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Quantitative Detection of *Clostridium tyrobutyricum* in Milk by Real-Time PCR▽†

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We developed a real-time PCR assay for the quantitative detection of *Clostridium tyrobutyricum*, which has been identified as the major causal agent of late blowing in cheese. The assay was 100% specific, with an analytical sensitivity of 1 genome equivalent in 40% of the reactions. The quantification was linear ($R^2 > 0.9995$) over a 5-log dynamic range, down to 10 genome equivalents, with a PCR efficiency of >0.946. With optimized detergent treatment and enzymatic pretreatment of the sample before centrifugation and nucleic acid extraction, the assay counted down to 300 *C. tyrobutyricum* spores, with a relative accuracy of 82.98 to 107.68, and detected as few as 25 spores in 25 ml of artificially contaminated raw or ultrahigh-temperature-treated whole milk.

Clostridium tyrobutyricum is a spore-forming anaerobic bacillus identified as the main spoiling bacterium responsible for late blowing due to butyric acid fermentation in hard and semihard cheeses (11, 12). This fermentation results from the germination of *C. tyrobutyricum* spores due to the anaerobic conditions generated in the cheese during its production (11). Late blowing results in deformation of the cheese loaf, the possible release of a foul-smelling inflammable substance (butyric acid) on cutting and, in some cases, an undesirable rancid taste. Damaged cheeses also contain several heterogeneously distributed cavities corresponding to the volume of gas produced and mass digested. *C. tyrobutyricum* spores are highly resistant to environmental conditions and contaminate the milk before cheese production. The main sources of contamination are thought to be silage, water, or unhygienic animal bedding (13). Late blowing occurs mostly in cheeses made with unpasteurized milk, but pasteurized milk cheeses may also be affected. The distribution of this problem within the cheese factory is heterogeneous, with only some batches or certain pieces within a batch contaminated and most problems occurring in spring and autumn, when temperatures are mild and humidity is high (5).

The routine diagnostic method used by analytical laboratories for detecting *C. tyrobutyricum* spores is a long and difficult process, involving most probable number counting and confirmation by checking the lactate fermentation capacity of cells from gas-positive tubes and a battery of additional tests, such as the examination of endospore position, carbohydrate fermentation profiling, and gas chromatographic analysis of volatile and nonvolatile organic acid by-products (4, 11). Furthermore, as several days are required for a visible change in turbidity and the appearance of gas with the displacement of paraffin, this method is difficult to adapt to the requirements of cheesemakers, as the milks from various sources have already been mixed and passed onto the production process by the time a positive result is obtained. Alternative DNA amplification-based methods for detecting the spores of this spoiling bacterium in milk or dairy products within 24 h have been devised to overcome this problem (6, 7, 12). The absolute quantification of contaminant microbiota in foodstuffs by real-time quantitative PCR (Q-PCR) is becoming increasingly common for diagnostic purposes in food microbiology (24). However, there is currently no available Q-PCR method for counting the spoilage microorganism *C. tyrobutyricum*. We report here the design and assessment of a Q-PCR assay for the identification and quantification of *C. tyrobutyricum* and its application to food samples.

Design and optimization of the *C. tyrobutyricum*-specific duplex *fla*-IAC Q-PCR assay. The assay targets a *C. tyrobutyricum* species-specific region of the flagellin gene (*fla*), encoding a protein involved in bacterial motility (2). Bacterial flagellins display considerable variation, which is useful for the identification of species (26), and the *C. tyrobutyricum fla* gene has been proposed as a good candidate for the design of PCR-based detection methods (2). The entire sequence of this gene (AJ242662) was 100% specific for *C. tyrobutyricum.* The most similar bacterium was *Clostridium novyi*, which was shown to be different from the target, with its score of 131 and an E value of 6×10^{-27}. Regions suitable for the design of *C. tyrobutyricum*-specific PCR primers and probes were identified by aligning all *fla* sequences deposited in public databases, using the CLUSTALW multiple alignment tool (European Bioinformatics Institute, EMBL; www.ebi.ac.uk). The primers *CTflaF* and *CTflaR* (Table 1) amplify a 100% specific 83-bp fragment from the coding sequence of the *C. tyrobutyricum fla* gene corre-

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TABLE 1. Oligonucleotides used in this study

