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Detection of butyric spores by different approaches in raw milks from cow, ewe and goat

M. Esteban [a], C. Díaz [a], J.P. Navarro [a], M.D. Pérez [a], M. Calvo [a], L. Mata [b], P. Galán-Malo [b], L. Sánchez [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 S.L. Bari 25 dpdo, 50197, Zaragoza, Spain

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ABSTRACT

Clostridium tyrobutyricum is described as the main causative agent of late blowing defect in cheese. Currently, there are no fast methods to detect this microorganism in raw milk, which would allow determining the use of milk for fresh or cured cheese. The technique commonly used is the Most Probable Number, which is laborious and non-specific. In this work, we present the optimization of a real-time PCR-based detection method for *C. tyrobutyricum* spores in raw milk samples. This novel approach extracts DNA in a semi-automatic system with magnetic beads. The applicability of the developed procedure has been tested in field milk samples from cow, ewe and goat (n = 202), allowing detection of low levels of butyric spores. Raw milk samples were also analyzed by microbiological culture in a selective medium for butyric bacteria, and positive colonies were identified by multiplex PCR and 16S rDNA sequencing. Apart from *C. tyrobutyricum*, other *Clostridium* spp. were identified, which should be considered for further development of detection methods.

1. Introduction

Late blowing defect (LBD) of cheese is caused by butyric fermentation and affects hard and semi-hard cheeses. Butyric acid bacteria (BAB) belonging to the genus *Clostridium* are responsible for LBD due to the germination of their spores and consequent growth of vegetative cells during cheese maturation, producing cavities, cracks and changes in flavor and taste, making affected cheeses unsuitable for commercialization.

Clostridium is a genus of rod-shaped, Gram-positive bacteria endospore-forming. Clostridia included in the BAB group produce butyric acid and are strictly anaerobic. *Clostridium* bacteria start the sporulation process to survive in life-threatening conditions and when they are suitable for growth, the germination of spores is activated to produce vegetative cells (Hutchison et al., 2014). The spores of BAB present in milk normally come from silage and can survive heat treatments applied to milk before cheese manufacturing. Implementing appropriate hygienic actions, such as udder cleaning, adequate milking routine, and good quality silages, is decisive in avoiding milk contamination with spores (Arias et al., 2013).

Several *Clostridium* species are implicated in LBD, though *C. tyrobutyricum* has been reported as the primary causative agent (Bassi et al., 2015; Morandi et al., 2021; Turchi et al., 2016). It has been demonstrated that there is significant heterogeneity in the rate of spore germination and gas production among different strains of the same species, which should be considered when assessing the potential risk of LBD (Podrzaj et al., 2020). The other BAB species that could contribute to LBD are *C. sporogenes*, *C. butyricum, C. beijerinckii, C. perfringens* and *C. tertium* (Feligini et al., 2014; Le Bourhis et al., 2007; Reindl et al., 2014). More research is needed about the prevalence of *C. tyrobutyricum* and other species in milk, also considering that several factors can determine the development of some species over others, such as seasonality, treatment of silage, and geographical location of dairy cattle (Calamari et al., 2018; Feligini et al., 2014).

Abbreviations: LBD, Late Blowing Defect; BAB, Butyric Acid Bacteria; MPN, Most Probable Number; qPCR, Real-time PCR; CECT, Colección Española de Cultivos Tipo; RCM, Reinforced Clostridium Media; TGE, Tryptone-Glucose-yeast Extract; PBS, Phosphate Buffered Saline; UDG, Uracyl DNA-Glycosylase; Ct, Cycle threshold; LOD, Limit of Detection; TBE, Tris-Borate-EDTA.

* Corresponding author. Departamento de Producción Animal y Ciencia de los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón (IA2), Universidad de Zaragoza-CITA, Miguel Servet 177, 50013, Zaragoza, Spain.
E-mail address: lousanchez@unizar.es (L. Sánchez).

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At present, there is no fast method for detecting *Clostridium* spores and vegetative cells in raw milk to prevent LBD. The routine method commonly used is the Most Probable Number (MPN), which consists of culturing several dilutions of milk samples, previously pasteurized (75 °C 10 min) to inactivate vegetative cells, in a specific liquid medium. Later, the samples are incubated at 37 °C for several days (3–10) with a paraffin or agar layer on top of culture tubes to create anaerobiosis. Samples are considered positive when paraffin or agar is detached from the surface of the medium by gases produced in the butyric fermentation. Finally, spore enumeration is based on the dilutions and MPN tables (Bergère & Sivelä, 1990). MPN is not an official method and is laborious and non-specific, as it can identify milk samples as positive due to the presence of other sporulated bacteria different from butyric clostridia. Although other microbiological methods were developed, such as culture on selective media for *Clostridium* bacteria (Jonsson, 1990), several days are also required for spore germination and bacterial growth. Recently, a new microbiological method has been developed by Brändle et al. (2018) that included a novel and patented medium named AmpMedia666 for *Clostridium* spore germination and growth. This method is based on the medium color change and the results can be achieved in 48 h. The concentration range of this method was set up between 75 and 59,000 spores/L, detecting *Clostridium* spores with high selectivity. Although this novel approach is faster than those previously developed, it does not allow making a rapid decision about the way to process milk for cheese manufacture. The method of Brändle et al. (2018) was used to analyze the Alpine milk quality used to make Austrian cheese. However, no correlation was found between spore concentration and LBD because of the low levels of *Clostridium* spores found, which were below or near the limit of detection (75 spores/mL) of the method (Burtscher et al., 2020).

