Evaluation of methods for DNA extraction from *Clostridium tyrobutyricum* spores and its detection by qPCR

Esteban, M.[a], Marcos, P.[a], Horna, C.[a,b], Galan-Malo, P[b]., Mata, L.[b], Pérez, M.D.[a], Calvo, M.[a], Sánchez, L[a].

[a] *Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón (IA2), Universidad de Zaragoza-CITA, Zaragoza, Spain*

[b] *ZEULAB, Bari 25 dpdo, 50197, Zaragoza, Spain*

HIGHLIGHTS

1. Effectiveness of methods for *C. tyrobutyricum* spore disruption was tested by qPCR.
2. Microwave treatment was found to be the best disruption method.
3. *C. tyrobutyricum* spores produced in agar are more resistant than those in liquid.
4. DNA from *C. tyrobutyricum* spores amplify later than that from vegetative cells.

ABSTRACT

Clostridium tyrobutyricum is the major agent that causes the blowing defect in cheese due to the germination of its dormant spores during the ripening stage. As a result, many of the affected cheeses show cavities and cracks, which cause the product loss in most cases. Nowadays, there is not a fast method capable of detecting milk contaminated with *C. tyrobutyricum* spores. The aim of this study has been to develop a fast and reliable method based on real time PCR (qPCR) to detect *C. tyrobutyricum* spores in raw milk. One of the main limitations has been to find a good procedure for the spore disruption to extract the DNA due to its high resistance. For this reason, different disruption methods have been tested, including chemical agents, bead beating, enzymatic and microwave treatment. Furthermore, an enzymatic treatment with subtilisin was applied for milk clarification and recovery of spores. The comparison of the assayed methods has been made using sterile milk spiked with *C. tyrobutyricum* spores, obtained in solid or liquid medium.

The results showed that microwave treatment followed by a standard DNA purification step was found to be the best disruption method. The Ct values obtained for spores were higher than those found for vegetative cells by qPCR, for the same quantity of DNA.

This difference could be due to the action of the Small Acid Soluble Proteins (SASP) in the DNA packaging of spores. Moreover, spores obtained in agar plate were found more resistant to disruption than those obtained in liquid medium. Subtilisin and microwave treatments were found to be successful for DNA extraction from *C. tyrobutyricum* spores in milk and subsequent identification by qPCR. However, the differences observed between the amplification of DNA from spores obtained in different media and from vegetative cells have to be taken into account to optimize a method for *C. tyrobutyricum* detection.

KEYWORDS

Clostridium tyrobutyricum; spores; real time PCR; milk.

1. Introduction

The late blowing defect in cheese is caused by bacteria from *Clostridium* genus and appears after 2-3 months of cheese ripening. During this period, clostridium spores germinate and ferment lactic acid, releasing butyric acid, acetic acid, carbon dioxide and hydrogen (Zhao et al., 2013). As a result of the pressure exerted by the gases produced, many of the affected cheeses form cavities and cracks, which cause the product loss in most cases. Many authors have reported *Clostridium tyrobutyricum* contamination and isolation from silages (Jonsson, 1990), cow milk (Bermúdez et al., 2016; Driehuis et al., 2016) goat milk (Reindl et al., 2014) and Grana Padano cheese (Bassi et al., 2015). However, other clostridia have been also isolated from raw milk, such as *C. sporogenes*, *C. butyricum, C. beijerinckii, C. perfringens* and *C. tertium* and contribute to late blowing defect in cheese (Feligini et al., 2014; Le Bourhis et al., 2007; Reindl et al., 2014). Therefore, *C. tyrobutyricum* is considered the main causative agent of late blowing defect in cheese (Klijn et al., 1995), though to a lesser extent other species could lead to the problem (Gómez-Torres et al., 2015; Le Bourhis et al., 2007). These bacteria are Gram positive, anaerobe and fermentative, producing butyric acid. The spores and vegetative cells of butyric acid bacteria can be present in raw milk used to produce cheese. The contamination of milk can originate in the feeding silage and take place during the milking process. This contamination can be the result of inadequate hygienic practices, because the spores go through the digestive tract and are excreted with faeces (Driehuis, 2013). However, while the vegetative cells are destroyed by the pasteurization process applied to milk prior to cheese-making, the spores can survive this treatment. Consequently, the spores can germinate and cause the late blowing defect

65 during the ripening stage, when the anaerobic conditions inside cheese are favourable
66 (Heyndrickx, 2011). This problem affects mainly hard and semi-hard cheeses, among
67 them, Grana Padano, Parmigiano Reggiano, and Manchego cheeses (Garde et al., 2011).

68 There are several microbiological methods that have been applied to detect the presence
69 of butyric acid-producing bacteria spores in milk, such as the Most Probable Number
70 (MPN) (Bergère and Sivelä, 1990). The MPN is based on a statistical method used to
71 estimate the number of butyric clostridia by culturing ten-fold dilutions of milk samples,
72 after being subjected to a previous heat treatment, and leaving the spores to germinate
73 and produce gas at 37 ºC, under anaerobic conditions. There are several variations of the
74 MPN method, though no universally accepted protocol exists. One of those variations is
75 the NIZO method (NEN, 2009), which is used in the Netherlands as a standard protocol.
76 Other methods used are the MPN Differential Reinforced Clostridium Media (DRCM),
77 the culture in Bryan and Burkey (BB)-broth and culture in RCM lactate-broth (Brändle
78 et al., 2016). However, all the MPN methods require long incubation periods for the
79 spores to grow and to produce the detachment of the paraffin or agar disposed on the
80 upper layer of the medium to create anaerobiosis.

81 Some selective microbiological methods have been also developed, such as culture in
82 modified Reinforced Clostridium Media (RCM), based on the addition of D-cycloserine
83 and neutral red to the agar. This method allows to identify the colonies of clostridia, but
84 must be completed with an additional lactate dehydrogenase test (Jonsson, 1990).
85 Another reported method combines the microfiltration of milk to retain and concentrate
86 the spores and the culture of the filter in modified RCM (Reindl et al., 2014). However,
87 all the described microbiological methods require several days of incubation and are not
88 completely specific, resulting in some cases, false positives due to the growth of other
89 microorganisms. A novel and fast microbiological test has been developed by Brändle
90 et al. (2018) showing high selectivity for *Clostridium* spores and a short incubation
91 time, of 48 h. In this study, a selective media called AmpMedia 666 was used, and the
92 concentration of spores was calculated by a reaction based on colour change, having a
93 limit of detection of 75 spores per litre, better than that achieved with the conventional
94 MPN method using Bryant and Burkey broth with resazurin and lactate.