Target	Primer	Type	Sequence[a]
C. tyrobutyricum fla gene	*CTflaF*	Forward	5′-CAGTTACAATTACGAGAACACATGGA-3′
	CTflaR	Reverse	5′-TGTACCACCAACTAAAGCAACATCA-3′
	CTflaP	TaqMan probe	5′-FAM-CGGCAGGAGCTACATTTGCTTCAACAG-TAMRA-3′
IAC chimeric DNA	*IACCTP*	TaqMan MGB probe	5′-VIC-CCATACACATAGGTCAGG-MGB-NFQ-3′
L. monocytogenes prfA gene	*IACCTF*	Forward	5′-CAGTTACAATTACGAGAACACATGGAGGCTCTATTTGC GGTC-3′
	IACCTR	Reverse	5′-TGTACCACCAACTAAAGCAACATCATCTTGATGCCATC AGGA-3′

[a] TAMRA, 6-carboxytetramethylrhodamine.

sponding to positions 539 to 621 (AJ242662). Blast-N tool v.2.2.14 (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) was used to confirm in silico that none of the selected oligonucleotides recognized any registered DNA sequence other than the target sequence.

One of the major barriers to the systematic introduction of Q-PCR-based methods for routine food analysis is the common occurrence of false-negative results due to the presence of PCR inhibitors in the sample. This is particularly true of analyses aiming to detect *C. tyrobutyricum* spores in dairy products, such as milk, which contains various substances that may cause bacterial contamination to be underestimated or the amplification reaction to be totally abolished (25). Our assay was designed to overcome this problem, using an internal amplification control (IAC). This IAC consisted of a nontarget nucleic acid fragment that is coamplified with the target sequence, with the same primers used for the test reaction, for the simultaneous assessment of PCR performance. When a negative signal is obtained for the target *fla* signal, the absence of a positive IAC signal indicates that amplification has failed (19). The IAC was constructed as previously described (22). The IAC consisted of a 121-bp chimeric DNA containing a portion (nucleotide positions 421 to 490) of the *Listeria monocytogenes* positive regulatory factor A (*prfA*) gene (14), flanked by the *C. tyrobutyricum*-specific *fla* gene sequences targeted by the *CTflaF* and *CTflaR* primers.

Primers and 6-carboxyfluorescein (FAM)-labeled *C. tyrobutyricum*-specific probe (Table 1) were optimized for Q-PCR assays, using as a template 1 ng of DNA from *C. tyrobutyricum* strain CECT 4011. The assays were carried out in a reaction volume of 20 μl containing 1× Universal PCR master mix (Applied Biosystems) on a 7500 real-time PCR system platform (Applied Biosystems) with the following program: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. Optimal conditions (300 nM for *CTflaF* and *CTflaR* primers and 100 nM for *CTflaP* probe) were the minimum primer and probe concentrations giving the lowest cycle threshold (C_T) value and the highest fluorescence intensity for a normalized reporter value (15). The optimal IAC probe concentration was determined experimentally, by carrying out Q-PCR in the presence of 10,000 IAC molecules, no *C. tyrobutyricum* DNA, and 100 nM FAM-labeled *fla* probe and with various amounts (from 25 to 250 nM) of VIC-labeled IAC probe (Table 1). The minimum probe concentration not resulting in an increase in C_T values was 100 nM. As excess IAC may inhibit the target-specific reaction, Q-PCRs were also carried out in the presence of various amounts of IAC (10,000, 1,000, 100, or 10 molecules per reaction) and various amounts of *C. tyrobuticum* DNA (equivalent to approximately 1×10^3, 100, 10, 2, or 1 genome equivalent). The maximum amount of IAC with no inhibitory effect on the *fla*-specific FAM signal was 100 copies of chimeric DNA.

Selectivity of the *fla*-IAC assay. The capacity of the *fla*-IAC Q-PCR assay to discriminate between target and nontarget bacteria was assessed using 1 ng of genomic DNA from 22 *Clostridium* spp. and 87 strains from 18 different non-*Clostridia* species, including various food-borne and environmental genera frequently found in milk and dairy products (see Tables S1 and S2 in the supplemental material). The *fla*-IAC Q-PCR assay was inclusive for *C. tyrobutyricum* and 100% exclusive for nontarget bacteria, as all *C. tyrobutyricum* strains tested gave a positive *fla* signal, whereas none of the 107 nontarget bacteria did. All reactions generated a positive IAC (VIC) signal, excluding the possibility that the absence of an *fla* (FAM) signal observed in non-*C. tyrobutyricum* isolates was due to PCR failure.

