PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

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Detecção e quantificação de células viáveis de *Bacillus sporothermodurans* e de *Bacillus cereus* em leite através de PCR convencional e de PCR em tempo real associadas ao propídio monoazida

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Prof. Dra. Sílvia Dias de Oliveira

Co-orientador: Prof. Dr. Carlos Alexandre Sanchez Ferreira

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Aprovado em 31 de outubro de 2012.

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Porto Alegre

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AGRADECIMENTOS

A realização desta tese de doutorado não teria sido possível sem a colaboração de diversas pessoas e instituições, às quais serei eternamente grata.

A Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), pela bolsa concedida via programa PROBOLSAS que possibilitou a realização deste doutorado.

A minha orientadora, professora Sílvia Dias de Oliveira pela oportunidade de desenvolver este projeto, pela atenção, orientação, ética, competência, incentivo e por sempre fazer com que eu buscasse o meu aperfeiçoamento. Agradeço também pelos valiosos ensinamentos transmitidos nesses anos de convívio.

Ao co-orientador deste trabalho, professor Carlos Alexandre Sanchez Ferreira, pelo apoio, incentivo e contribuições.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa, fundamental para a execução deste trabalho.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela bolsa concedida para realização do doutorado sanduíche na Universidad Politécnica de Cartagena (UPTC), em Cartagena, Espanha.

A UPCT e ao professor Pablo S. Fernandez, pela oportunidade de desenvolvimento de uma parte da pesquisa, além de todo apoio recebido durante minha estada em Cartagena, Espanha.

Aos colegas do laboratório, pelos momentos de discussão, troca de conhecimento, por toda ajuda fornecida na realização desta pesquisa e, principalmente, pela amizade.

Aos amigos, pela confiança e compreensão da importância dessa etapa em minha vida.

A toda minha família, que sempre apoiou meus estudos, pelo incentivo e paciência durante esta jornada.

Ao meu namorado Augusto Moro, meu mais especial agradecimento, pelo apoio incondicional em todas as minhas decisões, por estar comigo em todos os momentos, sempre me dando força e coragem, pelo amor e carinho em tempo integral.

A todos aqueles que, direta ou indiretamente, colaboraram com a realização deste trabalho, meus sinceros agradecimentos.

RESUMO

A presença de *Bacillus* spp. em leite representa um importante problema para a indústria de laticínios devido à sua capacidade de esporulação e à possibilidade de resistência do esporo ao tratamento térmico por ultra alta temperatura (UAT). O *Bacillus sporothermodurans* sobrevive ao sistema UHT, germinando e se multiplicando no leite estocado e, caso não seja corretamente quantificado e identificado, pode ultrapassar o limite estabelecido pela legislação para microrganismos mesófilos aeróbios, além de alterar a qualidade dos produtos lácteos quando em altas concentrações. Por outro lado, a contaminação de leite por *Bacillus cereus* constitui não somente uma importante causa de deterioração, mas também está associada com a ocorrência das síndromes emética e diarreica. Tradicionalmente, estes microrganismos são identificados e quantificados em alimentos através de técnicas clássicas de cultivo, mas métodos baseados na Reação em Cadeia pela Polimerase (PCR) também têm sido amplamente utilizados. Entretanto, a PCR não distingue células mortas de células viáveis, o que pode ser contornado com o emprego de intercalantes de DNA, como o propídio monoazida (PMA). O PMA se liga ao DNA derivado de células com membranas rompidas, impedindo suas amplificações na PCR, permitindo, assim, a detecção seletiva de células viáveis. Portanto, a presente tese teve por objetivo caracterizar a resistência térmica de *B. sporothermodurans*, bem como desenvolver métodos de detecção e quantificação de células viáveis de *B. sporothermodurans* e de *B. cereus* em amostras de leite através de PCR associada ao PMA. Tratamentos isotérmicos e não isotérmicos permitiram a determinação do perfil de resistência térmica de esporos de *B. sporothermodurans* ao processo UHT, predizendo que a 121°C foi encontrado um valor D entre 2 a 4 min. A detecção e quantificação seletivas de *B. sporothermodurans* e de *B. cereus* através de PMA-qPCR foram desenvolvidas utilizando o gene RNAr 16S e o gene da hemolisina como alvos, respectivamente. O tratamento com PMA a partir de cultura pura e leite UHT artificialmente contaminado foi padronizado através da PCR convencional para a detecção de células viáveis destes microrganismos. A inibição da amplificação de DNA de células mortas foi obtida na concentração de 30µg/mL de PMA. A padronização dos ensaios de qPCR foram realizados utilizando sondas de hidrólise (sistema TaqMan®) específicas para cada gene alvo. O limite de quantificação a partir de leite UHT artificialmente contaminado foi de 2,2 x 10^2 UFC/mL para *B. sporothermodurans* e de 7,5 x 10^2 UFC/mL para *B. cereus*. As técnicas foram aplicadas a 135 amostras de leite UHT de diferentes marcas comerciais, comparando com a metodologia clássica de cultivo para cada microrganismo. *B. sporothermodurans* e *B. cereus* foram, respectivamente, detectados em 14 (10,4%) e 44 (32,6%) das amostras analisadas pelos métodos moleculares desenvolvidos, e em 11 (8,1%) e 15 (11,1%) pelos métodos convencionais de cultivo. Os métodos de PMA-qPCR desenvolvidos neste estudo foram específicos e sensíveis para a detecção e quantificação de células viáveis de *B. sporothermodurans* e de *B. cereus*, mostrando-se aplicáveis para serem utilizados na avaliação de amostras de leite, reduzindo o tempo de análise deste produto. Além disso, os resultados demonstraram que *B.* cereus pode ser encontrado em leite tratado pelo sistema de UHT.

Palavras-chave: *Bacillus sporothermodurans*, *Bacillus cereus*, viabilidade, Propídio Monoazida (PMA), PCR em tempo real, leite UHT.

ABSTRACT

The presence of *Bacillus* spp. in milk is an important problem for the dairy industry due to their capability of sporulation and the possibility of spore resistance to heat treatment by ultra high temperature (UHT). *Bacillus sporothermodurans* survive to the UHT system, germinating and growing in stored milk and, if not correctly identified and quantified, can exceed the criterion established for mesophilic aerobic, besides altering the quality of dairy products when in high concentrations. On the other hand, contamination of milk by *Bacillus cereus* is not only an important cause of deterioration, but is also associated with the occurrence of diarrhea and emetic syndromes. Traditionally, these microorganisms are identified and quantified in food using conventional microbiological techniques, but the Polymerase Chain Reaction (PCR) based methods have been widely used for the same purpose. However, PCR cannot distinguish between viable and dead cells, which can be overcame with the use of DNA intercalating, such as propidium monoazide (PMA). PMA binds to DNA derived from cells with damaged membranes, preventing their amplification by PCR, allowing, thus, the selective detection of viable cells. Therefore, this thesis aimed to characterize the thermal resistance of *B. sporothermodurans* and to develop methods of detection and quantificatification of viable cells of *B. sporothermodurans* and *B. cereus* in milk samples by qPCR associated with PMA. Isothermal and non-isothermal treatments allowed the determination of the profile of heat resistance of *B. sporothermodurans* spores to heat UHT process, predicting that to 121°C was found a D value between 2 a 4 min. The selective detection and quantification of *B. sporothermodurans* and *B. cereus* by PMA-qPCR were developed targeting 16S rRNA gene and hemolysin gene, respectively. The treatment with PMA from pure culture and artificially contaminated UHT milk were standardized by end-point PCR for the detection of viable cells of these microorganisms. The inhibition of amplification of DNA from dead cells was obtained at a concentration of 30µg/mL PMA. The standardization of qPCR assays were performed using hydrolysis probes (TaqMan® system) specific to each target gene. The quantification limit from UHT milk artificially contaminated was 2.5 x 10^2 CFU/mL for *B. sporothermodurans* and 7.5 x 10^2 CFU/mL for *B. cereus*. The assays were applied to 135 samples of UHT milk of different commercial brands, comparing with the conventional method of cultivation for each microorganism. *B. sporothermodurans* and *B. cereus* were respectively detected in 14 (10.4%) and 44 (32.6%) of the samples by molecular methods developed, and in 11 (8.1%) and 15 (11.1%) by conventional culturing methods. The PMA-qPCR methods developed in this study were specific and sensitive for the detection and quantification of viable *B. sporothermodurans* and *B. cereus* cells, being applicable for the evaluation of milk samples, reducing the time for the analysis of this product. Furthermore, the results showed that *B. cereus* can be found in UHT milk.

Keywords: *Bacillus sporothermodurans*, *Bacillus cereus*, viability, propidium monoazida (PMA), real time PCR, UHT milk.

LISTA DE SIGLAS

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

DNA – Ácido Desoxirribonucléico (*Deoxyribonucleic Acid*)

EMA – Etídio Monoazida (*Ethidium Monoazide*)

HHRS – Esporos altamente resistentes ao calor (*Highly Heat Resistant Spores*)

MAPA – Ministério da Agricultura, Pecuária e Abastecimento

PCR – Reação em Cadeia pela Polimerase (*Polymerase Chain Reaction*)

PMA – Propídio Monoazida (*Propidium Monoazide*)

qPCR – Reação em Cadeia pela Polimerase quantitativa (*quantitative Polymerase Chain Reaction*)

RAPD – DNA polimórfico amplificado ao acaso (*Random Amplified Polymorphic DNA*)

REP-PCR – Reação em Cadeia pela Polimerase tendo como alvo sequências Palindrômicas Extragênicas Repetidas (*Repetitive Element Palindromic PCR*)

RNA – Ácido Ribonucléico (*Ribonucleic Acid*)

rRNA – Ácido Ribonucléico ribossomal (*Ribosomal Ribonucleic Acid*)

UAT – Ultra Alta Temperatura

UFC – Unidades Formadoras de Colônia

UHT – *Ultra High Temperature*

VBNC – Viável mas não Cultivável (*Viable But Non-Culturable*)

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Introdução

Objetivos

1.1 Introdução

O leite é um alimento de alto valor nutritivo, que apresenta um importante papel no desenvolvimento e na manutenção da saúde. Contudo, devido à sua composição, torna-se suscetível à colonização por um grande número de microrganismos, que pode causar modificações físico-químicas e sensoriais, além de representar problemas econômicos e de saúde pública (1-3).

A produção de alimentos lácteos microbiologicamente seguros depende grandemente da qualidade do leite *in natura*. No Brasil, o leite cru apresenta, de maneira geral, altas contagens de microrganismos aeróbios mesófilos, psicrotróficos e coliformes, indicando, assim, uma deficiência na higiene durante a sua produção, transporte e amazenamento (1, 4). Segundo Carvalho (5), a qualidade do leite brasileiro está abaixo dos padrões verificados em outros países, o que acaba refletindo em menor rendimento industrial dos derivados, redução da vida de prateleira e, consequentemente, menor qualidade dos produtos.

Desta forma, o controle da contaminação do leite *in natura* deve ser efetuado desde a ordenha até a obtenção do produto final, para garantir um alimento com qualidade higiênico-sanitária que atenda aos limites estabelecidos pelas legislações nacionais vigentes. No Brasil, as características microbiológicas e físico-químicas do leite, para controle do produto desde a sua produção, transporte, até sua chegada à indústria, são definidas através da Instrução Normativa n° 51 de 18 de setembro de 2002 (IN51) e da Instrução Normativa n° 62 de 29 de dezembro de 2011 (IN62) (6, 7).

Além disso, o beneficiamento de laticínios tem empregado tratamentos térmicos para contribuir com a segurança microbiológica desses produtos. Um dos processos térmicos utilizados na indústria de laticínios é o sistema UHT (*Ultra High Temperature*) ou UAT (Ultra Alta Temperatura), que visa à inativação de microrganismos presentes no leite, minimizando os riscos à saúde e aumentando o tempo de vida útil do produto.

O leite UHT tornou-se um produto de destaque pela sua facilidade de comercialização e de consumo. As indústrias produtoras de leite UHT conseguiram expressivas taxas de crescimento de vendas no período de 1991 a 2000. A participação do leite UHT no mercado de leite fluido subiu de 4,4% em 1990 para 68,8% em 2000 e, em 2005, representou 74% do leite fluido consumido no Brasil (3, 8). Em 2011, estimou-se que o leite UHT foi consumido em 89% dos domicílios brasileiros (9).

O Ministério da Agricultura, Pecuária e Abastecimento (MAPA) define o leite UHT como: "leite homogeneizado submetido, durante 2 a 4 segundos, a uma temperatura entre 130°C e 150°C, mediante um processo térmico de fluxo contínuo, imediatamente resfriado a uma temperatura inferior a 32°C e envasado sob condições assépticas em embalagens estéreis e hermeticamente fechadas" (10).

A eficácia do tratamento por UHT é influenciado pelo binômio tempo/temperatura, bem como pela carga inicial de microrganismos presentes na matéria prima (leite cru) (11-13). Dentre os vários microrganismos que podem contaminar o leite *in natura* por meio do solo, poeira, fezes e camas dos animais, equipamentos e utensílios higienizados inadequadamente, destacam-se as bactérias pertencentes ao gênero *Bacillus* (14, 15), que apresentam espécies capazes de produzir esporos que resistem ao calor empregado no processamento por UHT (16, 17).

A contaminação do leite UHT brasileiro por bactérias do gênero *Bacillus* tem sido determinada. Na análise de 32 amostras de leite UHT comercializadas na região de Ribeirão Preto (SP), foi observada a presença de *Bacillus* spp. em 10,5% (18). Em Belo Horizonte, 80 amostras de leite UHT foram analisadas, sendo que 33 (41,2%) apresentaram contagem de bactérias mesófilas aeróbicas entre 10^4 e 10^5 UFC/mL, das quais 93,1% pertenceram ao gênero *Bacillus* (19). Entre as espécies de *Bacillus* importantes na indústria de laticínios está o *Bacillus sporothermodurans*, que é capaz de produzir esporos altamente resistentes ao calor

(HHRS), o que confere a esta bactéria a capacidade de sobreviver em alimentos tratados pelo sistema UHT, podendo germinar e se multiplicar no produto estocado (16, 17, 20). A multiplicação de *B. sporothermodurans* pode alcançar o limite de 10^5 UFC/mL, levando à instabilidade do produto devido à produção de enzimas proteolíticas (17, 21). O *B. sporothermodurans* produz um dos esporos mais termorresistentes, uma vez que possui valor $D_{140°C}$ entre 3,4 a 7,9 segundos (20). A análise dos danos estruturais e da sobrevivência de esporos de *B. sporothermodurans* tratados pelo calor demonstrou que a inativação completa dos esporos ocorre somente após tratamento térmico a 130°C por 8 minutos (21). No entanto, o aumento da temperatura e/ou tempo de espera na tentativa de inativar esporos de *B. sporothermodurans* afeta as características organolépticas, bem como a qualidade nutricional do leite UHT (22). Portanto, a caracterização da resistência térmica de esporos de *B. sporothermodurans* através de tratamentos isotérmicos e não isotérmicos permite estimar a letalidade dos processos térmicos aplicados pelo sistema UHT.

O *B. sporothermodurans* tem sido isolado no leite UHT em diferentes regiões geográficas (16, 23-25), até mesmo em contagens que excedem aos critérios microbiológicos de aceitação para o leite UHT em alguns países (10, 26). No Brasil, a legislação estabelece que o leite UHT não deve conter microrganismos capazes de proliferar sob condições normais de armazenamento e distribuição, sendo que após a incubação da embalagem fechada a 35-37ºC, por 7 dias, a contagem de mesófilos aeróbios não poderá exceder ao limite de 10^2 UFC/mL (10). Apesar do *B. sporothermodurans* ser um mesófilo, de acordo com a IN 62 de 26 de agosto de 2003, quando ocorrer a identificação de *B. sporothermodurans* a contagem deste microrganismo deve ser subtraída da contagem total de mesófilos aeróbios (27). Esta diferenciação, provavelmente, se deva ao fato deste microrganismo não ser patogênico (16) e causar alterações físico-químicas somente após uma densidade de 10^5 UFC/mL (17). Entretanto, este bacilo tem sido isolado a partir de amostras de leite UHT produzido em várias

regiões brasileiras (18, 19, 28-30). Uma análise de amostras de leite UHT procedentes das regiões Sul, Sudeste e Centro-oeste, revelou que 45% apresentavam *B. sporothermodurans* acima de 10^2 UFC/mL, ou seja, acima dos padrões nacionais vigentes em relação à contagem de bactérias mesófilas (28). Neumann e colaboradores (31) analisaram 511 amostras de leite UHT de 11 diferentes marcas comercializadas no estado do Rio Grande do Sul, e 100% estavam de acordo com o padrão legal vigente, entretanto, 10,8% das amostras estavam contaminadas com *B. sporothermodurans*. A análise de 88 amostras de leite UHT de 11 diferentes marcas comercializadas na região do Alto Uruguai-RS revelou que 54,5% (seis marcas) estavam contaminadas por *B. sporothermodurans* (29).

