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Tesis Doctoral

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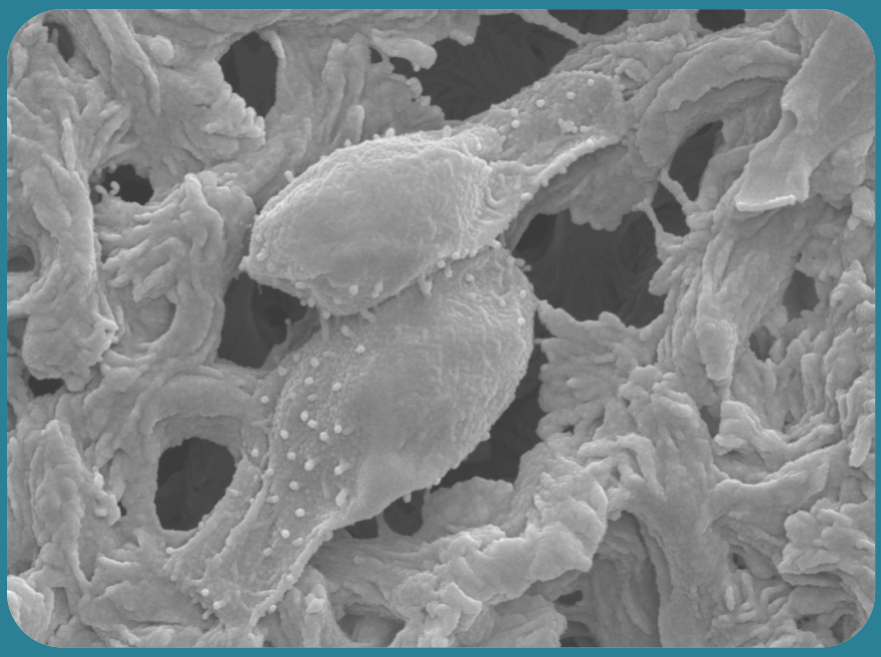

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Tesis doctoral

Development of a detection method for butyric spores based on Real-Time PCR and biomagnetic separation in milk

Desarrollo de un método de detección de esporos butíricos en leche basado en PCR a tiempo real y separación biomagnética

Autora: Miriam Esteban Pellejero

Directoras:

María Lourdes Sánchez Paniagua y Patricia Galán-Malo

El éxito depende del esfuerzo

-Sófocles

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Hoy me encuentro aquí, escribiendo los agradecimientos de mi tesis doctoral después de 5 años de trabajo y esfuerzo, que sin duda han merecido la pena. Llegar hasta aquí ha sido fruto del trabajo y la constancia, pero no sólo míos sino también de mis directoras de tesis doctoral.

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List of abbreviations

AILA: Asociación Interprofessional Lechera de Aragón

APS: ammonium persulfate

BB: bead beating

BCA: bicinchoninic acid

BMS: biomagnetic separation

CE: capture efficiency

CFU: colony-forming unit

cG3P: complete G3P

Ct: cycle threshold

CV: coefficient of variation

DNA: deoxyribonucleic acid

EFSA: European Food Safety Authority

FITC: fluorescein isothiocyanate

FRET: Förster or fluorescence resonance energy transfer

G3P: gene 3 protein

GMO: genetic modified organism

HRM: high-resolution melting

ITC: isothermal titration calorimetry

KF: KingFisher Duo Prime System

LAB: lactic acid bacteria

LAMP: Loop-Mediated Isothermal Amplification

LBD: late blowing defect

LOD: limit of detection

MFI: mean fluorescence intensity

MNPs: magnetic nanoparticles

MPN: most probable number

MW: microwave

MW: molecular weight

MWCO: molecular weight cut off

NCBI: National Center for Biotechnology Information

NPs: nanoparticles

PBS: phosphate buffered saline

PBS-T: phosphate buffered saline- Tween 20

PCR: polymerase chain reaction

PEG: polyethilenglycol

RCM: reinforced clostridium media

rDNA: ribosomal DNA

RT: room temperature

SAI: Servicio de Apoyo a la Investigación

SASPs: small acid-soluble proteins

GPR: germination proteinase

SD: standard deviation

SDi: standard deviation of differences

SDS: sodium dodecyl sulphate

SDS-PAGE: dodecyl sulphate polyacrylamide gel electrophoresis

TEMED: tetramethylethylenediamine

tG3P: truncated G3P

UDG: uracil-DNA glycosylase

UHT: ultra-high temperature

UV: ultraviolet

Summary

Late blowing defect (LBD) is produced in hard and semi-hard cheeses due to the fermentation produced by butyric bacteria, leading into changes on flavor, smell and also to the appearance of cracks. The cheeses with LBD cannot be commercialized, which produces important economic loses for dairy industry. Normally, these cheeses are destinated to melted cheese. Butyric bacteria are Gram positive and sporulated, and spores, the resistance form of these microorganisms, are very difficult to remove completely from raw milk because they can survive to several treatments. Nowadays, there is not a fast method to detect butyric bacteria in raw milk, which would allow to use contaminated raw milk in different dairy products except for hard or semi-hard cheeses in which LBD can take place. The routine microbiological method used to detect butyric bacteria in raw milk is the Most Probable Number (MPN), which provides results between two and seven days. For this reason, faster and more specific methods alternative to MPN method are needed to prevent LBD and reduce economic losses in dairy industry.

Although different species of butyric bacteria can produce LBD, *C. tyrobutyricum* is considered the main causative agent. The main objective of this thesis has been to develop a detection method for *C. tyrobutyricum* spores in raw milk based on Real-Time PCR and biomagnetic separation.

The first part of this thesis has consisted on the screening of different disruption methods to obtain pure genomic DNA from *C. tyrobutyricum* spores. An effective disruption is a key point to have enough DNA for qPCR analysis taking into account the resistance of spores in comparison with vegetative cells. From the results obtained, microwaves (MW) treatment and bead beating (BB) followed by column purification were selected. To facilitate the recovery of spores, milk was treated with subtilisin and after centrifugation, MW and BB treatment were applied to the precipitate of spores for further analysis by qPCR. Finally, MW treatment was found successful in UHT milk as a previous step to the qPCR analysis to detect *C. tyrobutyricum* spores.

Although MW treatment followed by column purification of DNA was found as the best choice in an aqueous buffer and UHT milk, the application of this method in cow raw milk samples coming from Spanish laboratories of milk quality control, gave results with low precision by qPCR. For this reason, a third method was evaluated based

on a commercial kit and automated system named King Fisher (KF) Duo Prime System. This method disrupts the spores based on BB and the released DNA is recovered with magnetic particles.

The calibration curve made with the KF method allowed to achieve better LOD and lower Ct by qPCR analysis than MW and column purification. After this verification step, 202 samples of cow, ewe and goat from three different geographical regions of Spain were analyzed to determine *C. tyrobutyricum* spore concentration. The spore levels found were in the range 10^2-10^3 spores/mL, although an important number of samples were below the LOD and could not be quantified. For this reason, this method was considered as qualitative, though qPCR precision was clearly improved by applying the KF method.

To verify *C. tyrobutyricum* presence in raw milk samples, they were cultured in a selective media for butyric bacteria composed of RCM with D-cycloserine and neutral red. The grown colonies were analyzed by multiplex PCR and 16S rDNA sequencing. The results obtained showed that other microorganisms, such as *Lactobacillus* and *Paenibacillus* can grow in the selective medium. *C. tyrobutyricum* was only isolated in cow milk and other species, such as *C. sporogenes* and *C. perfringens,* were identified suggesting that they could contribute to LBD in cheese. Moreover, the identified species of *Clostridium* were different depending on the milk type analyzed (cow, ewe or goat).

The second part of this thesis was focused on the development of a capture system based on magnetic particles functionalized with affine ligands. The results from qPCR analysis revealed that most of the samples analyzed were below the LOD, for this reason a step of spore capture directly from milk could be a good strategy to improve their detection. With this aim, the pCZS1 peptide obtained by Phage Display technique as affine for *C. tyrobutyricum* spores was evaluated. First, the affinity of pCZS1 for butyric spores was checked by isothermal titration calorimetry (ITC) and flow cytometry. After that, particles were functionalized with pCZS1 and *C. tyrobutyricum* spore capture was done in PBS and UHT milk. Positive results were achieved only in PBS and UHT milk treated with subtilisin. However, as the main objective was to apply the spore capture directly in milk, other ligands were assayed to achieve this goal.

The transmembrane G3P protein from M13 phage was expressed with pCZS1 attached to the N-terminal end to increase the exposition of the peptide to the spores. First, only the soluble domain of G3P, named as tG3P, together with pCZS1 were expressed. A second ligand was designed based on the complete structure of tG3P together with pCZS1, named as cG3P, and pCZS1. Both proteins were expressed in *Escherichia coli* cells and purified by affinity chromatography by applying a protocol to solubilize inclusion bodies. tG3P protein provided better yields than cG3P protein probably because the transmembrane domain affects the purification process. tG3P was evaluated by ITC and flow cytometry confirming its affinity for butyric spores. cG3P was only evaluated by ITC with a similar K_d value in comparison with tG3P but due to the stability problems of tG3P, only tG3P was functionalize to magnetic particles. *C. tyrobutyricum* spores were captured with particles functionalized with tG3P reporting low capture values in PBS and milk with no successful results. For this reason it was concluded that G3P protein did not provide any advantage over pCZS1.

The last part of this thesis has focused on the purification of polyclonal antibodies from the serum of rabbits immunized with *C. tyrobutyricum* spores. After the purification, Protein A and Protein G magnetic particles were functionalized with the specific antibodies. The capture efficiency for the spores was evaluated in PBS and UHT milk with both particles providing rates close to 90%. As a final application, the immunocapture was done in raw milk followed by qPCR detection. The DNA extraction from spores was made by MW treatment followed by column purification. Protein A particles reported better values in terms of amplification rates and Ct values than Protein G particles, confirming that the immunocapture of *C. tyrobutyricum* spores directly from milk, followed by qPCR detection is possible.

The results obtained in this thesis set up the basis to develop a detection method for *C. tyrobutyricum* spores in raw milk by qPCR revealing the critical points. Furthermore, an immunocapture system based on magnetic particles coated with antibodies is proposed to recover *C. tyrobutyricum* spores from raw milk for further qPCR detection.

Resumen

La hinchazón tardía (HT) se produce en quesos de pasta dura y semi-dura como consecuencia de la fermentación producida por bacterias butíricas, dando lugar a cambios en el sabor y aroma, y a la aparición de agujeros o roturas. Los quesos afectados por HT no pueden ser comercializados lo que produce pérdidas económicas en el sector de la industria láctea. Normalmente, estos quesos se destinan a queso rallado. Las bacterias ácido butíricas son Gram-positivas y esporuladas. Sus esporos, la forma de resistencia de estos microorganismos, son muy difíciles de eliminar completamente de la leche cruda porque pueden sobrevivir a una gran variedad de tratamientos. Actualmente, no existe un método rápido para detectar las bacterias butíricas en leche cruda, que permita decidir el destino final de la leche contaminada para elaborar diferentes productos lácteos, excepto para quesos de pasta dura y semi-dura en los que tiene lugar la HT. El método de rutina para detectar las bacterias butíricas es el Número Más Probable (NMP), en el cuál los resultados se obtienen entre dos y siete días. Por esta razón, métodos más rápidos y específicos alternativos al NMP, son necesarios para prevenir la HT y reducir las pérdidas económicas en la industria láctea.

Aunque diferentes bacterias butíricas pueden producir la HT, *C. tyrobutyricum* es considerado como el principal agente causante. El principal objetivo de esta tesis doctoral ha consistido en elaborar un método de detección para esporos de *C. tyrobutyricum* en leche cruda basado en PCR a tiempo real (qPCR) y bioseparación magnética.

La primera parte de esta tesis doctoral ha consistido en el cribado de diferentes métodos de ruptura de esporos de *C. tyrobutyricum* para obtener ADN genómico puro. Disponer de un método efectivo resulta clave para obtener suficiente cantidad de ADN para su análisis mediante qPCR, considerando la resistencia de los esporos en comparación con las células vegetativas. Teniendo en cuenta los resultados obtenidos, se seleccionaron los tratamientos por microondas (MW) y *bead beating* (BB), seguidos de una purificación del ADN mediante columna de sílica. Para recuperar los esporos, la leche se trató con subtilisina y después de una centrifugación, se aplicaron los tratamientos de MW y BB al precipitado de esporos para su posterior análisis mediante qPCR. Finalmente, el tratamiento de MW fue seleccionado como el más apto en leche UHT como paso previo a la detección de los esporos de *C. tyrobutyricum* mediante qPCR.