Real-time PCR (qPCR) has emerged in food microbiology as a powerful tool for identifying and enumerating food pathogens because is more specific, accurate and faster than the common microbiological methods (Martínez et al., 2011). Several approaches based on PCR or qPCR have been developed for detecting *Clostridium* spores and vegetative cells. In the case of *Clostridium* spores detection in milk, previous steps must be performed, such as sample clarification and efficient breakage of spores for DNA release (Esteban et al., 2020), considering the high resistance of bacterial spores, as has been reported for other bacterial species (Torok, 2003). The methods proposed by López-Enríquez et al. (2007), Bassi et al. (2013) and Arnaboldi et al. (2021) described DNA extraction and calibration of qPCR for quantification of *C. tyrobutyricum* spores in raw milk. Furthermore, Morandi et al. (2015) developed a triplex qPCR for the simultaneous detection of *C. tyrobutyricum*, *C. sporogenes* and *C. beijerinckii* spores in raw milk samples, and recently, the development of a triplex qPCR has been published for the detection of *C. butyricum*, *C. tyrobutyricum* and *C. sporogenes* in cheese (Şahiner et al., 2022). However, in many of these studies, the extraction of DNA had several incubation steps that made total processing very long, and the qPCR was only applied to the analysis of artificially contaminated milk samples. Nowadays, as far as we know, only two studies using field samples have been published (Arnaboldi et al., 2021; Bassi et al., 2013).

In this work, we present a novel method for extracting DNA from *C. tyrobutyricum* spores based on the application of bead beating followed by affine magnetic bead separation and qPCR detection. This method has been applied to measure the levels of *C. tyrobutyricum* spores in cow, ewe and goat raw milk samples from three Spanish dairy interprofessional laboratories. Moreover, the presence of other species of *Clostridium* and other bacteria has been analyzed by microbiological culture.

2. Materials and methods

2.1. Sporulation process and spore purification

The sporulation process was performed as described by Bassi et al. (2013) for *C. tyrobutyricum* CECT 4012T (Colección Española de Cultivos Tipo, Valencia, Spain) type strain and UZ01 wild strain isolated from contaminated field bovine milk. First, an inoculum of *C. tyrobutyricum* vegetative cells was grown in 9 mL RCM (Reinforced Clostridium Media) (Scharlau, Barcelona, Spain) in a tube sealed by a paraffin layer to create anaerobiosis. After 48 h of incubation at 37 °C, the culture was transferred into the sporulation system. The device was composed of a 500 mL flask bottle with 400 mL TGE (Tryptone-Glucose-yeast Extract) medium (Lavilla, 2008) for the culture of CECT 4012 T strain and RCM for UZ01 strain, and a dialysis membrane of 12–14 kDa molecular weight cut-off and 25 cm length Cellu Sep Membrane (Membrane Filtration Products, Seguin, USA) with 20 mL of TGE/RCM medium. The preculture was added to the sterilized dialysis membrane of the sporulation system and was incubated for up to 1 month at 37 °C.

Afterward, the culture was recovered and washed twice with sterile distilled water, followed by centrifugation at 4,000×*g* for 10 min at 4 °C. The precipitate containing spores and vegetative cells was incubated with lysozyme (Sigma Aldrich, St Luis, MO, USA) at a final concentration of 0.4 mg/mL for 24 h at 45 °C. After the lysis of vegetative cells, spores were separated from cellular debris with a Percoll® (Sigma Aldrich) gradient, as described before (Lavilla et al., 2010). Spores were collected from the bottom of the gradient, washed five times with distilled water and centrifuged at 13,000×*g* for 10 min.

Finally, spores were resuspended in 200 μL of phosphate buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4, and enumerated using a Thoma chamber under an optical microscope. The stock was kept at −20 °C.

2.2. Enzymatic treatment of milk and spore recovery

Raw cow milk aliquots of 1 mL were spiked with different concentrations of *C. tyrobutyricum* spores to achieve suspensions from 10^1 to 10^6 spores/mL. Serial dilutions were made from a 10^8 spores/mL suspension to minimize the sub-sampling error and milk was finally spiked with 100 μL of each dilution to reach the target concentration. Three DNA independent extractions were performed in three different days from 10^1 to 10^6 spores/mL suspensions in 1 mL raw milk and analyzed separately by qPCR. For milk digestion, a solution containing subtilisin was used as previously described (Esteban et al., 2020). Subtilisin was dissolved in a specific buffer, containing a detergent, at 1:28 (v/v) dilution. Both reagents are used routinely in the analysis of total bacterial count in milk by Bactoscan (Foss, Hilleroad, Denmark) and the exact composition is not disclosed by the company. The subtilisin solution was added to milk in equal volumes (1:1). Then, the mixture was incubated at 60 °C in a water bath for 30 min and centrifuged at 13,000×*g* for 30 min to obtain the spores in the precipitate.

2.3. Disruption of spores and DNA extraction

Disruption of the spores recovered from the precipitate, to extract and purify DNA was performed with MagMax™ total nucleic acid isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The precipitate obtained after milk digestion was dissolved in 100 μL of PBS and subjected to bead beating for 1 min at 6,500 rpm in a Precellys 24 homogenizer (Bertin Technologies SAS, San Quentin Yvelines Cedex, France) using the bead tubes provided by the kit.