Analytical sensitivity and quantification range of the *fla*-IAC Q-PCR assay. The achievement of low detection and quantification limits is a critical aspect in the design of molecular diagnostic methods for microbial contaminants of food. This goal is of particular interest in the case of *C. tyrobutyricum*, as it has been reported that as few as 50 spores/liter is sufficient to cause late blowing (3). The detection and quantification limits of the PCR assays were determined using genomic DNA isolated from early-stationary-phase cultures of *C. tyrobutyricum* strain CECT 4011 obtained under anaerobic conditions. Three independent PCRs were performed, with a range of DNA concentrations equivalent to approximately 1×10^5, 1×10^4, 1×10^3, 1×10^2, 10, 2, or 1 target molecule. Table 2 shows the mean C_T values for a total of nine PCR replicates (30 replicates for 10, 2, or 1 genome equivalent) from three independent experiments. Positive amplification in all PCR replicates of each DNA dilution was achieved when 10 or more target molecules were present, and 1 target molecule could be detected with a 40% probability (Table 2). The capacity of a Q-PCR assay to determine accurately the number of targets present in the sample depends on the linearity and efficiency of the PCR. Linearity relates to the ability of the method to generate results proportional to the amount of analyte present in the sample and is represented by the regression coefficient

TABLE 2. Determination of the detection and quantification limits of the Q-PCR assay with genomic DNA from *C. tyrobutyricum* strain CECT 4011[a]

Approx no. of genome equivalents/reaction	Confidence interval limit[b]		Signal ratio[c]	C_T[d]
	Lower	Upper		
fla				
1×10^5	99,643	100,358	9/9	21.98 ± 0.05
1×10^4	9,887	10,113	9/9	25.18 ± 0.09
1×10^3	964	1,036	9/9	28.93 ± 0.08
1×10^2	89	111	9/9	32.32 ± 0.11
1×10^1	7	14	30/30	35.63 ± 0.19
2	0	4	24/30	37.84 ± 0.06
1	0	2	12/30	38.52 ± 0.25
fla-IAC				
1×10^5	99,643	100,358	9/9	21.99 ± 0.02
1×10^4	9,887	10,113	9/9	25.35 ± 0.04
1×10^3	964	1,036	9/9	28.85 ± 0.06
1×10^2	89	111	9/9	32.44 ± 0.06
1×10^1	7	14	30/30	35.74 ± 0.15
2	0	4	21/30	37.75 ± 0.16
1	0	2	11/30	39.95 ± 0.56

[a] Nontemplate controls for both Q-PCR systems were negative (C_T values of 50 in all replicates). The overall slopes of the regression curve were −3.4436 and −3.4595 for the *fla* and *fla*-IAC Q-PCR systems, respectively, indicating PCR E of 0.952 and 0.946, respectively; the R^2 were 0.9995 and 0.9998, respectively.

[b] Calculated for the expected number of template molecules at each dilution at the 95% confidence level.

[c] Number of positive results out of 9 reactions (30 reactions for those containing 10, 2, and 1 genome equivalent per reaction).

[d] Cycle number at which fluorescence intensity equals a fixed threshold. Mean values ± standard errors of the mean are shown. The experimental results were statistically significant ($P < 0.05$), taking into account the unavoidable error associated with serial dilutions.

(R^2). Efficiency (E) is the capacity of the PCR to duplicate the amplicon molecules in each cycle and is calculated from the slope of the linear regression curve relating C_T and initial target nucleic acid concentration. The slopes of the linear regression curves calculated over a 5-log range were similar to the theoretical optimum of −3.32 (*fla*, −3.4436; *fla*-IAC, −3.4595) and showed amplification to be very efficient (*fla*, $E = 0.952$; *fla*-IAC, $E = 0.946$). Moreover, R^2 values were close to 1 (*fla*, 0.9995; *fla*-IAC, 0.9998), indicating that the assay was highly linear. Confidence intervals based on the standard deviations of C_T values did not overlap, down to 10 target molecules, indicating that reliable quantification was possible above this limit. These results are similar to those for other Q-PCR methods for other bacteria and eukaryotic organisms (8–10, 16, 17, 20, 22, 23).

