Development of a real-time PCR assay for direct detection and quantification of *Bacillus sporothermodurans* in ultra-high temperature milk

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ABSTRACT

In our study, a new and highly sensitive real-time PCR Taqman assay was developed for the direct and specific detection of *Bacillus sporothermodurans* in UHT milk. The target region was selected based on the 16S rRNA gene profiles of 11 *B. sporothermodurans* from GenBank. A standard curve was created using a reference strain of *B. sporothermodurans*, DSM 10599. A low limit of detection for *B. sporothermodurans* in UHT milk (10 cfu/mL) was obtained. Furthermore, a total of 110 UHT milk samples from several supermarkets were directly assessed to detect and quantify *B. sporothermodurans* using the real-time PCR Taqman developed. The *B. sporothermodurans* counts obtained were highly correlated with the microbial plate counts in the UHT milk samples. This is the first time that *B. sporothermodurans* has been quantified directly from UHT milk. This technique could be applicable as a routine tool for preventing the growth of these bacteria by allowing for the rapid screening of raw milk samples in dairy plants. As expected, the probability of bacterial growth in UHT milk packages increased with the *B. sporothermodurans* counts in the raw milk.

Key words: *Bacillus sporothermodurans*, real-time PCR Taqman, ultra-high temperature milk

INTRODUCTION

Bacillus sporothermodurans is an important nonpathogenic bacterium in the dairy industry that can produce highly heat-resistant endospores that can survive UHT milk processing (140°C for a few seconds). These spores may germinate during storage in UHT products and grow up to 10⁵ cfu/mL, causing instability due to their proteolytic activity (Klijn et al., 1997; Montanari et al., 2004). This is a common problem encountered in many dairy factories, reducing milk quality, shelf life,

and consumer acceptability (Pettersson et al., 1996). However, attempts to increase temperature or holding time could affect the organoleptic and nutritional qualities of the UHT milk (Claeys et al., 2001).

Bacillus sporothermodurans was first detected in UHT milk in Italy and Austria in 1985 and then also in Germany in 1990 (Hammer et al., 1995). This microorganism has also been found in sterilized milk and other products such as chocolate milk, evaporated milk, and reconstituted milk (Herman et al. 2000). Directive 92/46 of the European Union related to milk and dairy products establishes that the general bacterial plate counts of heat-treated milk products after 15 d of incubation at 30°C must be below 10 cfu per 0.1 mL (European Council, 1992). Therefore, the spores of *B. sporothermodurans* that survive after the sterilization process become an obstacle for dairy factories in meeting the legal requirements established by European legislation.

The detection of *B. sporothermodurans* is usually performed using culture techniques that include the use of brain heart infusion (BHI) agar after heating the sample 100°C for 30 to 40 min (Scheldeman et al., 2006). In addition, different molecular methods have been used to identify and characterize *B. sporothermodurans*. For instance, this bacterium has been identified by conventional PCR methods using specific primers based on the 16S rRNA gene (Scheldeman et al., 2002), which has regions with high variability for identifying *B. sporothermodurans* (Klijn et al., 1997). Likewise, a conventional PCR assay was modified to include propidium monoazide to detect viable *B. sporothermodurans* (Cattani et al., 2013). Although these studies have been conducted over the last decade, no attention has been paid to quantitative detection. Real-time PCR (RTi-PCR), which is widely used in food microbiology, is an accurate and rapid quantitative detection technique. For instance, *Staphylococcus* spp., *Coxiella burnetii*, and *Streptococcus agalactiae* (Zanardi et al., 2014; Van den Brom et al., 2015; Zhang et al., 2015) have all been verified using RTi-PCR. In one study, Tabit and Buys (2011) used RTi-PCR SYBR Green to detect *B. sporo-*

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thermodurans in a pure culture. However, this method has not been applied directly to milk samples, and no quantification was applied for this bacterium. Moreover, RTi-PCR Taqman assays have not been proven effective for the detection and quantification of *B. sporothermodurans* in either pure cultures or milk samples directly. Hence, the aim of this study is to develop a direct and accurate RTi-PCR Taqman assay for the direct rapid quantitative screening of *B. sporothermodurans* in UHT milk. So this method could not only be used to control large amounts of UHT milk batches but also to control a large amount of raw milk samples or environmental samples previously submitted to heat treatment to avoid this problem in the milk industry.