Afterward, the mixture was centrifuged at 13,000×*g* for 5 min. The supernatant containing the released DNA was recovered and processed in the KingFisher Duo Prime system (Thermo Fisher Scientific), using a special 96-well plastic plate. Briefly, the DNA solution obtained from the

lysis step (115 μL) was mixed with 65 μL of molecular biology grade isopropanol (Fisher Bioreagents™, Thermo Fisher Scientific) and 20 μL of bead mix (MagMax™ total nucleic acid isolation kit, Thermo Fisher Scientific) containing magnetic beads, and the mixture was added to wells. Then, the washing solutions were added to the specific rows and finally, the elution strip was filled with 90 μL of the elution solution.

For the automatic extraction of DNA, the MagMax pathogen DNA/RNA protocol was applied for 25 min. After extraction, DNA was recovered from the elution strip and centrifuged at 13,000×*g* for 5 min to remove the traces of magnetic beads used for DNA purification.

2.4. Raw milk samples

Raw milk samples from cow, sheep and goat were collected from the Asociación Interprofesional Lechera de Aragón (Movera, Spain), the Instituto Lactológico de Lekunberri (Lekunberri, Spain) and the Associació Interprofessional Lletera de Catalunya (Cambrils, Spain), respectively.

Samples were subjected to heat treatment at 80 °C for 10 min to inactivate bacterial vegetative cells. Then, samples were processed as described in sections 2.2 and 2.3 and analyzed by qPCR.

2.5. Quantitative real-time PCR (qPCR)

The samples containing spore DNA to be analyzed by qPCR were prepared by mixing 12.5 μL of Master Mix SYBR Green® Power Up (Applied Biosystems, Waltham, MA, USA), 1.5 μL of each forward and reverse primer and 7.5 μL of RNase/DNase free water (Invitrogen, Paisley, UK). The primers used in this study (*fla* primers) are designed for detecting *C. tyrobutyricum* and were published by López-Enríquez et al. (2007).

The qPCR steps were the following: an initial stage of 50 °C/2 min to activate the uracil DNA-glycosylase (UDG), an initial denaturation at 95 °C/10 min, 45 cycles of amplification with denaturation at 95 °C/15 s, hybridization at 60 °C and elongation at 60 °C/1 min. Each sample was tested in duplicate. The threshold cycle value (Ct), which is inversely proportional to the number of DNA copies, was obtained from the amplification curve. Samples were considered negative when Ct values were >40 according to recognized guidelines and previous studies (Burns & Valdivia, 2008; Bustin et al., 2009).

Raw milk samples that failed to amplify one duplicate in the first qPCR run were analyzed in a second qPCR by duplicate. When at least one duplicate of the second run was amplified, the sample was considered positive for *C. tyrobutyricum* (Ahmed et al., 2009).

The efficiency of qPCR for each condition was calculated according to the following formula: $E = -1 + 10^{-1/slope}$. The performance of qPCR was tested previously to detect *C. tyrobutyricum* spores in milk samples (see supplementary material).

The limit of detection (LOD) was set up according to the MIQE guidelines for qPCR (Bustin et al., 2009), as the concentration that can be detected with reasonable certainty (95% probability is commonly accepted).

2.6. Detection of butyric spores by microbiological culture

For detecting butyric spores in raw milk samples using microbiological analysis, a protocol based on the method described by Jonsson (1990) was applied. All milk samples analyzed by qPCR (being positive or negative) were cultured on a selective medium to verify the presence of *C. tyrobutyricum* spores. For milk enrichment, 500 μL of raw milk, previously heated at 80 °C for 10 min to inactivate vegetative cells, was added to 9 mL of RCM and incubated in anaerobiosis at 37 °C for 48 h.

Afterward, the enriched milk was subcultured on agar plates by spreading 100 μL of culture using the streaking technique. RCM agar was supplemented with D-cycloserine (Acros Organics, NJ, USA) at a final concentration of 200 μg/mL to avoid the growth of facultative anaerobe bacteria, such as those of the *Bacillus* genus. Neutral red (Sigma-Aldrich) was also added to the culture medium at a final concentration of 50 μg/mL as pH indicator.

Agar plates were incubated for 4 days at 37 °C in Gaspak jars (Anaerocult, Merck Millipore, Burlington, MA, USA) adding an AnaeroGen sachet (Thermo Fisher Scientific) to create anaerobiosis. Yellow brilliant colonies under UV-lamp were considered positive for butyric bacteria.

2.7. Multiplex PCR and 16S Sanger sequencing of colonies

Some positive colonies (brilliant yellow) grown in RCM selective media were amplified by multiplex PCR, as described before (Cremonesi et al., 2012), to identify which of them were butyric bacteria. Three positive colonies were collected and resuspended in 50 μL of sterile distilled water. For cell disruption, the samples were boiled for 15 min and then, they were subjected to −20 °C for 5 min. Later, samples were centrifuged at 10,000×*g* for 5 min to obtain a clean soluble phase with DNA, which was used directly for PCR. The primer sequences used for multiplex PCR are described in Table 1.

Samples for multiplex PCR were prepared by mixing 12.5 μL of PCR Master Mix 2× (Promega Biotech Ibérica, Madrid, Spain) with 0.5 μL of forward and reverse primers to detect four different species (*C. tyrobutyricum, C. butyricum, C. beijerinckii* and *C. sporogenes*) and 2 μL of DNA, completing the volume with 6.5 μL of DNase free water (Invitrogen) to 25 μL as final reaction volume.

The colonies that were negative by multiplex PCR were analyzed by 16S PCR and rDNA sequencing. Based on the results obtained, the 16S rDNA sequencing was the method selected to identify positive colonies found in the selective media. For this purpose, a standard PCR was performed using primers targeting the 16S ribosomal RNA following the protocol described by Guerrieri et al. (2020). The PCR products were identified by Sanger sequencing at the University of Zaragoza Sequencing Service (Spain).

