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Use of fluorescent CTP1L endolysin cell wall-binding domain to study the evolution of *Clostridium tyrobutyricum* during cheese ripening

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ABSTRACT

Clostridium tyrobutyricum is a bacteria of concern in the cheese industry, capable of surviving the manufacturing process and causing butyric acid fermentation and late blowing defect of cheese. In this work, we implement a method based on the cell wall-binding domain (CBD) of endolysin CTP1L, which detects *C. tyrobutyricum,* to monitor its evolution in cheeses challenged with clostridial spores and in the presence or absence of reuterin, an anti-clostridial agent. For this purpose, total bacteria were extracted from cheese samples and *C. tyrobutyricum* cells were specifically labelled with the CBD of CTP1L attached to green fluorescent protein (GFP), and detected by fluorescence microscopy. By using this GFP-CBD, germinated spores were visualized on day 1 in all cheeses inoculated with clostridial spores. Vegetative cells of *C. tyrobutyricum,* responsible for butyric acid fermentation, were detected in cheeses without reuterin from 30 d onwards, when LBD symptoms also became evident. The number of fluorescent *Clostridium* cells increased during ripening in the blowing cheeses. However, vegetative cells of *C. tyrobutyricum* were not detected in cheese containing the antimicrobial reuterin, which also did not show LBD throughout ripening. This simple and fast method provides a helpful tool to study the evolution of *C. tyrobutyricum* during cheese ripening.

1. Introduction

Clostridium tyrobutyricum, a gram-positive, anaerobic, spore-forming bacterium, is considered to be the principal cause of late blowing defect (LBD) of cheese, which generates severe economic losses for the cheese industry. This bacterium is capable of fermenting lactic acid with the production of butyric and acetic acids and gases such as carbon dioxide and hydrogen. The pressure of accumulated gases causes cracks and splits resulting in the appearance of texture and flavour defects during ripening, which are generally accompanied by unpleasant aromas and a rancid flavour (Garde et al., 2013). Recently, D'Incecco et al. (2018) suggested that, in addition to spores, vegetative cells may also represent a potential risk for the development of LBD in cheese, and highlighted the need to have low numbers of both vegetative cells and spores of *C. tyrobutyricum* in milk destined for hard cheese manufacturing, and also to have methods able to count both of them.

The detection and enumeration of vegetative cells of *Clostridium* from cheeses by plate counting remains a difficult task, mainly because vegetative forms of some clostridia species die rapidly after air/oxygen exposure despite any anaerobic measures taken during sample manipulation. Accordingly, traditional methods for detection of *Clostridium* in cheese are based on the determination of spore counts by heat treatment of cheese samples to destroy vegetative cells, followed by a "most probable number" enumeration, based on gas production after growing in an appropriate medium with lactate as a carbon and electron source, and incubating for a long period. As these techniques are laborious, time-consuming, and fail to differentiate among *Clostridium* spp. and even among spore-forming bacteria, methods based on PCR techniques have been proposed to detect dairy-related *Clostridium* spp. directly in cheese, without the need for strain isolation (Bassi et al., 2013, 2015; Cocolin et al., 2004; Klijn et al., 1995; Le Bourhis et al., 2005). More recently, Bassi et al. (2015) performed Illumina next generation sequencing of 16 S amplicons to investigate the community of clostridia involved in spoilage of Grana Padano cheeses suspected of blowing defects. However, DNA extraction methods used so far fail to distinguish between vegetative cells and spores, and may extract the free DNA released in the cheese paste during cell lysis, unless a propidium monoazide treatment was applied before DNA amplification (Erkus et al., 2016). Furthermore, these methods may have other difficulties such as a low DNA extraction efficiency due to the

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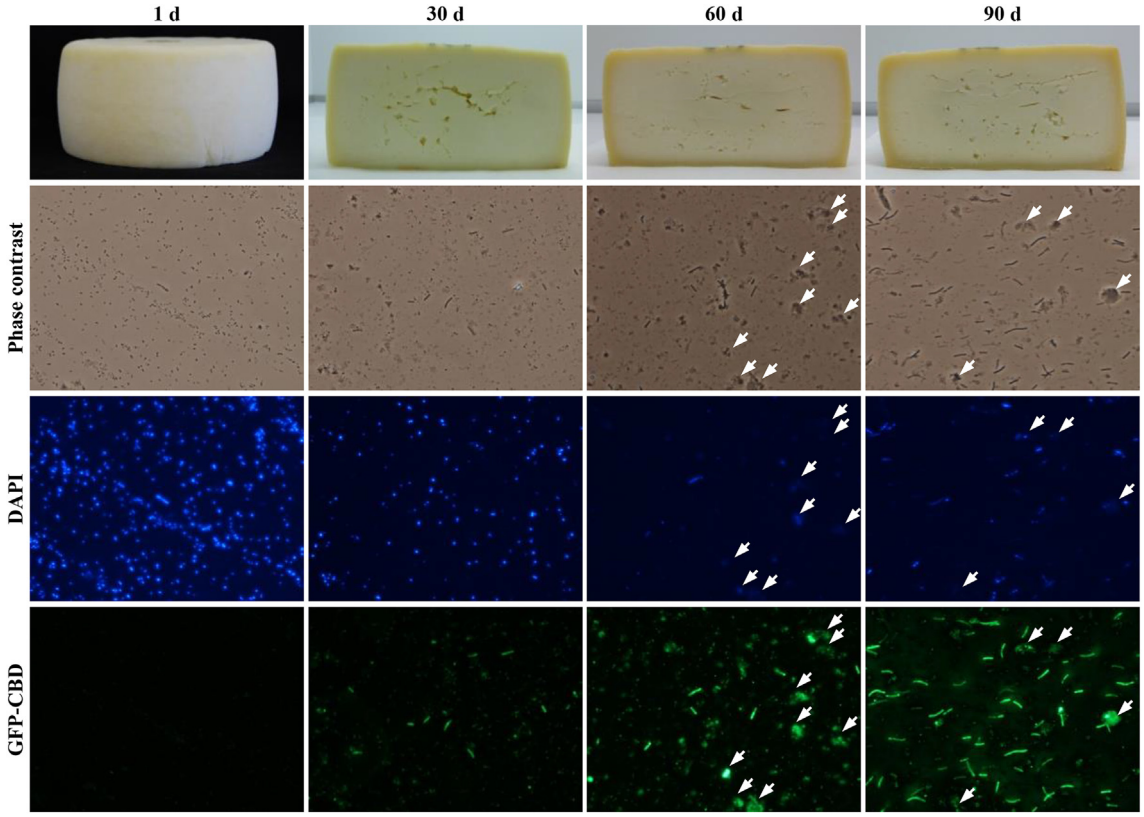