95 In the recent years, the efforts in developing alternative and fast methods for the
96 detection of *C. tyrobutyricum* have increased. Lavilla et al. (2010) published an article
97 describing a detection method based on flow cytometry using a fluorophore-labelled

98 polyclonal antibody against *C. tyrobutyricum* spores. In that study, the detection limit
99 was established in 10^3 spores/100 mL. The use of magnetic nanoparticles with specific
100 ligands, such as antibodies or affine peptides, were also proposed by Lavilla et al.
101 (2012) as novel methods for the detection of *C. tyrobutyricum* spores. Although those
102 methods are faster and highly specific, their introduction into the routine analysis is not
103 easy due to the high cost of the materials and equipment required.

104 Several methods based on molecular biology techniques have been developed as
105 alternative methods for bacterial detection. Thus, the use of real time PCR has
106 considerably increased in the last years for the detection of microbiological food agents
107 (Klein, 2002; Malorny et al., 2004), because is a fast and highly specific technique.
108 However, one challenge of PCR in the case of spores is to obtain pure genomic DNA,
109 which involves their effective disruption.

110 *Clostridium tyrobutyricum* has been enumerated in spiked milk and other matrices by
111 real time PCR with success (Bassi et al., 2013; López-Enríquez et al., 2007), showing a
112 good limit of detection. A multiplex real time PCR has been proposed (Morandi et al.,
113 2015) to identify and enumerate *C. beijerinckii*, *C. sporogenes* and *C. tyrobutyricum* in
114 artificially contaminated milk. However, it is important to point out that, to our
115 knowledge, there are not published studies about the identification and enumeration of
116 *Clostridium* spores by real time PCR in raw milk samples with natural contamination.

117 Previous studies have shown the great resistance of different types of spores to be
118 disrupted. It has been reported that spores frequently resist heat treatments, radiation,
119 chemical agents, and also enzyme treatments (Ávila et al., 2014) due to their complex
120 wall structure (D'Incecco et al., 2018).

121 Moreover, the way to obtain the spores influences the composition and resistance of
122 their cortex, as it has been proved in *Bacillus subtilis* (Abhyankar et al., 2016). In that
123 study, differences in the composition of the coat proteins of spores produced on solid
124 agar plates or in liquid medium were found, which were correlated with the thermal
125 resistance of the two types of spores. The spores produced on agar plates presented
126 higher resistance to thermal and wet heat treatments, and also germinated slower than
127 the spores produced in liquid medium (Rose et al., 2007).

128 The first aim of this study was to evaluate several methods for the disruption of
129 *Clostridium tyrobutyricum* spores to achieve pure DNA to be amplified by real time

130 PCR. The second objective of this study was to compare the disruption of spores
131 produced on agar plates with that of spores induced in liquid medium.

132 **2. Material and methods**

133 *2.1. Sporulation process*

134 *Clostridium tyrobutyricum* spores were obtained by two different methods: sporulation
135 on agar plates and in liquid medium. *Clostridium tyrobutyricum* CECT 4012 strain was
136 obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Bacteria
137 were inoculated in tubes with 10 mL of Reinforced Clostridial Medium (RCM)
138 (Scharlau, Barcelona, Spain), a 2 cm layer of paraffin was added on the top of medium
139 to create anaerobiosis and the tubes were incubated for 24 h at 37 ºC. Afterwards, 100
140 µL of bacterial suspension were spread uniformly over RCM agar plates. The plates
141 were incubated in Gaspak jars with the anaerobic gas generator AnaeroGen™ 2.5 L
142 (Oxoid, Basingstoke, UK) and an anaerobic indicator (Oxoid) for 7 days at 37 ºC. The
143 colonies were collected with a Digralsky spreader adding 4 mL of phosphate buffered
144 saline (PBS) consisting of 140 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM
145 KCl, pH 7.4.

146 The sporulation in liquid medium was carried out from a 24 h *C. tyrobutyricum* culture
147 in RCM medium as described before (Lavilla et al., 2010). Briefly, 1 mL of that
148 preculture was inoculated into 50 mL of fresh RCM medium with a paraffin layer of 4
149 cm to create anaerobiosis. After 24 hours, 50 mL of culture were inoculated onto a 12-
150 14 kDa dialysis membrane immersed into Tryptone-Glucose-yeast Extract (TGE)
151 medium. The device where the dialysis membrane was placed was composed of a glass
152 tube of 40 cm (height) x 7 cm (diameter) closed with a stopper drilled with three holes
153 fitted with tubes having different functions. One of the tubes was used to inoculate the
154 culture into the dialysis membrane, the second tube to pump in N_2 to create anaerobiosis
155 (5 or 10 min of N_2 bubbling are required for the best conditions) and the third one to
156 eliminate the residual gases originated from the bacterial growth. The culture was
157 incubated at least for 72 h and afterwards, the suspension of sporulated cells was
158 centrifuged at 4000 x g for 15 min at 4 ºC and washed three times with sterile distilled
159 water.

160 *2.2. Purification of spores*

161 The bacterial cells were lysed with lysozyme from egg white (Sigma-Aldrich, St. Louis,
162 MO, USA) added to the suspension at a concentration of 0.4 mg/mL and incubated for
163 24 h at 45 ºC in a total volume of 100 mL. Afterwards, the suspension was centrifuged
164 for 15 min at 4000 x g and 4 ºC. The supernatant was discarded, and the precipitate was
165 washed twice in the same conditions using sterile distilled water to eliminate the cellular
166 debris. The precipitate obtained was resuspended in 2 mL of sterile distilled water and
167 applied to a Percoll® gradient (Sigma-Aldrich) to separate the spores from vegetative
168 cells, as described before (Leuschner et al., 1999). The gradient was prepared with
169 different concentrations of Percoll®: 1.13, 1.11, 1.09, 1.07 and 1.05 g/mL. The different
170 solutions were placed in falcon tubes, adding 10 mL of each concentration, starting
171 from the densest and ending with the less dense. Afterwards, 2 mL of the lysate was
172 added on top of the gradient and it was centrifuged for 45 min at 4000 x g and 4 ºC.
173 After the centrifugation, the spores were collected from the bottom of the gradient,
174 washed 5 times with sterile distilled water and recovered by centrifugation at 13000 x g
175 for 10 min. The precipitate containing the spores was resuspended in 200 µL of PBS
176 and the concentration was determined by counting in a Thoma chamber under a phase
177 contrast microscope ECLIPSE E400 (Nikon, Tokyo, Japan). Finally, the spore
178 suspension was stored at -20 °C until use.

179 *2.3. Disruption of spores*

180 Taking into account the difficulty to extract the DNA from the spore core, required to
181 perform real time qPCR quantification, it was necessary to attempt different disruption
182 methods. For all the treatments, the spores were resuspended in PBS. These methods
183 were tested in three independent experiments on three different days.

184 *2.3.1. Microwave treatment*

185 The extraction of DNA by microwaves was carried on by using a GE87M-X microwave
186 oven (Samsung, Barcelona, Spain). For this treatment, 100 µL of 10^6 spores/mL
187 suspension were added in microwave resistant Eppendorf vials. The tubes were placed
188 in a microwave steamer and were heated for 15 min at 600 W.