Portanto, o principal problema em relação à contaminação de leite UHT pelo *B. sporothermodurans* está no aumento da contagem total de microrganismos mesófilos aeróbios viáveis, caso não seja realizada a correta detecção e quantificação deste microrganismo para, então, diferenciá-lo dos demais mesófilos, evitando que o limite estabelecido pela legislação seja ultrapassado. Assim, fica clara a importância de se buscar métodos rápidos, sensíveis e específicos que permitam a detecção e quantificação de *B. sporothermodurans*.

O *Bacillus cereus* é outra espécie de *Bacillus* que tem sido isolado de uma ampla variedade de alimentos processados e *in natura*, entre eles, leite e produtos lácteos, cereais, e alimentos prontos para consumo (32-37).

A presença de *B. cereus* em leite e produtos lácteos pode causar importantes problemas para a indústria de laticínios, pois além de ser um importante deteriorante causador de alterações sensoriais no leite, pode causar dois tipos de doenças de origem alimentar: a síndrome emética, provocada pela toxina cerulida pré-formada no alimento, e a síndrome diarréica, provocada por uma enterotoxina produzida no intestino do hospedeiro (38-40).

Acredita-se que a dose infectante de *B. cereus* necessária para causar doença de origem alimentar seja de 10^5 a 10^8 UFC/g ou mL de alimento (41, 42), embora alguns estudos

relatem que o consumo de alimentos que contenham concentrações acima de 10^3 UFC/g ou mL não é seguro (43-45).

A prevalência de *B. cereus* em leite e derivados tem sido reportada em diversos países. Bahout (46), ao analisar 60 amostras de leite UHT comercializadas no Egito, constatou a presença de *B. cereus* em 29,2% das 11 (18,3%) amostras positivas para *Bacillus* spp. Reyes e colaboradores (47) constataram a presença de *B. cereus* em 45,9% das 381 amostras de produtos lácteos secos utilizados pelo programa de alimentação escolar no Chile. Batchoun e colaboradores (35), ao avaliarem 22 amostras de iogurte adquiridos no comércio da Jordânia, demonstraram a presença de *B. cereus* em 61,3% das amostras analisadas. No Brasil, a contaminação de leite UHT com *B. cereus* também tem sido reportada por diversos autores. Rezende e colaboradores (48), ao avaliarem 120 amostras de leite UHT adquiridas no comércio da região de Ribeirão Preto (SP), detectaram a presença de *B. cereus* em 34,1% das amostras analisadas. Vidal-Martins e colaboradores (3) detectaram a presença de *B. cereus* em 11,8% de 110 amostras de leite UHT comercializadas em São José do Rio Preto (SP). Rezende-Lago e colaboradores (49) detectaram a presença de *B. cereus* em 13,3% das 30 amostras de leite UHT comercializadas na região de Ribeirão Preto (SP). Montanhini e colaboradores (37) observaram que 16,4% das 110 amostras de leite UHT coletadas no comércio dos Estados do Paraná, Santa Catarina e São Paulo estavam contaminadas com *B. cereus*.

A legislação brasileira não especifica limites para a presença de *B. cereus* no leite e nos derivados lácteos, com exceção para o leite em pó, em que é estabelecido um limite máximo de $5,0 \times 10^3$ UFC/g (50). No entanto, a legislação estabelece que o leite UHT, após 7 dias de incubação a 35-37°C, não deve apresentar microrganismos patogênicos e causadores de alterações físicas, químicas e organolépticas do produto, em condições normais de

armazenamento (50), a partir do que subentende-se que estes produtos devem estar livres de *B. cereus*.

As técnicas tradicionais para identificação e enumeração de microrganismos em alimentos envolvem o uso de meios de cultivo, além da confirmação através de testes bioquímicos e/ou sorológicos (51). No Brasil, os métodos analíticos oficiais para o controle microbiológico de produtos de origem animal e água, que inclui a contagem de microrganismos mesófilos aeróbios viáveis em produtos lácteos líquidos UHT, são estabelecidos pela Instrução Normativa n° 62/2003 (27). Porém, estes métodos podem ser extremamente trabalhosos, requerendo dias ou até semanas para produzir um resultado conclusivo. Adicionalmente, microrganismos podem estar em um estado fisiológico denominado viável mas não cultivável ("viable but non-culturable" – VBNC), no qual as células sofrem mudanças fisiológicas e morfológicas, que podem proporcionar um fenótipo de resistência, além de perderem a sua capacidade de crescer nos meios de cultivo (52-54).

Apesar de apresentarem limitações, as técnicas clássicas de cultivo e identificação são consideradas o padrão ouro para a detecção e quantificação de microrganismos em alimentos (55-57). Entretanto, diversos métodos moleculares desenvolvidos para a análise de DNA e RNA, especialmente utilizando a Reação em Cadeia pela Polimerase (PCR), vêm sendo desenvolvidos para a análise microbiológica de alimentos (55, 58, 59). A PCR tem-se mostrado útil para identificação de espécies bacterianas, principalmente para aqueles que dependem de uma caracterização fenotípica laboriosa, como é o caso do *B. sporothermodurans* e do *B. cereus*, pois a técnica pode ser utilizada sem a necessidade do isolamento da bactéria (39, 60-62). Entretanto, a PCR convencional não proporciona resultados quantitativos, somente qualitativos baseados na presença ou ausência de amplificação da sequência-alvo após eletroforese em gel. Por outro lado, a PCR em tempo real ou PCR quantitativa (qPCR) é capaz de monitorar as amplificações ciclo a ciclo através

de compostos fluorescentes presentes na reação que emitem fluorescência proporcional à quantidade de produto amplificado (63,64). Desta forma, é possível quantificar de forma precisa o número exato de moléculas presentes em determinada amostra. Os compostos químicos utilizados na qPCR podem ser agrupados em dois grupos: corantes intercalantes de DNA fita dupla, como por exemplo o *SYBR*® Green, e sonda com uma sequência específica, como por exemplo o sistema TaqMan®.

PCR convencional e qPCR tendo como alvo o gene RNAr 16S foram desenvolvidas para a detecção de *B. sporothermodurans* (25, 61). O protocolo desenvolvido por Scheldeman e colaboradores (61) foi validado pela utilização de uma coleção de *B. sporothermodurans* isolados de diversas fontes e também de uma coleção de outras espécies de *Bacillus* geneticamente relacionados ao *B. sporothermodurans*. No entanto, posteriormente foi descrito o *B. acidicola* (65), que apresenta total identidade com a região alvo dos oligonucleotídeos iniciadores desenhados para a detecção de *B. sporothermodurans*, amplificando um fragmento de mesmo tamanho daquele esperado para esta espécie. O mesmo par de oligonucleotídeos iniciadores foi, posteriormente, utilizado para a confirmação de *B. sporothermodurans* em colônias isoladas de leite cru e tratados por UHT através da qPCR empregando o *SYBR*® Green (25). Ambos objetivaram apenas a detecção deste microrganismo em colônias isoladas, sendo que a aplicabilidade dos métodos desenvolvidos na matriz leite não foi verificada pelos autores.

Diferentes estudos têm descrito a detecção de *B. cereus* em alimentos através da utilização de vários genes alvo, tais como genes que codificam para hemolisina, cereolida-*ces*, e RNAr 16S (66-70). Mais recentemente, protocolos que empregam qPCR para a detecção e quantificação de *B. cereus* em alimentos também têm sido relatados. Martínez-Blanch e colaboradores (45) desenvolveram um método empregando qPCR para detecção e quantificação deste microrganismo em ovos líquidos e em fórmula infantil contaminados

artificialmente. O ensaio teve como alvo o gene que codifica para a fosfolipase C específica para fosfatidilcolina C (pc-plc) e apresentou um limite de detecção de 6,0 X 10^1 UCF/mL. Posteriormente, outro protocolo de qPCR foi desenvolvido tendo gene RNAr 16S como alvo para a detecção e quantificação de *B. cereus* em alimentos a base de peixe e outros ingredientes, tais como clara de ovo, água, sal, amido de trigo e óleo de girassol, apresentando um limite de detecção de 1,65 X 10^2 UFC/mL (71).

No entanto, os estudos que descreveram métodos baseados na amplificação de DNA para a detecção e quantificação de *B. sporothermodurans* e de *B. cereus*, até o momento, não verificaram a viabilidade destes microrganismos (25, 45). A determinação da viabilidade bacteriana é uma questão fundamental para a aplicação de ferramentas baseadas em biologia molecular para a detecção de microrganismos em alimentos, principalmente naqueles que sofrem tratamentos térmicos e, ação antimicrobiana. A PCR não é capaz de distinguir entre DNA oriundo de células mortas do DNA pertencente a células viáveis, uma vez que, após a morte celular, o DNA pode permanecer íntegro no ambiente (72-74). Desta forma, métodos moleculares baseados na detecção de DNA tendem a superestimar a presença de células viáveis. Os agentes intercalantes de DNA, etídio monoazida (EMA) (75) e propídio monoazida (PMA) (76), têm sido associados à técnica de PCR com o objetivo de detectar seletivamente o DNA de células viáveis (77-81).

EMA e PMA possuem a capacidade de penetrar na membrana celular comprometida de células mortas e se ligar covalentemente ao DNA após foto-indução do grupo azida, inibindo a sua amplificação através da PCR. Por outro lado, o DNA de célula viável não sofre ação do agente intercalante, uma vez que a célula possui membrana celular intacta (76-82). Estes dois intercalantes têm-se mostrado úteis para diferenciação de células viáveis e mortas, tanto de bactérias Gram-positivas quanto de Gram-negativas (76). No entanto, estudos demonstraram que o EMA é um indicador limitado de viabilidade celular, pois é incorporado

também em células viáveis, levando à perda substancial de detecção de DNA oriundo de célula viável (78, 83).

A utilização de PMA associado a qPCR para detecção de microrganismos em alimentos tem sido relatada. Mamlouk e colaboradores (84) desenvolveram um ensaio associando o PMA à qPCR para detecção e quantificação de *Brochothrix thermosphacta* viáveis diretamente de camarões cozidos e salmão fresco. Os pesquisadores verificaram que o ensaio desenvolvido foi sensível e específico, com limite de detecção de 1,2 x 10^2 UFC/mL. Liang e colaboradores (74), empregando um método de PMA-qPCR para a detecção de células viáveis de *Salmonella* spp., apresentaram limite de detecção de 10^3 UFC/mL.

Portanto, a presença de bactérias cultiváveis e de bactérias que estejam no estado viável mas não cultivável pode ser detectada pela qPCR associada ao tratamento com PMA, inibindo a detecção de DNA proveniente de células mortas. Desta forma, o tratamento com PMA, tem o potencial de limitar a análise do DNA originário somente de células bacterianas com membrana celular intacta. Contudo, a utilização deste intercalante de DNA pode apresentar algumas limitações. Por exemplo, o tratamento com PMA pode não inibir completamente a amplificação de DNA das células mortas pela PCR quando as sequências alvo são curtas (79, 85, 86). Tal limitação pode ser contornada pela utilização do PMA associado a duas etapas de amplificação através de uma *nested*-PCR (85). Além disso, um grande número de variáveis deve ser levado em consideração na padronização do tratamento com PMA, tais como: determinação da concentração de PMA, método para obtenção de células mortas, tempo de incubação no escuro, tempo de foto-ativação pela luz halógena e potência da luz halógena. Tais fatores podem ser dependentes da concentração celular utilizada e da espécie microbiana analisada (87, 88). Adicionalmente, outro fator que desempenha um papel importante na eficiência do tratamento com PMA é a turbidez da amostra. Zhu e colaboradores (86) sugerem que o tratamento com PMA em amostras com

Unidades Nefelométricas de Turbidez (UNT) acima de 10 não inibem adequadamente a amplificação de DNA proveniente de células mortas e pode, portanto, produzir resultados falsos positivos devido a alterações na foto-ativação e/ou falhas na ligação do PMA ao DNA. Os autores observaram também que, o tratamento com PMA não foi eficaz em amostras que apresentaram concentração celular com densidade óptica (DO_{600nm}) acima de 0.8 (86).

Tendo em vista o tempo necessário para a detecção e identificação de *B. sporothermodurans* e de *B. cereus* a partir de amostras de leite através das técnicas tradicionais de cultivo, bem como a importância do leite na alimentação humana, torna-se indispensável garantir sua qualidade, principalmente em relação aos aspectos microbiológicos pelos riscos de veiculação de microrganismos patogênicos e deteriorantes. Assim, para superar possíveis desvantagens dos métodos de cultivo, incluindo a detecção e quantificação de VBNC, a qPCR combinada com o PMA pode ser utilizada para detectar e quantificar somente células viáveis de *B. sporothermodurans* e de *B. cereus* em leite UHT.

1.2 Objetivos

1.2.1 Objetivo Geral

Este trabalho teve como objetivo estabelecer métodos de detecção e quantificação de células viáveis de *B. sporothermodurans* e de *B. cereus* em amostras de leite através de PCR associada ao PMA, bem como caracterizar a resistência térmica de *B. sporothermodurans* a fim de estimar a letalidade do processamento através de UHT para este microrganismo.

1.2.2 Objetivos específicos

a) Caracterizar a resistência térmica de endósporos de *B. sporothermodurans* em sopa de legumes tratada pelo sistema UHT.

b) Padronizar uma *semi-nested* PCR para a detecção de células viáveis e endósporos de *B. sporothermodurans*, utilizando o gene RNAr 16S como alvo;

c) Estabelecer os limites de detecção da técnica de *semi-nested* PCR para detectar células viáveis e endósporos de *B. sporothermodurans*;

d) Detectar seletivamente células viáveis de *B. sporothermodurans* em leite através de *semi-nested* PCR associada ao PMA;

e) Padronizar um método de quantificação de células viáveis de *B. sporothermodurans* através de qPCR associada ao PMA;

f) Quantificar células viáveis de *B. sporothermodurans* por qPCR associada ao PMA em amostras de leite UHT em comparação com a metodologia clássica de cultivo para este microrganismo.

g) Quantificar células viáveis de *B. cereus* por qPCR associada ao PMA em amostras de leite UHT em comparação com a metodologia clássica de cultivo para este microrganismo.

Capítulo 2

Artigo Científico 1

Kinetic characterization of *Bacillus sporothermodurans* spores in liquid food under static and dynamic heating regimes

Artigo científico a ser submetido como artigo completo.

Kinetic characterisation of *Bacillus sporothermodurans* spores in liquid food under static and dynamic heating regimes

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ABSTRACT

Bacillus sporothermodurans produces highly heat-resistant endospores, surviving to ultra-high temperature processing. High heat resistant sporeforming bacteria are one of the main threats for the stability of shelf-stable, low-acid heat processed foods. At the same time they are the best indicators to establish the minimum requirements for heat processes, but in order to reduce existing heat treatments it is necessary to have precise scientific knowledge of the factors involved in their inactivation and good modelling tools that describe these kinetics. The aim of the present work was to study the inactivation kinetics in static conditions (isothermal) in a food substrate (vegetable soup) and to compare them with those performed under dynamic realistic heating profiles (1.5 and 2.5^{o}C/min) simulating processing conditions in the food industry. A thermoresistometer Mastia and spores of *Bacillus sporothermodurans* as sensor element have been used for this study. Results from these experiments have been modelled using a regression analysis for both the static and dynamic conditions. Inactivation parameters were estimated accurately and precisely when data of both dynamic profiles were combined.

Keywords: Thermal technologies; high heat resistance; *Bacillus sporothermodurans;* spore forming bacteria.

1 Introduction

The thermal inactivation of bacterial spores has been the topic of many studies due to their high heat resistance. *B. sporothermodurans* has been characterized to produce highly heat-resistant spores (HRS) that may survive UHT treatment (135 to 142°C for a few seconds) (Hammer et al., 1995; Pettersson et al., 1996). Due to their high heat resistance, *B. sporothermodurans* spores have been found to be resistant at temperature above 130°C with D_{140} ranging from 3.4 – 7.9 s and *z*-values ranging from 13.1 – 14.2 °C (Huemer et al, 1998).

In general, the safety of food products, such as vegetable soups, depends on the processes commonly applied to inactivate vegetative cells and highly heat resistant spores, combined with good manufacturing practices. The final aim is to produce a stable product that can be preserved for long periods at room temperature with the purpose of ensuring a high level of protection of consumer health. The application of heat is both an important method of preserving foods and a means of developing texture, flavour and colour of the treated product. However, thermal treatments are not always sufficient to inactivate all sporeforming bacteria, especially those that are highly heat resistant and non-pathogenic (Hornstra et al., 2009). An essential issue for food manufacturers is the effective application of thermal technologies without damaging other desirable sensory and nutritional qualities in a food product (Richardson, 2004). Therefore, the need to secure the microbiological quality and safety of food products has demonstrated interest in the use of mathematical models for quantifying and predicting the microbial behavior.