Fig. 1. Photographs of ewe milk cheese made with a mesophilic starter and spores of *Clostridium tyrobutyricum* INIA 68 showing late blowing defect from 30 d onwards, and evolution of cheese microbiota during ripening by phase contrast and fluorescence microscopy (blue fluorescence: DAPI staining of bacteria, green fluorescence: GFP-CBD labelling of *C. tyrobutyricum* cells). White arrows indicate fluorescent artefacts due to cheese residues/clostridial cell debris. Magnification x 400. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

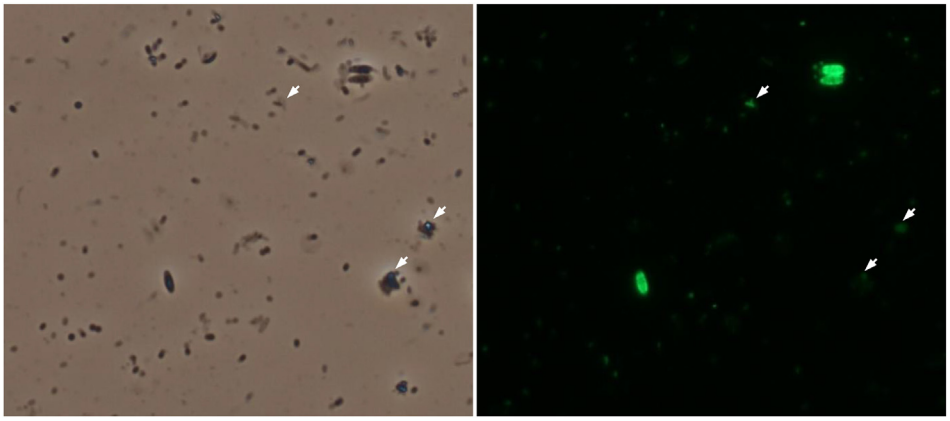

Fig. 2. Germinated spores of *Clostridium tyrobutyricum* in 1-day-old ewe milk cheese made with a mesophilic starter and spores of *C. tyrobutyricum* INIA 68 by phase contrast (left) and fluorescence microscopy (right: green fluorescence, GFP-CBD labelling of *C. tyrobutyricum* cells). White arrows indicate fluorescent artefacts due to cheese residues/clostridial cell debris. Magnification x 400. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interference of cheese components with chemical reagents necessary for DNA extraction, and/or a low PCR sensitivity as consequence of an insufficient elimination of these chemical reagents, many of which are PCR inhibitors (Bonaïti et al., 2006).

An innovative strategy to detect *Clostridium* spp. cells specifically has been studied by harnessing the properties of high-affinity cell wall-binding domains (CBDs) of bacteriophage endolysins (Gómez-Torres et al., 2018; Mayer et al., 2011). Endolysins are highly evolved enzymes encoded by bacteriophage genomes which are used to digest the bacterial cell wall from within at the terminal stage of the phage multiplication cycle (Loessner, 2005). They show a modular organisation of at least two distinct functional domains: an N-terminal enzymatically active domain, which catalyses bacterial cell wall break down, and a C-terminal CBD commonly responsible for the high specificity of the enzyme, targeting the protein to the ligands present in or on the bacterial cell wall (Pastagia et al., 2013). Numerous studies have demonstrated the potential of bacteriophage endolysins for the control and detection of food-borne pathogens and spoilage bacteria; however, very few studies actually investigate its use as detection system in food products (Schmelcher and Loessner, 2016). A CBD-based magnetic separation procedure has been evaluated for capture and detection of *Listeria monocytogenes* from artificially and naturally contaminated food samples (Kretzer et al., 2007; Schmerlher et al., 2010). Other techniques based on the high specificity and binding affinity of CBDs have been their use in enzyme linked immunosorbent assays for detecting *Staphylococcus aureus* in milk (Yu et al., 2015), and their use in surface plasmon resonance for detecting *Bacillus cereus* (Kong et al., 2015).