189 *2.3.2. Bead beating treatment*

190 The bead beating procedure was carried out following the method by Vandeventer et al.
191 (2011), with some modifications. For the spore lysis, 250 mg of 100 µm-diameter silica
192 beads and 625 µL of 10^6 spores/mL suspension were added in tubes of 2 mL. The

193 samples were bead beaten using a Mini-BeadBeater (BioSpec Products, Bartlesville,
194 OK, USA) at maximum speed for 1 min pulses, three times, with an interval of 30 s,
195 placing the tubes in ice between each pulse.

196 *2.3.3. DNA precipitation*

197 The DNA precipitation protocol was obtained from Sambrook (2001). A volume of 10
198 µL of sodium acetate was added to a 100 µL suspension of *C. tyrobutyricum* spores at a
199 concentration of 10^6 spores/mL previously disrupted. Then, 220 µL of 100% cold
200 ethanol was added and incubated for 1 h at -80 °C. Afterwards, the mixture was
201 centrifuged for 15 min at 13000 x g and 4 °C. The supernatant was discarded, and the
202 pellet was resuspended in 500 µL of 80% cold ethanol and centrifuged for 10 min at
203 13000 x g and 4 °C. Finally, the supernatant was discarded, and the pellet was air dried
204 and resuspended in 100 µL of filtered miliQ water.

205 *2.3.4. Proteinase K treatment*

206 The enzyme proteinase K was added at a final concentration of 2 mg/mL to 100 µL of
207 10^6 spores/mL suspension and was incubated for 30 min at 55 °C. Then, the proteinase
208 was inactivated, in order to avoid qPCR inhibition, by incubating the samples for10 min
209 at 90 °C.

210 *2.3.5. Heat treatment*

211 For the heat treatment, samples containing 100 µL of 10^6 spores/mL were incubated at
212 90 ºC for 20 min in a water bath.

213 *2.3.6. 2-Mercaptoethanol-lysozyme treatment*

214 This treatment, described in the study of Torok (2003), required the addition to 100 µL
215 of 10^6 spores/mL suspension of 90 µL of a buffer containing 3.6 M guanidine
216 hydrochloride (pH 2.8), 10% of 2-mercaptoethanol and 1 mg/mL lysozyme, in 10 mM
217 Tris-HCl buffer, pH 8, added with 1 mM EDTA and 5% Triton X-100,. The solution
218 was incubated for 1 h at 45 ºC. The final sample was purified using the GenElute™
219 Bacterial Genomic DNA Kit (Sigma-Aldrich) for DNA purification.

220 *2.3.7. Lysozyme-proteinase K-guanidine hydrochloride-Tween 20-Triton X-100*
221 *treatment*

222 The method reported by López-Enríquez et al. (2007) for the quantification of *C.*
223 *tyrobutyricum* using qPCR was followed with some modifications. To begin with, 200

224 µL of 10 mM Tris-HCl, pH 8, with 50 mM EDTA, and 10 µL of 100 mg/mL lysozyme
225 were added to 200 µL of a suspension containing 10^6 spores/mL. This mixture was
226 incubated for 1 h at 37 °C. Afterwards, one volume of 10 mM Tris-HCl, 1% SDS, 100
227 µg/mL of proteinase K was added and the mixture was heated for 1 h at 37 °C.

228 Then, one volume of 30 mM Tris-HCl buffer, pH 8, with 0.8 M guanidine
229 hydrochloride, 7.5 mM EDTA, 5% Tween 20 and 0.5% of Triton X-100 was added.
230 After incubation of 45 min at 65 ºC, the mixture was centrifuged for 10 min at 13000 x
231 g and the pellet was discarded. For protein elimination, a phenol-chloroform extraction,
232 adding one volume, was carried out and the resulting sample was transferred to columns
233 of the kit GenElute™ Bacterial Genomic DNA for DNA purification.

234 *2.3.8. DNA purification*

235 DNA from vegetative cells was isolated with the DNA purification kit GenElute™
236 Bacterial Genomic DNA following the instructions of the manufacturer referred to
237 Gram-positive bacteria.

238 The protocol for isolating the spore DNA, after applying one of the different disruption
239 methods, was adapted by omitting the lysis step required for the vegetative cells, to
240 evaluate the effectiveness of the disruption treatment. The final elution volume was
241 adjusted to 100 µL.

242 *2.4. Treatment of spore contaminated UHT milk for real time PCR analysis*

243 A 500 µl volume of commercial UHT whole milk, with 3,6% of fat and 3% of protein,
244 was spiked with 10^6/mL *C. tyrobutyricum* spores previously obtained, as described
245 above. Contaminated milk was treated with subtilisin solution, a non-specific protease
246 used to treat milk samples prior to enumeration of bacteria by BactoScan equipment
247 (Foss, Hilleroed, Denmark). A 500 µL volume of subtilisin solution was added to 500
248 µL of UHT spore contaminated milk. The mixture of milk and enzyme was heated at 60
249 ºC in a water bath for 1 hour and then, it was centrifuged at 13000 x g for 30 min at
250 room temperature. After centrifugation, milk fat and proteins were discarded, and the
251 spores located at the bottom of the vial were recovered. The spores were resuspended in
252 100 µl of PBS for microwave treatment or in 625 µl for bead beating. After the spore
253 disruption, DNA was extracted with the GenElute™ Bacterial Genomic DNA. Two
254 samples of milk were spiked with spores and analyzed by real time PCR in duplicate.
255 The experiment was repeated three times on three different days.

256 *2.5. Quantitative Real-Time PCR (qPCR)*

257 The reagents used for the SYBR Green qPCR assay were: 12.5 µL of Mastermix
258 SYBR® Green Power Up (Applied Biosystems, Waltham, MA, USA), 7.5 µL of
259 RNAses free water (Invitrogen, Paisley, UK) (9.5 µL in negative controls), 1.5 µL of
260 each primer and 2 µL of DNA sample.

261 The reagents used for the TaqMan qPCR assay were: 11 µL of TaqMan ™ Universal
262 PCR Master Mix (Applied Biosystems), 7 µL of RNAse-free water (9 µL in negative
263 controls), 1 µL of TaqMan ™ Copy Number Assays (Applied Biosystems) and 2 µL of
264 DNA sample.

265 The primers used in both assays were the CTfla, which target the flagellin gen of *C.*
266 *tyrobutyricum*, described in the study by López-Enríquez et al. (2007). Each sample was
267 tested on qPCR in duplicate.