These models can predict the microbiological safety and the shelf life of products under commercial conditions. In this way, the intensity of the thermal treatments can be calculated and, as a consequence, the microbiological safety and nutritional quality of the obtained food products can be estimated. It is common practice that microbial model parameters are obtained under isothermal conditions and they are then validated under

dynamic realistic conditions. For these validations the general method assumes that non-isothermal treatments are composed of successive isothermal segments of short duration, each one at a different temperature (Conesa et al., 2009). Nevertheless, recent reports have highlighted that inactivation model equations and their associated parameter values obtained under static conditions (e.g., acid, thermal, etc) cannot be used directly for predicting dynamic conditions (Janssen et al., 2008; Valdramidis et al., 2006). This can be attributed either to the model structure properties (Janssen et al., 2008), the induced stress resistance phenomena (Valdramidis et al., 2007; Velliou et al., 2011) or the co-existence of stress-sensitive and stress-resistant sub-populations (Van Derlinden et al., 2010). A way to tackle these phenomena is by implementing approaches in which parameter estimates are obtained under realistic dynamic environments (Peleg et al., 2003; Dolan et al., 2007; Valdramidis et al., 2008; Dogan et al., 2009). These studies have been assessed on dynamic (simulated) data of specific temperature profiles. Further establishment of parameter estimates that are product and process specific is imperative especially in the area of spore inactivation that defines the safety boundaries of a thermal process.

The main objective of this study was to characterize the microbial resistance of *B. sporothermodurans* spores in vegetable soup under static and dynamic temperature conditions and assess parameter accuracy and precision that can be used for designing optimal thermal processes of soup products.

2 Materials and Methods

2.1 Microorganism and spore crop preparation

B. sporothermodurans IC4 (Unilever Netherlands Sourcing Unit Oss) was isolated from Indian curry soup and was able to survive high heat treatments. In order to sporulate the microorganism, a freeze-dried sample was rehydrated in Nutrient Broth (NB) (Scharlau, Barcelona, Spain) and incubated at 37ºC for 12-14 h under continuous agitation. Once turbidity was evident, the culture was streaked to check purity on Nutrient Agar (NA) (Scharlau). Plates containing Fortified Nutrient Agar (FNA) (Mazas et al., 1995) were used as sporulation media. A bacterial suspension was prepared by flooding the NA plates, once incubated at 37ºC for 24 h, with buffered peptone water. This suspension was collected with sterile pipettes and used as inoculum. Plates containing the sporulation medium were inoculated with 0.2 mL of the suspension and allowed to dry under aseptic conditions in a laminar-flow cabinet. The plates containing sporulation media were then incubated at 37°C for at least 4 days, until a sporulation rate of at least 90% was accomplished. Spores were harvested and then centrifuged three times (5000 g for 10 min, at 4ºC). Spores of *B. sporothermodurans* were suspended in distilled water for thermal treatments. The spore suspension was stored at – 20°C until further use.

2.2 Vegetable soup

Vegetable soup treated by ultra high temperature (UHT) processing was purchased from a local supermarket. Ingredients were water, onions, carrots, leek, celery, olive oil and salt. Nutritional information (100 mL): energy, 9kCal/ 40kj; protein content 0.5 g; carbohydrates 1.4 g; fat 0.2 g; NaCl 0.4 g. The final pH was 6.2.

2.3 Heat treatment

All heat treatments were carried out in a thermoresistometer Mastia (Conesa et al., 2003) that can be programmed to perform isothermal and non- isothermal experiments. The instrument consists on a stainless steel vessel of 400 mL volume with a screw cap that contains an agitation shaft with a propeller, and several ports with screw caps to hold the pressure source, the inoculum injection port with a gas chromatography septum, the sampling tube and a thermocouple (Pt 100). The main vessel is pressurised through a manometer connected to the pressure source. The control of the temperature inside the thermoresistometer was done by the PLC, by means of a PID (Proportional Integral Derivative). The vessel was filled with 350 mL of the substrate, pressurized and then set to the treatment temperature selected. For isothermal treatments, the vessel was filled with sterile distilled water and, once the heating temperature had attained stability, 0.2 mL of the *B. sporothermodurans* spore suspension was injected. When vegetable soup was used as heating medium, the instrument was sterilized with distilled water, cooled, emptied and immediately filled with vegetable soup and heated to the temperature of treatment. The temperature was kept constant through all the experiment. The samples were collected into sterile test tubes at preset time intervals (Table 1), appropriately diluted and immediately plated and incubated. Before sampling, the contents of the sampling tube of the thermoresistometer were discarded. The temperatures from isothermal treatments were 118, 121, 124, and 127°C. Experiments were performed in triplicate for each temperature.

For non-isothermal treatments the procedure was similar, but the thermoresistometer was programmed to perform the selected temperature profile. The non-isothermal treatments for vegetable soup were run in a temperature range from 80 to 121^{o}C at a rate of 1.5^{o}C/min

and from 75 to 121°C at a rate of 2.6°C/min. These temperature profiles were selected to simulate typical processing conditions used in the food industry to process low acid soups. Population densities were determined by decimal serial dilutions of the samples in sterile peptone water, and were pour plated on Brain Heart Infusion (BHI) agar (Merck). The plates were incubated at 37°C for 48 h to determine the number of bacterial spores expressed in CFU/mL. All experiments were performed in triplicate for each of the tested profile.

2.4 Model development

A global identification technique (Valdramidis et al., 2005; van Zuijlenet al., 2010) was performed for both the isothermal and the dynamic data. Based on preliminary assessment of the isothermal data, which included regression analysis with a set of non-linear models, as those presented in GInaFiT (Geeraerd et al., 2005). The appropriate primary model structure appeared to be the classical log-linear. After integration of the Bigelow model in the log-linear model the following equation is obtained:

$$\frac{d\log_{10} N(t)}{dt} = -\frac{1}{D_{ref}} \cdot \exp\left(\frac{\ln 10}{z} \cdot (T - T_{ref})\right) \quad (1)$$

Herein, $\log_{10}N(t)$ represents the microbial cell density [log (CFU/mL)], D_{ref} is the decimal reduction time and z the thermal resistance constant. In the case of the dynamic temperature profiles, temperature evolution T, which was recorded every 5 s, was plugged into Equation 1. Linear interpolation was performed for estimating temperatures between the recorded values.

Three different types of parameter identification approaches were applied during the regression analysis of Equation 1: (i) all isothermal data was treated at once, (ii) the two dynamic experiments were studied separately and (iii) the two dynamic profiles were studied together. For more details also refer to the approach described by Valdramidis et al. (2008).

2.5 Regression and statistical analysis

All regression analysis was implemented by using the MatLab Optimisation Toolbox (The Mathworks Inc., Natick, MA, USA). The command of *lsqnonlin* was used to solve non-linear leas-squares problems and ode23s was the solver of the differential equations. Statistical analysis included the estimation of Sum of Squared Error (SSE), Mean Squared Error (MSE), Root Mean Squared Error (RMSE), estimation of the parameters standard error and the 95% confidence interval of the estimated parameters.

3 Results and discussion

Heat resistance *of B. sporothermodurans* was characterized over a wide range of temperatures (both for isothermal and non-isothermal treatments) in vegetable soup. Survival curves of *B. sporothermodurans* IC4 under isothermal treatment and dynamic conditions in vegetable soup for the different temperatures tested. The isothermal results (Figure 1) showed that due to the high resistance of the microorganism, temperature as high as 127°C was required with a treatment time of 3 min in order to achieve a 2.5-log microbial inactivation. At 121°C, a treatment time of 15 min was required to reduce microbial populations at 3-log cycles.

Table 2 and Figure 1 show the results of the regression analysis for the isothermal

data. The application of a one-step regression analysis of all the isothermal data prevents accumulation of fitting errors (Valdramidis et al., 2005). When the same spores were studied in water, microbial non-linearities appeared to be evident and were expressed by the presence of a shoulder effect (van Zuijlen et al., 2010). In the same work, the kinetic studies of the inoculated commercial soups have shown a variation depending on the type of product (mushroom, chicken, pea). The estimated parameter of the present work resulted in higher D_{121} values than those obtained from distilled water in which *B. sporothermodurans* IC4 was sporulated in mushroom soup agar. This is an expected result considering the protective effect of nutrients that are present in the soup.

Based on the model structure selection of the isothermal data, regression analysis was performed for the non-isothermal data by using the same model (Figure 2, Table 2). The selection of the same Equation 1, is on the basis that dynamic data are assumed to exhibit similar physiological responses, which will only be dependent on the specific applied temperatures. Some authors have highlighted the importance of working with model structures that incorporate the possible physiological adaptations, for example induced heat resistance (Valdramidis et al., 2007). In the current study this was not assessed, as the objective was to directly compare the regression methodologies applied as compared with the use of different type of data, i.e., isothermal and non-isothermal.

Initially, regression analysis of the dynamic data was performed separately, i.e., ramp1, ramp2. Despite the high fitting capacity of the model (ramp1 with RMSE=0.20, ramp2 with RMSE=0.10), the parameter estimates had very high standard errors and the D_{121} and z values were significantly different between the two ramps. This could be attributed to the experimental data and the information derived from them. Evidently, when these data are

combined together (ramps 1 and 2, Figure 3), the results obtained gave much more accurate parameters (low SE values) and models with high statistical performance (Table 2, Figure 3).

Previous studies have also shown that the more the microbial system is excited (in this case by temperature variations) the more the obtained information related to the microbiological responses (Valdramidis et al., 2008). In addition, these types of microbiological results can be generated more easily saving time and resources compared to the traditional methodology of the isothermal data approach. For example in the current study, on one hand there were 40 isothermal data collected by applying four separate temperature set-ups, on the other had 22 non-isothermal data were collected by applying only two separate temperature set-ups. It should also be highlighted that the estimated parameters (from the experimental sets of ramp1, ramp2) are derived from a set of dynamic experiments that cover the complete temperature range of a realistic industrial process environment (i.e., heating up, holding and cooling profile, as it happens typically in retort processing).

4 Conclusions

The implementation of regression analysis in which parameter estimates are obtained under dynamic environments is imperative in order to assess microbial inactivation kinetics in realistic conditions. Although this type of studies have been previously assessed on some dynamic (simulated) data of specific temperature profiles, further characterization of target microorganisms (spores for sterilization, or vegetative organisms for pasteurization) will be required in future research activities. This conclusion is drawn considering that inactivation parameters estimated under non-isothermal conditions give accurate and precise estimates.

Acknowledgements

Fernanda Cattani acknowledges Programa Capes/Fundação Carolina (Brazil) for financial support. This project was funded by Spanish "Ministerio de Ciencia e Innovación", ref. AGL 2010-22206-C02-02/ALI and Fundación SENECA, CARM, Spain ref 08795/PI/08.

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Table 1: Time sampling of isothermal treatments at each temperature.

Temperature (°C)	Intervals of time (min) of sample collection
118	0, 5, 10, 15, 20, 25
121	0, 1, 2.5, 3.45, 5, 6.5, 8, 10, 12.5, 15
124	0, 1, 2, 3, 4, 6
127	0, 1, 1.5, 2, 2.5, 3

Table 2: Parameter estimates, standard erros, 95% confidence intervals and statistical indices of the performed parameter identification techniques

All static data				
Parameters	estimate	SE	95% CI	95% CI
D_{121}	5.58	0.3412	4.89	6.28
z (degC)	10.37	0.6865	8.98	11.77
$\log_{10}N(0)_1$ (log10(cfu/mL))	4.66	0.1254	4.40	4.91
$\log_{10}N(0)_2$ (log10(cfu/mL))	4.80	0.1077	4.58	5.02
$\log_{10}N(0)_3$ (log10(cfu/mL))	4.54	0.0959	4.34	4.73
$\log_{10}N(0)_4$ (log10(cfu/mL))	4.54	0.1300	4.28	4.80
SSE, MSE, RMSE = 2.28, 0.07, 0.259				
Ramp1				
Parameters	estimate	SE	95% CI	95% CI
D_{121}	6.14	0.6412	4.74	7.53
z (degC)	6.00	0.2627	5.42	6.57
$\log_{10}N(0)_1$ (log10(cfu/mL))	4.25	0.0684	4.10	4.40
SSE, MSE, RMSE = 0.50, 0.04, 0.204				
Ramp2				
Parameters	estimate	SE	95% CI	95% CI
D_{121}	9.21	0.5253	8.04	10.38
z (degC)	21.91	0.7511	20.23	23.58
$\log_{10}N(0)_1$ (log10(cfu/mL))	4.46	0.0589	4.33	4.59
SSE, MSE, RMSE = 0.10, 0.01, 0.102				
Ramp1 and Ramp2				
Parameters	estimate	SE	95% CI	95% CI
D_{121}	5.11	0.3554	4.38	5.84
z (degC)	8.23	0.1454	7.93	8.53
$\log_{10}N(0)_1$ (log10(cfu/mL))	4.29	0.0530	4.18	4.40
$\log_{10}N(0)_2$ (log10(cfu/mL))	4.3157	0.0562	4.1997	4.4318
SSE, MSE, RMSE = 0.73, 0.03, 0.175				

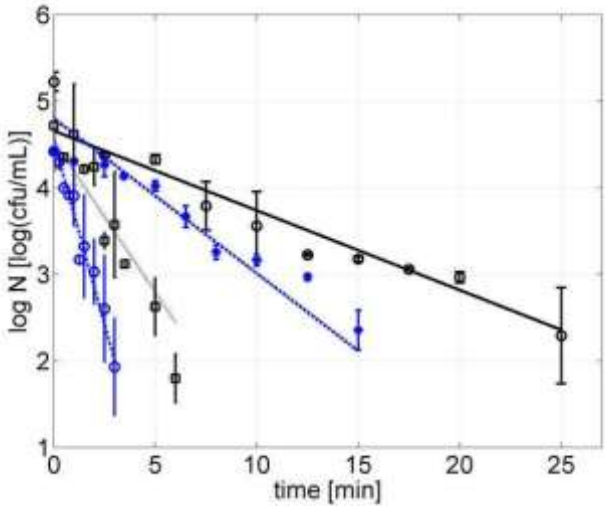

Figure 1. Regression analysis of all the static data by the use of Equation (1) at the following temperatures: 118 (●), 121 (●), 124 (○) and 127ºC (○).

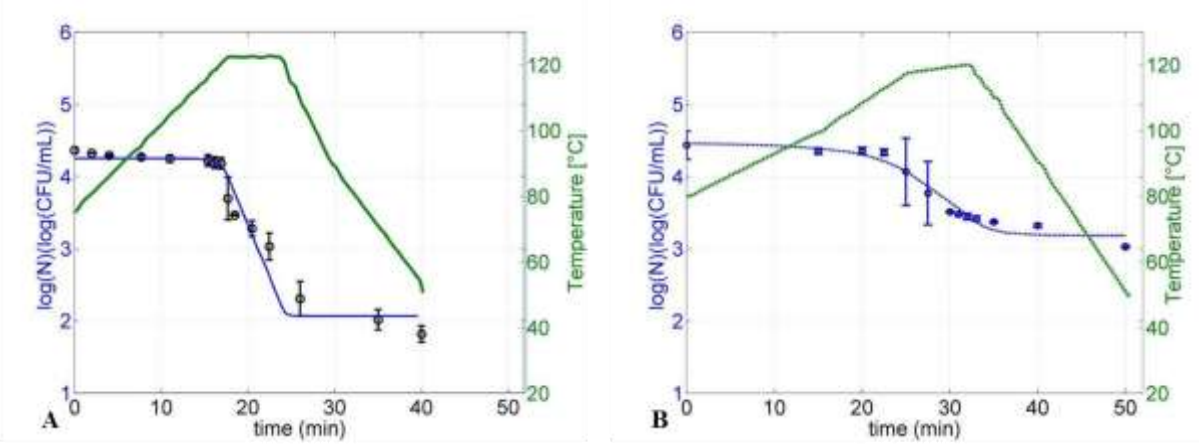

Figure 2. Regression analysis of data collected in ramp 1 (A) for a temperature profile from 80 to 121^{o}C and ramp 2 (B) for a temperature profile from 75 to 121^{o}C; by the use of Equation (1) in both cases. Temperature profile appears with a thicker line.