In a previous study, we described the ability of the CBD of the

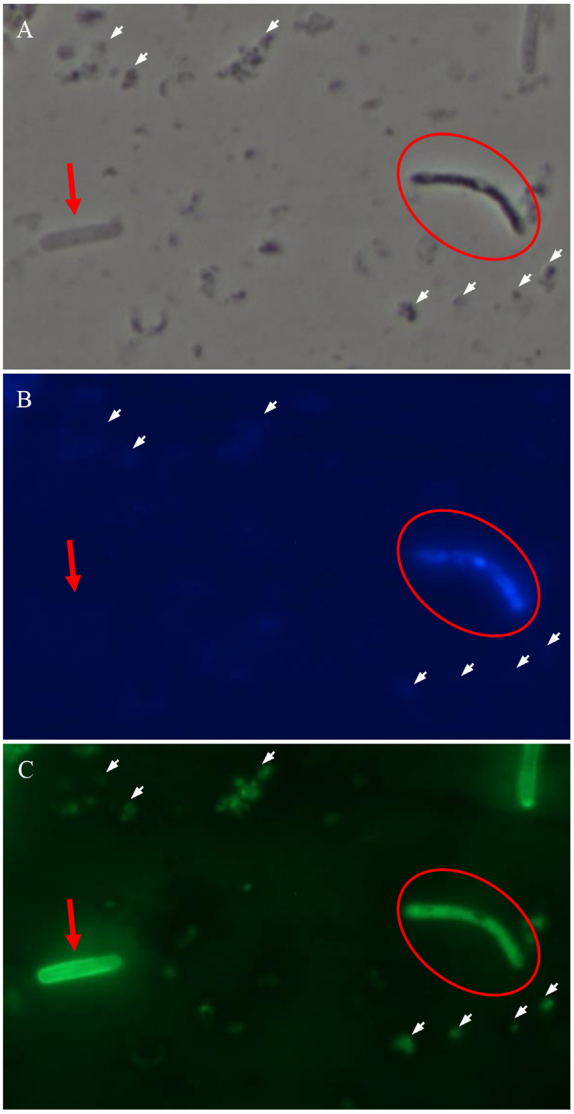

Fig. 3. Detail of *Clostridium tyrobutyricum* cells in 90-day-old ewe milk cheese made with a mesophilic starter and spores of *C. tyrobutyricum* INIA 68 by phase contrast (A) and fluorescence microscopy (B: blue fluorescence, DAPI staining of bacteria; C: green fluorescence, GFP-CBD labelling of *C. tyrobutyricum* cells). Red arrows point to a lysed cell of *C. tyrobutyricum* (not stained by DAPI) and red circles point to a partially lysed cell of *C. tyrobutyricum*. White arrows indicate fluorescent artefacts. Magnification x 1000. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CTP1L endolysin that targets *C. tyrobutyricum* (Mayer et al., 2010) attached to fluorescent green fluorescent protein (GFP-CBD) to bind to vegetative cells and spores of dairy-related *Clostridium* spp. (Gómez-Torres et al., 2018). In addition, *C. tyrobutyricum* cells were specifically labelled directly with GFP-CBD in the matrix of a LBD cheese. In this work, we have improved the labelling protocol and implemented the new protocol to monitor the evolution of *Clostridium* during ripening of a semi-hard ewe milk cheese made in the presence of spores of *C. tyrobutyricum* INIA 68, with or without reuterin as an anti-clostridial agent. Reuterin is an antimicrobial compound with activity against *Clostridium* species (Ávila et al., 2014; Garde et al., 2014), and it is produced in cheese by *Lactobacillus reuteri* INIA P572 in the presence of glycerol (Ávila et al., 2017).

2. Materials and methods

2.1. Cheese samples

Semi-hard Castellano cheeses were made as described previously by Ávila et al. (2017). Castellano cheese is an uncooked, pressed and ripened variety, produced in the Castilla y Leon region (Central Spain). Briefly, pasteurized ewe milk was distributed in four vats, each containing 50 L of milk. Commercial freeze-dried mesophilic lactic culture Choozit™ MA 11 from Danisco (Laboratorios Arroyo, Santander, Spain), consisting of *Lactococcus lactis* subsp. *lactis* and *cremoris* strains, was added to all vats (approximately 7 log cfu/mL milk). Spores of *C. tyrobutyricum* INIA 68 were inoculated in vats 2, 3 and 4 (approximately 5 log spores/mL milk) to cause cheese LBD, and *L. reuteri* INIA P572 was added at 0.1% (approximately 6 log cfu/mL milk) to vats 3 and 4. After 25 min of lactic cultures inoculation, rennet (18 mL/50 L milk, 1:15,000 strength; Laboratorios Arroyo) was added to all vats, and food-grade glycerol (> 99%, FCC, FG, Sigma-Aldrich) to vat 4 (30 mM final concentration) to promote reuterin synthesis by *L. reuteri*. The curds were cut 30 min after rennet addition into 6–8 mm cubes and scalded at 37–38 °C for 40–50 min. The whey was drained off and curds were distributed into cylindrical moulds. Cheeses were pressed at 1.5–2 atm until curd pH was 5.4, salted for 17 h at 12 °C in brine (190 g of NaCl/L), and ripened at 12 °C and 85% relative humidity for 90 d. One cheese of approximately 1 kg (for 1 d analyses) and three cheeses of approximately 3 kg (for 30, 60 and 90 d analyses) were obtained from each vat.