268 The amplification process, in the case of the SYBR Green qPCR, has an initial stage of
269 50 ºC/2 min, which activates the enzyme uracil-DNA-glycosylase (UNG). The
270 following steps of the amplification process are common for both types of qPCR, the
271 first step being an initial denaturation of 95 ºC/10 min, and afterwards 50 cycles of
272 amplification, with a denaturation of 95 ºC/15 s, hybridization at 60 ºC and elongation
273 at 60 ºC/1 min. The threshold cycle value (Ct) was obtained from the amplification
274 curve and was inversely proportional to the number of copies of DNA.

275 In the SYBR Green qPCR, after 50 cycles of amplification, a melting curve process
276 starts, which gives the melting temperature (Tm) of the DNA fragments that have
277 amplified.

278 The amplification products were also visualized on agarose gels stained with SYBR
279 Safe® (Invitrogen).

280 *2.6. Analysis of DNA amplification from vegetative cells and spores.*

281 Genomic DNA of vegetative cells was extracted from a precipitate obtained after
282 centrifugation of 1.5 ml of an overnight bacterial broth culture with GenElute Bacterial
283 Genomic DNA Kit. A step of DNA concentration with ethanol in the presence of
284 sodium acetate was needed as suggested by the kit manufacturer.

285 For extraction of DNA, 10^7 spores/mL produced in liquid medium were resuspended in
286 100 µl of PBS and subjected to microwave treatment and column purification. The step

287 of ethanol precipitation in the presence of sodium acetate was also needed, to achieve a
288 high amount of DNA.

289 The concentration of the DNA extracted from both types of spores was measured using
290 a NanoDrop 1000 (Thermo Fisher Scientific, CA, USA) and diluted to achieve the
291 following DNA concentrations: 30, 20, 10, 1, 0.1 and 0.01 ng/µl. 2 µl of each DNA
292 solution was analyzed by real time PCR in duplicate and in three independent assays.

293 The efficiency (E) of qPCR for vegetative cells and spores was calculated following this
294 formula: $E = 10^{-1/slope}$.

295 *2.7. Data analysis.*

296 The Ct values were obtained from the qPCR software Step One™ 2.3. version (Life
297 Technologies, Carlsbad, CA, USA). The Ct or threshold cycle value is the cycle number
298 at which the fluorescence generated in the amplification reaction surpasses the
299 fluorescence threshold, a fluorescent signal significantly above the background
300 fluorescence. The mean values and standard deviations of Ct values were calculated
301 with Microsoft® Excel (Microsoft Spain, Madrid). T-test was performed using
302 GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA),
303 www.graphpad.com.

304 **3. Results**

305 *3.1. Evaluation of the spore disruption methods.*

306 The main aim of this study was to find an efficient and reliable method to obtain the
307 highest amount of pure genomic DNA from *C. tyrobutyricum* spores to be amplified
308 and allow their detection. The results obtained by the disruption methods under
309 evaluation are shown in Table 1. Some of the methods were completed with a final step
310 of DNA purification by spin column and all were analyzed by qPCR. Two treatments
311 proved to be clearly more effective for spore disruption than the others. The best results
312 were obtained with microwave (MW) heating followed by DNA purification, resulting
313 in 100% of positive results with SYBR Green and 83.3% with the TaqMan probe. The
314 second most efficient disruption method was bead beating (BB) followed by DNA
315 purification, which gave an amplification percentage of 66.67% with SYBR Green and
316 50% with the TaqMan probe. The rest of the treatments analyzed were not considered in
317 further analysis because of the low percentage of amplification obtained.

318 In most of the procedures analyzed, SYBR Green allowed to achieve higher number of
319 amplified samples, in comparison with the TaqMan probe. Moreover, in the
320 experiments carried out with SYBR Green, the melting curve obtained after
321 amplification allowed confirming that the qPCR products corresponded with the
322 expected amplicon. The qPCR products of all the assays showed only one peak with a
323 Tm around 76 ºC, without the presence of other peaks indicating contamination or
324 primer dimerization. Therefore, the rest of the assays were carried out with SYBR
325 Green, as it proved to be the best method to detect genomic DNA of *C. tyrobutyricum*
326 spores by qPCR under the conditions of this study.

327 In all the assays performed, genomic DNA from *C. tyrobutyricum* vegetative cells was
328 included as positive control. It was observed that for the same amount of genomic
329 DNA, the DNA obtained from vegetative cells amplified at lower number of cycles than
330 the one obtained from spores. This fact will be described and discussed later on in this
331 section.

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346 Table 1. Effect of different disruption methods on the amplification by qPCR of *C. tyrobutyricum* spores (10^6 spores/mL) generated on agar plates. ND: not detected. The values represent the Ct mean ± standard deviation from three independent experiments with two replicates each (n=6). *Standard deviation is not included as only one sample amplified with this treatment.

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	SYBR Green reagent		TaqMan probe	
Treatment	**Ct value**	**% positive samples**	**Ct value**	**% positive samples**
Bead beating (BB)	41.74*	8.33	45.26 ± 1.76	16.67
Heat shock and BB	ND	-	44.10 ± 0.85	16.67
BB-DNA column purification	33.79 ± 3.08	66.67	37.36 ± 5.28	50.00
BB-DNA ethanol precipitation	39.99 ± 0.00	16.67	40.81*	8.33
BB-proteinase K	39.20*	8.33	ND	-
BB-microwaves (MW)	38.82 ± 0.09	16.67	ND	-
MW	39.48 ± 0.91	16.67	41.33*	8.33
Heat shock-MW	ND	-	ND	-
MW-DNA column purification	33.54 ± 2.05	100.00	37.53 ± 1.71	83.33
MW-DNA ethanol precipitation	36.54 ± 1.51	25.00	ND	-
MW-proteinase K	37.85 ± 3.05	16.67	ND	-
MW-bead beating	37.16 ± 4.93	25.00	44.42 ± 2.32	16.67
β-mercaptoethanol-lysozyme-MW-DNA purification	40.19 ± 6.65	33.33	40.96 ± 0.16	16.67
Lysozyme-proteinase K-guanidine hydrochloride-Tween 20-Triton X-100[(1)]	36.73 ± 1.40	75.00	40.93 ± 0.43	25.00

352 (1) Method based on López-Enríquez et al (2007)

The effectiveness of disruption methods on spores generated on agar plates and in liquid medium was compared. The spores generated on agar plates were found to be more resistant to disruption than those produced in liquid medium. The values of Ct shown in Table 2 correspond to the qPCR assays performed after treating liquid medium spores by microwaves and bead beating in comparison with those obtained on agar plates. Liquid medium spores amplified with a 100% in both methods tested and with significantly lower Ct value (28.58 ± 1.57) in the case of microwave treatment and column purification than that for agar plate spores (33.54 ± 2.05). Different results were obtained for agar plate spores with 66% of amplified samples after bead beating and column purification and with 100% after microwave and column purification. For the bead beating treatment and column purification no differences were found in Ct values between agar spores and liquid medium.