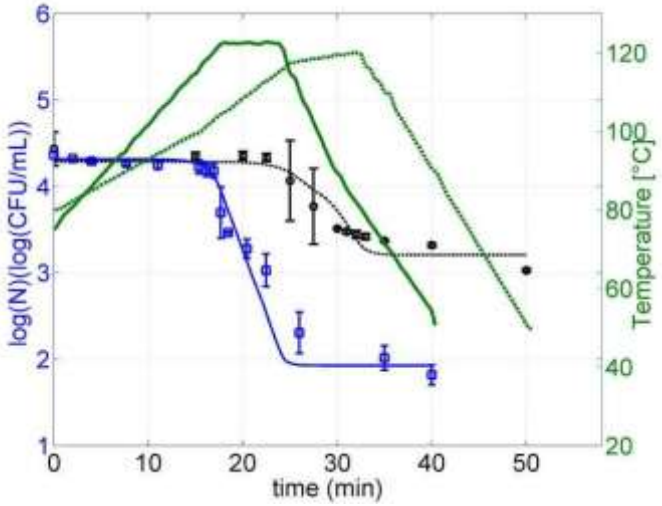

Figure 3. Regression analysis of both data collected during ramps 1 (– , line blue) and 2 (--, line black) by the use of Equation (1).Temperature profile appears with thick line.

Capítulo 3

Artigo Científico 2

Detection of viable cells of *Bacillus sporothermodurans* combining propidium monoazide with semi-nested PCR

Artigo científico submetido ao periódico *Food Microbiology*.

Fator de impacto: 3.283

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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.

Keywords: *Bacillus sporothermodurans*; semi-nested PCR; milk; detection; viable cells; propidium monoazide.

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; Nocker et al., 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; Yánez 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.

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 stearothermophilu*s (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 liter 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 X 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: EU 231617.1; 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 ºC), 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 x 10 cm (W x L) gel size, in 0.5 x 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 (~ 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 5,000 x *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 6,000 x *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: GU 238287 and JX 569192). 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; Nocker et al., 2007; Cawthorn et al., 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 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 et al., 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 ≤ 72ºC 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 72ºC 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 to 10), and its effect was not inhibited by milk components. The 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.

Acknowledgments

The authors thank the United States Department of Agriculture (USDA) for supplying seven *Bacillus* species strains and appreciate the financial support provided by Conselho Nacional de Pesquisa (CNPq).

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Figures

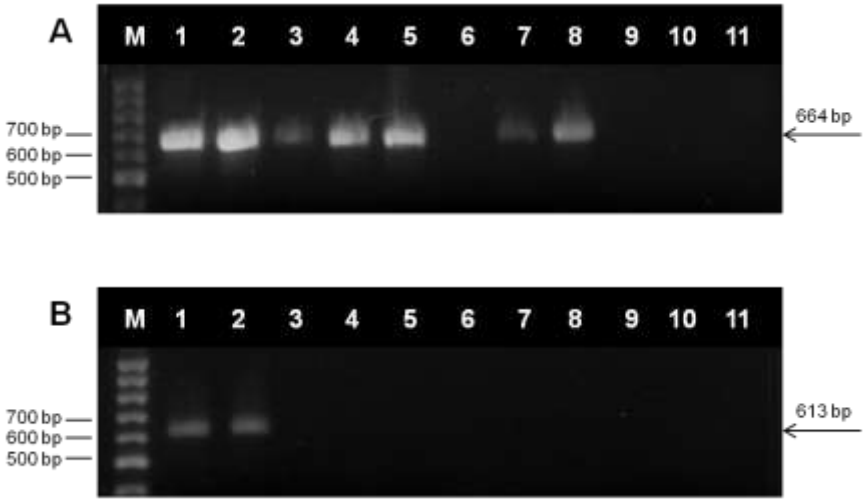


Fig. 1. (A) Detection of *Bacillus sporothermodurans* by PCR. Amplicons originated from genomic DNA of the following: (lane 1) *B. sporothermodurans* CBMAI 148; (lane 2) *B. sporothermodurans* CBMAI 155; (lane 3) *Bacillus cereus*; (lane 4) *Bacillus acidicola*; (lane 5) *Bacillus lentus*; (lane 6) *Bacillus 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.

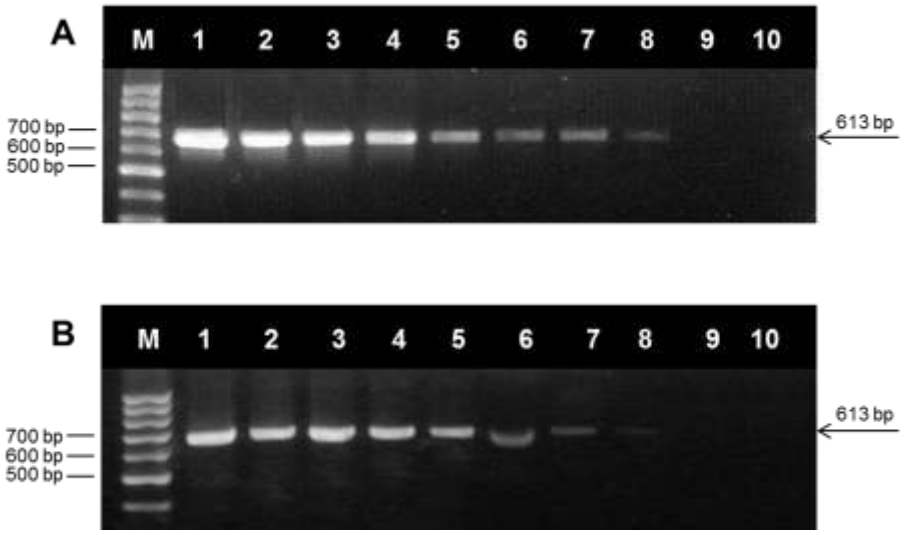


Fig. 2. Detection limit of *Bacillus sporothermodurans* by semi-nested touchdown PCR. Amplifications were performed from genomic DNA of (lane 1) *B. sporothermodurans* CBMAI 148 culture (4.0 x 10^7 CFU/mL); (lanes 2 to 9) ten-fold dilutions of a *B. sporothermodurans* CBMAI 148 culture (4.0 x 10^7 CFU/mL) 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.

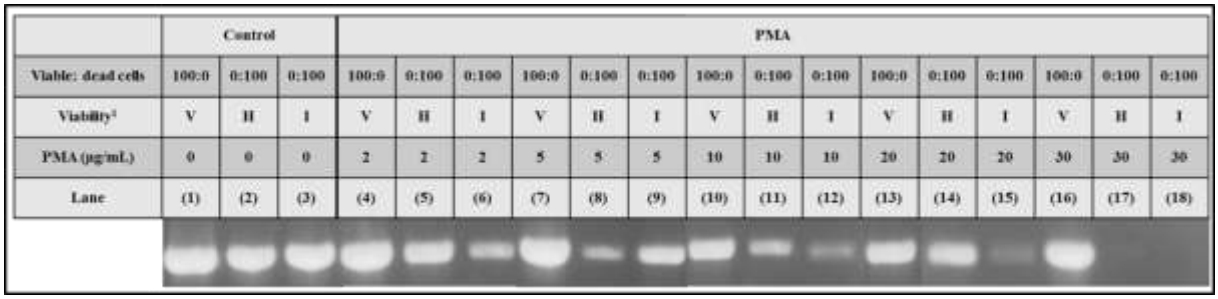


Fig. 3. The effect of different concentrations of propidium monoazide (PMA) for the detection of viable and dead (heat- or isopropanol-killed) *Bacillus sporothermodurans* CBMAI 148 cells by PCR. Lanes 1-3: control samples, without PMA treatment; lanes 4-6: 2 µg/mL PMA; lanes 7-9: 5 µg/mL PMA; lanes 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.

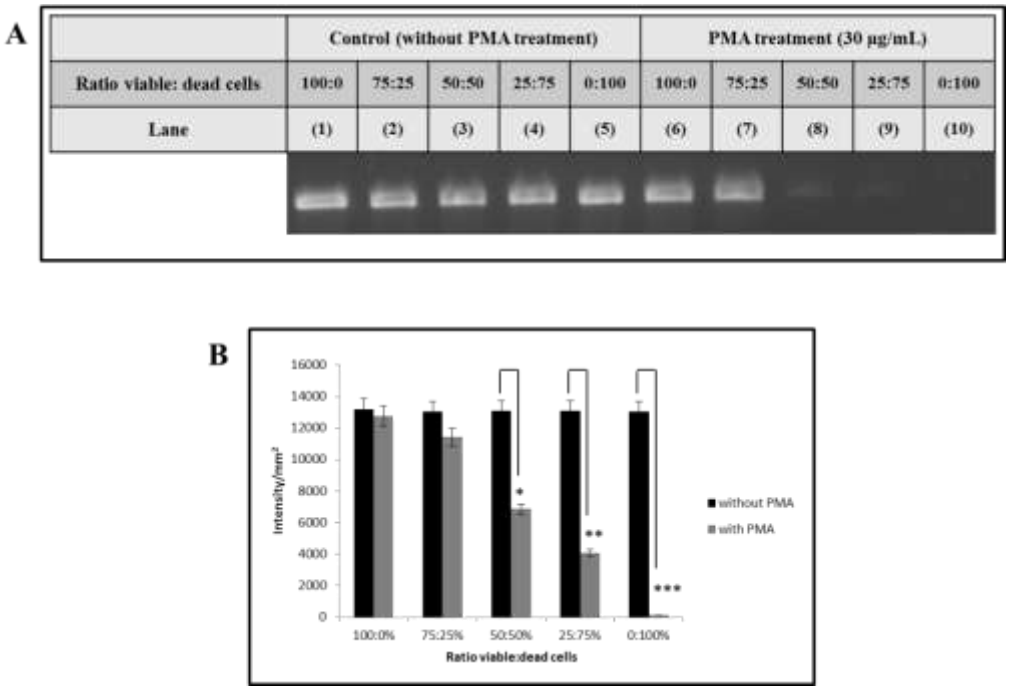


Fig. 4. The effect of propidium monoazide (PMA) treatment for the detection by PCR of viable and isopropanol-killed *Bacillus sporothermodurans* CBMAI 148 cells mixed at different ratios. **(A)** PCR products visualized on agarose gel stained with 0.5 µg/mL ethidium bromide under UV light. **(B)** Diagram 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.

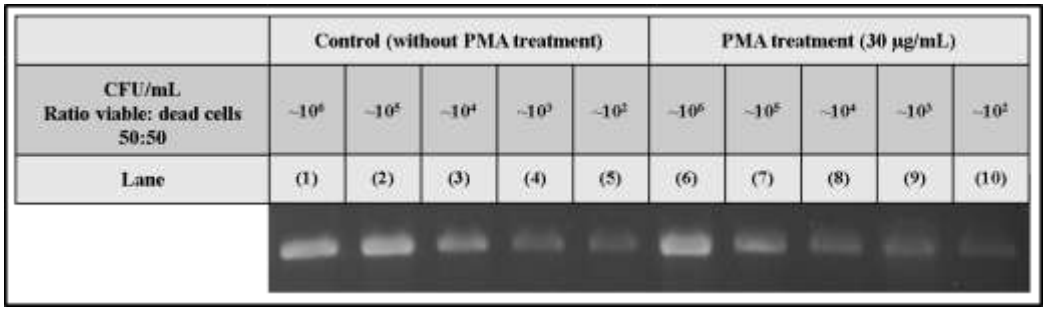
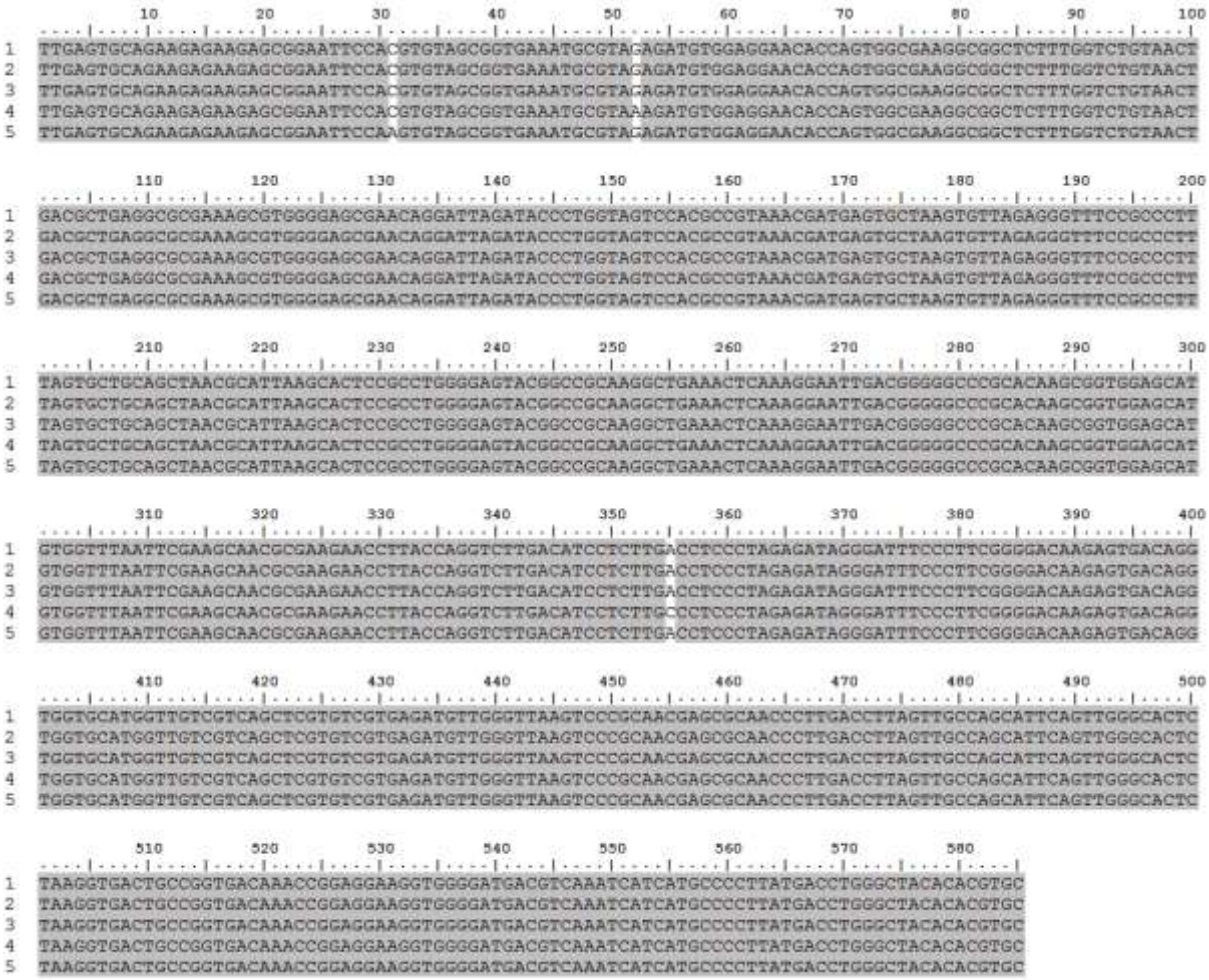
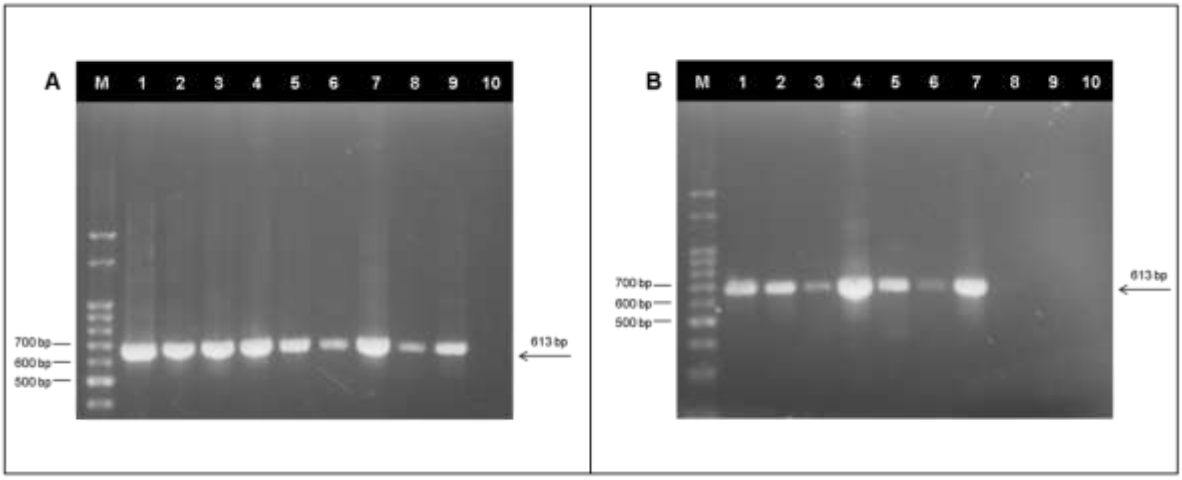


Fig. 5. Detection limit of the PMA-PCR to detect viable and isopropanol-killed *Bacillus sporothermodurans* cells in artificially contaminated milk. The ratio between viable and dead cells is 50:50.