Aunque el tratamiento de MW y la posterior purificación del ADN mediante columna, se seleccionó como el mejor método a aplicar en tampón acuoso (PBS) y leche UHT; la aplicación de este método en leches crudas procedentes de laboratorios de control de calidad de la leche españoles, dio una baja precisión en el ensayo de qPCR. Por esta razón, se evaluó un tercer método basado en un kit comercial y que precisa de un sistema automatizado denominado King Fisher (KF) Duo Prime System.

La curva de calibración realizada con el método KF permitió conseguir valores de Ct y un límite de detección (LD) más bajo en el ensayo de qPCR que el tratamiento de MW, como paso previo a la purificación del ADN con columna. Después de esta verificación, 202 muestras de vaca, oveja y cabra, procedentes de tres zonas geográficas españolas diferentes, fueron analizadas para determinar la concentración de esporos de *C. tyrobutyricum.* Los valores de concentración de esporos se encontraron en el rango de 10^2-10^3 esporos/mL, aunque un importante número de las muestras analizadas se encontraron por debajo del LD y no pudieron ser cuantificadas. Por esta razón, aunque la precisión de la qPCR mejoró claramente al aplicar el método de KF, este método se ha considerado como cualitativo.

Para verificar la presencia de *C. tyrobutyricum* en las muestras de leche cruda, éstas fueron cultivadas en un medio selectivo para bacterias butíricas que contiene D-cicloserina y rojo neutro. Las colonias fueron analizadas mediante PCR multiplex y secuenciación del 16S rADN. Los resultados mostraron la presencia de otros microorganismos, como *Lactobacillus* y *Paenibacillus*, que también podían crecer en este medio. *C. tyrobutyricum* solo fue aislado en leche de vaca, y otras especies como *C. sporogenes* y *C. perfringens*, fueron identificadas, sugiriendo que también podrían contribuir a la HT. Además, las especies de *Clostridium* identificadas, fueron diferentes dependiendo del tipo de leche analizada (vaca, oveja o cabra).

La segunda parte de esta tesis se ha centrado en el desarrollo de un sistema de captura de esporos basado en partículas magnéticas funcionalizadas con ligandos afines. Los resultados del análisis mediante qPCR de las leches crudas, había revelado que muchas de las muestras se encontraban por debajo del LD. Por esta razón, un paso de captura de los esporos directamente en la leche, podría ser una buena estrategia para mejorar la detección. Con este objetivo, se evaluó el péptido pCZS1 obtenido mediante

la técnica de Phage Display y afín por los esporos de *C. tyrobutyricum*. En primer lugar, la afinidad del péptido pCZS1 por los esporos butíricos, se analizó mediante Calorimetría de Titulación Isotérmica (CTI) y citometría de flujo. Después, se evaluó la capacidad de captura de esporos de *C. tyrobutyricum* en PBS y leche UHT por partículas magnéticas funcionalizadas con pCZS1. Los resultados de la captura fueron positivos en PBS y en leche UHT tratada con subtilisina. Sin embargo, el principal objetivo, consistía en aplicar la captura de los esporos directamente en leche, por lo que se evaluaron otros ligandos con el fin de conseguir este objetivo.

La proteína G3P transmembrana del fago M13, se expresó unida al péptido pCZS1 en el extremo N-terminal de la proteína para incrementar la exposición del péptido a los esporos. Primero, se expresó solo el dominio soluble de la proteína G3P junto con pCZS1, denominándola como tG3P. Después, se diseñó una segunda proteína formada por la G3P completa y el péptido pCZS1, denominándola cG3P. Ambas proteínas fueron expresadas en *Escherichia coli* y purificadas mediante cromatografía de afinidad aplicando un método para solubilizar los cuerpos de inclusión. La proteína tG3P tuvo mejor rendimiento que la cG3P, probablemente porque la parte transmembrana pudiera afectar al proceso de purificación. La proteína tG3P fue evaluada mediante CTI y citometría de flujo confirmando su afinidad por los esporos butíricos. La evaluación mediante CTI de la proteína cG3P, obtuvo un valor de K_d muy similar al obtenido para la proteína tG3P. Sin embargo, debido a los problemas de estabilidad de la proteína cG3P, las partículas magnéticas solo se funcionalizaron con la proteína tG3P. Los esporos de *C. tyrobutyricum* fueron capturados con las partículas magnéticas funcionalizadas con tG3P, pero se obtuvieron valores muy bajos de captura tanto en PBS como en leche UHT. Por esta razón, se concluyó que la proteína G3P no aportaba ninguna ventaja frente al péptido pCZS1.

La última parte de esta tesis se ha centrado en la purificación de anticuerpos policlonales procedentes del suero de conejos inmunizados con esporos de *C. tyrobutyricum.* Después de la purificación, los anticuerpos se unieron a partículas magnéticas con proteína A y G. La eficiencia de captura de los esporos con las partículas funcionalizadas con los anticuerpos se evaluó en PBS y leche UHT, obteniendo valores de recuperación cercanos al 90%. Como aplicación final, la inmunocaptura se realizó en

leche cruda y después se realizó el ensayo de qPCR para detectar los esporos. La extracción del ADN de los esporos se realizó mediante tratamiento de MW y purificación en columna. Las partículas con proteína A, proporcionaron valores de Ct y porcentajes de amplificación mejores que los ensayos realizados con las partículas con proteína G. Estos resultados confirman que la immunocaptura de esporos de *C. tyrobutyricum* directamente en leche y el posterior análisis mediante qPCR son posibles.

Los resultados obtenidos en esta tesis sientan las bases para el desarrollo de un método de detección de esporos butíricos en leche cruda mediante qPCR revelando los puntos críticos. Además, se propone un sistema basado en la inmunocaptura de esporos con partículas magnéticas funcionalizadas con anticuerpos para recuperar los esporos de *C. tyrobutyricum* de la leche cruda y para su posterior detección mediante qPCR.

Literature review

1. Late blowing defect

1.1. Description of the problem and incidence

Late blowing defect (LBD) is caused by butyric fermentation and affects to hard and semi hard cheeses during the ripening stage. Swiss and Dutch cheeses, such as Emmental, Gouda and Edam are frequently affected by this problem (Ledenbach & Marshall, 2009). Likewise, Italian hard cheeses such as Grana Padano and Parmiginano Reggiano are also described as being susceptible for LBD because of the long ripening stage. In Spain there are also varieties reported as being affected by this problem, such as Manchego (Garde et al., 2011), Vidiago (Rilla, 2003) and Genestoso (González et al., 2007) cheeses. LBD is one of the most common problems in cheese industry and causes important economic losses due to the appearance of cracks and changes in smell and taste, which are produced by butyric fermentation.

Nowadays, the incidence of LBD is not clear because many of the routine analysis performed in the laboratories of milk quality control do not include its detection. Although some of these laboratories analyze raw milk by the Most Probable Number (MPN) technique, the number of cheeses that are affected by LBD are difficult to be determined because they are not analyzed and reported with standardized procedures. However, it is considered that LBD has a high prevalence in hard and semi-hard cheeses, in countries such as Italy and Spain.

LBD defect is caused by bacteria that produce butyric fermentation. These microorganisms are anaerobic and form spores, which are one of the resistance life forms most difficult to be eliminated. The early detection of butyric acid bacteria is required to determine the destination of milk, considering that contaminated milk with butyric spores can be used to elaborate many dairy products, except to elaborate hard and semi-hard cheeses with long ripening stage.

1.2. Butyric acid bacteria

LBD is caused by butyric bacteria of the genus *Clostridium*. Some *Clostridium* species, such as *C. difficile*, have virulence factors responsible for the production of toxins which cause illness in humans (Poxton et al., 2001). However, butyric acid bacteria are completely harmless because they do not produce toxins or cause

pathogenicity. These bacteria are Gram positive bacilli, endospore forming and butyric acid producer. Butyric bacteria are strict anaerobes and their vegetative cells die in the presence of oxygen. However, their spores can survive in a variety of environments and harsh conditions, including extreme temperatures and presence of hydrolytic enzymes or DNA damage agents (Errington, 2003).

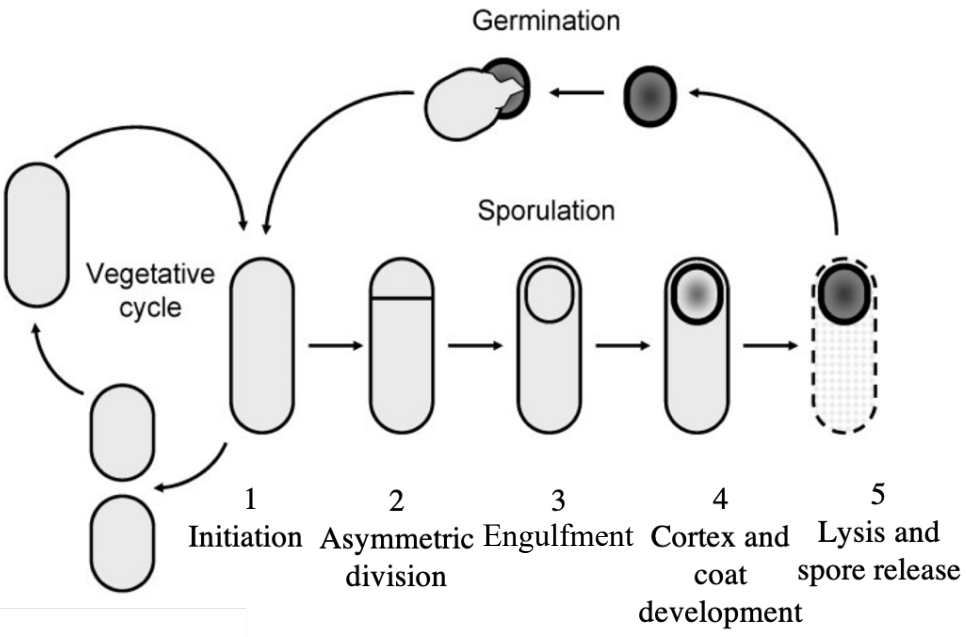

Figure 1. Cell cycle of *Bacillus* and *Clostridium* spores and vegetative cells. Adapted from Errington (2010).

Vegetative cells from the genera *Clostridium* and *Bacillus* start the sporulation process to survive life-threatening conditions (see **Figure 1**), such as starvation, DNA damage or presence of antibiotics (Hutchison et al., 2014). The first event on spore formation is DNA replication followed by an asymmetric division leading to a mother cell with a small forespore. This process is regulated by a group of transcription factors called SpoA0 and σ factors. After the asymmetric division, the mother cell engulfs the forespore, and in this step, DNA compaction and metabolic dormancy are described. The mother cell mediates the production of the cortex, as well as the inner and outer coats, to develop the mature spore. Finally, the mother cell is lysed releasing the mature spore to the environment (Paredes-Sabja et al., 2014).

Spores can start germination when environmental conditions are favorable (Errington, 2010). This process is induced by the presence of certain compounds named

as germinants, which activate the germination regulators (GR), triggering the signaling cascade in the spore. Although the main activating conditions for the germination of *C. tyrobutyricum* are L-lactate, L-alanine and low pH values (Bassi et al., 2009), other factors such as heat shock treatment might also be involved. Once germination has started, dipicolinic acid (DPA) chelated with Ca^{2+} is released from the spore core, activating the hydrolases contained in the cortex that degrade its peptidoglycans (Paredes-Sabja et al., 2014). After this event, the germinated spore starts to uptake water, due to changes in its permeability, in a process called swelling and, as a consequence, the spore becomes turgid and elastic (Hitchins et al., 1963). Once the germination process is completed, the resulting vegetative cells start binary division.

Spores of *Clostridiales* and *Bacillales* are one of the most known resistant forms of life because of their properties. Vegetative cells of Gram-positive bacteria have a peptidoglycan wall membrane and a cytoplasmatic membrane. However, spores have multiple layers that are responsible for their resistance. First, the exosporium is found as the outer layer of the spore and is composed by exclusive proteins (see **Figure 2**). This layer is not present in all the sporulated bacteria (for example *Bacillus subtilis* does not have exosporium) and there is no evidence about its importance on the spore resistance (Setlow, 2014). Underlying the exosporium is the spore coat composed by insoluble and cross-linked proteins. The spore coat acts as a main fender and contains protective enzymes, such as catalase and superoxide dismutase, which are able to inactivate toxic chemicals. The outer membrane is found under the coat. Although its association with the spore resistance is still unclear, the outer membrane is essential for spore formation. The next layers are the spore cortex and the germ cell wall. Neither of them have been found relevant for the spore resistance. However, the spore cortex contains lytic enzymes that are essential for spore germination. Underlying the germ cell wall is the inner membrane, which plays an essential role to protect the spore's core because of its impermeability to toxic compounds and water. The deepest layer is the central core, which has a fundamental role in the spore resistance due to the low water content and heat resistance. High levels of dipicolinic acid in association with Ca^{2+} ions are found in the spore core, being involved in DNA protection and spore dormancy. Finally, the spore core contains high levels of the called Small Acid Soluble Proteins (SASPs) saturating the

spore DNA and protecting it from radiation, enzymes, heat, genotoxic compounds and desiccation (Setlow, 2011).