Quantitative detection of *C. tyrobutyricum* in milk. The *fla*-IAC PCR assay was used for the identification and quantification of *C. tyrobutyricum* in artificially contaminated raw (directly transferred from a dairy factory) and commercial ultrahigh-temperature-treated (UHT) whole-milk samples. In three independent replicated experiments, 1 ml of water containing approximately 3×10^6, 3×10^5, 3×10^4, 3×10^3, 300, 150, 75, 25 or 3 *C. tyrobutyricum* spores was added to 50-ml centrifuge tubes, each containing 25 ml of raw milk or 25 ml of UHT whole milk. Spores were prepared as indicated by Herman et al. (7). The mixture was then thoroughly homogenized, and 3 ml of Triton X-100 (Sigma, Saint Louis, MO) and 1% (wt/vol) powdered trypsin (Sigma) were added to the artificially contaminated milk samples, which were then vortexed vigorously. The samples were incubated at 37°C for 30 min. The tubes were centrifuged at 4,600 × *g* for 30 min. The supernatant of each tube was carefully removed, and the pellet was resuspended in 1 ml of phosphate-buffered saline. The suspension was transferred into a 1.5-ml microcentrifuge tube and centrifuged at 15,700 × *g* for 5 min. The resulting bacterial pellet was resuspended in 200 μl of 10 mM Tris-HCl (pH 8.0), 50 mM EDTA, and 10 μl 100-mg/ml lysozyme (Sigma) and incubated for 1 h at 37°C. We then added 200 μl of a solution containing 10 mM Tris-HCl (pH 8.0), 1% sodium dodecyl sulfate, and 100 μg/ml proteinase K (Sigma) and incubated this mixture for a further hour at 37°C. Subsequently, 1 volume of a buffer containing 30 mM Tris-HCl (pH 8.0), 0.8 M guanidine hydrochloride, 7.5 mM EDTA, 5% (wt/vol) Tween 20, and 0.5% (wt/vol) Triton X-100 was added and the mixture was incubated for 45 min at 65°C. The mixture was centrifuged at 13,000 × *g* for 10 min and cooled to room temperature for extraction with chloroform, followed by DNA purification using the QIAquick kit (QIAGEN, Hilden, Germany). DNA was resuspended in 50 μl of 10 mM Tris-HCl (pH 8.0). We consistently detected as few as 75 *C. tyrobutyricum* spores per 25 ml of milk samples and as few as about 25 *C. tyrobutyricum* spores in more than 44% of PCR replicates for raw and UHT whole-milk samples (Table 3). These detection limits correspond to approximately 1 genomic unit per reaction (Table 3), similar to the limits obtained when genomic DNA isolated from *C. tyrobutyricum* was used as a PCR template. We then evaluated the use of this method for quantifying *C. tyrobutyricum* spores in milk. We constructed regression curves of the C_T values obtained with artificially contaminated milk samples and the corresponding numbers of *C. tyrobutyricum* spores inoculated. The correlation coefficients ($R^2 = 0.9928$ and $R^2 = 0.9995$ for raw and UHT whole-milk samples, respectively) demonstrated that the Q-PCR assay was linear over a range of 5 logs, down to 300 spores/25 ml of milk (Table 3). The PCR efficiencies obtained ($E = 1.068$ and and $E = 0.823$ for raw and UHT whole-milk samples, respectively) indicated that the performance of the Q-PCR assay was excellent (Table 3). Moreover, these values were similar to those obtained when purified *C. tyrobutyricum* genomic DNA was analyzed.