For amplicon visualization, a 3% agarose gel for multiplex PCR and 1.5% for 16S PCR in TBE 1× (Tris-Borate-EDTA buffer) were run and stained with SYBR Safe (Invitrogen). A DNA base pair marker (New England Biolabs, Ipswich, MA, USA) from 100 to 1000 bp was added. The gels were visualized under a UV lamp or transilluminator.

2.8. Data analysis

The Ct values were obtained using the qPCR software Step One™ 2.3. version (Life Technologies, Carlsbad, CA, USA). The mean values and standard deviations of Ct values were calculated using Microsoft® Excel version 16.44 (Microsoft Spain, Madrid, Spain).

16S ribosomal DNA sequence analysis was performed and results were exported to FASTA file with Chromas version 2.6.6 Technelysium Pty Ltd (South Brisbane, Australia). Sequences were aligned with BLAST (NCBI database).

3. Results and discussion

3.1. Real-time PCR calibration

The main problem associated with the detection of butyric spores by qPCR in milk is to be able to extract pure DNA from them. First, it is necessary to release spores from milk components; in our study, using a solution containing a detergent and a protease was very effective for that purpose. Furthermore, we used an approach not applied before for butyric spores for DNA extraction and purification, which consisted of breaking them by bead beating and isolating DNA by magnetic beads. On this basis, the main objective of our study was to validate a qPCR method to detect *C. tyrobutyricum* spores that included a novel method to extract DNA.

qPCR efficiency was calculated from the calibration curves obtained

Table 1
Pair of primers used for the qPCR and the identification of *Clostridium* species by multiplex PCR and for 16S rDNA sequencing. Fla: flagellin, ColA: collagenase, nifH: nitrogenase iron protein, hydA: hydrogenase, enr: 2-eonato reductase. [μM]: final primer concentration.

Species	Primer sequence	Amplicon length	Target gene	[μM]
C. tyrobutyricum	Fw: 5′-CAGTTACAATTACGAGAACACATGGA-3′ Rv: 5′-TGTACCACCAACTAAAGCAACATCA-3′	83 bp	*fla*	60
C. sporogenes	Fw: 5′-TTGGGATTTTGGGGATAACA-3′ Rv: 5′-TCCGTATCGTTGTCGTCTTG-3′	549 bp	*colA*	30
C. beijerinckii	Fw: 5′- TGACACGATTTTTCATTCTCCA-3′ Rv: 5′- TCCATTGCCTTAATGACAGGT-3′	448 bp	*nifH*	20
C. butyricum	Fw: 5′- ATGGGTTAGGCAAGCAGAAA-3′ Rv: 5′- GCTGGATCTGCCTTCTCATC-3′	312 bp	*hydA*	15
C. tyrobutyricum	Fw: 5′- TGGTGTTCCACAAGAAGCTG-3′ Rv: 5′-GCAGCTGGATTTACTGCACA-3′	210 bp	*enr*	15
16S rDNA Bacterial genus	Fw: 5′-GCGGCGTGCCTAATACATGC-3′ Rv: 5′-CTACGGCTACCTTGTTACGA-3′	1000 bp	*16 S ribosome*	20

for CECT 4012T and UZ01. For CECT 4012T the efficiency was 93.12%, as shown in Table 2. It is considered that good values for qPCR efficiency should be in the range of 90–100% (Bustin et al., 2009). The LOD achieved for this strain was set up in 10^4 spores/mL. The calibration curve initially prepared for *C. tyrobutyricum* strain type CECT 4012 was also compared with that obtained with the wild strain UZ01 isolated from raw cow milk.

As shown in Fig. 1, lower Ct cycles were obtained for UZ01 compared with those for CECT 4012. The linear range for UZ01 was found from 10^2 to 10^6 spores/mL, while the range obtained for CECT 4012 was from 10^3 to 10^6 spores/mL. Furthermore, higher efficiency was obtained for UZ01 under the same conditions, exhibiting a value of 96.50% whereas for CECT 4012 the efficiency was 93.12%. Moreover, the LOD obtained for UZ01 was considerably lower, of 10^2 spores/mL instead of 10^4 spores/mL for CECT 4012. These results showed that the wild strain was detected more easily than the type one. The UZ01 calibration curve was used to quantify *C. tyrobutyricum* spores in naturally contaminated milk samples because lower LOD was achieved for it. However, with the results obtained in this study, we cannot explain the differences found between both strains. Even if more research is needed to establish the cause of such differences, it is clear that spore resistance might be a decisive factor, influencing spore breakage and DNA recovery.

Table 2
Detection and quantification of two strains of *C. tyrobutyricum* spores in raw milk treated with subtilisin. Ct: cycle threshold. R^2: regression coefficient. LOD: limit of detection SD: standard deviation. n = 6. The linear equation was calculated in the linear range.