The SASPs are synthesized during spore formation and are degraded during germination by a specific protease called Germination Specific Protease (GPR); therefore, those proteins are not present in the vegetative form. It is possible that SASPs can compromise the release of spore DNA because they are bound to the double helix. In fact, it has been demonstrated that SASPs affect the DNase cleavage activity and can block or reduce the *in vitro* transcription process of different genes because they bind to DNA (Setlow et al., 1992). Despite the high resistance of spores to disruption, there are some studies performed on different bacteria, such as *Bacillus anthracis* (Torok, 2003) and *Bacillus cereus*, in which DNA has been obtained and amplified by Real-Time PCR with low limits of detection, such as 3-4 total spores (Martínez-Blanch et al., 2010).

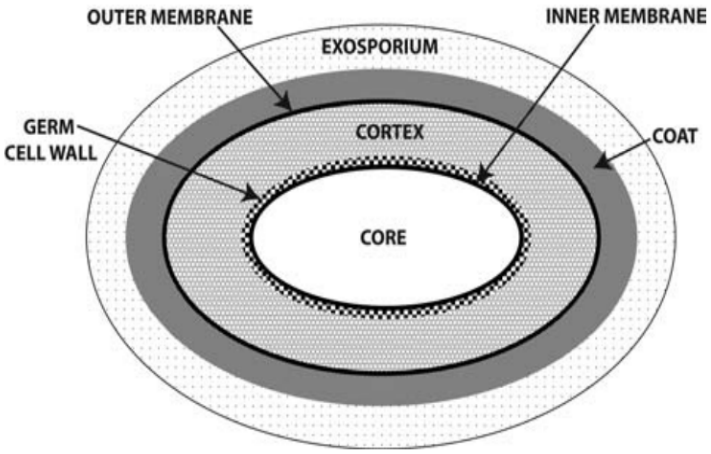

Figure 2. Spore structure of bacteria from *Bacillus/Clostridium* genera. Adapted from Setlow et al. (2011).

Moreover, the sporulation process influences the composition and resistance of the spore cortex, as it has been proved in *Bacillus subtilis* (Abhyankar et al., 2016). In this study, differences in the protein composition of the spore layers produced in solid agar plates and in liquid medium were found, which were correlated with the thermal resistance of the two types of spores. Rose et al. (2007) showed that spores from *Bacillus subtilis* produced in agar plates presented higher resistance to thermal and wet heat treatments, and also germinated slower than those produced in liquid medium.

There are many species of *Clostridium* spp. implicated in the LBD. However, *C. tyrobutyricum* has been reported as the main causative agent. The presence of *C. tyrobutyricum* seems to be a prerequisite for butyric fermentation and it has been isolated 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), appearing more frequently than other species. Other clostridia have been also isolated from raw milk, such as *C. sporogenes*, *C. butyricum, C. beijerinckii, C. perfringens* and *C. tertium*, which may contribute to LBD (Feligini et al., 2014; Le Bourhis et al., 2007a; Reindl et al., 2014). Nowadays, it is not clear if *C. tyrobutyricum* is the only predominant species causing LBD because in some publications it has been suggested that species such as *C. sporogenes, C. butyricum* and *C. beijerinckii*, were also significantly responsible for the problem (Brändle et al., 2016). *C. perfringens* and *C. sporogenes* were found as the main *Clostridium* spp in the case of ewe's milk (Turchi et al., 2016). There are some factors that can condition which are the principal species that lead to butyric fermentation. On one hand, the seasonality and the geographical site where the samples are collected (Feligini et al., 2014). On the other hand, the type of feed used for lactating animals; it has to be taken into account that the main source of nutrition for sheep is pasture (Umberger, 2009), contrary to many dairy cows that are usually fed with silage. The silage is one of the principal reservoirs of *Clostridium* spores and, consequently, cows will have higher levels of butyric contamination. In conclusion, although more research is needed about the prevalence of *C. tyrobutyricum*, it seems that many factors can affect the development of one species over others and the activity of different *Clostridium* spp., which lead to the final result of butyric fermentation during cheese ripening.

1.3. Butyric fermentation

LBD is caused by butyric fermentation that appears in the ripening stage, after several weeks of cheese production. The butyric spores of bacteria belonging to *Clostridium* genus can survive to heat treatments applied to milk during cheese manufacturing and can persist along the chain production, especially when the hygienic measures are not strict enough. Furthermore, vegetative cells represent also a potential risk because of their capability to sporulate during the vat process, as it has been

demonstrated in the Grana Padano cheese manufacturing (D'Incecco et al., 2018). Moreover, there are many cheeses made with raw milk and consequently, vegetative cells may remain active during its manufacturing.

Figure 3. Cheeses affected by LBD. On the left, an example of LBD (Drouin and Lafreniere, 2012). On the top right, LBD in aged cheese (D'amico, 2014). Below, the typical holes made by *C. sporogenes* in Emmental cheese, the white points and bad smell are characteristic of this species (Jakob, 2011).

During the ripening stage, butyric spores germinate because of the environment, pH and temperature, becoming vegetative cells (Leisen, 2002). Vegetative cells metabolize the lactic acid, liberating gas, butyric acid, CO_2 and H_2 (Drouin & Lafreniere, 2012). As a result of the pressure exerted by the gases produced, in many of the affected cheeses cavities and cracks are formed, as it is shown in **Figure 3**.

The main physical alterations caused in cheeses by butyric fermentation are bad smell and flavors produced by the released acids. Butyric acid provides cheese with sweaty, rancid and putrid odours impairing the characteristic flavor of ripened cheese. Other acids generated such as acetic and propionic acids, are considered to have a typical vinegar odour. Propionic acid also brings pervasive gas and burning smell (Curioni & Bosset, 2002).

The amount of acids generated as a consequence of butyric fermentation depends on the species involved (Le Bourhis et al., 2007b). As a result of all the

alterations caused by LBD, affected cheeses are not suitable for standard commercialization, and usually are derived to elaborate other products, such as grated or processed cheese (Sheelan, 2007).

1.4. Major pathways of butyric contamination

Although *Clostridium* spores are ubiquitous, due to their resistance to different environmental conditions, the main reservoir is soil. Crops are contaminated with spores during harvesting or plant growth. It is important to remark that the concentration of spores in soil usually determines the concentration in silage and finally, the concentration in milk (Driehuis, 2013). Then, crops suffer an acid lactic fermentation for silage production where pH decreases very quickly. However, if a high quantity of soil contaminants are present in the silage, there can be a buffering effect which delays and promotes the reduction of pH and the growing of butyric acid bacteria simultaneously. The secondary fermentation produced by butyric bacteria transforms the lactic acid into butyric acid, which leads to an increase of pH, thus favoring the anaerobic conditions and the growth of *Clostridium* bacteria and even their sporulation (Brändle et al., 2016). Then, when the silage is ingested by cows and the spores pass through the digestive tract, the spore concentration increases, especially in the intestine, and finally, the spores are excreted with the faeces. If the faeces are used as manure, the spores return to the soil and then to the crops.

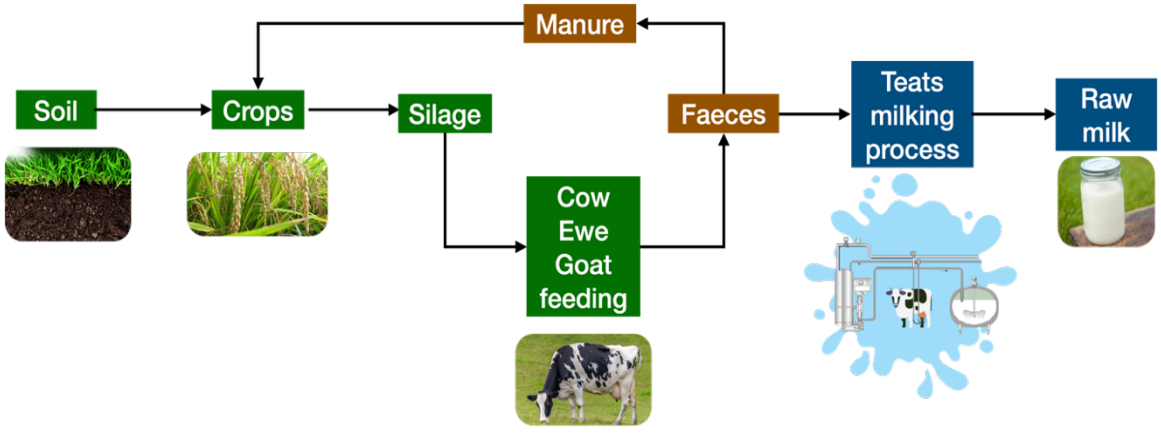

Figure 4. Contamination pathways of butyric spores from soil to raw milk. Adapted from Driehuis (2013).

The main contamination of milk occurs during the milking process (see **Figure 4**), since the spores adhere to teats, as a consequence of cows lying on the bedding, which

usually is fouled with faeces (Driehuis, 2013). For this reason, it is important to have good hygienic farming measures and is recommended to perform teat cleaning before milking (Magnusson et al., 2006).

The contamination of raw milk with butyric bacteria seems to be related with seasonality. Different authors have reported high concentrations of *Clostridium* spores in ovine milk in summer (Garde et al., 2011; Turchi et al., 2016). However, other studies have shown higher levels of contamination during winter, as a consequence of stabling and silage feeding of the animals during this period, the silage being the first cause of contamination (Salmerón et al., 2002). Nonetheless, further investigation is needed to understand the relationship between butyric spore contamination and seasonality.

2. Prevention of late blowing defect

The first action to prevent LBD is to guarantee a good microbiological quality of milk, an objective that must start at the farm. Different studies have suggested that the use of a cleaning method for teats before the milking process decreases considerably the number of spores in milk. Furthermore, farms in which the workers used gloves or applied some hygienic techniques, such as fore-stripping and pre-dipping, reported lower number of spore counts compared to farms where these measures were not used (Driehuis, 2013). However, certain hygienic practices are sometimes difficult to implement in the farms due to the high rhythm of work and to other factors.

To sum up, although the farms apply measures, such as cleaning teats during milking process, use of good quality silage and adequate stabling conditions to reduce *Clostridium* spore levels in milk, it is very difficult to eliminate them completely because of their ubiquitous presence.

The application of good dairy routine practices can be associated with mechanic and thermal treatments, or with the addition of inhibitors to avoid the germination of *Clostridium* spores and the growth of vegetative cells. These additional measures are explained in the next section.

2.1. Mechanical and thermal milk treatments

Pasteurization is a treatment often used in dairy industry to eliminate vegetative cells of bacteria. However, this treatment has secondary effects on milk, such as partial denaturation of whey proteins, enzymes and caseins, affecting directly to the quality of the final product. In the case of milk for cheese manufacturing, when the pasteurization treatment applied is high, caseins are partially denatured and because they are essential for milk coagulation, it has a negative impact on cheese production. The pasteurization method commonly applied to milk is *High Temperature Short Time (HTST)*, a treatment at 72°C for 15 s that does not affect milk aptitude for coagulation. However, *Clostridium* spores are not inactivated by this treatment (Grant et al., 1998).

On the other hand, bactofugation is the physical method frequently used in cheese factories to eliminate bacteria and spores by centrifugation. The first bactofuge was developed for the elimination of *C. tyrobutyricum* spores (Waes & Heddeghem, 1990). Typically, centrifugation is performed at 9000 x *g* of centrifugal force, taking less than one second for milk to pass through the centrifuge. Usually, the centrifugation is performed at warm temperature (52-58°C) to have milk more fluid to achieve an efficient removal of spores (Gésan-Guiziou, 2010). Although bactofugation removes in a single centrifugation around 97% of spores, low levels of remaining spores in milk could lead to produce the LBD (Waes & Heddeghem, 1990).