2.2. Extraction of cheese microbiota

In order to improve our previous protocol for detecting *Clostridium* into the cheese matrix by means of GFP-CBD (Gómez-Torres et al., 2018), a new step that consisted of a previous extraction of cheese microbiota was included and we utilised much larger cheese samples. Five grams of cheese sample were homogenised with 10 mL of PBS (pH 7.4) at 40 °C in a Stomacher 400 (A. J. Seward Ltd, UK). Then, 1 mL of each homogenate was centrifuged at 100 × *g* for 3 min at 4 °C to precipitate cheese solids and the supernatant was recovered and centrifuged at 6000 × *g* for 5 min at 4 °C. After removing the cheese fat with a swab and discarding the supernatant, the pellet was resuspended in 100 µL of PBS buffer (pH 7.4). This suspension, containing bacterial cells, was used for the GFP-CBD binding assays.

2.3. Detection of C. tyrobutyricum and total bacteria in the cheese microbiota by microscopy imaging

For the detection of *C. tyrobutyricum* cells in cheese, we produced recombinant GFP-CBD of CTP1L endolysin and negative control GFP-linker as described previously (Gómez-Torres et al., 2018). The DNA-specific dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) was used for the analysis of the total bacterial population of cheese. Prior to binding assays, 10 µL of the suspension containing cheese bacterial cells were mixed with 100 µL of DAPI solution at a concentration of 5 µg/mL for 20 min at 25 °C in dark conditions. Subsequently, the mixture was centrifuged at 6000 × *g* for 5 min at 4 °C and pellet was washed once with PBS buffer (pH 7.4) and resuspended in the original volume of PBS buffer. Then, these 10 µL bacterial suspensions were mixed with an equal volume of GFP-CBD endolysin or GFP-linker at a final concentration of 3.0 µM, incubated at 25 °C for 20 min and washed twice with PBS buffer. Finally, samples were viewed with a Nikon Eclipse 50i microscope equipped for phase contrast and fluorescence, DS Camera Head DS-Fi1 and NIS-Elements 2.34× version imaging software. Fluorescence was excited selecting the appropriate excitation and emission filters for GFP detection (495 and 530 nm) and DAPI staining (364 and 454 nm).