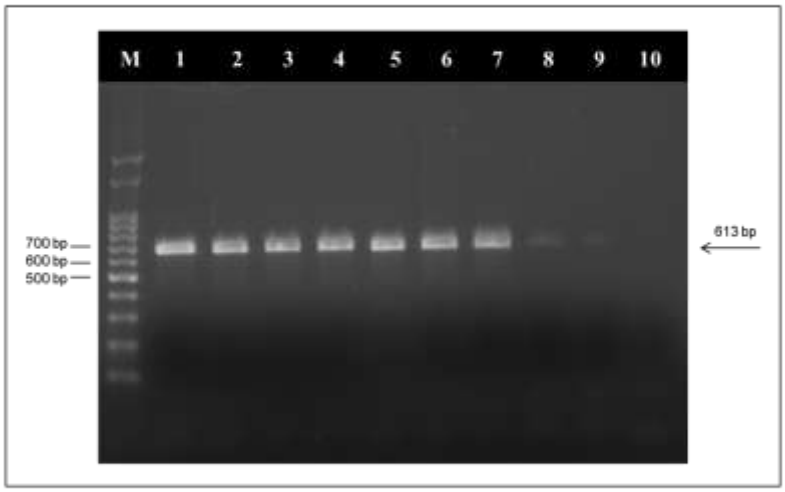
Supplementary Figures



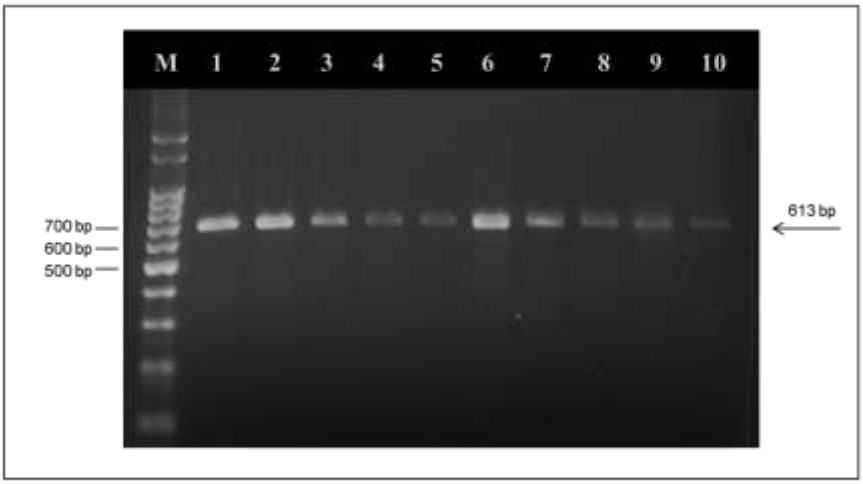
Supplementary Fig. 1. Alignment of the 16S rDNA nucleotide sequences of (1) *Bacillus sporothermodurans* CBMAI 148 (GenBank ID: GU 238287), (2) *B. sporothermodurans* CBMAI 155 (GenBank ID: JX 569192), (3) *B. sporothermodurans* LMG 17897 (GenBank ID: emb|AJ302941.1|), (4) *B. sporothermodurans* LMG 17883 (GenBank ID: emb|AJ302942.1|), and (5) *Bacillus acidicola* TCCC27037 (GenBank ID: gb|EU231617.1|). The similarity indexes are indicated in grey boxes.



Supplementary Fig. 2. (agarose gels corresponding to Fig. 3) The effect of the treatment with different concentrations of propidium monoazide (PMA) for the detection of viable and dead (heat- or isopropanol-killed) *Bacillus sporothermodurans* CBMAI 148 cells by PCR. **(A).** Amplicons originated from genomic DNA of: lanes 1-3 (without PMA): viable (1), heat killed (2) isopropanol killed cells (3); lanes 4-6 (2 µg/mL): viable (4), heat killed (5), isopropanol killed cells (6); lanes 7-9 (5 µg/mL): viable (7), heat killed (8), isopropanol killed cells (9); and lane 10: negative control (without template DNA); (M) 100 bp DNA ladder. **(B).** Amplicons originated from genomic DNA of: lanes 1-3 (10 µg/mL): viable (1), heat killed (2), isopropanol killed cells (3); lanes 4-6 (20 µg/mL): viable (4), heat killed (5), isopropanol killed cells (6); lanes 7-9 (30 µg/mL): viable (7), heat killed (8), isopropanol killed cells (9); and lane 10 – negative control (without template DNA); (M) 100 bp DNA ladder.



Supplementary Fig. 3. (agarose gels corresponding to 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. Lanes 1 to 5 (without PMA) correspond to amplicons from cell suspensions mixtures containing viable cells at 100%, 75%, 50%, 25% and 0%; lanes 6 to 10 (30 µg/mL of PMA) correspond to amplicons from cell suspensions mixtures containing viable cells at 100%, 75%, 50%, 25% and 0% .



Supplementary Fig. 4. (agarose gels corresponding to Fig. 5) Detection limit of the PMA-PCR to detect viable and isopropanol-killed *Bacillus sporothermodurans* cells in artificially contaminated milk. Lanes 1 to 5 correspond to amplicons from cell suspensions in milk (10^6 to 10^2 CFU/mL) without PMA treatment; Lanes 6 to 10 correspond to amplicons from cell suspensions in milk (10^6 to 10^2 CFU/mL) with 30 µg/mL of PMA.

Capítulo 4

Artigo Científico 3

Detection and quantification of viable *Bacillus sporothermodurans* in milk by PMA-qPCR

Artigo científico a ser submetido ao periódico *Food Research International*.

Fator de impacto: 3.150

Detection and quantification of viable *Bacillus sporothermodurans* in milk by PMA-qPCR

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ABSTRACT

Bacillus sporothermodurans produces highly resistant spores that can survive ultra high temperature (UHT) treatment in milk and germinate during the storage time. Currently, the quantification of this microorganism is performed by the traditional culture-based method, which is laborious and time consuming. Therefore, this study proposes a quantitative real-time PCR (qPCR) associated with a viability marker as a faster alternative to enumeration of viable *B. sporothermodurans* cells in UHT milk. Propidium monoazide (PMA) was chosen to detect only viable cells because of its ability to bind DNA from dead cells, preventing its amplification by PCR. The developed PMA-qPCR method showed to be specific for *B. sporothermodurans*, and presented a quantification limit of 2.2 x 10^2 CFU/ml, taken into account the losses that occur during the process of the milk samples. A total of 135 UHT milk samples were analyzed and *B. sporothermodurans* was found in 14 (10.4%) and 11 (8.1%) according PMA-qPCR and culturing method, respectively, not presenting a significant difference between both methods ($p = 0.083$). The positive samples showed counts ranging from 1.6 x 10^2 to 1.1 x 10^3 CFU/ml by PMA-qPCR and culturing method, not reaching quantities able to cause spoilage of the product. Therefore, the PMA-qPCR method could be of potential application in specific detection and accurate quantification of viable *B. sporothermodurans* cells in milk.

Keywords: *Bacillus sporothermodurans*; viability; UHT milk; propidium monoazide; qPCR.

1. Introduction

Bacillus sporothermodurans is a bacterium able to produce highly heat resistant spores, which are able to survive the industrial high temperature of UHT processing (130-140 °C for 4 s) (Hammer, Lembke, Suhren, & Heeschen, 1995; Pettersson, Lembke, Hammer, Stackebrandt, & Priest, 1996). Once the spores germinate, they may grow in stored UHT milk and cause instability due to their proteolytic activities and reduce shelf life (Klijn et al., 1997; Tabit & Buyes, 2010). Furthermore, *B. sporothermodurans* spores have been found to be more resistant than other heat resistant spores at temperatures above 130 °C with D_{140} ranging from 3.4 – 7.9 s (Huemer, Klijn, Vogelsang, & Langeveld, 1998). Unfortunately, the alternative of increasing the temperature and/or holding time to inactivate *B. sporothermodurans* spores in UHT milk can affect the organoleptic and nutritional quality of the product (Claeys, Ludikhuyze, & Hendrickx, 2001).

B. sporothermodurans is a non-pathogenic microorganism and, although it can affect the quality of dairy products when in counts above 10^5 CFU/ml (Klijn et al., 1997), there is no maximum limit for its presence in milk according to the legislations of several countries (FSANZ, 2001; Mercosul, 1995; USPHS/FDA, 2003). However, the *B. sporothermodurans* contamination can exceed the microbiological standard for viable aerobic mesophilic microorganisms (maximum 10^2 CFU/ml) required by the legislation of some countries, such as those belonging to European Union (EU) and Brazil (Anonymous, 1992; Brasil, 1997), which can result in considerable economic losses.

The detection and enumeration of *B. sporothermodurans* in milk is usually performed by traditional methods, including growth in culture media and confirmation by biochemical tests for bacterial identification (Pettersson et al., 1996; Brasil, 2003; Scheldeman, Herman,

Foster & Heyndrickx, 2006). These traditional procedures are laborious and time-consuming, and the phenotypic tests may lead to inaccurate results due to the poor growth characteristic of the organism (Scheldeman, Herman, Goris, De Vos & Heyndrickx, 2002), what can be circumvented by DNA-based PCR techniques that have been widely applied to detect microorganisms in food (Beneduce, Fiocco & Spano, 2007; Naraveneni & Jamil, 2005; Postollec, Falentin, Pavan, Combrisson & Sohier, 2011). However, DNA-based methods cannot differentiate between viable and dead bacterial because DNA can remain intact after cell death (Josephson, Gerba, & Pepper, 1993; Nogva, Drømtorp, Nissen, & Rudi, 2003). To overcome this limitation, nucleic acid intercalating dyes, such as propidium monoazide (PMA), have been used to treat samples prior to the PCR assays. PMA permeates through the damaged cell membrane of dead cells and binds to DNA, avoiding its amplification, while it has not been demonstrated to penetrate into viable cells (Nocker, Cheung, & Camper, 2006). Several studies have demonstrated that PMA has been used to inhibit the DNA amplification from a wide range of microorganisms including bacteria (Nocker, Sossa, Burr, & Camper, 2007; Cawthorn & Witthuhn, 2008; Taskin, Gozen, & Duran, 2011; Liang et al., 2011; Mamlouk et al., 2012), fungi (Vesper et al., 2008), a protozoan (Brescia et al., 2009), and viruses (Fittipaldi, Codony, Adrados, Camper, & Morató, 2010; Parshionikar, Laseke, & Fout, 2010). In our previous study (Cattani, Ferreira & Oliveira, submitted manuscript), we have presented the combination of PMA treatment with end-point PCR for the specific detection of viable *B. sporothermodurans* cells and showed applicability for the specific detection of viable cells from UHT milk. However, the quantitative analysis of the contamination by viable *B. sporothermodurans* through qPCR has not yet been investigated.

Thus, the aim of this study was to develop a qPCR associated with PMA treatment to quantify viable *B. sporothermodurans* cells, and determine its applicability in UHT milk.

2. Materials and methods

2.1 Bacterial strain and culture conditions

B. sporothermodurans CBMAI 148, obtained from the Brazilian Collection of Microorganisms from the Environment and Industry UNICAMP-CPQBA, *B. cereus* ATCC 33018, and *B. acidicola* (NRRL B-23453), *B. lentus* (NRRL NRS-1262), *B. firmus* (NRRL B-14307), *B. circulans* (NRRL B-378), *B. coagulans* (NRRL NRS-609) provided by the United States Department of Agriculture (USDA), were grown in Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) overnight at 37 °C.

2.2 Pre-treatment of samples with propidium monoazide

PMA (Biotium Inc., Hayward, California) was dissolved in 20% dimethyl sulfoxide (DMSO) (Nuclear, São Paulo, Brazil), and added to 500 µL of UHT milk samples to make final concentration of 30 µg/mL. The tubes were then placed in dark at room temperature for 10 min to allow the PMA to penetrate the dead cells and bind to the DNA. Next, the tubes were placed in crushed ice, and exposed to light for 10 min at 15 cm distance using a 500 W halogen light source (Osram, São Paulo, Brazil).

2.3 DNA isolation

Bacterial genomic DNA from pure culture or milk was extracted as described by Rademaker and de Bruijn (1997). DNA was ressuspended in a final volume of 50 µl of MilliQ water.

2.4 TaqMan-based qPCR assay

Specific probe and primers were designed with the Primer Express 3.0 software (Applied Biosystems, Foster City, USA) targeting the 16S rRNA gene to amplify a 200 bp-fragment. The forward primer (5'-AGAAGAGCGGAATTCCAC-3') and reverse primer (5'-AGCTGCAGCACTAAAGGGC-3') were used in combination with the TaqMan probe (5'-FAM- AGCGGTGAAATGC-MGBNQF- 3'). Amplification mixtures for qPCR contained 8 µl 2x Path-ID qPCR Master Mix (Ambion®, *Life Technologies,* USA), 0.4 µM of each primer (Invitrogen), 0.15 µM of the TaqMan-probe (Applied Biosystems) and 2 µl of template DNA, in a final volume of 20 µl. qPCR was performed on Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems) using an initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 68 °C for 45 s. No-template negative controls and the standard calibration curve was included in each run. Reactions were performed in duplicate.

The specificity of the qPCR developed assay was tested against six other different species of *Bacillus*: *B. cereus, B. acidicola, B. lentus, B. firmus, B. circulans* and *B. coagulans*.

In addition, the effectiveness of the PMA treatment combined with qPCR was further evaluated with viable and dead cells treated with PMA as described by Cattani, Ferreira & Oliveira (submitted manuscript). Briefly, *B. sporothermodurans* viable and isopropanol-killed

cells were treated with 30 µg/ml of PMA and, subsequently, submitted to DNA extraction. After the qPCR analysis, ΔCt values were calculated by the following equation: Ct value for PMA treated cells − Ct values for untreated cells.

2.5 Standard calibration curves

The standard curve to calibrate qPCR was generated using ten-fold dilutions of plasmid DNA harboring the target insert of *B. sporothermodurans*. The target fragment was cloned into the pGEM-T Easy vector systems (Promega, Madison, Wi, USA) according to the manufacturer's instructions. The recombinant vectors were used to transform Top10 *Escherichia coli* strain by heat shock (42 °C for 45 s), that were spread on Luria Bertani (LB) agar with ampicillin (100 µg/ml) and incubated at 37 °C for 12 h. The grown colonies were selected and inoculated in LB broth with ampicillin (100 µg/ml) at 37 °C for 12 h with shaking at 150 rpm. Plasmid DNA was purified using a PureYield™ *Plasmid Miniprep* kit (Promega), separated by electrophoresis in 0.6% agarose gel stained with ethidium bromide (0.5 µg/ml), and visualized under UV light. The corresponding DNA-fragment was isolated from agarose gel and purified using Quick gel extraction kit (Promega). The concentration of extracted plasmid DNA harboring the target insert was determined by fluorimetry (Qubit, Invitrogen, Carlsbad, CA, USA), the absolute numbers of plasmid molecules calculated as previously reported (Yun et al., 2006), and 10-fold serial dilutions were prepared, ranging from 10^7 to 10^1 plasmid copies per PCR. A standard curve was generated by plotting the DNA amount (mathematically adjusted to the supposed copies/ml) against the Ct value exported from StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

A second standard curve was constructed considering all steps required to perform the PMA-qPCR assay to analyze *B. sporothermodurans* contamination in milk. Then, a *B. sporothermodurans* CBMAI 148 overnight culture in BHI, containing 3.5 x 10^7 CFU/ml, was inoculated in 9 ml of milk and 10-fold dilutions were prepared in UHT milk. All milk dilutions were treated with PMA, in duplicate. DNA from 500 µl aliquot of inoculated milk was extracted as described above. The standard curve was generated by plotting the DNA amount (expressed in its correspondent cell concentration in CFU/ml) against the Ct value exported from the equipment.

Considering that the developed qPCR assay in this study is influenced by the fact that the 16S rRNA gene is present in multiple copies of the genus *Bacillus* ranging from seven to fourteen copies as revealed genomes sequenced to date (http://ribosome.mmg.msu.edu/rrndb/search.php) and, to our knowledge, there is no data in the literature concerning the exact number of 16S rRNA gene copies per cell of *B. sporothermodurans*, we estimate the number of gene copies of *B. sporothermodurans* comparing the amplification from the cell-based curve with the standard plasmid DNA-based curve.