Another non-thermal method proposed to reduce *Clostridium* spores is microfiltration of milk through a membrane at low operating temperatures and pressure, with a typical range of pore size between 0.2 and 5 µm (Boor & Fromm, 2006). By selecting correctly the pore size, a 99% reduction of spores can be achieved. As it has been reported, vegetative cells are retained in a 1.4 µm size pore, but spores may require a smaller pore (Griep et al., 2018). It has been reported that a 0.8 µm pore size is able to retain the spores for their further enumeration by microbiological culture (Reindl et al., 2014) . Another disadvantage of microfiltration is that this procedure can only be applied to skim milk, since the microorganisms are in the same size range as the milk fat globules (Gésan-Guiziou, 2010). On the other hand, if the pore size used is very small the amount of milk proteins could be significantly reduced due to a partial retention of casein micelles (Griep et al., 2018).

Emerging technologies, such as high pressure (HP) processing have been applied to inactivate pathogenic or spoilage microorganisms (Grant et al., 1998). In a recent work, HP processing was performed at different pressures for 10 min to inactivate *C. tyrobutyricum* in semi-hard cheeses at 7 days after manufacture (Ávila et al., 2016). For this purpose, raw ewe milk was spiked with 10^5 spores/mL of *C. tyrobutyricum* to promote butyric fermentation. The results showed that HP-treated cheeses were not affected by LBD compared to those untreated, verifying the effectiveness of post-manufacturing HP treatment.

Nevertheless, although some thermal and mechanical methods are quite promising, in practice they are expensive and require special equipment and operators. Moreover, most of them modify the physical properties of milk components and, consequently, the characteristics of the final product. For this reason, manufacturers usually add some inhibitors of *Clostridium* germination and growth to avoid LBD.

2.2. Addition of *Clostridium* inhibitors

Clostridium growth inhibitors are compounds that inhibit the germination of spores and the proliferation of vegetative cells. The main advantage of the addition of inhibitors is their easy use since no specific conditions or equipment are required.

The inhibitors more commonly added to cheese are nitrates (E251 and E252). The concentration of nitrates is regulated in the EU by Regulation (EC) 1333/2008 on food additives, setting the limit for its addition to milk for cheese manufacturing in 150 mg/kg. The nitrates are transformed into nitrites by bacteria during cheese ripening, and these nitrites have the potential to inhibit the germination of spores (Gray et al., 1979). The reaction between the aromatic amino acids of cheese proteins and the nitrite could lead to formation of nitrosamines, which are potentially carcinogenic. Actually, the EFSA recommendation is to add the minimum possible concentration of nitrates to decrease the potential risk for consumers (EFSA, 2017).

Lysozyme derived from hen egg-white (E1105) is routinely added in cheese industry to avoid the growth of vegetative cells of butyric bacteria, but is not effective for the inhibition of spore germination (Bester & Lombard, 1990). The action mechanism of lysozyme is to hydrolyze the ß1-4 glycosidic linkages of the peptidoglycan wall

promoting bacterial lysis. The European regulation has not set a maximum concentration of lysozyme to be added to foods; consequently, is the only inhibitor allowed as *quantum satis*. The addition of lysozyme works quite well when the spore concentration is low, less than 500 spores/L. However, if the spore level is higher lysozyme addition is not recommended due to three reasons. First, lysozyme has been described in combination with other parameters as L-lactate and low pH, as a promoter for *C. tyrobutyricum* spore germination (Bassi et al., 2009). Second, high doses of lysozyme inhibit lactic bacteria activity and, consequently, the production of lactic and propionic acids, which are desirable in cheese ripening (Brändle et al., 2016). In additon, lysozyme is a potential allergen, since different studies have proved acute allergy reactions after the consumption of cheese containing egg lysozyme (Schneider & Pischetsrieder, 2013).

Another method for prevention of LBD, is the addition of some antimicrobial substances named as bacteriocins, which are produced by lactic acid bacteria (LAB) (Rilla, 2003). Bacteriocins act as bactericidal or bacteriostatic compounds, depending on the type. The bacteriocin called nisin (E234), produced by *Lactococcus lactis* and some *Streptococcus* strains, is commonly used in cheese manufacturing and is the only one licensed as food preservative (Silva et al., 2018). Other new bacteriocins as reuterin, generated by *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri)*, have been proposed as good inhibitors for controlling LBD (Gómez-Torres et al., 2014). Even though, further investigation is needed for implementing other bacteriocins as food preservatives in dairy products, because, bacteriocins can also inhibit the growth of LAB, which are essential for cheese ripening (Silva et al., 2018).

Although there are many inhibitors to control LBD, even high amounts of these substances cannot avoid butyric fermentation completely, as it has been explained before. Moreover, the Regulation (EU) 1169/2011 on the provision of food information for consumers, declares that the incorporation of additives must be informed in the product label. In addition, the use of additives or inhibitors are not allowed in certain cheeses, such as those having Protected Designation of Origin (PDO). Furthermore, consumers may reject food products with certain additives, such as nitrates, which can be potentially carcinogenic.

For these reasons and considering that butyric spores and vegetative cells are non-pathogenic for humans, the desirable option would be to develop a fast and easy method capable of detecting *Clostridium* spores in milk before cheese processing. The detection of spores would allow manufacturers to choose the most appropriate use for milk, considering that contaminated milk can be destined to manufacture other dairy products instead of mature cheese.

3. Detection and enumeration of *Clostridium* spores and vegetative cells

Butyric fermentation is easy to be detected in cheese due to the characteristic gases, flavors and cracks that are produced. However, it is important to avoid the LBD and its consequences by controlling the microbiological quality of milk. In this section the detection methods that have been developed for *Clostridium* spores enumeration and detection are explained, from the microbiological methods to the immunological ones, and also revising the new methods based on molecular biology, such as the polymerase chain reaction (PCR).

3.1. Microbiological methods

3.1.1. The Most Probable Number (MPN) method

The first techniques developed to detect *Clostridium* spores were based on the germination and growth of the microorganisms present in milk. One of the routine methods performed for *Clostridium* spore detection is the Most Probable Number Method (MPN) (Bergère & Sivelä, 1990).

In the MPN method, the first step is pasteurization of milk sample (75°C for 10 min) to inactivate the vegetative cells that could be present. After heat treatment, the sample is cultured on a specific medium for the germination, selection and growth of *Clostridium* spores. Depending on the expected number of spores, several dilutions are made, and the tubes are incubated at 37°C for several days (3-10) with paraffin or agar layer on top of medium to create anaerobiosis. Milk samples are considered as positive when the detachment of paraffin or agar due to the gases produced in the butyric fermentation is visible. Finally, spore enumeration can be estimated based on the dilution factor applied to milk samples and MPN tables (Bèrgere & Silvëla, 1990).

However, there is not an official MPN method and as a consequence there are multiple MPN procedures that differ in the protocol and culture media (Brändle et al., 2017). One of the commonest MPN techniques used is the one based on sample culture in Bryant and Burkey broth (BB-broth), being one of the most accredited media (Brändle et al., 2016). Other media developed are based on the addition of lactate to promote spore germination as the RCM (Reinforced *Clostridium* media)-lactate broth (Fryer & Halligan, 1976).

Although the MPN method is used as a routine technique in laboratories for *Clostridium* spore enumeration, the main disadvantages are the following:

- MPN is not a selective method for *Clostridium* spores; therefore, other facultatively anaerobic spore forming bacteria as *Bacillus subtilis,* which are usual in milk, can grow in the media used for this technique, giving false positive results.
- Dilutions of milk samples for culture and several days of incubation are required for the detection of spores because the germination must take place and may be slow.
- The limit of detection of MPN method set up in 30 spores/L, considering as a reference the NIZO MPN procedure (NEN, 2009), is an added disadvantage because a low number of spores produce the LBD. Moreover, the results are based on the estimation of the number of spores, which can be overestimated due to the growth of other bacteria (Jackob, 2011).
- To sum up, MPN is not a selective, fast and reliable method and is quite laborious.

Despite its limitations, the MPN method can estimate the number of *Clostridium* spores in a milk sample giving information about the quality of hygienic measures of the farm and usually is the selected method by milk quality control laboratories. However, there are other selective procedures based on microbiological culture to grow and isolate butyric acid bacteria.

3.1.2. Other detection methods based on microbiological techniques

To avoid the non-specific growth of microorganisms in RCM, some authors have developed specific media with supplements to detect easily *Clostridium* spores. As an example, the RCM agar medium proposed by Jonsson (1990) supplemented with D-cycloserine and neutral red, can be useful to detect butyric spores. The antibiotic avoids the growth of *Bacillus* spp. and neutral red allows identifying *Clostridium* colonies, as they appear with brilliant yellow color under a UV lamp. This method, could be completed with the lactate dehydrogenase test, which gives positive results only for *C. tyrobutyricum* (Jonsson, 1990).

Another procedure that combines a microbiological method with microfiltration was developed by Bourgeois et al. (1984) and modified later by Reindl et al. (2014). In the second study, goat milk was previously digested with trypsin, Triton X-100, subtilisin solution and Tween-80 for 30 min in an ultrasonic water bath at 38°C. Digested milk was ultra-filtrated through a 0.8 µm size pore, and then, the filter was transferred onto modified RCM supplemented with D-cycloserine and neutral red (Jonsson, 1990). The results were promising with a good detection limit set up in 25-50 spores/mL.

Another method that has been proposed is the culture on a "double tube" system described by Ali & Fung (1990) for *C. perfringens* and implemented for *C. tyrobutyricum* by Schmidt et al. (2000). The incubation time in this method is 6 hours shorter than the culture in Gaspak jars, though it requires at least 2 days for colony counting.

The novel enumeration method proposed by Brändle et al. (2018) includes a patented medium named as AmpMedia666 used for *Clostridium* germination and growth. First, milk sample is pasteurized and then, the specific medium is added to an automated AMP-6000®-APS pipetting device (SY-LAB), where the germination and growth takes place. After 48 h, the results based on colour change are analyzed by the equipment. The range of detection achieved with this procedure was set from 75 to 59,000 spores/L and is able to detect *Clostridium* spores with an enhanced selectivity. This new method has the main advantage of being automated, faster and more accurate

than the MPN method. However, 48 h are still needed in this method for spore germination and bacterial growth.

In brief, several microbiological methods have been developed placing the efforts on improving the sensitivity, selectivity and operation time. However, most of them are time consuming because they require the germination of spores and the growth of vegetative cells and they are not completely specific for butyric bacteria, because other microorganisms can grow in their media. In recent years, other methods have been developed to detect butyric spores, such as immunochemical and molecular biology methods, showing higher specificity and sensitivity.

3.2. Immunochemical methods

The immunoassays for bacterial detection are based on the reaction between an antigen and an antibody and one of the most common and routinely used technique is the ELISA (Enzyme-Linked-Immunosorbent-Assay). There are many variations of the technique, but focusing on the sandwich ELISA, the principle of the assay is to capture the antigen with an specific antibody bound to a solid phase (i.e. well of the microtiter plate) and to detect it with an specific antibody labeled with a detection molecule (enzyme, fluorophore, etc). In the indirect format, an antibody labeled with an enzyme or a fluorophore targeting the detection antibody is used. If an enzyme is used in the final step its substrate is added to obtain the signal. The absorbance or fluorescence can be measured with a plate reader. The ELISA technique has the main advantage of being fast, quantitative and accurate. The main application of ELISA in food industry is to test food quality by detecting allergens or contaminants and it has worked quite well for this purpose (Fuller et al., 2006).

The methods based on ELISA can detect easily, with lower LOD, small molecules as allergens, but the values of LOD are higher when this assay is applied to bacteria. Thus, the LOD found for the detection of *Salmonella enterica* applying an ELISA was found to be around 10^6 cfu/ml (Galikowska et al., 2011) using bacteriophages as recognition agents. Another study described the detection and quantification of *E. coli* O157:H7 strain by paper-based ELISA technique using antibodies (Pang et al., 2018). This approach uses a Whatman filter paper as a support instead of a plate and the LOD was

set up in 10^4 cfu/mL. Although both methods were fast and specific, the LOD achieved was high. The ELISA assay works quite well for small molecules as proteins, allergens or toxins that are in solution, but the performance is very different for bacterial detection. For this reason, this technique is not commonly used for this purpose. The problem of the low sensitivity of this technique makes this procedure inviable for spore detection because low concentrations of spores in milk can lead to LBD.

However, many efforts have been made in order to find an immunological assay capable of detecting *Clostridium* spores. The first study developed an ELISA assay against *C. tyrobutyricum* with a high LOD of 10^4 spores/ml (Brändle et al., 2016; Lembke, 1984).

Flow cytometry was also evaluated for detection of *C. tyrobutyricum* spores. Lavilla et al. (2010) developed a fast method using polyclonal antibodies to detect *Clostridium* spores with a LOD of 10^3 spores/100 mL in raw milk. This novel method can be easily applied in spiked milk samples in less than 2 h. However, it is important to remark that this technique was not applied to detect spores in field raw milk samples.