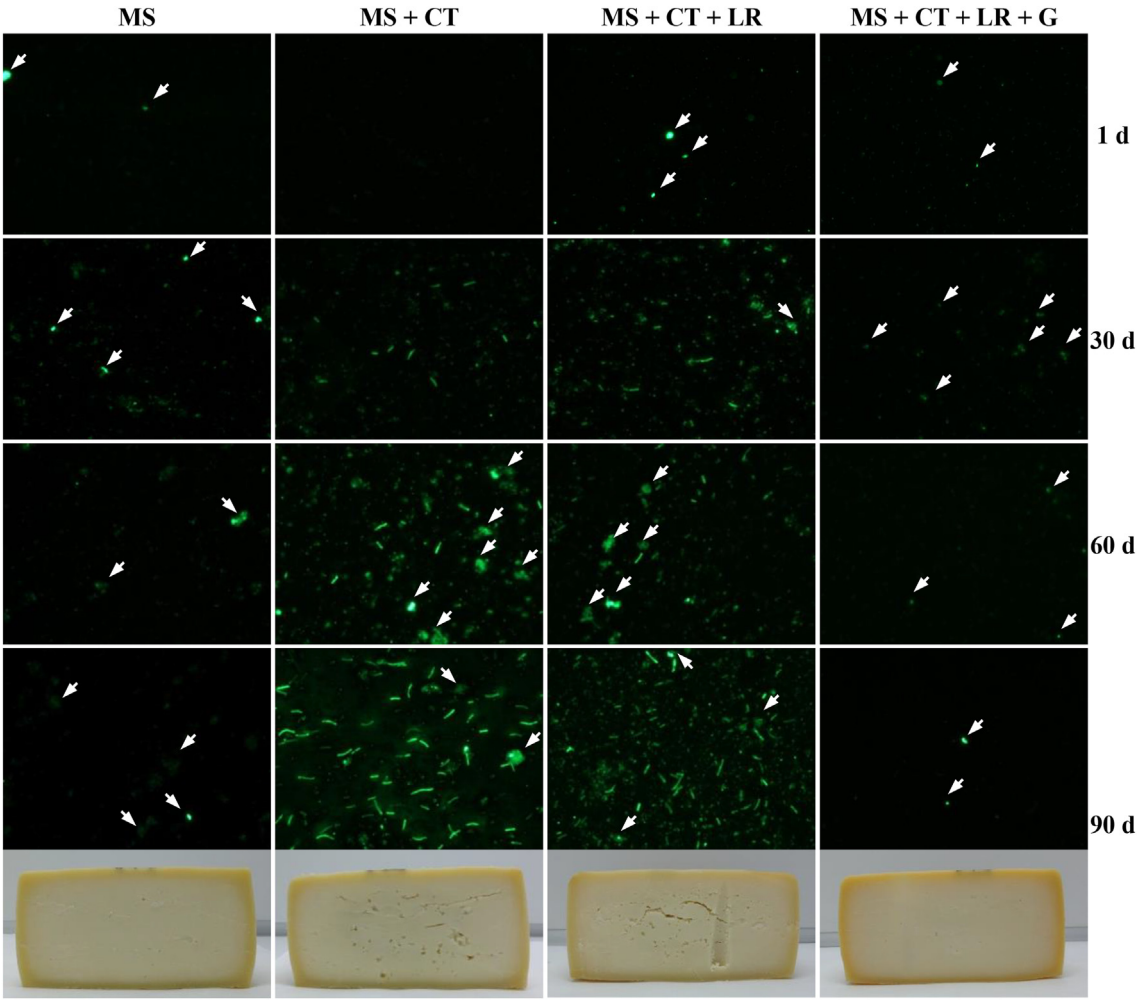

Fig. 4. Photographs of 90 day-old ewe milk cheeses made with a mesophilic starter (MS), with or without spores of *Clostridium tyrobutyricum* INIA 68 (CT), *Lactobacillus reuteri* INIA P572 (LR) and glycerol (G), and evolution of *C. tyrobutyricum* INIA 68 during cheese ripening by GFP green fluorescence (GFP-CBD labelling of *C. tyrobutyricum* cells). White arrows indicate fluorescent artefacts. Magnification x 400. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4. Microbiological and chemical analysis of cheeses

Lactococci, lactobacilli and spore counts of cheese samples were determined by the plate count method as described by Ávila et al. (2017). Determinations of reuterin, butyric acid, cheese pH and dry matter content were carried out as described by Gómez-Torres et al. (2014).

3. Results and discussion

3.1. Changes in microbial composition during ripening of a cheese with late blowing defect

In a previous work, we showed that GFP-CBD of CTP1L was able to specifically bind to the vegetative cells of *Clostridium* in the matrix of an 8 month-old LBD cheese made with milk artificially contaminated with spores of *C. tyrobutyricum* INIA 68 (Gómez-Torres et al., 2018). However, in that work clostridial spores labelled with GFP-CBD in the cheese matrix remained undetected by fluorescence microscopy, most probably because of their low numbers (2.45 log spores/g). In addition, cheese solids prevented the observation of bacteria by phase contrast microscopy and hindered cell detection by fluorescence microscopy. With the aim of studying *C. tyrobutyricum* behaviour during cheese ripening, in this work, we have improved our protocol by using larger cheese samples and introducing a preceding step that consisted of a simple extraction of the total bacterial population from cheese samples. The new protocol allowed us to visualise cheese bacteria by phase contrast and fluorescence microscopy.

Fig. 1 shows the evolution of the microbiota of cheese made with a mesophilic starter and spores of *C. tyrobutyricum* INIA 68 during 90 d of ripening after DAPI staining of the total bacterial population (blue cells) and specific GFP-CBD labelling of *C. tyrobutyricum* (green cells). On day 1, coccus-shaped bacteria, belonging to starter lactococci, were detected in cheese by phase contrast and DAPI staining, but *C. tyrobutyricum* vegetative cells labelled with GFP-CBD were not observed. However, upon detailed observation of several microscope fields (10–15) we were able to detect some germinated spores of *C. tyrobutyricum* (Fig. 2). Germinated spores are characterized by lacking refringency and having an elongated shape beginning to look like vegetative cells, which indicates that spores are in the outgrowth stage of germination. Most recently, spores of *C. tyrobutyricum,* sealed within dialysis tubes, were kept in the vat/cheese during the manufacture and ripening of Grana Padano cheese (D'Incecco et al., 2018). Similarly to our findings in cheese extracts, scanning and transmission electron microscopy analysis of the content of dialysis tubes revealed spore germination at the end of curd acidification (~48 h), and these spores had a larger core and a smaller exosporium with respect to dormant spores (D'Incecco et al., 2018).

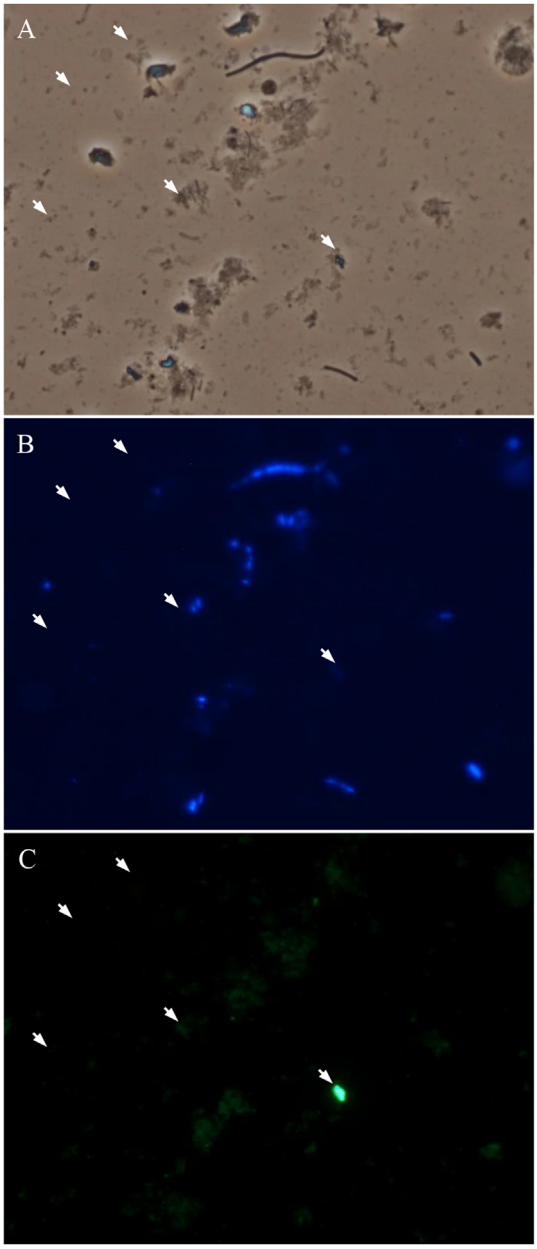

Fig. 5. Detail of lactobacilli cells in 90-day-old ewe milk cheese made with mesophilic starter by phase contrast (A) and fluorescence microscopy (B: blue fluorescence, DAPI staining of bacteria; C: green fluorescence, GFP-CBD labelling of *C. tyrobutyricum* cells). White arrows indicate fluorescent artefacts. Magnification x 400. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