Relative accuracy of quantification of *C. tyrobutyricum* spores in milk. The effective implementation of any alternative method in routine laboratories requires a demonstration of its equivalence to the accepted reference method in terms of relative accuracy (18, 20, 21). Relative accuracy is defined as the closeness of agreement between the results obtained by an accepted method and those obtained by an alternative method (1). A relative accuracy of 100% indicates total agreement between the alternative and reference methods. The C_T values obtained from raw and UHT whole-milk samples artificially contaminated with *C. tyrobutyricum* were extrapolated to the corresponding standard regression curve, and the resulting theoretical numbers of *C. tyrobutyricum* spores were compared with those obtained by standard plating techniques (Table 3). Relative accuracy values ranged between 91.74% and 108.80%, indicating a high degree of correspondence between the quantitative results obtained by the reference method (number of *C. tyrobutyricum* spores/ml as determined by standard plating)

TABLE 3. Quantitative detection of *C. tyrobutyricum* in milk

Approx no. of *C. tyrobutyricum* spores/25 ml[a]	Approx no. of *C. tyrobutyricum* genome equivalents/reaction[b]	Signal ratio[c]	C_T value[d]	% Relative accuracy[e]
Commercial UHT whole milk				
3×10^6	1.2×10^5	9/9	22.79 ± 0.31	99.26
3×10^5	1.2×10^4	9/9	26.34 ± 0.05	100.90
3×10^4	1.2×10^3	9/9	30.29 ± 0.29	100.21
3×10^3	1.2×10^2	9/9	34.04 ± 0.36	101.36
3×10^2	12	9/9	38.11 ± 0.34	96.92
150	6	9/9	39.70 ± 0.25	NA[f]
75	3	9/9	39.34 ± 0.38	NA
25	1	4/9	40.41 ± 0.61	NA
3	0	0/9	>50	NA
0[g]	0	0/9	>50	NA
Raw milk				
3×10^6	3×10^4	9/9	23.91 ± 0.13	95.30
3×10^5	3×10^3	9/9	26.16 ± 0.08	102.78
3×10^4	3×10^2	9/9	29.03 ± 0.04	107.68
3×10^3	30	9/9	32.54 ± 0.04	105.68
3×10^2	3	9/9	36.22 ± 0.25	82.98
150	2	9/9	37.23 ± 0.34	NA
75	1	9/9	38.51 ± 0.58	NA
25	1	4/9	39.27 ± 0.43	NA
3	0	0/9	>50	NA
0[g]	0	0/9	>50	NA

[a] The results of three independent experiments with three PCR replicates used in each are shown. For commercial UHT whole milk, the overall efficiency was 0.823, and the linearity (R^2) was 0.9995. Raw milk was directly collected from a dairy factory. For raw milk, the overall efficiency was 1.068, and the linearity (R^2) was 0.9928.

[b] The estimated number of *C. tyrobutyricum* genome equivalents in each PCR run, assuming 100% DNA extraction efficiency, is shown. For commercial UHT whole milk, each reaction mixture contained 2 μl of a DNA preparation of 50 μl extracted from 25 ml whole UHT milk. For raw milk, each reaction mixture contained 5 μl of a DNA preparation of the 1:10 dilution of the initial 50 μl extracted from 25 ml whole UHT milk.

[c] Number of positive results out of nine reactions.

[d] Cycle number at which fluorescence intensity equals a fixed threshold. Mean values ± standard errors of the mean are shown. The experimental results were statistically significant ($P < 0.05$), taking into account unavoidable error associated with serial dilutions.

[e] Degree of correspondence between the results obtained by the standard plating technique (log *C. tyrobutyricum* spores/ml) and those obtained by the *fla*Q-PCR method (log *C. tyrobutyricum* genome equivalents/ml).

[f] NA, not applicable.

[g] Noncontaminated milk.

and the results obtained by the *fla*-IAC Q-PCR method over a wide (at least 5 logs) dynamic range (Table 3).

In conclusion, we describe here the first reliable and sensitive real-time PCR-based method for quantifying *C. tyrobutyricum* spores in dairy products, incorporating an IAC to assist interpretation of the results obtained. It has an excellent quantification capacity, as defined by its wide dynamic quantification range (at least 5 orders of magnitude) linearity ($R^2 > 0.9995$), PCR efficiency ($E = 0.946$), and quantification limit (down to 10 *C. tyrobutyricum* genome equivalents). When combined with simple detergent and enzymatic treatment of samples before centrifugation and nucleic acid extraction, it provides a highly efficient quantitative analysis of large volumes of whole milk, with detection down to 25 spores in 25 ml of raw or UHT whole milk, with an excellent relative accuracy to the reference microbiological method. This method therefore provides a promising alternative to traditional microbiological methods for the rapid, sensitive detection of *C. tyrobutyricum* spores in dairy products that could be easy to adopt in food analysis laboratories.

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