2.6 Sample preparation

The developed PMA-qPCR assay, in parallel with traditional microbiological method, was used to analyze 135 UHT milk samples from different lots and brands. The samples were bought from local supermarkets during the period of October 2011 to January 2012. All samples were initially incubated for 7 days at 36 ± 1 °C and analyzed in parallel through traditional microbiological method and qPCR associated to PMA treatment. For the molecular

analysis, two aliquots of each milk sample were collected and submitted to DNA extraction with and without prior PMA treatment.

2.7 Detection of *B. sporothermodurans* in milk by culturing

The procedure for the enumeration of *B. sporothermodurans* in UHT milk was performed according to Brasil (2003), with some modifications. Briefly, after the incubation mentioned above, aliquots of 0.1 ml of each milk sample and two decimal dilutions (10^{-1} and 10^{-2}) were spread on BHI (Merck) agar modified by addition of 1 mg/ml of vitamin B_{12} (Vetec Química Fina Ltda, Rio de Janeiro, Brazil) (Pettersson, et al., 1996), in duplicate. All plates were incubated at 30 °C for 72 h for colony count. Colonies reported as typical (small, smooth, off-white to beige, and no soluble pigment) were enumerated and selected for further biochemical (esculin hydrolysis, glucose fermentation, nitrate reduction, catalase, oxidase and urease production) and morphological (Gram stain) identification.

2.8 Statistical analysis

In order to compare the frequencies of positive samples for the presence of *B. sporothermodurans* obtained by the PMA-qPCR, qPCR without PMA and culturing method, Cochran's Q test was employed followed by pairwise comparisons using the Bonferroni correction. One-way ANOVA, succeeded by Tukey's post-hoc test, was used to evaluate 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 (α) was set to 0.05 for all tests.

3. Results and discussion

In the present study, a qPCR assay combined with PMA was developed and applied to determine the prevalence of viable *B. sporothermodurans* cells in UHT milk. At first, the qPCR was tested for specificity against other *Bacillus* species and positive results were obtained only for *B. sporothermodurans* presence. In order to develop a method able to select only viable cells, a PMA treatment was then associated to the qPCR assay and, thus, viable and dead cells of *B. sporothermodurans* quantification experiments were evaluated. The ΔCt value for viable cells was 0.25 (Table 1) indicating that PMA did not influence viable cells detection. In contrast, the ΔCt value for dead cells was 15.92 (Table 1), demonstrating that PMA reduced amplification of DNA derived from dead cells with efficiency of 99.99%, and not inhibiting the amplification of target DNA from viable cells of *B. sporothermodurans*.

For the detection and quantification of *B. sporothermodurans* by PMA-qPCR method, plasmid DNA- and cell-based standard curves were obtained. The plasmid DNA standard curve showed an amplification efficiency of 87.5% with a good quantitative accuracy (r^2= 0.9958) and allowed us to determine the quantification limit of the designed qPCR in 2.7 x 10^3 DNA copies/ml (Fig 1A). Nevertheless, to establish the real sensitivity of the PMA-qPCR method, the second standard curve was constructed using artificially contaminated UHT milk with a known number of *B. sporothermodurans* and submitted to the PMA treatment and DNA extraction for qPCR analysis. The results showed a detection limit of 3.5 x 10^2 CFU/ml with an amplification efficiency of 88.8% and determination coefficient (r^2) of 0.9986 (Fig. 1A). Based on the data obtained, it was possible to estimate the number of copies of the16S rRNA gene, which is consistent with twelve copies per cell, and, therefore, it could be concluded that the actual detection limit of PMA-qPCR assay for detection and quantification

of viable *B. sporothermodurans* in milk was 2.2 x 10^2 CFU/ml (Fig. 2B). Such analysis took into account the estimated loss of genetic material during the processing of milk samples, which has been previously determined for another *Bacillus* species as approximately 38% using the same PMA treatment and DNA extraction protocols (Cattani, Barth Jr, Nasário, Ferreira & Oliveira, submitted manuscript).

In order to investigate the usefulness of the PMA-qPCR method, a total of 135 UHT milk samples were evaluated for the presence of viable *B. sporothermodurans*. After incubation at 36°C for 7 days, no UHT milk samples showed visible changes such as bloating, casting or coagulation. Thus, all samples were submitted to molecular and culturing methods. The PMA-qPCR and the traditional microbiological technique detected viable *B. sporothermodurans* in 14 (10.4%) and 11 (8.15%) UHT milk samples, respectively, showing no statistically significant difference ($p = 0.083$) between both methods. The occurrence of *B. sporothermodurans* found in milk was similar to those described by Neuman, Salvatori, Majolo and Froder (2010) evaluating 511 UHT milk samples by culturing methods. However, a number quite larger than these was found previously by Busatta, Valduga and Cansian (2005) in 88 UHT milk samples analyzed. The quantitative analysis allow us to find Ct values for the 14 positive milk samples ranging between 36.0 and 39 cycles, which corresponded to 1.6 x 10^2 to 1.1 x 10^3 CFU/ml (Table 2), not reaching a quantity able to cause spoilage of the product (Klijn et al., 1997).

Twenty-four (17.8%) samples were positive by qPCR for the detection and quantification of *B. sporothermodurans* without PMA treatment, resulting in significant difference between qPCR and PMA-qPCR methods ($p = 0.002$). These results clearly illustrated that qPCR without a viability marker significantly overestimated the number of *B.*

sporothermodurans present in the milk samples in comparison with the PMA-qPCR assay. The selective quantification of viable cells is of special concern for the samples that suffered some antimicrobial treatment, such as UHT process. In this case, the milk is heated up to high temperature for a few seconds killing vegetative bacterial cells. However, DNA from these dead bacterial cells can serve as template during PCR amplification resulting in false positive results (Josephson et al., 1993; Wolffs, Norling & Radstrom, 2005; Liang et al., 2011; Mamlouk et al., 2012). Thus, the PMA treatment can inhibit the amplification of DNA from dead cells and enable the qPCR to quantify specifically viable *B. sporothermodurans* cells present in UHT milk.

In conclusion, the PMA-qPCR assay developed in this study was found to be a sensitive and specific method, providing a good estimation of viable *B. sporothermodurans* cells contamination in milk. This assay also presents the advantage of obtaining results in a short time when compared to the traditional culture method, and it could be of great value for the dairy industry to quantify this microorganism in UHT milk.

Acknowledgments

Financial support was provided by MAPA-SDA/CNPq (Conselho Nacional de Pesquisa), Brazil, and Fernanda Cattani received a scholarship from PROBOLSAS/Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

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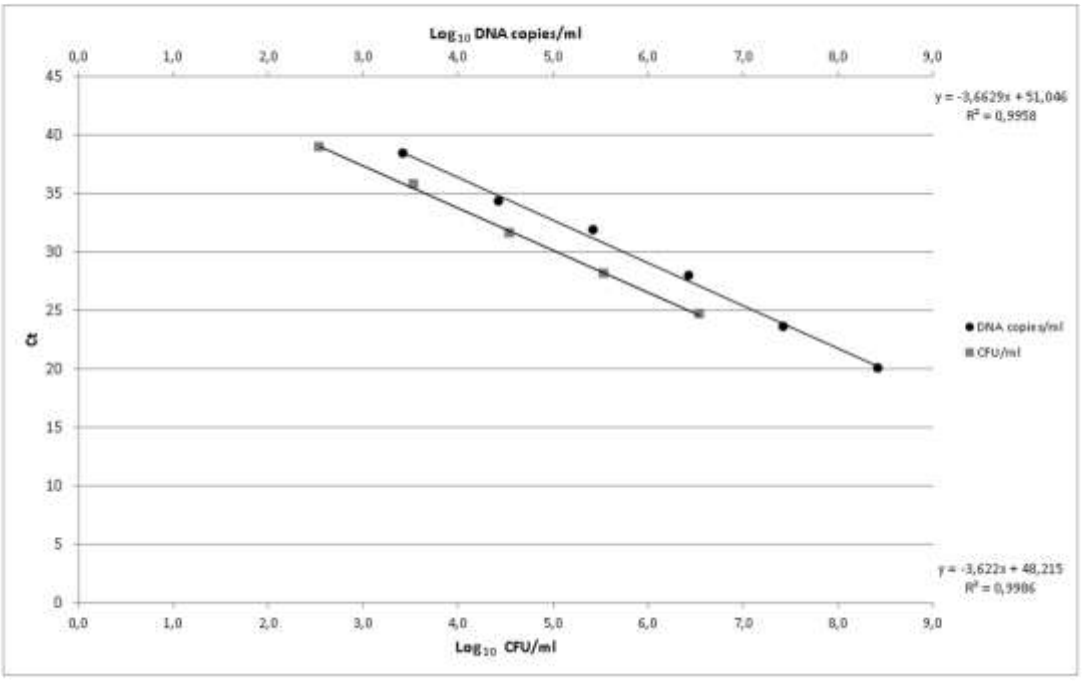
Figure

A


Log10 DNA copies/ml
0,0 1,0 2,0 3,0 4,0 5,0 6,0 7,0 8,0 9,0
45 40 35 30 25 20 15 10 5 0
Ct
y = -3,6629x + 51,046
R² = 0,9958
DNA copies/ml
CFU/ml
y = -3,622x + 48,215
R² = 0,9986
Log10 CFU/ml

B

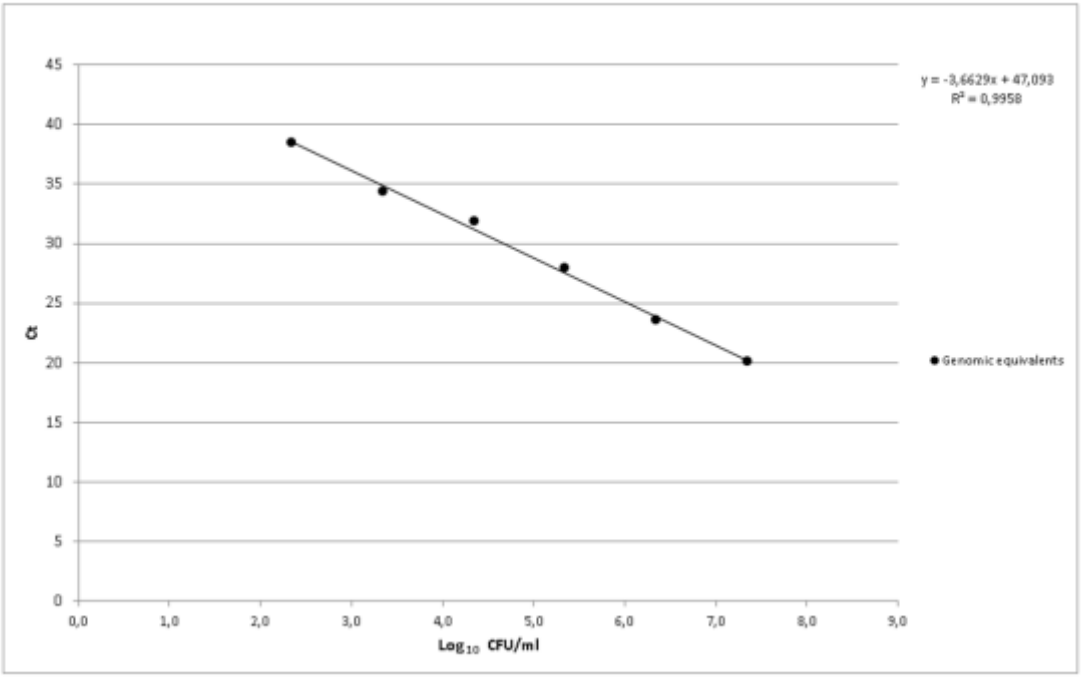

45 40 35 30 25 20 15 10 5 0
Ct
y = -3,6629x + 47,093
R² = 0,9958
Genomic equivalents
0,0 1,0 2,0 3,0 4,0 5,0 6,0 7,0 8,0 9,0
Log10 CFU/ml

Figure 1. Standard curves used to calibrate the PMA-qPCR method. (A) Plasmid DNA and cell-derived standard calibration curves for the *B. sporothermodurans* PMA-qPCR assay. Standard curve of 10-fold serial dilution of: (●) plasmid DNA (from 2.7 x 10^8 to 2.7 x 10^3 of DNA copies/ml); (■) 10-fold serial dilution of *B. sporothermodurans* in artificially inoculated milk (from 3.5 x 10^6 to 3.5 x 10^2 CFU/ml). (B) Standard curve generated by the interpolation of the cells-derived curve and the plasmid DNA curve.

Tables

Table 1

Effect of PMA treatment on cycle threshold (C_t) values obtained in quantitative PCR assay from viable or dead *Bacillus sporothermodurans* derived from a culture with cell density of 10^7 CFU/ml.

Cells	Ct values				
	NT	SD	T	SD	ΔCt
Viable	22,20[a*]	0.56	22,45[a]	0.6	0.25
Dead	22,03[a]	0.58	37,95[b]	0.35	15.92

[*]Different superscripts indicate statistically different values (p<0.05).
NT, samples not treated with PMA
T, samples treated with PMA
SD, standard deviations

Table 2

Quantification of viable *Bacillus sporothermodurans* in UHT milk by real time PCR associated with PMA and conventional culture method.

CFU/ml	Real-time PCR		Traditional method
	Ct[a] range	n° samples	n° samples
$\geq 4.0 \times 10^2$ - $\leq 1.1 \times 10^3$	$36.0 \leq Ct \leq 38.0$	6	6
$\geq 1.6 \times 10^2$ - $\leq 3.9 \times 10^2$	$38.1 \leq Ct \leq 39.0$	8	5
not detectable	> 39.0 or undeterminated[b]	121	124
Total number of analyzed samples		135	135

[a]Ct=cycle threshold; [b]Ct >39.0 or undetermined was assumed as absence of *B. sporothermodurans* detection.

Capítulo 5

Artigo Científico 4

Real-time PCR associated with propidium monoazide for quantification of viable *Bacillus cereus* in milk

Artigo científico submetido ao periódico *International Journal of Food Microbiology*.

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Specific quantification of viable *Bacillus cereus* in milk by qPCR

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Running title: Quantification of viable *Bacillus cereus*

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ABSTRACT

Bacillus cereus is an important spore-forming pathogen that is associated with foodborne diseases and can be detected in food using culturing methods, which can be laborious and time-consuming. The aim of this study was to develop a quantitative real-time PCR (qPCR) combined with a propidium monoazide (PMA) treatment to specifically analyze the contamination of UHT milk by viable *B. cereus* cells. Thirty µg/mL 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 x 10^2 CFU/mL of milk. To evaluate the applicability of the developed assay, 135 UHT milk samples were analyzed and compared to the culture-based method, and *B. cereus* was detected in 44 (32.6%) and 15 (11.1%) samples, respectively. The evaluation of microorganism counts in UHT milk using both methods indicates that infective dose may be reached depending on the product storage conditions. Thus, the PMA-qPCR assay developed in this study was found to be a sensitive method that was able to supply an accurate estimation of the contamination of viable *B. cereus*. The application of this method in the food industry could provide great value through the quantification of this foodborne pathogen, which represents an important public health risk.

Keywords: *Bacillus cereus*; viability; propidium monoazide; UHT milk; real-time PCR.

1. Introduction

Bacillus cereus is a spore-forming Gram-positive bacterium that is ubiquitously found in the environment and has been isolated from a wide variety of foods (Dierick et al., 2005; Fricker et al., 2007; Ankolekar et al., 2009). These foods include raw, pasteurized and ultra high temperature (UHT) milk (Notermans et al., 1997; Christiansson et al., 1999; Bahout, 2000; Banykó and Viletelová, 2009; Batchoun et al., 2011; Montanhini et al., 2012). This microorganism is undesired in the food industry due to its spoilage capability and pathogenic potential (Ankolekar et al., 2009). Consumption of food contaminated with *B. cereus* has been implicated in outbreaks of emetic and diarrheal syndrome (Lund and Granum, 1996; Ehling-Schulz et al., 2004; EFSA, 2005; Arnesen, et al., 2008).

Traditionally, enumeration of *B. cereus* 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; Brasil, 2003; ISO 7932, 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; Alessandria et al., 2010; 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 non-viable cells is amplified, then the bacteria 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 extracellular DNA and DNA from dead and/or membrane-compromised cells (Nocker et al., 2006; Nocker et al., 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). However, to our knowledge, the effectiveness of PMA has not yet been evaluated for *B. cereus*, which would be of value from a food safety perspective.

We describe a quantitative real-time PCR (qPCR) combined with PMA treatment that is specific and sensitive in the detection and quantification of viable *B. cereus* directly from naturally contaminated UHT milk samples.

2. Materials and methods

2.1. Bacterial strains and culture conditions

B. sporothermodurans CBMAI 148 was obtained from the Brazilian Collection of Microorganisms from the Environment and Industry UNICAMP-CPQBA. *B. acidicola* (NRRL B-23453), *B. lentus* (NRRL NRS-1262), *B. firmus* (NRRL B-14307), *B. circulans* (NRRL B-378) and *B. coagulans* (NRRL NRS-609) were provided by the United States Department of Agriculture (USDA). All strains were cultivated in Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) at 37 °C for 24 h.