Sample	Log (spores/mL)	Ct mean	SD	Signal ratio
Raw milk (subtilisin treated) CECT4012	1	37.2	–	1/6
	2	37.7	1.12	2/6
	3	36.9	0.77	5/6
	4	33.5	0.18	6/6
	5	30.0	0.16	6/6
	6	26.5	0.13	6/6
Linear range	10^4-10^6 spores/mL			
Linear equation CECT 4012	y = −3.49x+47.50 R^2 = 1 LOD 10^4 spores/mL qPCR efficiency 93.12%			
Raw milk (subtilisin treated) UZ01	1	34.3	3.38	4/6
	2	34.1	1.37	6/6
	3	31.1	2.06	6/6
	4	27.4	1.84	6/6
	5	23.8	1.74	6/6
	6	20.7	2.07	6/6
Linear range	10^2-10^6 spores/mL			
Linear equation UZ01	y = −3.40x+41.04. R^2 = 0.99 LOD 10^2 spores/mL qPCR efficiency 96.53%			

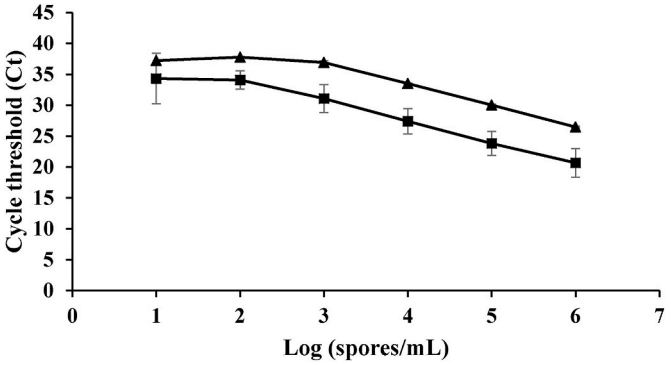

Fig. 1. qPCR calibration curves for amplification of *C. tyrobutyricum* in cow raw milk samples spiked with spores. Type strain CECT 4012 (▲) and UZ01 strain (■).

3.2. Analysis of raw milk samples from cow, ewe and goat by qPCR

Once the performance of the method previously described was evaluated according to recognized guidelines (see supplementary material), raw milk samples from cow, ewe and goat were analyzed to verify the presence of *C. tyrobutyricum* spores.

In this study, a total of 68 raw milk samples from cow, 86 from ewe and 48 from goat were analyzed by qPCR (n = 202). Raw milk samples from the three species were cultured in RCM selective media to compare the results with those obtained by qPCR.

As shown in Fig. 2, from the total number of milk samples analyzed

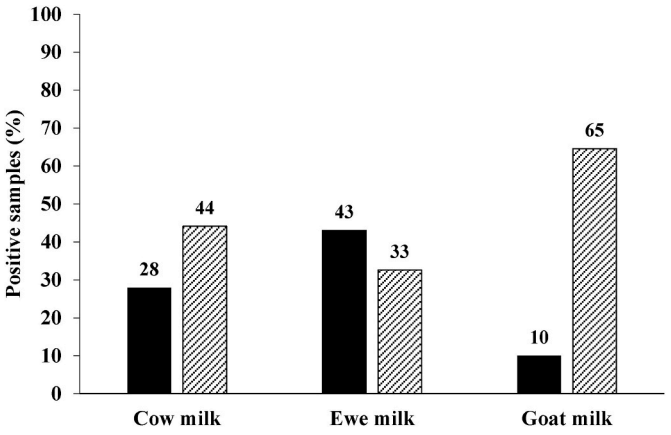

Fig. 2. Percentage of positive samples for *C. tyrobutyricum* by qPCR considering Ct < 40 (■) and positive by culture in RCM selective medium (▨). The raw milk samples analyzed were from cow (n = 68), ewe (n = 86) and goat (n = 48).

for each species, a percentage of 28% and 43% of the cow and ewe milk samples, respectively, were positive by qPCR, while only 10% goat milk samples were found positive. As has been previously indicated, samples were considered positive for *C. tyrobutyricum* by qPCR when Ct values were <40. The melting curve was obtained to determine the melting temperature of the selected amplicon and coincided for all samples. The concentration of spores in milk samples was estimated with the linear equation obtained in the qPCR calibration for UZ01 strain and the Ct value obtained for each sample. All the raw milk samples considered positive presented Ct cycles in the range of 33–39. For cow milk samples, the spore concentrations found were between 4.5 and 214 spores/mL. These results indicate that the levels of *C. tyrobutyricum* spores in some samples were lower than the LOD of 10^2 spores/mL obtained by the proposed method. Of the total qPCR positive cow samples, only 26% could be quantified because in the rest of the samples, the Ct cycles were out of the linear range. The mean concentration of *C. tyrobutyricum* spores in cow milk samples was 138 ± 74 spores/mL. These results are similar to those published by Bassi et al. (2013). In the mentioned work, samples from different points of the production chain, such as feces, raw milk, cheese, and curd, were analyzed by TaqMan qPCR. The levels of contamination of raw milk with *C. tyrobutyricum* spores and vegetative cells obtained by Bassi et al. (2013) were set in the range of 100–1000 CFU/mL, in the same order as that found in our study. However, the percentage of positive samples for *C. tyrobutyricum* spores found in our study is higher than that reported by Arnaboldi et al. (2021), which was 15.28% of the total samples analyzed and the levels of spores were in the range of 363–1508 spores/L.

A high number of positive samples for *C. tyrobutyricum* spores by qPCR were found in the ewe milks analyzed, with a value of 43%. When estimating the number of spores in the positive ewe samples, only 11% could be quantified correctly because the rest gave values below the LOD (<10^2 spores/mL). The mean concentration of spores in positive ewe samples was 3689 ± 3412, much higher than the values obtained in cow milk samples.

Of the goat milks analyzed, only 10% of samples were positive for *C. tyrobutyricum* spore contamination. From these positive samples, 80% could be quantified, showing levels of 986 ± 1161 spores/mL.

The reproducibility was found to be low in those milks that were amplified only in one duplicate. These milk samples were subjected to a second qPCR under the same conditions as those performed in the first run, and only 40% were finally confirmed as positive. We assume that a certain percentage of samples may not be detected by qPCR because they have spore levels below the LOD of the method and/or because butyric spores have high resistance to breakage, which can make the extraction of DNA more difficult.