Table 2. Effect of microwaves and bead beating followed by column purification for disruption of *C. tyrobutyricum* spores (10^6 spores/mL) generated in agar plate and liquid medium, on their amplification by qPCR with SYBR Green. The values represent the Ct mean ± standard deviation from three independent experiments with two replicates each (n=6).

Treatment	**Agar plate spores**		**Liquid medium spores**	
	Ct value	**% amplified samples**	**Ct value**	**% amplified samples**
MW-DNA column purification	33.54 ± 2.05	100	28.58 ± 1.57*	100
BB-DNA column purification	33.79 ± 3.08	66.67	33.23 ± 1.37	100

*Significant differences for p□0.05 between agar plate and liquid medium spores

3.2. Detection of spores from solid or liquid media added in UHT milk.

With the aim of getting one step closer to the real application of the methods proposed in this study, we tested the two most efficient disruption procedures on UHT milk spiked with *C. tyrobutyricum* spores instead of PBS. The results obtained, shown in Table 3, have been expressed as the percentage of spore spiked UHT milk samples showing amplification of genomic DNA.

378 With both disruption methods we observed the same behavior as described in PBS, with
379 100% amplification of DNA from samples with spores produced in liquid medium and
380 lower Ct values compared to samples with spores produced in solid medium. This fact
381 should be considered in order to develop and validate a quantification method for *C.*
382 *tyrobutyricum* spores in milk.

383 Moreover, the subtilisin method was found very efficient to recover spores from UHT
384 milk and for the subsequent steps and real time PCR analysis. It allowed the clear
385 separation of a fat layer, a liquid interphase and a precipitate containing the spores. As it
386 is shown in the supplementary material, the percentage of spores recovered from milk
387 digested with subtilisin was higher than that obtained with a simple centrifugation.
388 However, it is important to point out that higher Ct values were found in UHT milk
389 treated with subtilisin in all the conditions tested, except for liquid medium spores
390 treated by bead beating and DNA column purification.

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392 Table 3. Effect of the most efficient disruption methods on spores produced in solid or
393 liquid media added to UHT milk; in both methods a final step of column purification
394 was used. qPCR was carried out with SYBR Green. The values represent the Ct mean ±
395 standard deviation from three independent experiments (n=6).

Treatment	**Agar plate spores**		**Liquid medium spores**	
	Ct value	**% amplified samples**	**Ct value**	**% amplified samples**
MW-DNA column purification	36.16 ± 1.20	58.33	30.13 ± 0.97	100
BB-DNA column purification	38.20 ± 1.18	33.33	30.52 ± 1.33	100

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397 The qPCR products were analyzed by agarose gel electrophoresis to verify that the size
398 of DNA amplicons was the same after applying the different disruption methods. The
399 results obtained are shown in Figure 1, where it can be observed the expected size of the
400 amplicons, around 90-100 bp, regardless the disruption method used. Furthermore, we
401 observed differences in the amount of DNA amplified from solid medium spores,
402 compared to liquid medium spores, which correlates well with the lower Ct found for
403 solid medium spores.

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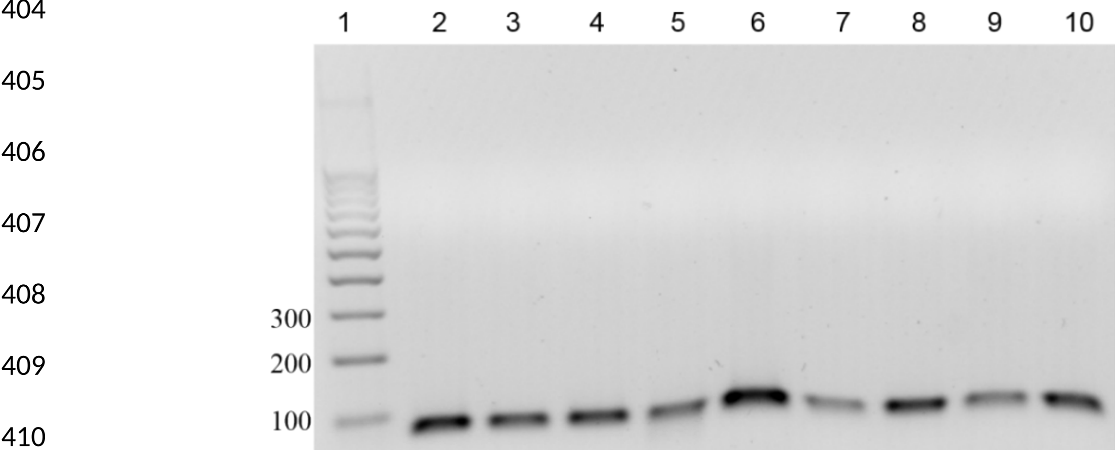

411 Figure 1. Analysis by agarose gel electrophoresis of qPCR products (82 bp) obtained
412 from the amplification of DNA extracted from *C. tyrobutyricum* spores by different
413 methods. Lane 1, molecular weight base pair; Lane 2, vegetative cells; Lanes 3-6, PBS
414 spiked with spores: Lane 3, DNA from solid medium spores treated by BB (CP)*; Lane
415 4, liquid medium spores treated by BB (CP)*; Lane 5, solid medium spores treated by
416 MW (CP)*; Lane 6, liquid medium spores treated by MW (CP)*. Lanes 7-10, UHT
417 milk spiked with spores and treated with subtilisin: Lane 7, solid medium spores treated
418 by BB (CP)*; Lane 8, liquid medium spores treated by BB (CP)*; Lane 9, solid medium
419 spores treated by MW CP)*; Lane 10, liquid medium spores treated by MW (CP)*.
420 *With a final step of DNA column purification (CP)

421 *3.3. qPCR analysis of C. tyrobutyricum spores and vegetative cells*

422 DNA from vegetative cells and liquid medium spores was extracted and purified by the
423 same method, and the amplification of the same amount of DNA from each extraction
424 was compared. As it can be observed in Figure 2, the results clearly show the
425 differences in DNA amplification, as the DNA from vegetative cells amplified at lower
426 number of cycles than that from spores. Furthermore, the lowest quantity of DNA from
427 vegetative cells that could be detected was 0.02 ng, while no less than 0.2 ng could be
428 detected from spores. The standard curves obtained indicate that there is a difference of
429 about 10 cycles in the Ct values between DNA amplification from vegetative cells and
430 that from liquid medium spores, and this difference is maintained for all the
431 concentrations assayed. Consequently, the slope of the two regression lines is similar,
432 indicating that the difference is not due to a random effect. The qPCR efficiency
433 obtained for vegetative cells was 96.84%, whereas for spores it was only of 59.23%.

434 The melting curves obtained for the amplification of DNA from liquid medium spores
435 and vegetative cells showed Tm mean values very similar, of 76.18 ± 0.23 and 76.49 ±
436 0.07, respectively.