B. cereus ATCC 33018 was grown overnight in BHI broth (Merck) at 37 °C, corresponding to a concentration of 10^8 cells/mL. Dead cells were obtained from two

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 cell suspension followed by incubation for 30 min at room temperature. The absence of viable cells from both strategies was confirmed by spread plating 100 µL aliquots of cells on BHI agar.

2.2. Optimization of PMA conditions

PMA (Biotium Inc., Hayward, USA) was dissolved in 20% dimethyl sulfoxide (DMSO) (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. Afterwards, tubes were exposed to light for 10 min at a 15-cm distance using a 500 W halogen light source (Osram, São Paulo, Brazil). Additionally, to determine the PMA effectiveness 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.

2.3. DNA extraction

Bacterial genomic DNA from pure culture or milk was extracted as described by Rademaker and de Bruijn (1997). The extracted DNA was resuspended in a final volume of 50 µL of MilliQ water.

2.4. End-point PCR

The optimization of PMA treatment was performed via end-point PCR using oligonucleotides targeting the hemolysin gene of *B. cereus* (Wang et al., 1997). PCR was performed in a solution containing 1.5 U of *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany), 1 X PCR buffer (Fermentas), 3 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (Fermentas), 0.8 µM 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, USA) 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. PCR products were visualized on 1% agarose gel (Invitrogen) in TBE 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 the Student's t-test using IBM® SPSS® Statistics version 20 (Somers, New York, USA). A *p*-value of less than 0.05 was considered to be statistically significant.

2.5. Sample preparation

A total of 135 UHT milk samples from different lots and brands were purchased from local retail markets during the period between October 2011 and January 2012.

All samples were analyzed in parallel using 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.

2.6. Detection of B. cereus in milk by culturing

Milk samples were incubated for 7 days at 36 ± 1 °C in order to favor spore germination. Subsequently, aliquots of 25 mL of each sample were taken aseptically and then diluted 10-fold in 0.1% peptone saline (pH 7.0). All dilutions were spread on *B. cereus* selective agar base mannitol egg yolk polymyxin B (MYP) (Himedia, Mumbai, India) in duplicate and incubated at 30 °C for 24-48 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 (Brasil, 2003). *B. cereus* ATCC 33018 was used as a reference culture.

2.7. Standard calibration curves

A standard curve was generated using 10-fold dilutions of plasmid DNA harboring the target insert of *B. cereus* by qPCR. The 185-bp fragment of the hemolysin gene was cloned into the pGEM-T Easy vector system (Promega, Madison, USA), 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 (LB) agar with ampicillin (100 µg/mL) and incubated for 12 h at 37 °C. Colonies were selected and inoculated in LB 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 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 Ct value exported from the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

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 33018 in BHI containing 7.5 x 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. 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 (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).

2.8. qPCR

A TaqMan qPCR method for the specific detection and quantification of *B. cereus* 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. qPCR using the primers and the probe was optimized with Path-ID qPCR Master Mix (Ambion®, Life Technologies, USA) in a final volume of 20 µL that contained 10 µL 2 X Path-ID qPCR Master Mix, 0.6 µM of each primer, 0.2 µM 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. In every analysis, a negative control was included by using 2 µL of MilliQ 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 six different species of *Bacillus*: *B. sporothermodurans*, *B. acidicola*, *B. lentus*, *B. firmus*, *B. circulans* and *B. coagulans*.

2.9. Statistical analysis

To compare the *B. cereus* positive sample frequencies obtained by the PMA-qPCR, qPCR without PMA and culturing method, Cochran's Q test was employed followed by pairwise comparisons using the Bonferroni correction. One-way ANOVA, succeeded 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 (α) was set to 0.05 for all tests.

3. Results and discussion

In the present study, we report a useful PMA-qPCR assay to allow the specific detection of viable *B. cereus*, enabling the DNA-based method to accurately 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 ($p<0.05$) (Fig. 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. A PMA concentration of 30 µg/mL was used in the remaining experiments, as it was shown to completely inhibit the PCR amplification from dead cell DNA (Fig. 1, lanes 17 and 18). By contrast, we detected specific amplifications from PMA-treated viable cell DNA, non-treated viable cells (Fig. 1, lanes 1, 4, 7, 10, 13 and 16) and non-treated dead cells (Fig. 1, lanes 2 and 3). Furthermore, we showed that the PMA associated to 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 (Fig. 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 the *B. cereus* contamination in some foods because certain levels of this microorganism may be acceptable, which indicates the need to use a quantitative method. We associated the standardized PMA treatment with the qPCR to specifically quantify viable *B. cereus* cells. To evaluate the effect of PMA on qPCR-amplifiable DNA from viable or dead cells, ΔC_t (C_t value for PMA treated cells − C_t values for untreated cells) values were calculated. The ΔC_t values for viable and dead cells were 0.83 and 14.93, respectively (Table 1), which indicates that PMA showed no interference in the detection of viable *B. cereus* using the qPCR assay developed here.

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 in this study was designed to specifically detect *B. cereus*, which was confirmed by the absence of amplification from the DNA of six different strains and species of *Bacillus*.

The enumeration of *B. cereus* 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 x 10^2 DNA copies/mL (Fig. 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 us a quantification limit of 7.5 x 10^2 CFU/mL (Fig. 3A). Because 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 (Fig. 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 loss was then considered when determining the quantity of viable *B. cereus* cells in UHT milk samples. The developed method was then compared to the traditional culturing technique by analyzing 135 UHT milk samples. The qPCR without PMA treatment detected *B. cereus* in 78 samples (57.8%), and the PMA-qPCR method detected 44 positive samples (32.6%) (C_t ranging from 26.9 to 37.0), presenting a significant difference ($p<0.001$) (Table 2). The PMA treatment likely inhibited the amplification of a large amount of DNA from cells that were killed during the UHT processing, which avoided an overestimation of *B. cereus* cells when using qPCR and, thus, did not overvalue potential health risks. All 15 samples that were *B. cereus*-positive according to the culture-based method were also detected by PMA-qPCR. Additionally, qPCR-PMA was able to detect the presence of *B. cereus* in 29 additional samples, showing a significant difference ($p<0.001$) when comparing both methods. Thus, these results indicate that the PMA-qPCR method was far more sensitive than the culturing method, which, at least partially, can be due to the lack of sensitivity of the selective media used to identify *B. cereus* (Ehling-Schulz et al., 2004; Fricker et al., 2008; Reekmans et al., 2009; Ceuppens et al., 2010). The MYP medium, which is commonly used to detect *B. cereus*, can present weak reactions and is unable to detect *B. cereus* strains without lecithinase activity (ISO 2004). 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 also viable but non-culturable (VBNC) microorganisms. The VBNC 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, Wery et al., 2008).

Analyzing these data in a quantitative perspective, it was found that 44 UHT milk samples contained *B. cereus* 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 days before the analyses, and, therefore, cell counts do not correspond to those originally present in the samples. However, these data show that raw milk can be contaminated with *B. cereus* spores that survive to 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 dose required to cause foodborne disease (Gilbert and Kramer, 1986; Granum and Lund, 1997; Granum, 2002; Arnesen et al., 2008; Martínez-Blanch et al., 2009; Salustiano et al., 2010). Moreover, according to European regulation (EC nº 2073/2005), foods in general should not contain microorganisms, their toxins or metabolites in quantities that present an unacceptable risk for human health. This guideline is similar to the Brazilian requirements for UHT milk, which should not present pathogenic microorganisms (Brasil, 2001). Thus, at least 28.1% and 11.1% of the UHT milk samples analyzed using the PMA-qPCR assay and traditional culture methods, respectively, did not meet the established standards.

An important advantage of the method developed in this study is that it accomplishes a rapid, specific and sensitive evaluation of *B. cereus* levels in UHT milk, detecting it at numbers lower than the minimum infectious dose. This method represents a more realistic and accurate analysis for food products, which is crucial to lower costs and improve food-health security.

Acknowledgements

Financial support was provided by MAPA-SDA/CNPq (Conselho Nacional de Pesquisa), Brazil, and Fernanda Cattani received a scholarship from PROBOLSAS/Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS).

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Tables

Table 1

Effect of PMA treatment on cycle threshold (C_t) values obtained in quantitative PCR assay from viable or dead *Bacillus cereus* derived from a culture with cell density of 10^8 CFU/mL.

Cells	Ct values				
	NT	SD	T	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

[*]Different superscripts indicate statistically different values (p<0.05).
NT, samples not treated with PMA
T, samples treated with PMA
SD, standard deviations

Table 2

Quantification of viable *Bacillus cereus* in UHT milk by real time PCR associated with PMA and bacterial culture method.

CFU/mL	Real-time PCR		Traditional method
	Ct[a] range	n° samples	n° samples
$\geq 3.9 \times 10^4$ - $\leq 3.8 \times 10^5$	$23.6 \leq Ct \leq 27.0$	3	3
$\geq 3.9 \times 10^3$ - $\leq 3.8 \times 10^4$	$27.1 \leq Ct \leq 30.8$	10	9
$\geq 3.8 \times 10^2$ - $\leq 3.8 \times 10^3$	$30.9 \leq Ct \leq 34.4$	25	3
$\geq 1.8 \times 10^2$ - $\leq 3.7 \times 10^2$	$34.5 \leq Ct \leq 37.0$	6	0
not detectable	> 37.0 or undeterminated[b]	91	120
Total number of analyzed samples		135	135

[a]Ct=cycle threshold; [b]Ct >37.0 or undetermined was assumed as absence of *B. cereus* detection.

Figures

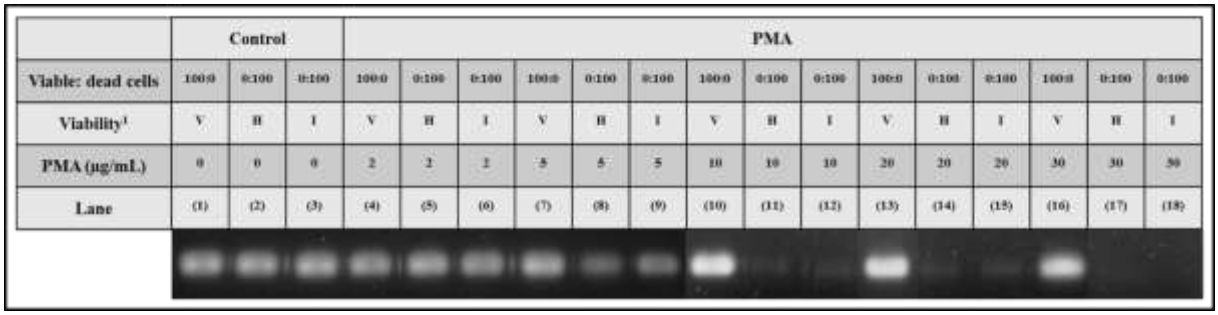


Figure 1. The effect of different concentrations of PMA on the detection of viable and dead (heat- or isopropanol-killed) *Bacillus cereus* cells using PCR. Lanes 1-3: control samples, without PMA treatment; lanes 4-6: 2 µg/mL PMA; lanes 7-9: 5 µg/mL PMA; lanes 10-12: 10 µg/mL PMA; lanes 13-15: 20 µg/mL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

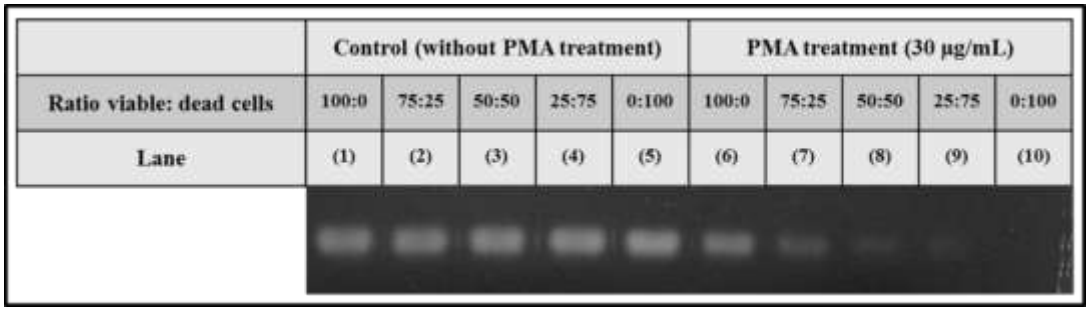
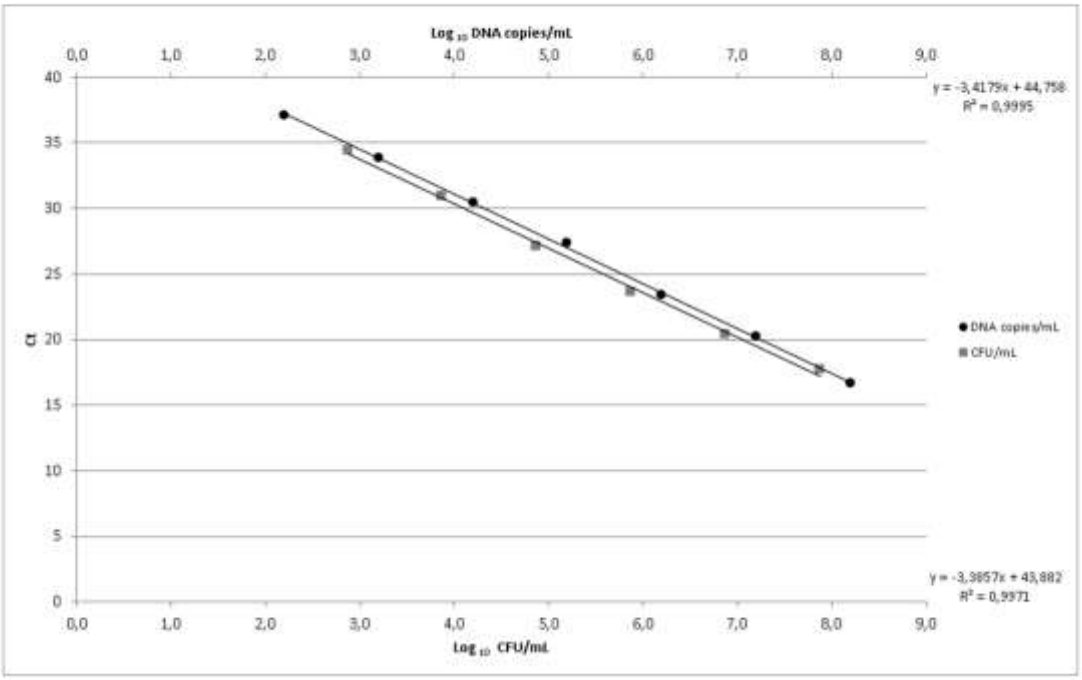


Figure 2. The effect of PMA treatment on the PCR-based detection of viable and isopropanol-killed *Bacillus cereus* cells mixed at different ratios.