The levels of *C. tyrobutyricum* spores found in our study are not very far from those reported in other studies using the MPN method. Thus, Driehuis et al. (2016) detected values between 0.04 and 25 spores/mL in cow milk and other authors found concentrations in the range of 0.24–240 spores/mL in ewe milk (Garde et al., 2011; Turchi et al., 2016). Those data come from studies performed in different countries, the Netherlands, Spain and Italy, respectively. Moreover, the variability in those levels can be due to differences in the type of animal feeding and hygienic practices.

Based on the results obtained in this study and the spore values reported for raw milk samples, the method proposed here may be considered qualitative, although the first approximation was conceived as quantitative. At low levels of spores, particularly for values <10^2 spores/mL, our approach can only discern between positive and negative samples. Even though the method is quantitative for artificially spiked milk with spores, in field samples it would be quantitative only for highly contaminated milk.

3.3. Analysis of raw milk samples cultured in RCM selective media

For the study of *Clostridium* spp. in raw milk samples, they were cultured in a selective medium after being subjected to heat treatment to inactivate vegetative cells, as described above. Raw milk samples were considered positive for butyric bacteria when yellow and brilliant colonies grew on RCM agar supplemented with D-cycloserine and neutral red, and were seen fluorescent under UV light. As previously indicated for qPCR analysis, the raw milk samples cultured in selective media were 68 from cow, 86 from ewe and 48 from goat (n = 202).

The results obtained from cow, ewe and goat milk samples cultured in RCM selective media are presented in Fig. 2. We found that 44% of cow milk samples were positive in the selective media showing yellow and fluorescent colonies, as these features have been described to be specific to clostridia (Jonsson, 1990). For ewe milk samples, the percentage of samples giving positive colonies was 33%, lower than that in cow milk. The percentage of goat milk samples with positive colonies was higher than that of the other species, being 65%.

The coincidence between the results obtained using both methods (microbiological culture and qPCR) was 14.70% in the case of cow milk samples, 7% for ewe and 8.3% for goat. This percentage was calculated based on the positive samples in both assays compared with the total number of samples analyzed (see supplementary material section 3). Thus, a substantial number of samples displayed different results depending on the type of analysis. It is important to note that the RCM agar with D-cycloserine and neutral red is selective for all *Clostridium* spp. This fact suggests that the high percentage of non-coincident results could be due to the growth of different *Clostridium* spp. in the culture media, which could not be detected by qPCR since the primers used were specific for *C. tyrobutyricum.* Similarly, Arnaboldi et al. (2021) described different positive rates for *C. tyrobutyricum* by qPCR and MPN, being 15.28% in the former and 85.41% in the latter. Therefore, our results indicate the presence of bacteria belonging to other genera in the milk samples analyzed.

Multiplex PCR and 16S rDNA sequencing were performed to elucidate whether bacteria from other genera than *Clostridium* can grow in the selective media and to understand better the results obtained.

3.4. Analysis of Clostridium spp. by multiplex PCR and 16S rDNA sequencing

To evaluate the presence of other *Clostridium* species and as a first approach, multiplex PCR was performed in 22 cow milk samples that were positive in the selective culture media and were also analyzed by qPCR. This multiplex PCR used was developed by Cremonesi et al. (2012) and four different species of clostridia were analyzed in the same PCR: *C. tyrobutyricum, C. sporogenes, C. beijerinckii* and *C. butyricum.*

The results obtained applying multiplex PCR showed 72% negative samples of the total. However, using this method we could identify *Clostridium* species in 28% samples analyzed, corresponding 14% to *C. tyrobutyricum* and 14% to *C. sporogenes* (Fig. 3). These results indicate that other species different from those identified in the multiplex PCR could be also present in milk samples and grow in the selective agar medium.

With the aim of being one step closer to identifying the species responsible for gas and butyric acid production, negative samples by multiplex PCR and positive samples by microbiological culture were subjected to a standard PCR with specific primers for the 16S rDNA gene. The sequencing of this gene allowed identifying the genus and species of the bacteria obtained from the colonies isolated in the selective medium. A total number of 62 raw milk samples (25 from cow, 11 from ewe and 26 from goat) of the total positive samples in RCM selective media were analyzed by 16S rDNA. The results at the genus level are shown in Fig. 4.

The 16S rDNA sequencing revealed three main genera in the colonies isolated from cow milk samples: *Clostridium, Paenibacillus* and *Lactobacillus*. In the colonies isolated from ewe and goat milks only *Clostridium* and *Lactobacillus* could be identified. As could be expected, *Clostridium* was found as the predominant genus in cow (48%) and ewe milks (55%), whereas *Lactobacillus* was identified in 88% of the analyzed colonies

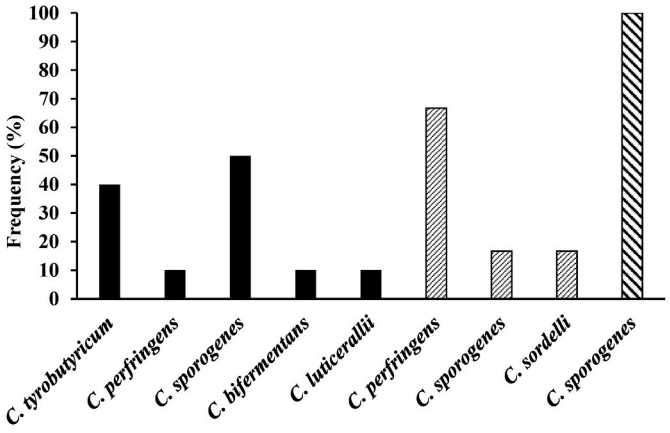

Fig. 3. Frequency and distribution of *Clostridium* spp. in colonies isolated from cow (■), ewe (▨) and goat (▧) milk samples cultured in RCM selective medium and identified by 16S rDNA sequencing and multiplex PCR. N = 20.