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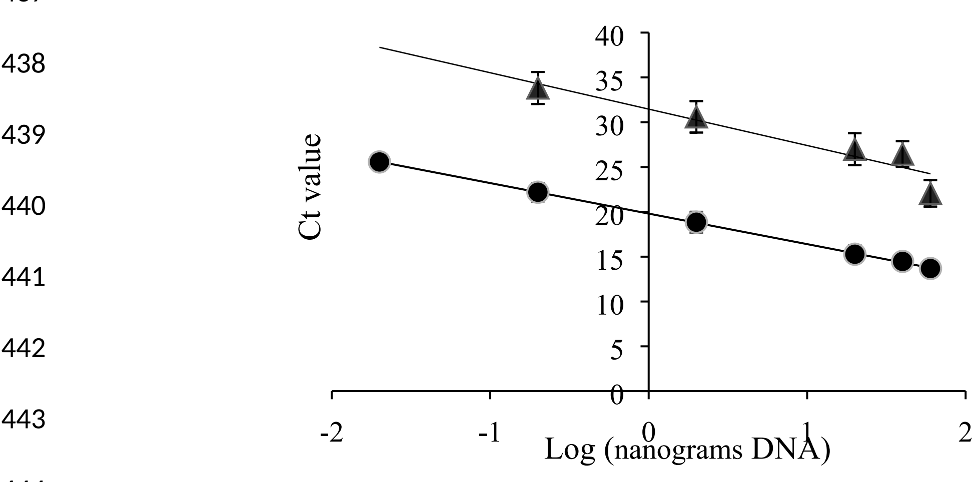

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445 Figure 2. Standard curve obtained by qPCR for amplification of DNA from *C.*
446 *tyrobutyricum* vegetative cells (●) and liquid medium spores (▲). The equation for
447 standard curve of vegetative cells is y = -3.406x + 18.779 with R^2= 0.9997 and for
448 spores y = -4.0583x + 30.241 and R^2=0.8991.

449

450 **4. Discussion**

451 *C. tyrobutyricum* is the main agent that causes the late blowing defect in cheese. The
452 cause of this spoilage derives from the presence of spores and vegetative cells in milk
453 used for cheese-making process. Moreover, cheeses made with milk previously heat
454 treated, could be affected because of the heat resistance of spores (Loessner et al.,
455 1997). The spores are still viable after heat treatment because of their resistant structure
456 that surrounds their core. Actually, the spores of various species of *Bacillus* and
457 *Clostridium* genera are the most resistant known life forms (Setlow, 2004). For this
458 reason, developing a suitable and rapid method to detect *C. tyrobutyricum* spores is
459 essential. However, due to the nature of spores, to find an efficient disruption method to
460 extract their DNA for quantification by qPCR is a real challenge. To our knowledge,
461 this is the first study to compare different procedures to find a reliable and efficient
462 method for *C. tyrobutyricum* spore disruption and DNA extraction.

463 The results obtained demonstrated that the best procedure for an efficient DNA
464 extraction from the spores of the *C. tyrobutyricum* strain tested in this study, was the
465 microwave treatment followed by spin column DNA purification. This final step is
466 essential to achieve a pure quantity of DNA for a reliable analysis.

467 In this study, we have proved that the DNA from *C. tyrobutyricum* spores obtained in
468 solid medium (agar plates) is more difficult to be amplified by qPCR than that from
469 spores obtained in liquid medium, suggesting that the former could be more resistant to
470 disruption than the latter. To our knowledge, this finding has not been reported before,
471 because the majority of previous studies related with the detection of *C. tyrobutyricum*
472 by qPCR have been made with liquid medium spores. However, there are some studies
473 on other sporulated bacteria, such as *Bacillus subtilis*, which indicate different
474 resistance to some treatments, such as heat (Abhyankar et al., 2016) or several
475 chemicals (Rose et al., 2007), between spores produced on agar plates and those
476 produced in liquid medium. These divergences have been explained by differences in
477 the cross-linking of spore coat proteins and in the inner membrane composition.
478 Therefore, it would be useful to know the structure of wild spores present in milk and
479 their ability to be amplified by qPCR amplification. This is a very important point that
480 has not yet been discussed, because it is difficult to reproduce the sporulation of
481 vegetative cells as it happens in nature, compared with that developed in the laboratory.
482 In addition, the analysis of raw milk samples with natural contamination by qPCR is
483 needed to complete the investigation because there are no published data about this
484 issue.

485 We also studied the extraction of DNA from *C. tyrobutyricum* spores in UHT milk for
486 qPCR analysis, with the aim of mimicking the real conditions. The main challenge to
487 achieve an efficient method for detecting *C. tyrobutyricum* spores in milk is to find a
488 simple and reliable method to separate and concentrate the spores that are distributed in
489 milk phases. We propose an effective method for milk digestion with subtilisin enzyme.
490 This non-specific protease is routinely used to count the total bacteria present in milk
491 with the Bactoscan equipment (Foss Electric) in the Milk Quality Control Laboratories.
492 Our results show that this method is a good approach to develop a fast procedure for *C.*
493 *tyrobutyricum* spore detection. The treatment of UHT milk with subtilisin allows its
494 effective digestion, thus obtaining a clean pellet of spores, compared to the non-treated
495 milk, which can be subjected to disruption by different methods, as we have tested in

496 the present study. This is a novel approach, considering that other digestion procedures
497 previously reported have higher number of steps (López-Enríquez et al., 2007) or are
498 based in a simple centrifugation (Morandi et al., 2015), which presents the problem that
499 a percentage of spores can be retained in the fat layer. As far as we have tested in UHT
500 and raw milk, subtilisin treatment allows recovering almost all the spores in the pellet,
501 as we have checked by plate count (Supplementary material).

502 In addition, we compared the Ct values obtained by qPCR when analyzing the same
503 amount of DNA derived from vegetative cells and from spores. This finding has not
504 been previously reported in *Clostridium* spores analyzed by qPCR. We observed that
505 the amplification of DNA from vegetative cells gave considerably lower Ct values than
506 that obtained for the DNA from spores, indicating that DNA may not have the same
507 degree of condensation. This fact could be explained by the great influence that SASP
508 have in the condensation of spore DNA; therefore, making it less available for the qPCR
509 reaction or other enzyme reactions (Lee et al., 2008; Setlow et al., 1992; Wetzel and
510 Fischer, 2015). These proteins are not present in vegetative cells and therefore, their
511 DNA is more accessible for amplification. It has been reported that the DNA of *C.*
512 *perfringens* spores is less available for the DNase I digestion (Raju et al., 2006) and also
513 that the SASP influence the transcription of certain genes as demonstrated in *B. subtilis*
514 (Setlow et al., 2000). These proteins protect the DNA and make it highly condensed and
515 are only cleaved by the action of the Germination Protease (GPR) and the YyaC
516 protease during the germination process. The action of the SASP proteins on the spore
517 DNA might interfere with the qPCR analysis resulting in high Ct values. The influence
518 of SASP also could depend on the gene selected for qPCR analysis, because of the
519 irregular distribution of SASP in the genome (Setlow et al., 2000).