Considering the low amounts of *Clostridium* spores in milk, a step of concentration could be useful for the detection. For this purpose, magnetic nanoparticles coated with a specific affine peptide obtained by the Phage Display technique were developed (Lavilla et al., 2012). The peptide obtained exhibited high affinity and specificity for *Clostridium* spores and the capture efficiency (CE) was clearly successful in a simple buffer as PBS. This method would be useful to recover the spores from raw milk samples without the steps of digestion and centrifugation.

Although the immunological methods have been tested for *Clostridium* identification and enumeration, the main problem is still the high LOD. Therefore, in recent years, molecular biology has emerged as a fast method alternative to microbiological and immunological methods, showing higher specificity and sensitivity.

3.3. Detection methods based on molecular biology

3.3.1. Detection methods based on the Polymerase Chain Reaction (PCR)

One of the most popular and routinely used molecular procedure for the detection of microorganisms is the Polymerase Chain Reaction or PCR. In the last few years, PCR has been developed for many purposes, such as detection of microorganisms

causing food poisoning or pathogen diagnosis. This procedure consists in the exponential amplification of a DNA fragment from the genomic DNA of the target microorganism. The bands of amplicons or DNA fragments obtained are visualized in agarose gels stained with a DNA-binding fluorophore (ethidium bromide or SYBR Safe® more used lately) under a UV lamp. For this reason, different studies have been published for the detection and enumeration of *Clostridium* spores based on the PCR technique. However, certain limitations were also found in this procedure, such as the need of a previous milk clarification and concentration of spores, the limit of detection and the difficulty in finding an efficient breakage procedure for DNA release, considering the high resistance of *Clostridium* spores.

The first molecular approach described for the detection of *C. tyrobutyricum* spores was reported by Herman et al. (1995) and consisted in a double PCR named as nested PCR. In that work, a previous digestion of milk was performed followed by a spore concentration by centrifugation. *C. tyrobutyricum* spores were broken by three microwave treatments at 700 W for 5 min. After DNA extraction, a precipitation was made to achieve higher concentration. PCR was carried out in a first step with the primers targeting the 16S-23S rRNA of *C. tyrobutyricum* and the PCR product obtained was amplified in a second PCR, reaching a LOD of 3-30 spores/100 mL of milk. The same protocol was applied for cheese samples spiked with *C. tyrobutyricum* vegetative cells obtaining a LOD of 20-750 cfu/g (Herman et al., 1997). However, although this approach worked quite well, the nested PCR involved two PCR that means long time for sample processing and testing.

Multiplex PCR is a variant of the technique in which different genes are amplified using their specific primers in the same reaction. This is a good approximation to detect different species of *Clostridium,* considering that although *C. tyrobutyricum* is the main species responsible for LBD, other species are also involved. For this reason, Morandi et al. (2015) developed a multiplex PCR to detect at the same time vegetative cells and spores of *C. tyrobutyricum*, *C. butyricum, C. beijerinckii* and *C. sporogenes*, reporting a 10^2 cfu/mL LOD. Reference strains of those species obtained from silages, raw milk and Grana Padano were analyzed in order to test the specificity of the method. However, an

enrichment step was needed for the analysis and, therefore, the PCR could not be applied directly in milk samples naturally contaminated with *Clostridium* spores.

In the last few years, the PCR technique has evolved to be faster and more accurate with the development of real-time or quantitative PCR (qPCR). The basis of qPCR is the same than that of standard PCR, but amplification of the DNA fragment is recorded in Real-Time by the addition of a fluorophore that binds to the double helix of DNA. By this way, the exact cycle of DNA amplification can be set up giving the possibility of comparing samples with different concentrations. Moreover, the visualization of amplicons in agarose gels is not required. This qPCR allows the quantification and is very interesting to detect and enumerate bacterial cells from a great variety of matrices.

The first qPCR developed for detection of *Clostridium* spores was described by López-Enríquez et al. (2007), who detected *C. tyrobutyricum.* The proposed method was able to detect above 25 spores in 25 mL of raw milk and UHT milk, and to quantify a broad range of spore concentrations. However, the protocol described for milk clarification previous to PCR had multiple steps, being a time-consuming procedure not appropriate for a routine laboratory control.

Another method based on qPCR was proposed by Bassi et al. (2013) for detection of *C. tyrobutyricum* spores and vegetative cells in animal feed, faeces, milk and cheese. Food samples and faeces spiked with spores and vegetative cells were previously homogenized and then, DNA was extracted using the bead beating (BB) technique. The spore and vegetative cell quantification range by this procedure was established from 10 to 10^6 cfu/mL, reporting a LOD of 10 cfu/mL. The main advantages of this procedure are the selectivity of the primers used (*pta* primers) which target the flagellin gene, and the efficient DNA extraction from different complex matrices.

qPCR can be adapted for the detection of two or several genes of interest to perform a multiplex qPCR. To apply this technique is necessary to have different primers with a specific fluorophore bound to each one and is only viable for the Taqman® technology based on molecular probes. This approximation was carried out by Morandi et al. (2015), achieving the detection of spores in milk spiked with three different *Clostridium* species: *C. tyrobutyricum, C. beijerinckii* and *C. sporogenes.* In this work, the

recovery of spores from spiked milk was made by simple centrifugation. Then, the precipitate obtained was subjected to lysozyme treatment and finally the spores were broken by microwave heating.

A multiparametric PCR was developed by Postollec et al. (2010). The aim of this study was to detect the principal species of spore-forming bacteria involved in food spoilage. This qPCR could detect several species at the same time studying the total bacterial microbiota from different samples. However, the main disadvantage of this method is that a pre-enrichment step is needed to carry out the analysis.

Although several efforts have been made to design a reliable detection method for *Clostridium* spores based on qPCR, there are only two published studies showing results in raw milk samples obtained for routine quality control, and in milk samples suspicious of being contaminated with butyric spores. In the first study, 60 samples from the agri-food chain, including raw milk, were analyzed by qPCR targeting *C. tyrobutyricum,* and 52 samples (86.6%) were positive (Bassi et al., 2013). In the second study, 144 raw milk samples were tested for *C. tyrobutyricum* contamination by qPCR (Arnaboldi et al., 2021). The results from this study revealed that 15.28% of the total samples were found positive. These studies demonstrate that qPCR is a reliable tool for *C. tyrobutyricum* detection in milk samples.

3.3.2. Other molecular approaches for the identification of Clostridium spp. involved in LBD

Molecular biology has allowed the identification of *Clostridium* population in different samples. In this line, the work developed by Cocolin et al. (2004) analyzed the *Clostridium* species present in cheeses with LBD signs. The study is based on PCR for amplification of the V1 region from 16S rRNA. After PCR, the DNA products were subjected to a denaturing gradient gel electrophoresis (DGGE) in polyacrylamide gel for the sequence specific separation of the PCR products. The main objective of the procedure was to separate the DNA fragments based on their composition in denaturing conditions, for each species a specific DNA fragment was found. By this way, it was possible to distinguish four species: *C. tyrobutyricum, C. butyricum, C. sporogenes* and *C. beijerinckii* in cheese samples. Another variant of this procedure was used to identify

the changes in the *Clostridium* population during cheese ripening and to study the contribution of each species to the development of LBD (Le Bourhis et al., 2007a).

Another method described is the automated ribosomal intergenic spacer analysis PCR (PCR-ARISA) based on the amplification by PCR of the 16S-23S intergenic space region by a fluorescent labeled primer (Fisher & Triplett, 1999). The amplification of the target gene is performed by conventional PCR with labeled primers. Afterwards, an automated system reads the fluorescence intensity and the fragment length of the amplicons showing the abundance of different spacer regions for each community. The identification of *C. tyrobutyricum* vegetative cells in raw milk was performed by this method to prevent LBD, showing a LOD of 3 genome equivalents per 25 mL of milk (approximately 1 cfu/25 mL) (Panelli et al., 2013). A further application of PCR-ARISA was to monitor the *Clostridium* population over time in raw milk and curd of Grana Padano cheese, as it has been reported by Feligini et al. (2014).

Although the PCR-ARISA is a reliable method to study the bacterial communities in complex matrices, new technologies as the next generation sequencing (NGS) have been developed displacing the purpose of the approaches described before. The 16S rRNA NGS was applied by Bassi et al. (2015) to study the bacterial community of Grana Padano cheeses with LBD. In this work, NGS, Real-Time PCR and DGGE technique were analyzed in order to find the main bacteria causative of LBD. The results revealed a predominance of *C. tyrobutyricum* and *C. butyricum* in cheese and milk samples.

Although, new methodologies as NGS or standard PCR techniques for the study of bacterial populations have worked quite well, qPCR is still the first choice to develop a method for *Clostridium* spores enumeration and detection. The main advantages of this technique compared with the others are speed of analysis, high-throughput, and no need of post-PCR steps.

4. Real-Time PCR

4.1. Basics and principles of Real-Time PCR (qPCR)

PCR was first developed by Kari B. Mullis in 1990, and as it has been explained before, this procedure can make unlimited copies of DNA fragments. For the exponential amplification, a pair of primers or small DNA fragments targeting the DNA are used in

combination with a thermostable polymerase, named *Taq* polymerase, from the bacteria *Thermus aquaticus*. In a first step, the double helix of DNA is denatured by heating at 95°C, then the temperature is reduced to the hybridization temperature and the primers are aligned to the target sequence. After annealing, the *Taq* polymerase starts adding dNTPs (deoxynucleotides tri-phosphate) to the complementary DNA strand. This procedure can be repeated a high number of cycles, usually between 30-40, providing a high amount of the target DNA (Mullis, 1990).

Real-Time PCR or quantitative PCR (qPCR) is a variation of PCR with the main advantage of being quantitative, allowing the determination of the PCR cycle during the amplification. For this purpose two chemistries are used, including DNA intercalating agents as SYBR® Green I and molecular probes. During the reaction, the fluorescence is measured and the instrument plots the fluorescence against the cycle number in an amplification curve (Arya et al., 2005). This plot represents the accumulation of the PCR product during the reaction (see **Figure 5**). The Ct or cycle threshold is the number of PCR cycles at which the fluorescence signal crosses the threshold, showing a statistically significant increase compared with the basal signal of the reaction.

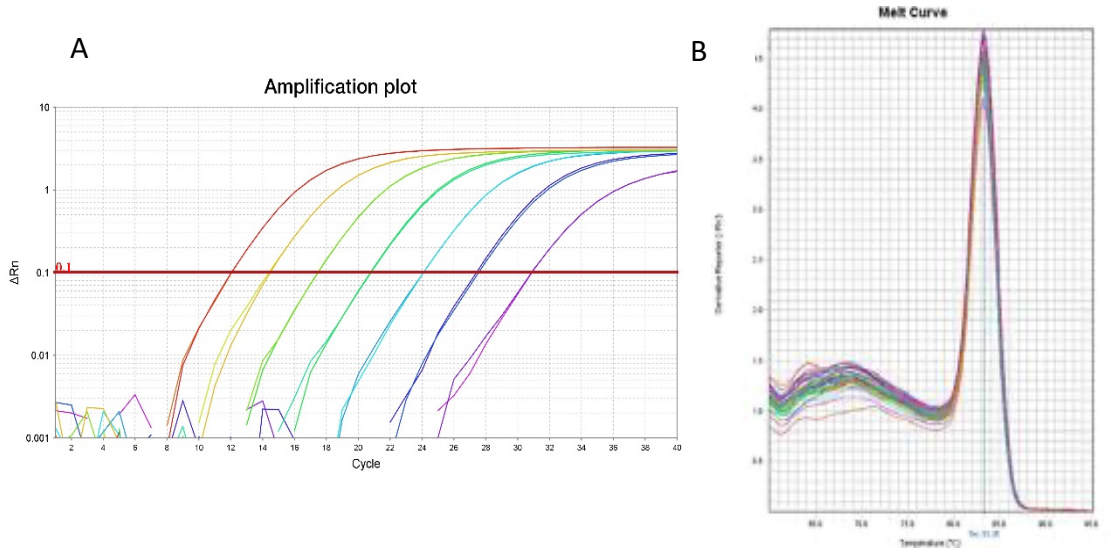

Figure 5. A) Amplification plot obtained for 10-fold series. B) Typical melting curve obtained for an amplicon after qPCR assay (Life Technologies, 2014).

The calibration or standard curve of qPCR are made with known concentrations of DNA template. The generated plot is made with the logarithm of known target concentrations (x-axis) against the Ct value for each concentration (y-axis). From the

calibration curve relevant information can be derived, as qPCR efficiency, correlation coeficient and Y-intercept.

qPCR efficiency is defined as the ratio of the number of target gene molecules at the end of a PCR cycle divided by the number of target molecules at the start of the same PCR cycle (Life Technologies, 2014). Theoretically, qPCR efficiency should be 100% because at maximum capacity the target sequence can double at each cycle because DNA has two strands. However, qPCR efficiency can display variations due to the composition of the amplicon or the conditions of the reaction. On the other hand, the presence of inhibitors can lead to an increase in efficiency. Moreover, qPCR reactions should have an efficiency between 90-100%, according to official guidelines (Bustin et al., 2009).