MATERIALS AND METHODS

Primer-Probe Design and Real-Time Conditions

A new design for a probe and primers was applied for the detection and quantification of *B. sporothermodurans* using the RTi-PCR reaction (forward, 5'-ACCAGGTCTTGACATCCTCTTGA-3'; reverse, 5'-ATGCACCACCTGTCACTCTTGT-3'; and probe, 5'-CTAGAGATAGGGATTTC-3'). The target sequence was selected based on the 16S rRNA gene profiles of 11 *B. sporothermodurans* from the National Center for Biotechnology Information (NCBI) database. These sequences were aligned

together using CLUSTAL X software (Larkin et al., 2007; accession numbers KC618495.1, NR_118833.1, NR_118832.1, NR_026010.1, JX569192.1, GU238287.1, AJ302942.1, AJ302941.1, AB190039.1, FJ188308.1, and HQ713507.1). A conserved alignment region targeting *B. sporothermodurans* was applied in the design of the primers and the Taqman probe using Primer Express software v2.0 (Applied Biosystems, Foster City, CA). Based on the alignments of the *B. sporothermodurans* sequences with reference sequences, primers and an MGB Taqman probe were designed using fragments of 76 bp as part of a 16S rRNA gene. Amplification by real-time PCR was performed using ABI PRISM 7000 (Applied Biosystems) under the following conditions: 10 min at 95°C, followed by 45 cycles of 15 s at 95°C for denaturation, and 1 min at 60°C for annealing and extension. Then, a 25-μL reaction volume was amplified, including 7.5 μL of template DNA, 12.5 μL of Environmental Master Mix, 900 nM of each primer, and 200 nM of the Taqman probe. All materials for the reaction were obtained from Applied Biosystems.

The specificity of the probe and primers were tested experimentally using positive reference strains and a negative control panel from different bacteria (Table 1).

Standard Curve

The developed primers and Taqman probe were employed using triplicate amplifications of the reference

Table 1. Real-time PCR Taqman assay specificity for detection of *Bacillus sporothermodurans*¹

Strain	Origin	Results ²
<i>Bacillus sporothermodurans</i> DSM No. 10599	DSM	(+)
<i>B. sporothermodurans</i> LMG 17668	LMG	(+)
<i>Bacillus acidicola</i> DSM 14745	DSM	(-)
<i>Bacillus coagulans</i> DSM 1	DSM	(-)
<i>Bacillus subtilis</i> ATCC 11774	ATCC	(-)
<i>B. subtilis</i> ATCC 9524	ATCC	(-)
<i>Bacillus cereus</i> ATCC 14893	ATCC	(-)
<i>Bacillus licheniformis</i> ATCC 14580	ATCC	(-)
<i>Bacillus megaterium</i> ATCC 25848	ATCC	(-)
<i>Bacillus thuringiensis</i> ATCC 33679	ATCC	(-)
<i>Pseudomonas fragi</i> ATCC 4973	ATCC	(-)
<i>Enterococcus faecalis</i> LMG20863	LMG	(-)
<i>E. faecalis</i> CECT 481	CECT	(-)
<i>Enterococcus thailandicus</i>	Lhica Lab	(-)
<i>Enterococcus casseliflavus</i>	Lhica Lab	(-)
<i>Staphylococcus caprae</i>	Lhica Lab	(-)
<i>Streptococcus uberis</i>	Lhica Lab	(-)
<i>Lactobacillus casei</i>	Lhica Lab	(-)

¹DSM were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. LMG: Belgian Coordinated Collections of Microorganisms. ATCC: American Type Culture Collection. CECT: Collection Española de Cultivos Tipo. Lhica Lab: from our laboratory, the Hygiene Food Inspection and Control laboratory.