After 30 d of ripening, phase contrast microscopy shows the growth of bacilli-shaped bacteria in cheese in addition to coccus bacteria, and fluorescent microscopy showed that these bacilli corresponded to *C. tyrobutyricum* vegetative cells. *Clostridium* cells extracted from cheese showed weak or no DAPI staining; a portion of the cells appeared lysed (Fig. 3), which may cause the release of their DNA into the cheese matrix. Scanning and transmission electron microscopy by D'Incecco et al. (2018) of the content of dialysis tubes with spores of *C. tyrobutyricum* kept in the vat during cheese making and ripening, showed faint cells in autolysis together with spores after the brining phase (20 d) until the end of ripening (6 months). DAPI staining of cells from a 48 h C. *tyrobutyricum* culture, stained *in vitro* or after addition to one cheese made without spores and treated according to our protocol, was successful with no observed lysis. Another possible explanation for weak DAPI staining of clostridial cells from cheese might be that the cell stage or the environment renders the cells less permeable to the stain. From 60 d onwards, only bacilli cells were observed by phase contrast and fluorescence microscopy. Strong fluorescent labelling of *Clostridium* cells with GFP-CBD was found but again not all cells were stained with DAPI (Figs. 1 and 3). The number of lysed cells increased with cheese age. We estimated, by counting the number of lysed and non lysed cells from several photographs, that the percentage of lysed cells was approximately 30, 40 and 75% at 30, 60 and 90 days, respectively.

Microscopic examination also showed the appearance of irregular shaped and sized fluorescent artefacts (Figs. 1–3). After comparing fluorescence microscopy images with phase contrast, fluorescent artefacts may be associated with residues coming from cheese, which contains multiple constituents that are naturally occurring fluorescent, such as aromatic amino acids, vitamin A and riboflavin. In addition, GFP-CBD may bind to cell debris from lysed *C. tyrobutyricum* cells. However, fluorescent artefacts lack the sharp outline of intact cells.

3.2. Evolution of C. tyrobutyricum INIA 68 in experimental cheeses with or without reuterin during ripening

The growth of *C. tyrobutyricum* INIA 68 during ripening of the four experimental semi-hard cheeses made in the presence of its spores, with or without reuterin as an anti-clostridial agent, is shown in Fig. 4 using GFP-CBD labelling specific for *Clostridium* cells. Phase contrast and DAPI micrographs were similar for all 1-day-old cheeses, showing coccus-shaped bacteria (not shown). As expected, *Clostridium* cells were not detected in cheese made only with the mesophilic starter throughout ripening, although some bacilli-shaped bacteria were observed in 90-day-old cheese by phase contrast and DAPI fluorescence microscopy after searching many fields (5–10) of view (Fig. 5).

Sparsely germinated spores of *C. tyrobutyricum* were detected in extracts from all 1-day-old cheeses made with clostridial spores, but only after observation of several microscope fields. From 30 d onwards, clostridial vegetative cells were readily detected by GFP-CBD in extracts from cheese made with the starter and *C. tyrobutyricum* INIA 68 spores, and from those made with the starter, *C. tyrobutyricum* spores and *L. reuteri*. In addition, the number of fluorescent *Clostridium* cells increased throughout ripening time in these cheeses. For cheese made with starter, spores of *C. tyrobutyricum* INIA 68 and *L. reuteri* INIA P572, phase contrast and DAPI images throughout ripening were similar to those of the cheese made with starter and spores of *C. tyrobutyricum* INIA 68 (Fig. 1). In addition, some bacilli-shaped bacteria were detected in 1-day and 90-day-old samples of this latter cheese after detailed observation of several microscope fields (5–10). However, vegetative cells of *Clostridium* were not detected during ripening of cheese that contained *L. reuteri* INIA P572 and glycerol (Fig. 4), and only lactococcal cells were visible by phase contrast and blue fluorescence microscopy (not shown).

3.3. Correlation between the microscopy imaging studies and microbiological and chemical determinations in cheeses

Table 1 shows lactococci, lactobacilli and clostridial spore counts during cheese ripening. Lactococci achieved high counts in all cheeses at the initial stages of ripening, with means values for all cheeses of 9.61 and 8.22 log cfu/g at 1 and 30 d, respectively (Table 1). These results relate well with those obtained by phase contrast microscopy at 1 and 30 d (Fig. 1) when coccoid cells were prevalent. Lactococci counts in cheeses declined from 60 d onwards when they were also not detectable by microscopy. The loss of lactococci viability together with the absence of coccus-shaped cells suggests that lactococci lysed during the last stages of cheese ripening. At 1 and 30 d, lactobacilli counts corresponded to *L. reuteri* since they were only detected in cheeses made with this microorganism. However, from 60 d onwards,

Table 1
Lactococci, lactobacilli and spore counts[1] in semi-hard ewe milk cheese made with reuterin-producing *Lactobacillus reuteri* INIA P572, spores of *Clostridium tyrobutyricum* INIA 68 and glycerol.