520 In the current study, the lowest amount of DNA from liquid medium spores being
521 detected was 0.2 ng, with a Ct mean value of 33.82, which corresponds to
522 approximately 10^5 genome equivalents, while for the same amount of DNA from
523 vegetative cells the amplification presented Ct mean values of 25.57. The lowest
524 amount of DNA detected for vegetative cells was set up in 10^{-6} nanograms of genomic
525 DNA equivalent to approximately 1 genome. These results indicate that higher amounts
526 of DNA have to be extracted to detect the spores than to detect the vegetative cells. This
527 fact increases considerably the limit of detection for *Clostridium* spores, which are more
528 resistant and consequently more difficult to be disrupted than vegetative cells.

529 Moreover, the slope of the regression line obtained for amplification of vegetative cell
530 DNA by using the *CTfla* primers was -3.406, very similar to the value reported in the
531 original study, of -3.443 (López-Enríquez et al., 2007), with an excellent linearity in
532 both studies. However, the linearity of the regression line obtained for DNA spore
533 amplification (R^2=0.8991) in our study differed from the linearity obtained in that by
534 López-Enríquez et al. (2007), probably due to the experimental differences between the
535 procedures.

536 The results reported in this study have revealed the difficulty of detecting DNA of
537 clostridial spores by qPCR when vegetative cells are present, since vegetative cells
538 amplified at lower cycles than spores. Therefore, in a mixture of DNA from clostridial
539 spores and vegetative cells, the DNA from vegetative cells would amplify earlier by
540 qPCR due to its nature, thus hiding the presence of clostridial spores. Due to this fact it
541 is very important to inactivate the vegetative cells that could be present in raw milk in a
542 first step, with a heat treatment of 90 ºC for 10 minutes. It could be also adequate to
543 treat the samples with DNase in order to detect only the DNA from spores after
544 disruption (Morandi et al., 2015). In our experimental conditions, the Percoll gradient
545 used for spore purification separated very well the vegetative cell debris from the
546 spores. In addition, the subtilisin treatment applied in our method washes off the
547 possible contamination of DNA from vegetative cells, as it migrates to the soluble phase
548 that is eliminated.

549 To sum up, the results obtained in our study show clearly the difficult challenge of
550 detecting *C. tyrobutyricum* spores by qPCR. On the one hand, the spore structure makes
551 it complicated to develop a good method for disruption and DNA extraction; and on the
552 other hand, there are no specific DNA extraction kits for bacterial spores. Furthermore,
553 we have demonstrated for the first time that in the case of *C. tyrobutyricum* the way in
554 which the spores are produced exerts great influence on the efficiency of detection.
555 Further studies are necessary to apply the best method identified in this study to the
556 analysis of real raw milk samples and to compare results obtained with the results of
557 this study, with the main objective of determining what kind of spores are present in the
558 dairy farm environment.

559 **4. Conclusion**

560 The subtilisin digestion of milk combined with microwave treatment followed by DNA
561 column purification has been proved as the best procedure for detecting *C.*

562 *tyrobutyricum* spores by qPCR in milk among all the methods tested. Furthermore, in
563 this study we have proved that the way the spore is produced affects its detection by
564 qPCR. We found that spores from liquid medium amplified earlier than those produced
565 on agar plates. Differences between the amplification of genomic DNA from vegetative
566 cells and from spores were also found, the second one showing higher Ct values. The
567 DNA of spores might be highly condensed due to SASP, which might limit the action of
568 Taq polymerase during the amplification reaction. This problem does not occur with the
569 vegetative cells, because their DNA is more accessible.

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576 **7. Bibliography**

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AUTHOR DECLARATION TEMPLATE

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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Author	**Affiliation**	**Date**	**Signature**
Miriam Esteban	Universidad de Zaragoza, Zaragoza, Spain	1/08/2019	
Pilar Marcos	Universidad de Zaragoza, Zaragoza, Spain/	1/08/2019	

Cristina Horna	Universidad de Zaragoza, Zaragoza, Spain ZEULAB	1/08/2019	
Patricia Galán	Universidad de Zaragoza-CITA, Zaragoza, Spain	1/08/2019	
Luis Mata	Universidad de Zaragoza-CITA, Zaragoza, Spain	1/08/2019	
María Dolores Pérez	Universidad de Zaragoza-CITA, Zaragoza, Spain	1/08/2019	
Miguel Calvo	Universidad de Zaragoza-CITA, Zaragoza, Spain	1/08/2019	
Lourdes Sánchez	Universidad de Zaragoza-CITA, Zaragoza, Spain	1/08/2019	María Lourdes Sánchez Paniagua Firmado digitalmente por María Lourdes Sánchez Paniagua Nombre de reconocimiento (DN): cn=María Lourdes Sánchez Paniagua, o=Universidad de Zaragoza, ou=Departamento de Producción Animal y Ciencia de los Alimentos, email=lousanchez@unizar.es, c=ES Fecha: 2019.07.29 11:02:35 +02'00'

AUTHOR STATEMENT

On behalf of all the authors I wish to inform about their contribution to this publication.

Esteban, M.: Investigation; Methodology; Writing – review & editing

Marcos, P.: Investigation; Methodology

Horna, C.: Investigation; Methodology

Galan-Malo, P.: Conceptualization; Investigation; Methodology; Writing – review & editing

Mata, L.: Conceptualization; Investigation; Methodology; Writing – review & editing

Pérez, M.D.: Conceptualization; Formal analysis; Writing – review & editing

Calvo, M.: Conceptualization; Formal analysis

Sánchez, L.: Funding acquisition¸ Supervision¸ Conceptualization; Investigation; Methodology; Writing – review & editing

Lourdes Sánchez	Universidad Zaragoza-CITA, Zaragoza, Spain	15th December 2019	María Lourdes Sánchez Paniagua	Firmado digitalmente por María Lourdes Sánchez Paniagua Nombre de reconocimiento (DN): cn=María Lourdes Sánchez Paniagua, o=Universidad de Zaragoza, ou=Departamento de Producción Animal y Ciencia de los Alimentos, email=lousanchez@unizar.es, c=ES Fecha: 2019.07.29 11:02:35 +02'00'

SUPPLEMENTARY MATERIAL

1. Calibration curve for real time PCR with *CTfla* primers.

The calibration curve for the real time PCR with *CTfla* primers was done with 10-fold dilutions of DNA from vegetative cells of *C. tyrobutyricum* CECT 4012.