²(-) and (+) are indicators for *B. sporothermodurans* detection according to standard curve.

strain of *B. sporothermodurans* (DSM No. 10599) for a standard curve. Serial dilutions of reference strain cultures (10^8 cfu/mL) were prepared after inoculating UHT milk with the reference strains, with concentrations ranging from 10^{-1} to 10^{-7} cfu/mL as determined by plate counts, in BHI agar. The UHT milk used to construct the standard curve was previously tested for the presence of *B. sporothermodurans*, using the methodology described here, with no positive result.

Then, 700 μ L of UHT milk was inoculated with the reference strain, and DNA was directly extracted using the Prep SEQ extraction kit. Subsequently, 10^{-1} to 10^{-7} dilutions were surface plated on to BHI agar plates (Merck, Darmstadt, Germany). These plates were then incubated at 37°C for 48 h.

UHT Milk Samples, Reference Strains, and Microbial Plate Counts

One hundred ten UHT milk samples were collected from 17 UHT manufacturers (A–Q) that had been bought from supermarkets. Of these, 98 samples were from cow milk, whereas 12 samples were from goat and sheep milk. The UHT milk samples were distributed in 2 groups: 61 samples of full-fat and 49 samples of skim milk. In addition, 2 reference strains of *B. sporothermodurans* (DSM No. 10599 and LMG 17668) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and the Belgian Coordinated Collections of Microorganisms, respectively. To make the initial dilution, 1 mL from the UHT milk samples was aseptically mixed with 9 mL of 0.1% peptone water (Merck). Appropriate dilutions were prepared (10^{-1} to 10^{-6}) and subsequently surface-plated on BHI agar plates, followed by incubation at 37°C for 48 h (Merck). After the incubation period, 2 colonies were randomly selected from 22% (5 out of 23) of the positive UHT milk samples using conventional microbiological counts. All of these colonies (10 colonies) were subjected to functional and conventional microbiological tests (colony and bacterial morphology, Gram staining, catalase, and oxidase) and identified by molecular methods as is described in the following paragraph.

Molecular Identification of Milk Isolates

The DNA templates were extracted from 3-d-old pure cultures from BHI agar plates that had been identified by amplification and sequencing of the 16S rRNA gene. Amplification of 800-bp fragment from the 16S rRNA gene was performed with p8FPL (5'-AGTTT-GATCCTGGCTCAG-3') and p806R (5'-GGACTAC-

CAGGGTATCTAAT-3') universal primers (McCabe et al., 1999), in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) under the following conditions: (1) initial denaturing step at 94°C/7 min; (2) 35 cycles of denaturation (94°C/60 s), annealing (55°C/60 s), and extension (72°C/60 s); and (3) final extension at 72°C/15 min (McCabe et al., 1999). The same primers used for PCR were also employed to sequence both strands of the PCR products. Sequencing reactions were analyzed using an automatic sequencing system (ABI 3730XL DNA Analyzer, Applied Biosystems) with the POP-7 system and reviewed carefully using Chromas software (Griffith University, Queensland, Australia). All 16S rRNA sequences obtained were compared with the GenBank database of the NCBI as reference data (accession numbers of these sequences: NR_118833.1, NR_118832.1, and NR_026010.1).

DNA Extraction for RTi-PCR

Bacillus sporothermodurans was inoculated in BHI and incubated at 37°C for 48 h. Afterward, 700 μ L of broth was taken in a clean tube and centrifuged at $13,000 \times g$ for 4 min. The DNA templates were subsequently extracted from the pellets using a Rapid Spin Samples Preparation Kit, Prep SEQ (Applied Biosystems), as per the manufacturer's instructions, to extract the DNA from the bacteria and UHT milk samples. The DNA was then stored at -20°C.

Quantitative Detection of *B. sporothermodurans* in UHT Milk Samples

Direct screening for *B. sporothermodurans* in retail UHT milk samples was performed by RTi-PCR Taqman amplification using a new probe and primers. The DNA was extracted from 700 μ L of each UHT milk sample, following the previously described procedures. The same UHT milk samples quantified by RTi-PCR were subsequently surface-plated on BHI agar, followed by incubation at 37°C for 48 h.