	Age	Cheeses[2]			
		MS	MS + CT	MS + CT + LR	MS + CT + LR + G
Lactococci	1 d	9.65 ± 0.05^{aD}	9.59 ± 0.01^{aD}	9.67 ± 0.05^{aD}	9.54 ± 0.01^{aD}
	30 d	8.19 ± 0.05^{aC}	8.12 ± 0.08^{aC}	8.61 ± 0.19^{bC}	7.96 ± 0.15^{aC}
	60 d	7.01 ± 0.08^{aB}	6.99 ± 0.08^{aB}	7.01 ± 0.05^{aB}	7.13 ± 0.04^{aB}
	90 d	6.47 ± 0.08^{bA}	6.14 ± 0.10^{aA}	6.53 ± 0.05^{bA}	6.52 ± 0.02^{bA}
Lactobacilli	1 d	ND^{aA}	ND^{aA}	7.59 ± 0.08^{bC}	7.32 ± 0.12^{bC}
	30 d	ND^{aA}	ND^{aA}	6.20 ± 0.06^{cA}	3.77 ± 0.08^{bAB}
	60 d	7.27 ± 0.07^{dB}	4.87 ± 0.05^{bC}	6.96 ± 0.05^{cB}	3.53 ± 0.06^{aA}
	90 d	7.55 ± 0.08^{bC}	4.14 ± 0.15^{aB}	7.84 ± 0.01^{bB}	4.06 ± 0.11^{aAB}
Spores	Milk	ND^{aA}	4.90 ± 0.28^{aC}	4.98 ± 0.02^{aC}	4.96 ± 0.01^{aC}
	Curd (2 h)	ND^{aA}	5.69 ± 0.06^{aD}	5.73 ± 0.03^{aD}	5.62 ± 0.02^{aD}
	6 h	ND^{aA}	3.18 ± 0.01^{aB}	3.02 ± 0.03^{aB}	3.03 ± 0.05^{aB}
	1 d	ND^{aA}	2.92 ± 0.42^{aAB}	2.63 ± 0.35^{aAB}	2.83 ± 0.21^{aAB}
	30 d	ND^{aA}	2.35 ± 0.01^{aA}	2.45 ± 0.09^{aAB}	2.38 ± 0.06^{aA}
	60 d	ND^{aA}	2.40 ± 0.02^{aA}	2.43 ± 0.18^{aAB}	2.42 ± 0.06^{aAB}
	90 d	ND^{aA}	2.42 ± 0.06^{aA}	2.32 ± 0.03^{aA}	2.52 ± 0.19^{aAB}

[1]Mean ± SD (n = 2) of duplicate determinations, expressed as log cfu/mL of milk or log cfu/g of curd/cheese. ND: not detected (< 1.40 log cfu/g of cheese for lactobacilli and lactococci counts and < 0.40 log cfu/mL of milk or < 0.40 log cfu/g of curd/cheese for spore counts). Means in the same row followed by different lowercase letters differ significantly ($P < 0.01$). Means in the same column followed by different capital letters differ significantly ($P < 0.01$).
[2]MS: mesophilic starter, CT: *C. tyrobutyricum* INIA 68, LR: *L. reuteri* INIA P572, G: glycerol.

Table 2
Symptoms of late blowing defect (LBD)[1], concentration of butyric acid[2], pH[2] and dry matter[2] in semi-hard ewe milk cheese made with reuterin-producing *Lactobacillus reuteri* INIA P572, spores of *Clostridium tyrobutyricum* INIA 68 and glycerol.

	Age (d)	Cheeses[3]			
		MS	MS + CT	MS + CT + LR	MS + CT + LR + G
LBD	1	No	No	No	No
	30	No	Yes	Yes	No
	60	No	Yes	Yes	No
	90	No	Yes	Yes	No
Butyric acid	1	ND^{aA}	ND^{aA}	ND^{aA}	ND^{aA}
	30	ND^{aA}	0.82 ± 0.02^{bB}	1.13 ± 0.02^{cB}	ND^{aA}
	60	ND^{aA}	2.65 ± 0.02^{bC}	3.45 ± 0.03^{cC}	ND^{aA}
	90	ND^{aA}	8.00 ± 0.03^{bD}	8.98 ± 0.05^{cD}	ND^{aA}
pH	1	5.13 ± 0.03^{aA}	5.10 ± 0.01^{aA}	5.12 ± 0.01^{aA}	5.29 ± 0.01^{bA}
	30	4.92 ± 0.12^{aA}	4.98 ± 0.10^{aA}	5.00 ± 0.17^{aA}	5.25 ± 0.07^{aA}
	60	5.00 ± 0.01^{aA}	5.22 ± 0.03^{abA}	5.42 ± 0.07^{bA}	5.27 ± 0.04^{abA}
	90	4.88 ± 0.09^{aA}	5.21 ± 0.01^{abA}	5.54 ± 0.03^{bA}	5.47 ± 0.09^{bA}
Dry matter, %	1	56.01 ± 1.06^{aA}	55.77 ± 0.37^{aA}	55.67 ± 0.67^{aA}	55.89 ± 0.17^{aA}
	30	61.43 ± 0.40^{aB}	61.10 ± 0.80^{aB}	61.58 ± 0.52^{aB}	60.76 ± 0.10^{aB}
	60	61.24 ± 1.09^{aAB}	64.87 ± 0.89^{aB}	65.27 ± 1.49^{aB}	62.69 ± 1.02^{aB}
	90	63.64 ± 0.47^{aB}	61.66 ± 0.66^{aB}	63.66 ± 0.45^{aB}	63.43 ± 0.59^{aB}