Other parameters as the correlation coefficient give information about the linearity of the standard curve. The Y-intercept corresponds to the theoretical limit of the reaction and is useful to have a direct measure of sensitivity.

The DNA binding dye SYBR Green I is one of the cost effective options for performing qPCR because is combined with the standard primers in the assay. This dye is intercalated into the double strand of DNA (dsDNA), allowing a strong fluorescence signal, but it has the main disadvantage of being non-specific. As a consequence, non-target amplifications or primer dimer artefacts can be recorded as positive in qPCR.

After running the qPCR, the melting curve or dissociation curve is obtained. The melting curve represents the change in fluorescence observed when dsDNA, with dye as SYBR Green I incorporated, dissociates or “melts” into single stranded DNA as the temperature of the reaction raises. The temperature at which 50% of DNA is denatured is known as melting temperature (Tm). Tm permits the indentification of primer dimers or non-target amplification products because each amplicon has a specific Tm, according to the GC content, length and other factors (see **Figure 5B**). Nowadays, the High Resolution Melting (HRM) curve is performed for the genotyping of known variants because this new technology can distinguish between single polymorphisms, DNA methylation and mutations (Farrar & Wittwer, 2017). The HRM requires a qPCR detection system with high thermal stability, a dedicated software for HRM analysis and

improved DNA binding dyes known as third generation dyes. These dyes, which include EvaGreen and LCGreen, bind DNA uniformly and saturate it without promoting the inhibition of DNA polymerase, as it occurs with SYBR Green I. This new technology allows identifying different strains of microorganisms and detecting rare diseases at genomic level.

In the last few years the development of hydrolysis probes, such as TaqMan™, has raised as an alternative to DNA binding dyes. These probes are based on primers targeting the sequence of interest and a fluorescent probe (see **Figure 6**). The first function of hydrolysis probes is the 5'-3' exonuclease activity of the *Taq* polymerase, this means that polymerase is able to degrade the DNA downstream of DNA synthesis. The other key mechanism is the Förster or fluorescent energy resonance transfer (FRET), which can be defined as the reduction of the emission of one fluorophore due to the near presence of another dye. This is commonly known as "quenching", the second dye being the quencher of the first one. During the starting of qPCR the probe is quenched due to the close presence of the two dyes. However, when the primers and the probe are bound covalently to the target sequence, the *Taq* polymerase starts amplification and cleaves the probe due to its 5'-3' exonuclease activity. Consequently, the reporter dye is released, and no quenching effect is found at this stage (Butler, 2012).

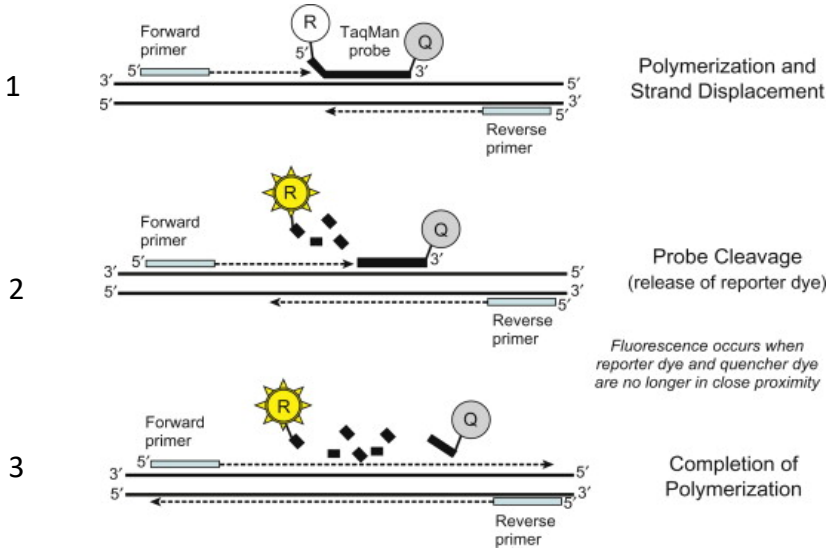

Figure 6. Action mechanism of hydrolysis probes. 1) The primers and the probe are aligned to the target sequence and the polymerization starts. 2) The *Taq* polymerase cleavages the probe due to its 5'-3' exonuclease activity. Therefore, the reporter dye is liberated. 3) The polymerization is completed, and the fluorescence rises (Butler, 2012).

The hydrolysis probes have supposed an important advance in qPCR chemistry because the melting curve step is not needed due to the high specificity of the probe. Moreover, TaqMan probes are normally used for multiplex qPCR for detecting different targets in the same qPCR reaction, with different fluorophores for each amplicon. Multiplex qPCR can be performed with SYBR Green I and melting curve analysis, though the specificity and simplicity of the TaqMan probes make them the perfect candidates for this type of qPCR (Arya et al., 2005).

Although the TaqMan probes are the most common, other hydrolysis probes can be used for qPCR. Molecular beacons and scorpions are a type of probes designed to adopt a hairpin or a stem loop structure, which permits the fluorescent dye and the quencher to be closer allowing the FRET phenomenon. The sequence of the hairpin is complementary to the target sequence, and during the annealing the structure of the loop changes due to the hybridization. In this stage the reporter dye and the quencher are not close, and the FRET is suppressed; therefore, fluorescence is highly increased. Molecular beacons and scorpions are commonly used for multiplex qPCR (Ng et al., 2005).

qPCR is a reliable and high specific technique that has revolutionized the molecular biology field. This method has been implemented for the diagnosis of virus, bacteria and pathogens due to its sensitivity, specificity and reduced time of analysis. Moreover, qPCR has been also used in food microbiology for the detection of microorganisms related with food safety and quality.

4.2. Real-Time PCR in food control

The qPCR has been introduced in the last few years in food microbiology for the detection of a variety of foodborne pathogens, genetic modified organisms (GMO), allergens and viruses. Nowadays, food analysis is needed for the control of food quality and safety.

In the case of foodborne pathogens, although sterilization methods can eliminate nearly all of them, these treatments are not adequate for the processing of some foods due to the changes in the physicochemical and nutritional properties. The

quality control of raw materials with fast and specific methods, such as qPCR, which ensure the absence of foodborne pathogens are good options for food industry.

Microbiological methods, such as plate count or culture of the target microorganism in a selective media, are usually applied to identify spoilage bacteria. However, as it has been described before, although microbiological culture is an easy technique it is not fast and it does not distinguish between species and strains and is not always completely specific.

One of the main limitations of the application of qPCR for detection and enumeration of foodborne pathogens has been their concentration in the raw material. The concentration of target bacteria is usually below the limit of detection. To avoid this problem, a pre-enrichment step is generally performed to increase the number of bacteria and, consequently, the quantification cannot be done directly. Even though, the combination of pre-enrichment with qPCR analysis is faster than microbiological assays that require long incubation times (see **Figure 7**) (Martínez et al., 2011).

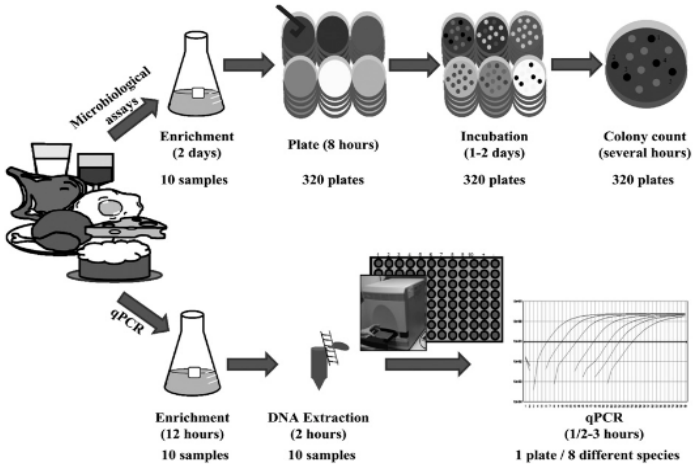

Figure 7. Comparison between traditional microbiological methods and qPCR for the enumeration of eight spoilage microorganisms in 10 samples. The microbiological culture needs 320 plates vs one plate of qPCR (Martínez et al., 2011).

Moreover, different qPCR assays have been proposed to detect and quantify spoilage microorganisms involved in food industry. These assays include the detection of yeasts, molds, a diversity of bacteria, such as lactic acid bacteria (LAB), acetic acid bacteria, *Enterobacteriaceae*, biogenic amine producer bacteria, and even viruses as bacteriophages. The control by qPCR of LAB and yeasts, which are involved in the fermentation of many foods and beverages, is useful to monitor their growth.

Nowadays, several qPCR commercial kits are becoming available for the detection and quantification of spoilage microorganisms. For example, the Foodproof Detection System for *Enterobacteriaceae* (Merck, Darmstadt, Germany) that can detect the presence of *Enterobacteria* by qPCR in food manufacturing as a control of hygienic quality. Another interesting qPCR kit developed by Merck is the Foodproof Beer screening kit (Biotecon diagnostics GmbH, Postdam, Germany) that allows the identification of 25 spoilage microorganisms found as contaminants for beer. For the correct detection, a pre-enrichment step is needed requiring from 30 min to 1 h, and the qPCR takes about 2 h to be completed (Martínez et al., 2011).

To sum up, qPCR can be considered as a new technique for the detection and enumeration of food spoilage microorganism, which is still in development. One of the main challenges is the variety of matrices where the microorganisms can be detected. However, there are many kits that can extract the DNA from many different matrices (cheese, milk, faeces, beer). Another important drawback is the qPCR LOD and the starting concentration of the bacteria that is usually low. To avoid this problem a pre-enrichment step could be performed and consequently, the quantification could not be done directly in the starting material. The automation and the new technologies, which are being implemented, can lead to improve DNA extraction and identification of microorganisms by qPCR.

The qPCR technique can be applied in food control in many fields, as the detection of antibiotic resistance strains, allergens or identification of genetic modified microorganisms (GMOs) (Salihah et al., 2016). Hereafter, successful examples of qPCR application to food control are explained.

One of the recent applications of qPCR in food analysis is identification of bacterial species with the aim of detecting adulteration and authenticity of food products. The HRM curve analysis enables to identify different Tm peaks in the melting curve of multiplex qPCR. This new technique can distinguish between mutations and polymorphisms. The HRM has been used in food technology to detect adulterants (maize, hazelnut and sunflower oils) in olive oil at levels above 10% (Vietina et al., 2013). Furthermore, this new technique can also be applied for the detection of adulterations in meat products (Drummond et al., 2013).

The detection of allergens is an important area in food traceability and quality control. Nowadays, methods commonly used for allergen detection are ELISA and lateral flow techniques. However, qPCR has also been applied successfully in this area, achieving LOD comparable with the traditional methods. qPCR has been used for the detection of allergen coding sequences for kiwi, peach and apple in processed foods with the main advantage of being a species-specific multiplexed assay (Graziano et al., 2018). Another efficient assay is based on the amplification of the 18S RNA in fish to identify traces. This analytical approach was free from cross-reactivity problems, in comparison with other assays, such as ELISA (Salihah et al., 2016).

The detection of GMOs is an important issue that concerns food safety and quality. Nowadays, qPCR used for the detection of GMOs in food is based on the amplification of genes that are usually present in GMOs. These genes are the 35S promoter gene from cauliflower mosaic virus (CaMV) and the nopaline synthetase terminator (T-NOS) from *Agrobacterium tumefaciens* (Mano et al., 2018). These genetic elements are commonly used in GMOs. There are many available commercial kits for the detection of GMOs in food matrices as the one developed by Roche named as Light-Cycler GMO Screening Kit. This kit detects simultaneously the 35S and T-NOS in a multiplex qPCR and it has shown excellent results in soya and Bt maize.

The diagnosis of enterotoxin producing bacteria has also been detected and evaluated by qPCR showing accurate results. Shiga toxin-producing-*E. coli* (STEC) and *Salmonella,* both considered as foodborne pathogens, have been identified by Dupont™ Bax® System (Dupont, Wilmington, DE, USA) (Wasilenko et al., 2014). This system perfoms a qPCR analysis for the different genes found in enterotoxin-producing *E. coli* serotypes. Although a pre-enrichment of the bacteria is needed for the correct quantification, a fast, simple and reliable assay is offered.