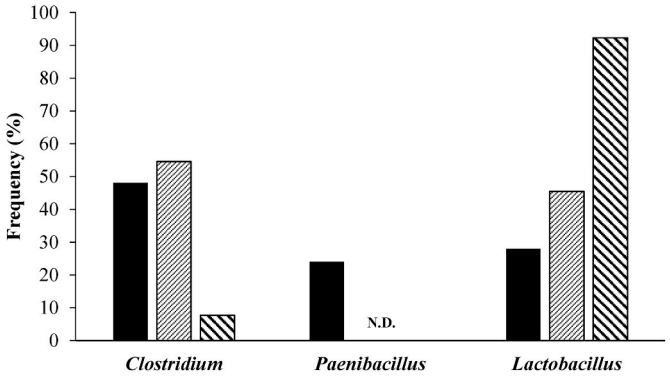

Fig. 4. Frequency and distribution of *Clostridium*, *Paenibacillus* and *Lactobacillus* found in cow (■), ewe (▨) and goat (▧) milk cultured in RCM selective medium and identified by 16S rDNA sequencing and multiplex PCR. N = 62. N.D.: not detected.

from goat milks. These results demonstrate that the agar medium normally used to isolate *Clostridium* spp. is not completely specific and other microorganisms can grow on it, probably avoiding the germination of *Clostridium* spores and the subsequent proliferation of vegetative cells. Moreover, it is important to remark that the colonies identified as *Lactobacillus* by 16S sequencing could be distinguished from the colonies of butyric bacteria because they were less brilliant under UV light and the color was orange-yellow (see supplementary material). This differential feature must be considered when the microbiological culture method is performed to identify *Clostridium* contamination in milk as a complementary method to PCR.

A significant percentage of the colonies isolated from cow milk samples (24%) were identified as *Paenibacillus,* suggesting that these bacteria are present in cow raw milk samples in a relevant proportion and grow in selective media for *Clostridium*. Two species of *Paenibacillus* were identified as *Paenibacillus macerans* and *Paenibacillus thermophilus*. *Paenibacillus* is a Gram-positive or Gram variable endospore-forming, and aerobic or facultative anaerobic bacteria (Sáez-Nieto et al., 2017). Because of being a sporulated bacterium, *Paenibacillus* can easily survive milk pasteurization, which explains its resistance to the heat treatment that was applied in this study to milk samples before culture.

Lactobacillus is a Gram-positive, non-spore forming, aero-tolerant or anaerobic bacteria. In our study, *Lactobacillus* was found to be the predominant bacteria in goat milk, constituting 92% of the total colonies that could be detected by 16S rDNA sequencing (n = 26) and the second predominant genus in cow milk (28%). Some authors have reported this microorganism as a resistant microorganism, surviving after ewe milk pasteurization (Salmerón et al., 2002). The identified *Lactobacillus* spp. were *Lactobacillus paracasei* (being the most frequent) and *Lactobacillus casei.* Other species isolated with lower frequency were *Lactobacillus rhamnosus, Lactobacillus parabuchneri*, *Lactobacillus gallinarum* and *Lactobacillus zeae.*

Based on the results obtained, the microbiological culture used in this study was found to be rather non-specific for butyric bacteria, because two other genera, *Paenibacillus* and *Lactobacillus*, were able to grow. Although this selective medium has been extensively used for butyric bacteria, other specific media should be considered as that recently developed by Brändle et al. (2018).

As shown in Fig. 3, several *Clostridium* spp. were identified from the colonies isolated in RCM supplemented with D-cycloserine and neutral red. The most predominant *Clostridium* species in bovine milk was *C. sporogenes* (50%) followed by *C. tyrobutyricum* (40%). Other published studies revealed that 58% (Bermúdez et al., 2016) and 78% (Brändle et al., 2018) of the colonies isolated from cow milk corresponded to *C. tyrobutyricum*, and lower percentages were obtained for *C. sporogenes* with a frequency of 17 and 11%, respectively. Other species found in our study were *C. perfringens*, *C. luticeralli* and *C. bifermentans*. These minor species were also found by other authors in cow milk (Bermúdez et al., 2016). In the ewe milk samples analyzed, the main species found was *C. perfringens* with a frequency of 67%; *C. sporogenes* and *C. sordellii* were also isolated but in a lower percentage. Our results are similar to those obtained by Turchi et al. (2016), who identified 56% of the isolated colonies as *C. perfringens* and 44% as *C. sporogenes.* Additionally, Arias et al. (2013) and Garde et al. (2011) found the relevant presence of *C. sporogenes* in ovine milk. However, *C. sordellii* was not found by other authors in a meaningful proportion in bovine, ovine or goat milk. In our study, only *C. sporogenes* was found in goat milk, while a high percentage of the colonies were identified as *Lactobacillus*.