All determinations and quantifications of calibration curves were performed in triplicate, whereas the quantification of pure cultures and UHT milk samples was performed in duplicate. Cycle threshold (C_T) values were plotted against counts (in log cfu/mL) of the reference bacteria used. The efficiencies (E) of the reactions were calculated from linear regression curves based on the formula described by Klein (2002). The E was calculated by the equation:

$$E = [10^{(-1/\text{SLOPE})}] - 1.$$

Statistical Analysis

The data were compared using the nonparametric Mann–Whitney U-test. All statistical analyses were carried out using the IBM SPSS Statistics Package (version 19, 2010, IBM Corporation, Somers, NY).

RESULTS

Primers and Probe Design and Specificity

A new design for a Taqman probe and primers was generated for direct detection and quantification of *B. sporothermodurans* in UHT milk samples. A total of 110 samples of commercially available UHT milk were purchased from 17 different manufacturers and tested for *B. sporothermodurans*. Our results showed a high specificity in the RTi-PCR Taqman for *B. sporothermodurans* using positive reference strains and a negative control panel from other species (Table 1).

According to the generated standard curve (Figure 1), C_T values >38.03 were considered as no amplification in the extracted DNA. In addition, the limit of detection for this assay was 1 log cfu/mL of UHT milk. The standard curve also showed a good correlation coefficient in the linear regression analysis (0.98) for extracted DNA. The curve slope for *B. sporothermodurans* was -3.1536 , while the E of the reactions was 107%, which was calculated from the linear regression curve.

Quantitative Detection of *B. sporothermodurans* in UHT Milk Samples

Bacillus sporothermodurans was detected and quantified in the UHT milk samples according to the standard curve. The accuracy of the Taqman probe assay was confirmed by comparing the values from the bacterial

counts with the plotted values (C_T), using the standard curves from the extracted DNA from BHI broth.

Thirty-two out of 110 of the retail UHT milk samples were contaminated with *B. sporothermodurans*. Although 17 manufacturers were used, only UHT milk samples from 5 were positive for *B. sporothermodurans*. Furthermore, 9 out of the 32 positive UHT milk samples, from 2 manufacturers (B) and (K), were not detected by conventional plate counts on BHI agar. However, these patterns of quantification, using RTi-PCR Taqman and bacterial plate counts, did not reveal a significant difference ($P > 0.05$). *Bacillus sporothermodurans* isolates were confirmed by amplification and subsequent sequencing of the 16S rRNA gene. All the colonies showed rod-shaped cells, positive to the Gram reaction, catalase, and oxidase tests. As shown in Table 2, *Bacillus sporothermodurans* was also found in UHT goat milk samples. Despite the apparent difference between the number of *B. sporothermodurans* detected in UHT milk full-fat samples in comparison with skim milk samples, these differences were not statistically significant ($P > 0.05$) using the RTi-PCR method or the standard plate count method.

DISCUSSION

Bacillus sporothermodurans has been incriminated in massive contaminations of UHT milk products since the mid-1980s. However, it is difficult to isolate this microorganism from raw milk using conventional microbiological methods due to the existence of highly competitive microbiota (Scheldeman et al., 2006). Consequently, some studies have developed conventional PCR techniques to detect *B. sporothermodurans* (Herman et al., 1998; Scheldeman et al., 2002). Real-time PCR molecular techniques have not been used to quantify *B. sporothermodurans* directly from UHT milk samples. For instance, Tabit and Buys (2011) used a SybrGreen RTi-PCR technique to detect *B. sporothermodurans*, but only in pure cultures. Furthermore, RTi-PCR Taqman chemistry has not been reported yet in the scientific literature for the study of *B. sporothermodurans* for either pure culture or UHT milk samples. In addition, this method has been reported in the scientific literature to achieve higher sensitivity and specificity than the SybrGreen method (Lurchachaiwong et al., 2008).

