with propidium monoazide (PMA) and bacterial culture method

| | PMA-qPCR | | Culture method[1] |
|---|---|---|---|
| Count (cfu/mL) | Ct[2] range | No. of samples | (no. of samples) |
| $\geq 3.9 \times 10^4$ to $\leq 3.8 \times 10^5$ | $23.6 \leq$ Ct $\leq 27.0$ | 3 | 3 |
| $\geq 3.9 \times 10^3$ to $\leq 3.8 \times 10^4$ | $27.1 \leq$ Ct $\leq 30.8$ | 10 | 9 |
| $\geq 3.8 \times 10^2$ to $\leq 3.8 \times 10^3$ | $30.9 \leq$ Ct $\leq 34.4$ | 25 | 3 |
| $\geq 1.8 \times 10^2$ to $\leq 3.7 \times 10^2$ | $34.5 \leq$ Ct $\leq 37.0$ | 6 | 0 |
| Not detectable | >37.0 or undetermined[3] | 91 | 120 |
| Total number of analyzed samples | | 135 | 135 |

[1]Cultural method quantified specifically *Bacillus cereus* sensu stricto.

[2]Ct = cycle threshold.

[3]Assumed to indicate absence of *B. cereus*.

Concerning the UHT milk analyses, it was found that 44 samples contained *B. cereus* group species in concentrations ranging from approximately $10^2$ to $10^5$ cfu/mL (Table 2). In this context, it is important to note that the samples were incubated at 36°C for 7 d before the analyses, and therefore, cell counts do not correspond to those originally present in the samples when were purchased on the market. This incubation simulates storage conditions found in many places of tropical weather. However, these data show that raw milk can be contaminated with *B. cereus* group spores that survive the UHT treatment. Consequently, as temperature and time of storage until consumption vary widely, it may be inferred that UHT milk can provide growth conditions for *B. cereus* to reach infective doses required to cause foodborne disease (Granum and Lund, 1997; Granum, 2002; Stenfors Arnesen et al., 2008), as enumerated in 3 UHT milk samples by both methods (Table 2).

## CONCLUSIONS

Propidium monoazide combined with qPCR may be useful for the selective detection of *Bacillus cereus* group species viable cells, which is particularly important in thermally treated dairy products. Therefore, these microorganisms can be promptly detected, preventing contaminated milk from being packaged and stored, perhaps, in conditions such that infective doses may be achieved.

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