C. sporogenes has been found in our study as the main species, independently of the type of milk, suggesting that this microorganism may have an important role in milk contamination and, consequently, in the development of LBD (Turchi et al., 2016). This result is in agreement with that reported by Arnaboldi et al. (2021), who found *C. sporogenes* as the predominant species in field milk samples analyzed using the MPN technique and multiplex PCR. In that study, positive samples for *C. tyrobutyricum* by qPCR were also positive for *C. sporogenes* by MPN and multiplex PCR. However, the second main species enumerated was *C. perfringens*, with a frequency of 25%. At present, the contribution of *C. perfringens* to LBD is unclear due to the lack of data on the germination and growth of this microorganism in cheese (Turchi et al., 2016). However, in general terms, *C. perfringens* is not considered a principal causative agent of LBD by many authors because its isolation has not been reported in cheese with this defect (Garde et al., 2011; Lycken & Borch, 2006).

In this study, *C. tyrobutyricum* was detected only in bovine milk samples and was the third main species, in terms of frequency, found in all the milks analyzed. This fact reveals that species of *Clostridium* present in milk used for cheese manufacture can differ depending on the geographical origin of milk, the type of milk analyzed and the feed used for lactating animals, as many authors previously reported (Brändle et al., 2018; Reindl et al., 2014; Turchi et al., 2016). This fact must be considered to develop a detection method based on qPCR. The presence of several *Clostridium* spp. in milk may condition the development of qPCR by selecting several primers or applying multiplex qPCR. A previous study to know the predominant species in the region of interest would be necessary to have optimal results in detecting butyric spores.

This study aimed to develop a fast and efficient method for detecting *C. tyrobutyricum* spores in raw milk samples. As described previously, raw milk was digested with a solution containing detergent and subtilisin, which facilitated the recovery of spores after centrifugation. The combination of bead beating with a semi-automatic method that extracts DNA with magnetic beads allowed purifying the DNA for qPCR. The

whole protocol described here allowed determining the levels of *C. tyrobutyricum* spores in 1 mL of raw milk in less than 5 h. As described previously, the processing time is critical to estimating *C. tyrobutyricum* spore levels in raw milk to determine the final milk destination. The first qPCR developed to detect *C. tyrobutyricum* spores in raw milk needed multiple enzymatic steps and a final purification to obtain pure DNA, which took at least 4 h besides the time for qPCR (López-Enríquez et al., 2007). The most recent study by Arnaboldi et al. (2021), in which *C. tyrobutyricum* spores are detected in raw milk samples extracts the DNA with an enzymatic protocol that also needs 4 h of incubation before qPCR. Although this last protocol was found successful in detecting and quantifying *C. tyrobutyricum* spores in field milk samples, in terms of processing time, the method we propose for DNA extraction is faster and semi-automatic, which means a clear improvement for future implementation in the analysis of a high number of samples. Moreover, ours is the first study to analyze *C. tyrobutyricum* contamination by qPCR in field raw milk samples of three dairy species (cow, ewe and goat) from three different geographical locations in Spain. Additionally, we have revealed the great differences in *C. tyrobutyricum* spore levels and bacterial species depending on the origin and type of milk.

Recently, a promising approach has been developed based on Loop-Mediated Isothermal Amplification (LAMP) (Cecere et al., 2021). In this study, 20 mL of raw milk sample was digested to recover spores, and DNA was extracted by heating. After DNA extraction, isothermal amplification was performed and the results were revealed by color change (naked eye). The entire protocol is simple and allows having results in around 3 h; however, it would be necessary to evaluate and validate this method in field raw milk samples.

Furthermore, in this study, several *Clostridium* species were also identified in milk samples using RCM as selective culture media and 16S rDNA sequencing to verify qPCR results. However, we have confirmed that it is very complicated to establish a correlation between methods, because there is no selective medium for isolating butyric bacteria. The 16S rDNA sequencing confirmed that other bacteria, such as *Lactobacillus* and *Paenibacillus*, can grow in RCM, and probably a similar situation may occur when analyzing milk samples by MPN.

4. Conclusions

The novel approach developed in this study to detect *Clostridium tyrobutyricum* spores is based on milk digestion, magnetic separation and purification of DNA followed by real-time PCR. This method has a LOD of 10^2 spores/mL for *C. tyrobutyricum* wild strain spiked raw milk samples. This advance considerably reduces the time required for the detecting and quantifying *C. tyrobutyricum* in less than 5 h, which is relatively fast-short compared with the microbiological methods normally used and those based on qPCR.

The analysis of raw milk samples of cow, ewe and goat has revealed that the concentration of *C. tyrobutyricum* is in the range of 10^2-10^3 spores/mL. This fact can be explained by the low levels of butyric spores in milk due to the good hygienic practices normally applied in dairy cattle and by the presence of other *Clostridium* spp. that could not be detected because only the specific primers for *C. tyrobutyricum* were used. The high LOD of qPCR could be a limitation of the method because low levels of spores in raw milk can lead to LBD.

The predominant species found in our study was *C. sporogenes,* identified in the milk of the three animal species analyzed, followed by *C. perfringens* and *C. tyrobutyricum*. The development of a multiplex qPCR designed to detect these three *Clostridium* species would increase the number of positive samples and would achieve more coincidence between the results of microbiological methods and those obtained by qPCR.

CRediT authorship contribution statement

M. Esteban: Writing – original draft, preparation, Methodology, Investigation, Data curation. **C. Díaz:** Methodology, Investigation, Software, Data curation. **J.P. Navarro:** Methodology, Investigation, Software, Data curation. **M.D. Pérez:** Writing – review & editing. **M. Calvo:** Writing – review & editing. **L. Mata:** Conceptualization, Methodology. **P. Galán-Malo:** Methodology, Investigation, Data curation, Writing – review & editing. **L. Sánchez:** Conceptualization, Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

On behalf of all the authors, I 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109298.

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