[1]LBD symptoms: cheese swelling, irregular eyes, cracks, off-odours.
[2]Mean ± SD (n = 2) of duplicate determinations, butyric acid is expressed as mg/g of cheese dry matter. ND: not detected. Means in the same row followed by different lowercase letters differ significantly ($P < 0.01$). Means in the same column followed by different capital letters differ significantly ($P < 0.01$).
[3]MS: mesophilic starter, CT: *C. tyrobutyricum* INIA 68, LR: *L. reuteri* INIA P572, G: glycerol.

lactobacilli counts comprised both *L. reuteri* and adventitious lactobacilli as they were detected in all cheeses.

Spores of *C. tyrobutyricum* were only recovered from cheeses made with milk artificially contaminated with them, and spore counts by plate counting were similar in these three cheeses (Table 1). The beginning of the germination of clostridial spores had an early onset since mean spore counts decreased from 5.68 log cfu/g ($4.79{\cdot}10^5$ spores/g) in 2 h-cheese curds to 3.07 log cfu/g ($1.17{\cdot}10^3$ spores/g) in 6 h-cheeses (after pressing) denoting, in turn, that the vast majority of spores ($4.78{\cdot}10^5$ spores/g) were germinating. In the outgrowth stage of germination, spores lose heat resistance and cannot be detected by plate counting (determined after heat-shock at 80 °C for 20 min). Similarly, D'Incecco et al. (2018) observed spore germination at the end of curd acidification (48 h) in the content of dialysis tubes with spores of *C. tyrobutyricum* kept in the vat during Grana Padano cheese making and ripening. In all our cheeses, mean spore counts remained practically constant from 1 d to 90 d, suggesting that little sporulation occurred during ripening. As novel production of spores did not occur during cheese ripening, spore count was not a good indicator of LBD in this cheese variety. Clostridial spores were not observed by microscopy throughout cheese ripening since spore plate counting was very low (lower than 2.92 log cfu/g, $8.32{\cdot}10^2$ spores/g). However, we were able to detect germinated spores of *C. tyrobutyricum* at day 1 in all cheeses made with clostridial spores (Fig. 2), due to their higher numbers (~10^5 spores/g). Overall, the microscopic results match with the decrease of spore counts during cheese manufacture (Table 1).

From 30 d onwards, vegetative cells of *C. tyrobutyricum* INIA 68 were observed in cheeses made with clostridial spores, except where

both the reuterin producer *L. reuteri* INIA P572 and glycerol were added. Reuterin production in that cheese also prevented LBD (Table 2, Fig. 3). Cheese made with clostridial spores, *L. reuteri* and glycerol showed a visual aspect very similar to cheese made without spores, and butyric acid was not detected during ripening (Table 2). These results are in accordance with the microscopic study in which clostridial cells were not observed in these two cheeses. On the contrary, the other two cheeses made with clostridial spores showed LBD and butyric acid from 30 d onwards (Table 2, Fig. 3) agreeing with the detection of *Clostridium* cells by microscopy (Figs. 1 and 3).

To date, attempts have been made to detect *C. tyrobutyricum* from cheeses with LBD using different qualitative PCR based methods (Cocolin et al., 2004; Klijn et al., 1995; Le Bourhis et al., 2005). In addition, a real-time quantitative PCR was applied for C. *tyrobutyricum* quantification in Grana Padano cheese with LBD (Bassi et al., 2015), with counts ranging from 0.30 to 7.50 log cfu/g – cells and spores together. Although the present method is not quantitative, the lactococci results showed that coccus-shaped bacteria were detected by microscopy in all microscope fields only when lactococci counts by plate counting were ≥8 log cfu/g; given this, we estimate that *C. tyrobutyricum* may have reached counts ≥ 8 log cfu/g in cheeses with LBD.

To our knowledge, this is the first time that a microscopy-based technique has been applied for the study of the development of *C. tyrobutyricum* in cheese during ripening. An advantage with respect to PCR methods is this technique allows us to visualise clostridial cells directly and with information on their cellular state that enables to explore the behaviour of *C. tyrobutyricum* in cheese production. For example, the observed lysed state of clostridial cells would explain the failure to isolate or count vegetative cells of *Clostridium* from cheeses with a high degree of LBD, despite the fact that anaerobic measures were taken during sample manipulation. The fact that the limit of detection is high and that detection varies between strains (Gómez-Torres et al., 2018) means that this technique is not currently suitable for the prevention of LBD, but we have shown that it can be used to add knowledge of how the spoilage organism behaves in the cheese environment.

4. Conclusion

We report an improved detection method for *C. tyrobutyricum* based on the high specific ability of the CBD of CTP1L endolysin to bind to clostridial cells, and its application to study the evolution of *C. tyrobutyricum* during cheese ripening in the presence or absence of an anti-clostridial agent. Results showed that it is a promising simple, fast and highly selective technique, and provides the basis for further development of a CBD-based sensitive and quantitative detection method for *C. tyrobutyricum*.

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