The RTi-PCR Taqman has been successfully applied for the quantitative detection of specific genes in different foods (Ladero et al., 2010; Guarddon et al., 2011) or microorganisms, such as *Salmonella* in milk, yogurt, and cheese; *Listeria monocytogenes* in raw milk (El-Sharoud, 2015; Paul et al., 2015); and *Bacillus cereus*, *Bacillus licheniformis*, and *Bacillus subtilis* in other

Figure 1. Standard curve of real-time PCR (RTi-PCR) of 10-fold serial dilution of *Bacillus sporothermodurans*. Cycle threshold (C_T) values are plotted against colony-forming unit count.

Table 2. Quantitative detection of *Bacillus sporothermodurans* in UHT milk samples¹

UHT milk manufacturer ²	Total UHT milk samples (110)				% of positive samples	Mean counts (log cfu/mL)							
	Full fat (61)		Skim (49)			Full fat		Skim					
	N	Positive ²	N	Positive		Plate count	±SD	RTi-PCR	±SD	Plate count	±SD	RTi-PCR	±SD
(A) Cow milk	12	10	12	7	15	5.07	2.2	5.27	1.9	3.43	1.4	3.47	1.6
(B) Cow milk	7	4	7	3	6	4.48	2.1	5.15	2.2	4.10	1.6	4.31	1.69
(C) Cow milk	6	1	6	0	1	—	—	—	—	—	—	—	—
(D) Cow milk	3	0	3	0	0	—	—	—	—	—	—	—	—
(E) Cow milk	3	3	3	0	3	4.3	2.2	4.58	2.3	—	—	—	—
(F) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(G) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(H) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(I) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(J) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(K) Cow milk	2	1	2	0	1	0	—	1.9	—	—	—	—	—
(L) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(M) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(N) Cow milk	2	0	2	0	0	—	—	—	—	—	—	—	—
(O) Goat milk	3	0	0	0	0	—	—	—	—	—	—	—	—
(P) Goat milk	3	3	0	0	3	5.13	0.1	5.21	0.1	—	—	—	—
(Q) Sheep milk	6	0	0	0	0	—	—	—	—	—	—	—	—

¹Log cfu/mL of real-time (RTi) PCR is according to cycle threshold (C_T) values obtained by RTi-PCR and standard curve of *B. sporothermodurans*.

²Positive detection of *B. sporothermodurans* according to the RTi-PCR and standard curve.

food products (Fernández-No et al., 2011). It is in this context that a reliable and sensitive RTi-PCR Taqman assay for the direct and specific detection of *B. sporothermodurans* in UHT milk products was developed in the present study.

A new design for a Taqman probe and primers was generated for the direct detection and quantification of *B. sporothermodurans* in UHT milk. Our method showed a good fit curve ($r \geq 0.98$) and a slope value (-3.1536) close to the theoretical optimal value (-3.32 ; Higuchi et al., 1993), indicating that the assay appropriately quantified *B. sporothermodurans*. The efficiency of our assay was 107%. In RTi-PCR assays, the accepted range of efficiency is 95 to 110% (West et al., 2007). This is because efficiency could be slightly decreased or increased by compounds presented in the sample matrix (Martínez-Blanch et al., 2009; Svec et al., 2015). The detection limit of the RTi-PCR Taqman assay was 10 bacteria per mL (1 log cfu/mL) of *B. sporothermodurans* in UHT milk samples, which confirms the sensitivity of this method for detecting and quantifying the target. Other authors have shown detection limits in food of above 1 log cfu/mL, as in the case of *B. cereus*, with a limit in food of 60 cfu/mL as reported by Martínez-Blanch et al. (2009) using a RTi-PCR Taqman assay. It is interesting to note that the detection limit for our new method complies with EU legislation for milk and dairy products (European Council, 1992), as well as other legal limits required in other countries.

In this study, RTi-PCR Taqman assays were used for first time to detect and quantify *B. sporothermodurans* in UHT milk samples via corresponding C_T values (Table 2). The C_T values were slightly lower than corresponding values of bacterial plate counts. However, a good correlation between bacterial plate counts from UHT milk samples and the RTi-PCR Taqman assay has been shown. These higher values obtained by the PCR method could be a result of the detection of DNA from dead bacterial cells. Additionally, and probably due to the same cause, some UHT milk samples from certain manufacturers also gave positive results in the RTi-PCR Taqman analysis but not on the plate counts (Table 2). Similar results related to higher counts using real-time methodology were also obtained by other authors with respect to conventional plate counts (Reichert-Schwilinsky et al., 2009) depending on the stress rate of the bacterial cells. Other authors tried to explain negative plate counts in detecting these spore-forming species as the result of inhibitory compounds, which are used in some factories, such as nisin and potassium sorbate, on spores and vegetative cells of *B. sporothermodurans* or in ultra-high-pressure homogenization treatments (Amador Espejo et al., 2014; Aouadhi et al., 2015).