A


Log 10 DNA copies/mL
0,0 1,0 2,0 3,0 4,0 5,0 6,0 7,0 8,0 9,0
40 35 30 25 20 15 10 5 0
Ct
y = -3,4179x + 44,758
R² = 0,9995
DNA copies/mL
CFU/mL
y = -3,3857x + 43,882
R² = 0,9971
Log 10 CFU/mL

B

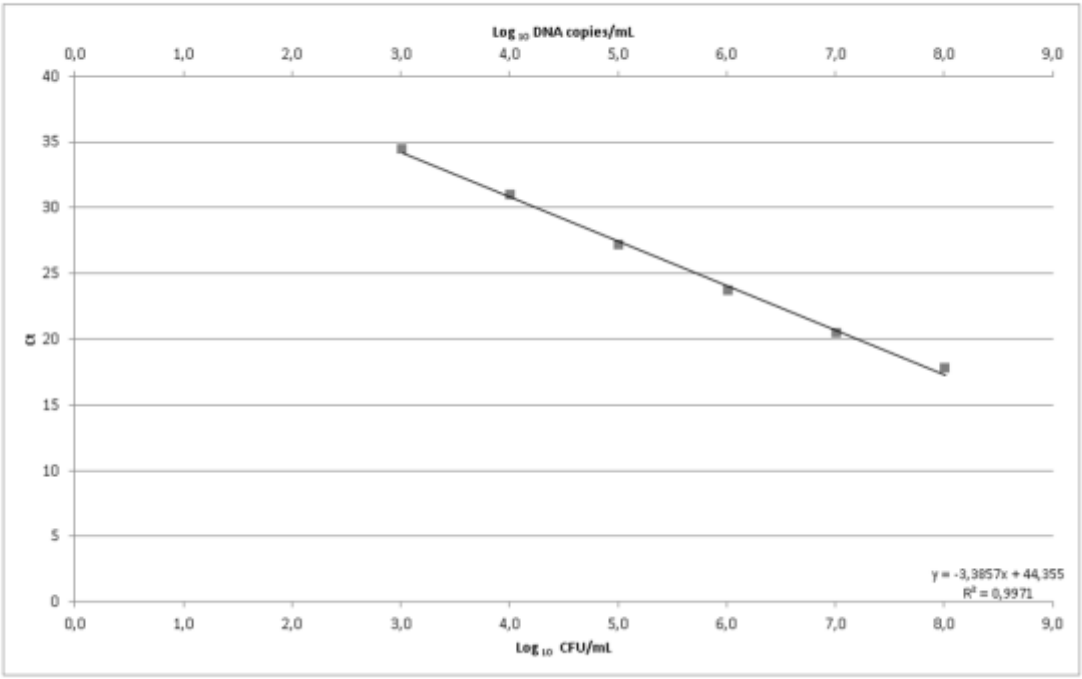
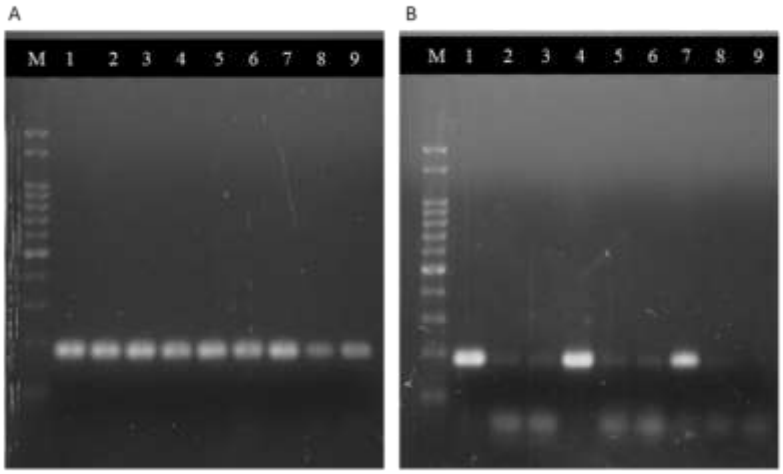
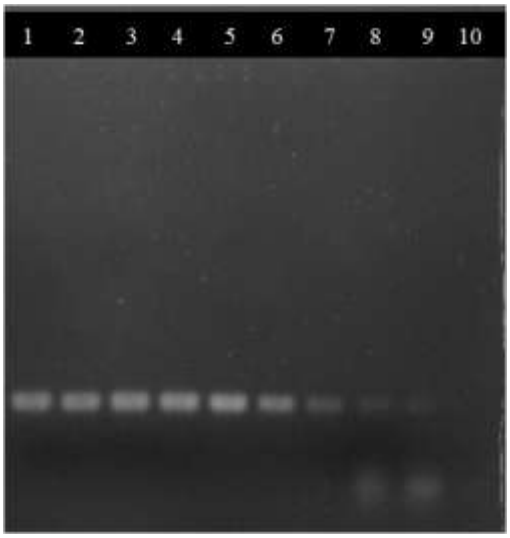

Log 10 DNA copies/mL
0,0 1,0 2,0 3,0 4,0 5,0 6,0 7,0 8,0 9,0
40 35 30 25 20 15 10 5 0
Ct
y = -3,3857x + 44,355
R² = 0,9971
Log 10 CFU/mL

Figure 3. Standard curves used to calibrate the PMA-qPCR method. (A) Plasmid DNA and cell-derived standard calibration curves for the *B. cereus* PMA-qPCR assay. A standard curve of 10-fold serial dilutions of (●) plasmid DNA (from 1.6 x 10^8 to 1.6 x 10^2 of DNA copies/mL) and (■) 10-fold serial dilutions of *B. cereus* in artificially inoculated milk (from 7.5 x 10^7 to 7.5 x 10^2 CFU/mL). (B) The standard curve generated by the interpolation of the resulted linear equations for the cells-derived curve and the plasmid DNA curve.

Supplementary Figures



Supplementary Fig. 1. (agarose gels corresponding to Fig. 1) The effect of different concentrations of PMA treatment for the detection of viable and dead (heat- or isopropanol-killed) *Bacillus cereus* ATCC 33018 cells by PCR. **(A)**. Amplicons originated from genomic DNA of the following: lanes 1-3 (without PMA) - viable (1), heat killed (2) isopropanol killed cells (3); lanes 4-6 (2 µg/mL PMA) - viable (4), heat killed (5), isopropanol killed cells (6); lanes 7-9 (5 µg/mL PMA) - viable (7), heat killed (8), isopropanol killed cells (9); lane 10 - negative control (without template DNA); (M) 100 bp DNA ladder. **(B)**. Amplicons originated from genomic DNA of: lanes 1-3 (10 µg/mL) - viable (1), heat killed (2), isopropanol killed cells (3); lanes 4-6 (20 µg/mL PMA) - viable (4), heat killed (5), isopropanol killed cells (6); lanes 7-9 (30 µg/mL PMA) - viable (7), heat killed (8), isopropanol killed cells (9); lane 10 – negative control (without template DNA); (M) 100 bp DNA ladder.



Supplementary Fig. 2. (agarose gels corresponding to Fig. 2) The effect of PMA treatment for the PCR detection of viable and isopropanol-killed *Bacillus cereus* ATCC 33018 cells mixed at different ratios. Lanes 1 to 5 (without PMA) correspond to amplicons from cell suspension mixtures containing viable cells at 100%, 75%, 50%, 25% and 0%; lanes 6 to 10 (30 µg/mL of PMA) correspond to amplicons from cell suspensions mixtures containing viable cells at 100%, 75%, 50%, 25% and 0%.

Capítulo 6

Depósito de Patente

6.1 Depósito de Patente

Os resultados obtidos nesta tese foram objeto de pedido de patente sendo depositada junto ao Instituto Nacional da Propriedade Industrial (INPI).

Título da Invenção: Métodos de detecção e quantificação de microrganismos viáveis em amostras de alimentos.

Data Depósito: 03/08/2012

Número de Depósito: BR1020120195127.

Período de Sigilo: 18 meses

DECLARAÇÃO DE DEPÓSITO DE PEDIDO DE PATENTE

Declaramos para os devidos fins, que na data de três de agosto de 2012, foi depositado junto ao Instituto Nacional da Propriedade Industrial - INPI, o pedido de patente intitulado *Método de Detecção e Quantificação de Microorganismos Viáveis em Amostras de Alimentos*, sob o número BR102012019512 7, de titularidade da União Brasileira de Educação e Assistência – UBEA e de inventoria dos profs. Sílvia Dias de Oliveira e Carlos Alexandre Sanchez Ferreira e da aluna de doutorado Fernanda Cattani.

Porto Alegre, 08 deoutubro de 2012.

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Capítulo 7

Considerações Finais

7.1 Considerações finais

Dentre os microrganismos que podem sobreviver no leite tratado pelo sistema de UHT devido à produção de esporos resistentes ao calor, destacam-se o *B. sporothermodurans* e o *B. cereus*. O *B. sporothermodurans*, embora não seja patogênico, pode causar deterioração e, assim, reduzir a qualidade do leite tratado por UHT e de derivados lácteos quando encontrado em altas concentrações. Por outro lado, a contaminação de leite por *B. cereus* constitui não somente uma importante causa de deterioração, mas também está associada com a ocorrência das síndromes diarreica e emética devido à ingestão de alimentos contaminados.

O *B. sporothermodurans* produz esporos altamente termorresistentes, possuindo valores D_{140} de 3,4 a 7,9 segundos (20), o que possibilita que este microrganismo resista às altas temperaturas utilizadas pelo tratamento térmico empregado para a conservação de alimentos. A análise dos danos estruturais e da sobrevivência de esporos de *B. sporothermodurans* tratados pelo calor demonstrou que a inativação completa do esporo ocorre somente após tratamento térmico a 130°C por 8 minutos (21). Neste trabalho, a resistência térmica do *B. sporothermodurans* IC4 foi caracterizada por diferentes temperaturas (tratamentos isotérmicos e não isotérmicos) em água destilada, leite UHT e em sopa de legumes tratada pelo sistema UHT. Os perfis de resistência observados tanto nos tratamentos isotérmicos, como nos não isotérmicos aplicados a diferentes temperaturas permitiram confirmar a elevada resistência dos esporos a altas temperaturas. Esta etapa do trabalho foi viabilizada devido à aprovação do estágio de doutorado sanduíche em Cartagena, Espanha, no período de novembro de 2010 a maio de 2011, junto à CAPES e à Fundação Carolina. Os laboratórios da Universidade Politécnica de Cartagena possibilitaram o acesso a equipamentos de alta tecnologia, com destaque para o Termoresistômetro Mastia (Patente espanhola

200302529), que não está disponível no Brasil, utilizado na área de Tecnologia de Alimentos da Faculdade de Engenharia Agrícola para auxiliar a indústria de alimentos a melhorar os tratamentos térmicos de seus produtos.

B. sporothermodurans pode germinar a partir dos esporos resistentes ao processamento por UHT e se multiplicar no leite estocado e, caso não seja detectado corretamente, pode ultrapassar o limite estabelecido pela legislação para microrganismos mesófilos aeróbios. Desta forma, o estabelecimento de um método de detecção e quantificação rápido e eficaz de células viáveis de *B. sporothermodurans*, bem como de *B. cereus*, que ainda possui o agravante de ser patogênico, é relevante para uma melhor qualidade e segurança do leite UHT destinado à população, além de evitar possíveis prejuízos às indústrias.

Tradicionalmente, a identificação e a enumeração de *B. sporothermodurans* e de *B. cereus* em alimentos são realizadas através de técnicas clássicas de cultivo. Estas técnicas são baseadas nos aspectos morfológicos e bioquímicos dos microrganismos, podendo apresentar variabilidade pela ação de fatores ambientais sobre a expressão gênica e risco de interpretações errôneas devido à semelhança fenotípica e genotípica existente entre as espécies do gênero *Bacillus*, bem como requerem vários dias para a obtenção dos resultados. Adicionalmente, estas técnicas podem não detectar microrganismos que sejam metabolicamente ativos, mas que não sejam cultiváveis (VBNC).

Métodos moleculares, como a PCR, podem constituir alternativas às técnicas clássicas de cultivo, oferecendo vantagens sob alguns aspectos. Entretanto, os métodos baseados em PCR podem não ser capazes de distinguir entre DNA de células viáveis e mortas, levando a resultados falso-positivos e, consequentemente, a uma superestimação do

número total de células (75). A utilização do PMA pode contornar esta limitação, uma vez que este se liga a moléculas de DNA oriundas de células com membranas danificadas, impedindo sua amplificação na PCR e permitindo a detecção seletiva do DNA de células viáveis (76).

Neste sentido, esse trabalho desenvolveu métodos baseados em PCR associada ao PMA para detecção e quantificação de *B. cereus* e de *B. sporothermodurans*, utilizando o gene da hemolisina e o gene RNAr 16S como alvos, respectivamente.

Na primeira etapa, foi desenvolvido um método para detecção específica de *B. sporothermodurans*, utilizando-se como ponto de partida um protocolo baseado em PCR convencional previamente descrito para identificação deste microrganismo em leite (61). No entanto, como esse método não apresentou a especificidade esperada, foi desenhado um oligonucleotídeo iniciador interno para ser utilizado em uma *semi-nested touchdown* PCR, que detectou somente DNA oriundo da espécie alvo, mostrando-se um protocolo específico. Este método também mostrou elevada sensibilidade para a detecção de células vegetativas e esporos de *B. sporothermodurans* tanto em cultura pura, como em leite, o que nos indica a possibilidade da utilização deste protocolo diretamente na matriz láctea, assim como a sua utilização na etapa de identificação deste microrganismo durante a execução das técnicas clássicas de cultivo. A partir do protocolo desenvolvido, procurou-se associar o PMA para a detecção seletiva de células viáveis, sendo otimizada a concentração de 30 μg/mL como ideal para a marcação de células mortas sem inibir a amplificação de DNA oriundo de células viáveis.

Embora o método desenvolvido tenha se mostrado sensível para a análise direta do leite, sem enriquecimento prévio, ele expressa resultados qualitativos, não sendo possível

realizar a quantificação precisa de microrganismos presentes em uma determinada amostra. Tal limitação foi contornada com o desenvolvimento de um método que utilizou qPCR associada ao PMA como marcador de viabilidade celular, mantendo a mesma sensibilidade observada para a PCR convencional. Além disso, a qPCR permite a análise direta, em uma única etapa, eliminando a necessidade da detecção dos produtos amplificados por eletroforese, diminuindo a possibilidade de contaminações cruzadas. A utilização de qPCR para determinar a ocorrência de *B. sporothermodurans* em leite tratado por UHT já havia sido descrita (25), mas não foi associada à determinação de viabilidade, não distinguindo DNA de células viáveis daquele de células mortas. A detecção seletiva de células viáveis é de suma importância para a análise de leite tratado termicamente, como pode ser observado pelos resultados obtidos a partir das análises das amostras de leite através de PMA-qPCR e qPCR não associada ao PMA. A qPCR sem PMA detectou um número significativamente maior de amostras positivas para *B. sporothermodurans*, provavelmente detectando também DNA oriundo de células mortas presentes nas amostras analisadas, derivadas, provavelmente, do tratamento por UHT aplicado ao leite.

O método desenvolvido foi aplicado em amostras comerciais de leite tratadas por UHT, mostrando que 10% foram positivas com quantidade superior a 10^2 UFC/mL. Estes resultados nos indicam que se o *B. sporothermodurans* não for corretamente identificado e quantificado, pode ser erroaneamente incluído na contagem de bactérias mesófilas aeróbias viáveis, o que poderia levar as amostras a estarem em desacordo com os padrões brasileiros vigentes (10, 27). Cabe ressaltar que o método de cultivo utilizado para isolamento, identificação e enumeração de *B. sporothermodurans* em leite foi conduzido conforme preconizado por Brasil (27), porém, além da semeadura em profundidade prevista pelo

MAPA, paralelamente, foi realizada a semeadura em superfície, uma vez que este microrganismo é aeróbio. Os dados apresentados neste trabalho que demonstraram a ausência de diferença significativa entre PMA-qPCR e método clássico de cultivo para a quantificação de *B. sporothermodurans* levaram em consideração somente a semeadura em superfície. Quando comparamos a contagem em profundidade com a contagem através da semeadura em superfície, bem como com a PMA-qPCR, observamos que a técnica de cultivo preconizada pelo MAPA subestima significativamente a contagem de *B. sporothermodurans*, o que pode levar ao aumento dos valores expressos na contagem de mesófilos aeróbios totais. Desta forma, se tivéssemos estabelecido a comparação da PMA-qPCR desenvolvida neste estudo para a análise de leite UHT com o cultivo em semeadura em profundidade, teríamos atribuído uma eficácia comparativa irreal ao método molecular. Esta análise comparativa dos métodos clássicos de cultivo não foi apresentada inicialmente neste trabalho, pois consta em um artigo que está em fase de redação que apresenta interesse para publicação em revista de circulação nacional.

O método desenvolvido para detecção e quantificação de *B. cereus* associando PMA à qPCR mostrou-se altamente específico e sensível para a análise da contaminação de leite por este microrganismo. A análise das amostras de leite tratado por UHT pelo método molecular desenvolvido evidenciou um número considerável de amostras contaminadas por *B. cereus* (32,6%). Destas, três amostras possivelmente atingiram contagens capazes de causar doenças de origem alimentar. Considerando que a legislação brasileira para leite UHT não permite a presença de microrganismos patogênicos, todas as amostras que apresentaram contaminação estavam em desacordo com a legislação vigente. Além disso, o método molecular desenvolvido e o método de cultivo determinaram a presença de células viáveis de *B. cereus*

em quantidades compatíveis com a dose infectante atribuída a este microrganismo após um período de incubação de uma semana a 36ºC, condição, muitas vezes, encontrada durante a estocagem deste produto nos pontos de venda. Desta forma, os resultados apresentados neste trabalho podem ser utilizados como um alerta para as indústrias de laticínios e para os órgãos oficiais de controle de produtos de origem animal, no sentido de que o *B. cereus* deva ser investigado nos leites tratados por UHT, uma vez que é um microrganismo potencialmente patogênico.

Adicionalmente, os resultados evidenciaram também que tanto a presença de *B. sporothermodurans*, como de *B. cereus* em leite tratado por UHT reforça a necessidade de um controle rigoroso na obtenção da matéria-prima e, em todas as etapas do processamento pelo sistema UHT. Além disso, padrões microbiológicos específicos de leite cru destinado ao processamento de leite por UHT, no que diz respeito à presença destes microrganismos, contribuiriam para a melhoria da qualidade do produto final.

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