5. Biomagnetic separation based on nanoparticles

5.1. A brief introduction to nanoparticles

In the last years, the use of nanoparticles (NPs) has increased notably due to their application in different fields, such as drug delivery, capture of images by magnetic

resonance (bioimaging), new treatments against cancer disease and biosensing (De et al., 2008).

NPs can be naturally synthetized by many organisms, such as bacteria, fungi, actinomycetes and algae. However, NPs used for biotechnological applications are usually synthetized by chemical procedures. NPs can be synthetized from different materials depending on the final application. For example, gold NPs are commonly used in lateral flow technique due to their unique optical properties, chemical stability and binding capacity for biomolecules. These NPs are usually coated with specific antibodies or ligands (Pan et al., 2018).

Silver NPs (AgNPs) have been found to be a potential antibacterial agent due to their ability of being accumulated in the cell wall with the subsequent pore formation and cell death. This fact has been demonstrated in assays performed with *E.coli* treated with AgNPs (Zhang et al., 2016). Moreover, AgNPs exhibit antifungal and antiviral activity. Even though these are the most important roles of AgNPs, other applications have been studied as anti-inflammatory, anti-angiogenic and anti-antitumor activities. Moreover, AgNPs are involved in biosensing drug delivery or water treatment (Zhang et al., 2016).

Table 1. Principal applications of MNPs in medical, environmental and food technology areas.

Scientific field	Principal applications	Source
Medical therapy and diagnosis	• Chemotherapy: MNPs as a chemotherapeutic carrier • Hyperthermia treatment • Gene therapy and magnetofection • Bioimaging	(Mohammed et al., 2017)
Environmental sciences	• Contaminant removal • Remediation • Water treatment • Biosensing	(Mohammed et al., 2017)
Food technology	• Enzyme immobilization • Protein purification (lysozyme and human serum albumin purification) • Food analysis • Capture or bioseparation from complex matrices • Biosensing	(Cao et al., 2012)

Magnetic nanoparticles (MNPs) are nanomaterials made of magnetic components such as iron, nickel or cobalt, among others. Among them, ferrite nanoparticles composed by a core of magnetite (Fe_3O_4) or maghemite (Fe_2O_3) are the most used for biotechnological applications. MNPs have multiple applications that are summarized in **Table 1**.

Even though all nano and microparticles are quite interesting, in this revision we have focused on MNPs applied to biomagnetic separation of microorganisms. Biomagnetic separation (BMS) is one of the emergent techniques for capture, enrichment or removal of microorganisms, such as bacteria, from a variety of complex samples as blood, serum, milk, food matrices and soil, among others. Moreover, it provides a separation without cell modification unlike other common methods such as centrifugation or filtration (Šafařík & Šafaříková, 1999). For the reasons explained before, BMS is a promising protocol to recover bacterial cells, as a previous step for their detection and enumeration.

5.2. Superparamagnetic particles: synthesis and properties

Superparamagnetic particles are the best candidates for biomedical and clinical applications because of their good properties. Superparamagnetism is a distinctive behavior of single-domain nanoparticles, originated from the fast flipping process of the total magnetic moment due to thermal energy (Goya, 2021). As a consequence, the superparamagnetic particles develop a magnetic moment in the presence of a magnetic field, though they are redispersed in the absence of it, as it is shown in **Figure 8**. Particles that exhibit superparamagnetism do not interact with each other in the absence of a magnetic field, avoiding the formation of undesirable aggregates (Fields et al., 2016).

Ferromagnetism, contrary to superparamagnetism, means that the particles have a permanent magnetic moment. Because of this, magnetic particles are oriented under the influence of a magnetic field, but they have a remanent magnetic moment in the absence of it and they aggregate in suspension. This situation is not desirable for cell bioseparation or other applications.

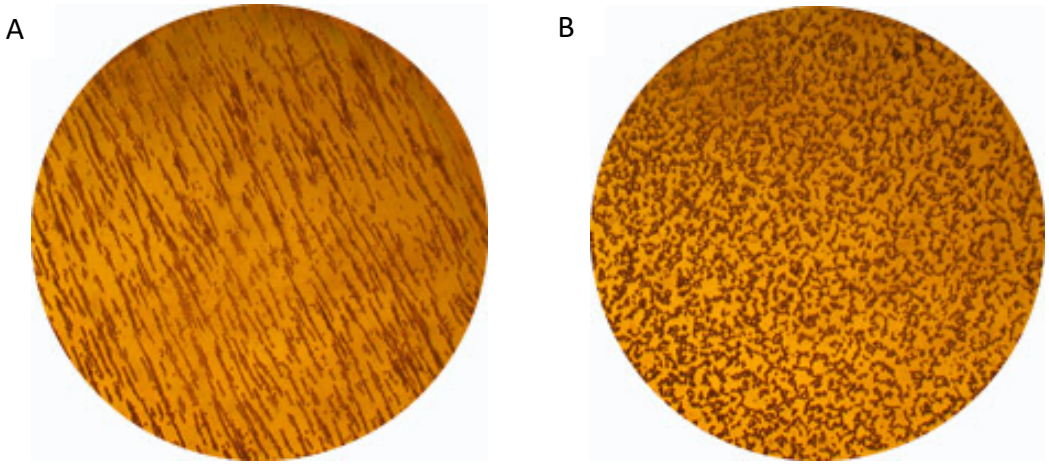

Figure 8. A: Superparamagnetic particles in the presence of a magnetic field. B: Superparamagnetic particles in the absence of a magnetic field (Chemicell, 2022).

The method most commonly used for the synthesis of iron-oxide NPs is the co-precipitation of ferrous and ferric ions in a basic medium using salts, such as iron (III) or chloride iron (II) sulphate (Fields et al., 2016). The co-precipitation occurs by the formation of a nucleus that grows due to the diffusion of ferric ions from the solution to the surface of the growing crystal. Although co-precipitation is by far the selected method for the synthesis of MNPs, it is very important to control all the parameters involved in the synthesis to obtain monodisperse nanoparticles (Akbarzadeh et al., 2012). Moreover, the size of the crystal must be controlled to obtain nanoparticles below the superparamagnetic size threshold of 20 nm. For this reason, sometimes the micro-emulsion technique is used for synthesizing MNPs. This method provides greater control of the nanoparticle size because is determined by the micelle size (Fields et al., 2016).

Although, 10-20 nm MNPs are used for many applications, magnetic bioseparation of targeted molecules requires magnetic particles of higher size. This is due to the strong magnetic fields that must be applied for correct an efficient separation from the matrix at a reasonable time scale. Because of this fact, the size of the particles used in the laboratory is in the range from 100 nm to 1000 nm. The synthesis of microparticles starts at the addition of nanoparticles into micron and submicron size structures. Micron-sized particles retain the superparamagnetic properties and are

more responsive to magnetic fields than the individual nanoparticles, allowing a fast separation (Fields et al., 2016).

After the synthesis process, magnetic beads are coated to prevent aggregation and degradation considering that magnetic particles made of iron are sensitive to oxidation. To prevent this undesirable situation and to increasing chemical stability, the particles are coated with organic polymers, surfactants, or inorganic layers such as silica, which is commonly used in magnetic particles. The silica shell protects the magnetic core but also prevents undesired interactions with the functional groups added to the particle (Akbarzadeh et al., 2012). Other coatings that are added to MNPs are neutral polymers as polyethilenglycol (PEG) or dextran, which have demonstrated their biocompatibility for medical applications. However, the coating surface not only provides protection and stability to the particle, it affords functional groups, such as amine or carboxyl, to act as anchor points for the attachment of functional ligands as proteins, peptides and antibodies (Fang & Zhang, 2009).

The superparamagnetic micro and nanoparticles coated with specific ligands can be used for the isolation of cells and microorganisms from complex matrices, such as food. The binding of specific ligands can be done, as it is performed on a solid support, through hydrophobic interactions, electrostatic binding as hydrogen bonds or ionic exchange, and by covalent binding. In the following section, biomagnetic separation (BMS) and the different ways and strategies to bind ligands to micro and nanoparticles is explained.

5.3. Biomagnetic separation (BMS): principles and ligands

As it has been exposed before, magnetic particles coated with selective ligands can be used for the efficient separation of different cells, such as bacteria. There are two different ways to perform BMS, named as positive or negative selection. The negative selection consists of the removal of the contaminant cells from a mixture. One of the limitations of this method can be the low concentration of the target cell in the sample. This type of separation can lead to an unpurified sample if the removal is not completely efficient. Positive selection is the isolation of target cells from the mixed suspension. Moreover, two main methods can be used for purification of a target by BMS based on

coated particles: direct and indirect. The direct method allows the capture of the cell with nanoparticles coated with the ligand. The indirect method first adds the ligand and incubates it with the target cell, and after the incubation and washing, the nanoparticles are added to capture the cell-affinity ligand complexes (Šafařík & Šafaříková, 1999). The direct method is commonly used for a detection purpose, due to shorter incubation times and ready-to-use nanoparticles. In **Figure 9**, the main steps of direct biomagnetic separation are explained.

Biomagnetic separation of bacterial cells usually consists in the following steps (Šafařík & Šafaříková, 1999):

1. MNPs with the affinity ligand are added to the mixture with the target microorganism or molecule. The sample is incubated for 30-60 min in rotation to ensure the interaction between particles and microorganisms and complex formation. Later, a magnet separator is applied to recover MNPs with the microorganism of interest bound.
2. After the recovery of microorganism-MNPs complexes, they are washed to eliminate the unwanted cells or other components of the sample that might bind non-specifically to MNPs.
3. Finally, the microorganisms bound to MNPs can be cultured because the separation does not affect their viability, or can be disrupted for a variety of applications such as electrophoresis, qPCR or sequencing. However, for certain applications a separation of the microorganisms from magnetic particle is needed, some of them are explained below. After the detachment of target, magnetic particles can be recovered with a magnetic separator and cells remain in the supernatant. This method allows re-using magnetic particles.

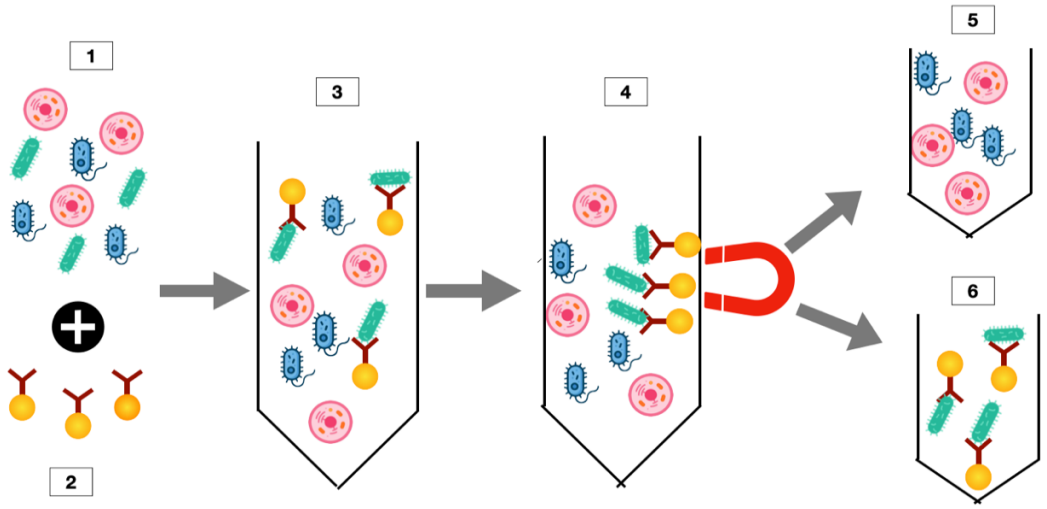

Figure 9. Schematic representation of the BMS process. 1) Complex sample containing the target bacteria (green). 2) MNPs coated with the ligand (antibody, specific peptides or proteins). 2) Mixing and incubation of the complex sample with MNPs. In this stage the MNPs bound the target bacteria. 4) The application of a magnetic field separates MNPs with the target bacteria from the sample. 5) The supernatant is discarded or kept for later analysis. 6) The MNPs with the target bacteria are separated.

For BMS of bacteria, there are different ligands that have been used successfully. In the following sections the main ones are described.