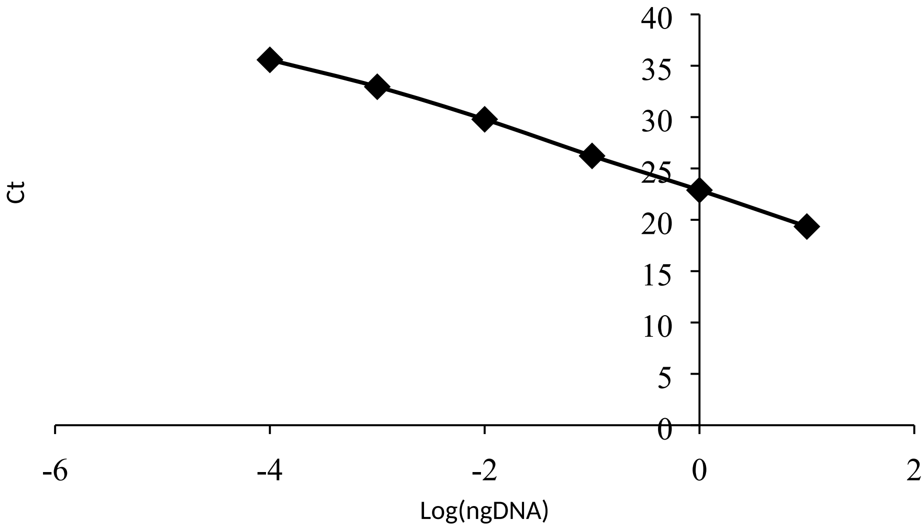

Figure 1. Calibration curve for *CTfla* primers with genomic DNA from from *C. tyrobutyricum* vegetative cells CECT 4012.

Table 1. Determination of the quantification and limit of detection of real time PCR for genomic DNA obtained from *C. tyrobutyricum* vegetative cells CECT 4012.

Log ngDNA	*Approx. Genome eq. calculated*	*Ct mean*	*SD*	*Percentage of positive reactions*
1	3.03E+06	19.34	0.24	6/6
0	3.00E+05	22.88	0.12	6/6
-1	3.00E+04	26.22	0.15	6/6
-2	3.00E+03	29.78	0.18	6/6
-3	3.00E+02	32.95	0.12	6/6
-4	3.00E+01	35.57	0.53	6/6
-5	3.00E+00	35.93	0.72	6/6
-6	1.00E+00	37.41	0.95	6/6
-7	Less than 1	38.52	1.29	4/8

$y= -3.2834x + 22.869 \quad R^2= 0.9978$

Each dilution of genomic DNA was analyzed by duplicate in real time PCR in three different experiments in different days (n=6). The lowest concentration of DNA was

repeated one more time in order to find the detection limit. The genome equivalents were calculated approximately with the following formula where x is the nanograms of DNA and N is the length of the genomic DNA:

$$\text{number of copies (molecules)} = \frac{X \text{ ng} * 6.0221 \times 10^{23} \text{ molecules/mole}}{(N * 660 \text{ g/mole})^{\dagger} * 1 \times 10^{9} \text{ ng/g}}$$

Figure 2. Genome equivalents calculation from Calculations: Converting from nanograms to copy number, https://eu.idtdna.com/pages/education/decoded/article/calculations-converting-from-nanograms-to-copy-number

For the genomic length we used the *Clostridium tyrobutyricum* strain W428 chromosome, complete genome (3,011,209 bp circular DNA). GenBank accession number: CP016280.1.

According to "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR experiments", the efficiency of real time PCR was set up in 98.79%. Robust and precise qPCR assays are usually correlated with high PCR efficiency (Bustin et al., 2009) and efficiency should be 90-105% (Kralik and Ricchi, 2017). The linear dynamic range was established between 10 total nanograms of DNA and 10^{-4} total nanograms of DNA (30 genome equivalents). The sensitivity is expressed as the Limit of Detection (LOD) (Bustin et al., 2009) was established in 10^{-6} total nanograms of DNA or approximately 1 copy number of genomic DNA.

The specificity of the qPCR is given by the specificity of the primers, and those were checked previously by López-Enríquez et al. (2007). In that study they tested different clostridial species and, other no clostridial bacteria, the primers were found specific for *C. tyrobutyricum* (see supplementary material of the published article). Moreover, we have checked also by an in-silico study in NCBI primer-BLAST, only one sequence was found with a length of 82bp and targeting *C. tyrobutyricum* (GenBank code: AJ242662). The melting curve was analyzed in each experiment and only one peak with Tm = 76.61 ± 0.10 was obtained.

The repeatability (intra-assay variation) was calculated as the CV (Coefficient of Variation) between the replicates for each sample. The CV was set up using the following formula CV = (SD*100)/ X as SD the standard deviation and X the mean Ct

obtained as CV≤ 5.8%. Repeatability must be CV≤25% (Broeders et al., 2014; Kralik and Ricchi, 2017)

The reproducibility (inter-assay variation) was calculated as the CV between runs with the following formula CV = (SD*100)/ X as SD the standard deviation and X the mean Ct obtained for each dilution. The CV obtained was CV≤ 1.49%. Reproducibility must be CV≤25% (Broeders et al., 2014; Kralik and Ricchi, 2017).

2. Spores recovery from milk treated with subtilisin and untreated.

The efficiency of the spore recovery with the subtilisin treatment was analysed by colony counting. With this objective, 18 mL of UHT whole milk were spiked with 10^6 total spores or 2.22E+05 spores/ml. Two bottles of contaminated UHT milk were treated with subtilisin (18 ml of subtilisin and buffer) and other two bottles were set up as the control without subtilisin. The bottles containing the subtilisin were incubated for 1 hour at 60ºC followed by a centrifugation of 13000 g. The control bottles were subjected to the same conditions.

After the centrifugation, the fat layer and the pellet were visible. The fat layer was recovered and resuspended in 10 mL of PBS as the pellet obtained. For the pellets a dilution in PBS was needed to count the colonies (10^{-3} dilution). For the fat layer of the untreated milk a 10^{-2} dilution was done and the fat layer of the subtilisin treated milk was cultured directly. For each dilution or condition, 100 µl of suspension was spread on RCM agar plates. After 24 h of incubation in anaerobic conditions at 37ºC the colonies were counted. The results obtained are the following:

Table 2. Results obtained from the separation of 10^6 total spores in UHT milk treated with subtilisin and untreated. The experiment was done by duplicate in three different days n=6. P value 0.05

Milk	Percentage of spores retained in fat layer (%)	Spores in the fat layer (CFU/mL)
Treated with subtilisin	0.053 ± 0.089	5.15E+02 ± 5.77E+02
Non treated	5.771 ± 6.481	6.25E+04 ± 4.37E+04

As it is shown in the results, a high number of CFU/ml were found in the fat layer of milk untreated compared with the milk digested with subtilisin. Although the standard deviation was high, significant differences were found for the number of spores retained

between the two cases. However, the percentage of spores retained in the fat layer was not found significant due to the variation between the experiments.

3. Bibliography.

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