However, in contrast to conventional PCR techniques (Scheldeman et al., 2002) and SybrGreen RTi-PCR chemistry (Tabit and Buys, 2011), RTi-PCR Taqman, which shows higher sensitivity and specificity, might be sufficiently effective for the specific quantification of *B. sporothermodurans* in UHT milk regarding safety and quality assessment.

Milk quality, good hygiene, and farm management practices are frequently monitored according to total bacterial count. Among the other microbiology-based tests that are carried out to assess the quality and safety of milk and dairy products are those designed to quantify thermotolerant bacteria and specific pathogens (Gopal et al., 2015). However, *B. sporothermodurans* has never before been quantitatively detected directly in raw or UHT milk. Positive results in UHT milk samples taken from supermarkets confirmed that contamination with this species before the thermal treatment can negatively affect credibility and competitiveness in the dairy industry. According to the results obtained, this method could be used in dairy factories to control microorganisms in packaged UHT milk through the rapid screening of raw milk for high numbers of these thermoresistant bacteria before use in UHT milk production.

Bacillus sporothermodurans was only detected in samples from 5 manufacturers, with high numbers of *B. sporothermodurans* detected in 4 of these positive brands (Table 2), indicating the need to control these microorganisms at the source. High incidences of *B. sporothermodurans* were also reported by Tabit and Buys (2011). It is possible to explain the high mean count as part of the UHT process, with heat-resistant spores occurring more frequently in indirect UHT as compared with direct UHT processing (Scheldeman et al., 2006), in addition to milk contamination on the farm (Vaerewijck et al., 2001). These spore-forming bacteria have a negative effect on the quality, shelf life, and consumer acceptability of UHT milk (Pettersson et al., 1996; Herman et al., 1998). However, it is possible to control these problems by using our RTi-PCR Taqman assay to screen the spores or vegetative cells of *B. sporothermodurans* in a large number of raw milk samples in a rapid way (less than 2 h) to identify dairy farms with high amounts of these bacteria. In any case, it should be pointed out that this method was carried out in UHT milk samples to prove the validity of RTi-PCR Taqman assay counts as compared with standard plate counts in milk samples naturally contaminated with these bacteria. The possible validation of the method by using natural raw milk would be very complex due to the presence of numerous other contaminant bacteria, and the absence of selective culture media to detect *B. sporothermodurans*.

The results showed that the mean count of *B. sporothermodurans* was higher in UHT full-fat milk compared with samples of skim UHT milk (Table 2). Although no significant difference was found, the highest count in full-fat milk could be due to the ability of fat to relieve the effect of high temperatures on *B. sporothermodurans* (Ahmed et al., 1995). Likewise, no significant difference was found in the quantification of *B. sporothermodurans* using RTi-PCR Taqman and bacterial plate counts. This has confirmed the success of the RTi-PCR Taqman method for routine monitoring of *B. sporothermodurans* in dairy factories.

CONCLUSIONS

A new molecular assay was developed as a rapid, accurate, and direct method of quantitatively detecting *B. sporothermodurans* in UHT milk samples. Moreover, this method could help in tracing the abundance of *B. sporothermodurans* in raw milk samples or even in samples taken from the plant environment. The usefulness of this method was demonstrated using real UHT milk samples taken from supermarkets, proving that there are samples of UHT milk containing these bacteria in which conventional microbiological methods could not directly detect the presence of *B. sporothermodurans*, although detection was possible using the method presented here. An accurate quantification of *B. sporothermodurans* is important for dairy factories to obtain efficient sterilization and stable UHT products.

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