1. Antibodies: the antibodies have been used for many techniques such as flow cytometry, ELISA and lateral flow techniques. For this reason, the antibodies were the first choice for BMS from the beginning, called in this case immunomagnetic separation. The antibodies have worked very well with different nanoparticles. There are many strategies to couple proteins to nanoparticles based on physical adsorption or covalent crosslinking. Physical adsorption is the easiest approach for antibody binding; however, this process is random and not controllable, leading to lower biological activity of the molecule. For the covalent crosslinking, there are four functional groups available: primary amines (NH_2), carboxyls (-COOH), sulfhydryls (SH), and carbonyls (CHO) (Fields et al., 2016). The important point of this procedure is to ensure the Fc binding of the antibody to the nanoparticle to have the correct orientation. However, the affinity binding has been also used for this purpose, attaching the antibodies to protein G from *Streptococcus* spp. or protein A from *Staphylococcus aureus*. The binding of antibody to protein A/G gives the correct orientation of the molecule due to the capture of the Fc

region (CD bioparticles, 2022). This condition provides theoretically the perfect binding, contrary to the situation of covalent attachment. In the protocol of covalent binding, the antibody molecules can achieve different orientation; however, the higher number of molecules bound per particle usually allows that enough antibodies are correctly oriented on the particle surface (see **Figure 10**).

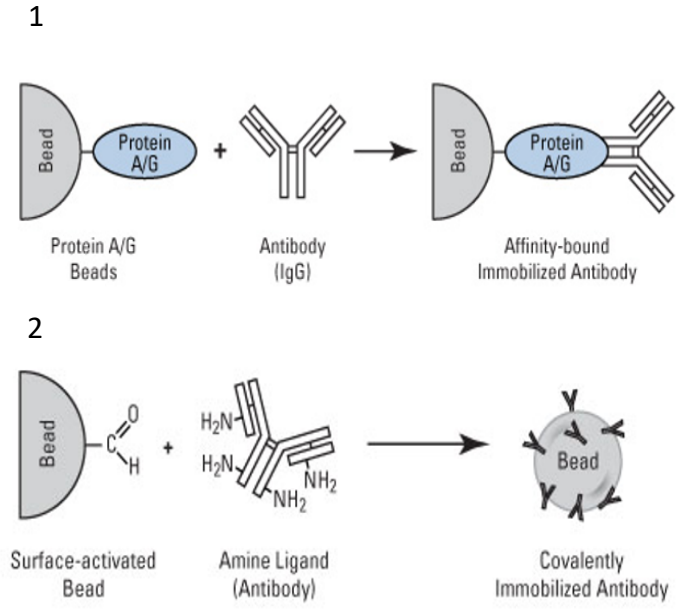

Figure 10. Schematic representation of the antibody binding to protein A/G beads (1). The antibodies are bound by the Fc region. In the carboxylated bead the antibodies are attached with different orientations because the amine groups are distributed all over the whole molecule (CD bioparticles, 2022).

In immunological techniques, IgG are the immunoglobulins most commonly used because they are present in blood in high levels. Therefore, the purification of antibodies is usually performed from blood of immunized animals, such as rabbits and ewes. In the last few years, immunoglobulin fragments such as Fab or the engineered single-chain variable fragments (ScFv) have emerged as an alternative to the whole molecule of IgG, facing some of their limitations as the formation of undesirable complexes. One of these examples is the Fab ligand, which is composed by one of the Fab immunoglobulin region and it has been reported as a good alternative because it can be bound to the nanoparticles by conjugation of the hinge thiol, allowing its correct orientation. Moreover, the engineered antibody fragments and the discovery of new antibody fragments by the Phage Display technique have been a great advance. This approach

has evolved as a promising tool for the discovery of new affine ligands from peptides to antibody fragments. In the Phage Display method, a random library of peptides/proteins is used. The peptides are expressed (displayed) as fusion proteins of the coat M13 phage protein. As a result, the library is composed by a high number of phages, each one with a different genotype. Then, the recombinant phages are exposed to the ligand and after washes to remove the unbound phages, the remaining ones are eluted and amplified in *E.coli*. This process called biopanning emulates the natural selection, and after several rounds the recombinant phages are selected (Mimmi et al., 2019). The antibody fragments obtained by this protocol have shown greater selectivity, higher binding capacity and more favorable pharmacokinetic properties. The Phage Display is very extended in the biomedical and biosensor field (Richards et al., 2017).

Proteins: native proteins and engineering proteins are often coupled to nanoparticles for different applications. The procedure is the same described for the antibody conjugation and usually covalent binding is performed. However, the development of new affine matrices has opened new options in this field. It is usual that engineered proteins expressed in *E. coli* carry a polyhistidine tag (His-tag) for its purification by affinity chromatography. The development of nickel or cobalt nitriloacetate (Ni/Co-NTA) nanoparticles, which bind proteins by the His-tag, is being used for protein purification but also for other procedures as binding of proteins for downstream applications. The application of the His-tag as a purification method for this type of proteins have worked very well in complex matrices as cell crude lysates (Kim et al., 2007). Moreover, these particles have also been tested for biosensing, showing high efficiency. One of the advantages of this procedure is the known location of the binding site of the protein, which can be oriented in the correct way due to the binding by the polyhistidine-tag (see **Figure 11**). In addition, this binding is easy and fast, and the protein can be easily eluted with 500 mM imidazole (Magdeldin & Moser, 2012). The protein elution allows re-using the nanoparticles.

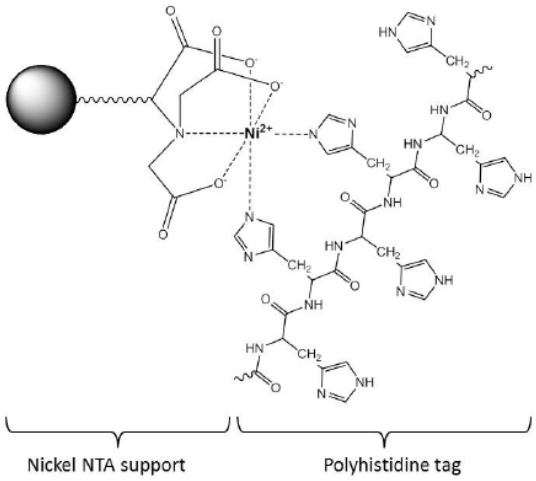

Figure 11. Complex of the nickel (II) NTA support with a polyhistidine tagged protein (Magdeldin & Moser, 2012).

3. Peptides: the development of the Phage Display technique has allowed the discovery of new ligands such as affine peptides. Peptides are smaller chains of aminoacids which can be used as antibodies or proteins for bacterial bioseparation from different sources as water or food. The biopanning process performed in Phage Display identifies affine peptides against the target antigen. Peptides can be used as ligands to coat nanoparticles easily by procedures as those described for antibodies and proteins. Despite their short size, peptides have proved similar sensitivity than antibodies for bacterial bioseparation using MNPs (Steingroewer et al., 2007). One of the main advantages of affine peptides is their simple synthesis and that the use of experimental animals is not necessary as in the production of antibodies.

5.4. BMS in food microbiology: trends and applications

Biomagnetic separation is being used in food microbiology for the capture of food pathogens, such as *Salmonella* and *E.coli,* and food spoilage microorganisms. The method normally used to identify these bacteria is the microbiological culture. However, in the last few years new approaches have been developed in order to reduce incubation times and laborious processing. These new techniques include immunological based methods, such as ELISA or lateral flow technique, nucleic acid amplification methods as PCR, qPCR and LAMP and biosensors. Furthermore, a pretreatment of food sample is needed before applying the technique to detect the microorganisms, digestion,

centrifugation and filtration, being the treatments usually applied. Although these treatments are cheap and easy to implement in dairy industries, the main disadvantage is their non-specificity. To resolve this problem, separation based on magnetic nanoparticles is being applied at a research level (Wang et al., 2020).

There are many factors that affect the capture efficiency (CE) of bacteria by magnetic nanoparticles. One of them is the size of the particles used for the analysis. Although particles of 1 μm or larger are desirable for a rapid recovery with the magnet, it has been demonstrated by many authors that small nanoparticles, of 500 nm or lower, are more effective in capturing the bacteria (Chen & Park, 2018; Du et al., 2018). The explanation is that a high number of small nanoparticles provides a larger surface area and more binding sites for the ligands that capture the bacteria (Wang et al., 2020). However, although this observation has been made by different authors there are studies in which magnetic particles of large size have been used successfully with high CE (Wei et al., 2016).

Another important factor that affects biomagnetic separation is food matrix where the separation takes place. Different authors have observed that biomagnetic separation can be successful in aqueous buffers, such as PBS, but the results obtained change when the assay is performed in food matrices. For example, Zheng et al. (2016) reported that the maximum CE achieved for *Salmonella* when the assay was performed in mung bean sprout was 38.2%, in comparison with that obtained in pure cultures of 72%. They concluded that the nanoparticles bound to the non-specific microbiota, which caused low CE. Milk has been also evaluated as a matrix for biomagnetic separation, and similar observations were found. Kim et al. (2014) measured CE for *Salmonella enteritidis* in spiked PBS and they obtained high percentages (90%); however, lower CE (< 30%) was found in milk. Milk is a complex medium consisting of proteins, fat globules and other molecules, which may interfere with the capture of target bacteria.

Despite the interferences that may cause food matrix and considering that many other factors have an influence, such as incubation time, number of MNPs and bacterial concentration, many efforts have been made to develop reliable bacterial capture systems based on MNPs (see **Figure 12**).

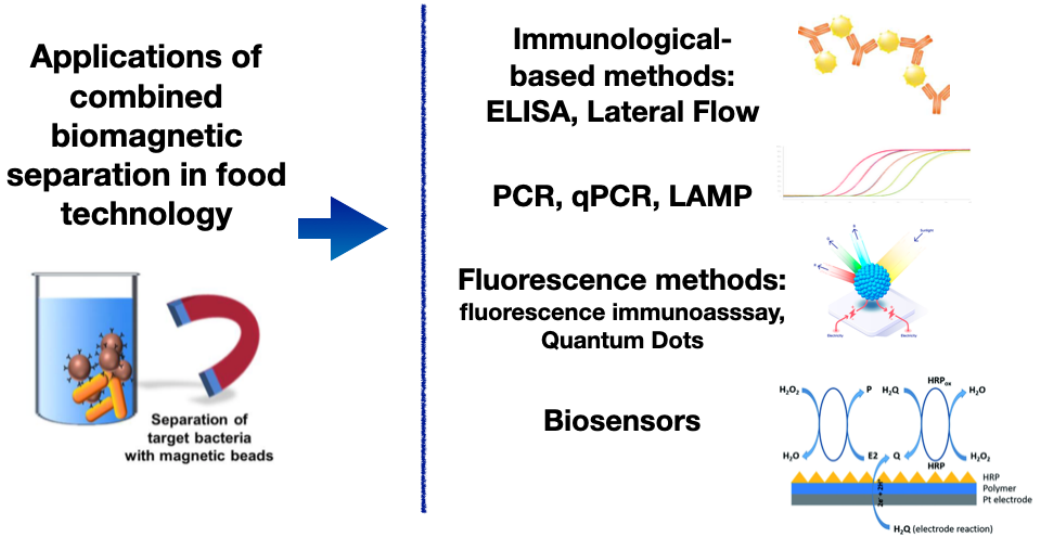

Figure 12. Biomagnetic separation and principal assays used in combination for detection of food microorganisms. Adapted from Whang et. al (2020).

Immunomagnetic separation has been combined with ELISA assay to detect *Salmonella* in skimmed milk powder within 21 h. This procedure was found to be as efficient as the commercial ELISA assays available and even faster. However, a pre-enrichment of the microorganism was needed for an accurate detection (Mansfield & Forsythe, 2000).

Biomagnetic separation followed by detection with lateral flow assay has been performed in the case of *Bacillus* spores. For this purpose, *Bacillus anthracis* spores (Wang et al., 2015) were captured from milk, baking soda and starch with magnetic beads coated with specific antibodies, without pre-treatment of the sample. Then, magnetic beads with the bound spores were located in the lateral flow strip to identify and quantify the spores. Moreover, Fisher et al. (2009) developed a protocol in which the spores of *Bacillus anthracis* were captured from milk samples by beads coated with antibodies and then, the bacteria were eluted and put directly onto the lateral flow strip. The combination of magnetic separation with lateral flow assay provided better LOD than using directly the lateral flow assay. In both methods the LOD was found between 10^4 cfu-10^5 cfu per mL of milk or gram of dairy product tested.

In the case of *C. tyrobutyricum* spores an specific peptide obtained by the Phage Display technique and bound to MNPs was compared with antibody coated particles

(Lavilla et al., 2012). In this study, peptide-nanoparticles were found to achieve better CE and stability compared with the antibody-nanoparticles. The particle size and coating method were found very critical to have good results. Although the results obtained in this study were quite promising, the CE was only evaluated in PBS and not in milk.

Although BMS works well with immunology-based methods, in some studies the combination of BMS with PCR (conventional PCR or qPCR) or LAMP has resulted in lower detection limits than those obtained with the antibody-based techniques. As an example, Vinayaka et al. (2019) developed an immunomagnetic separation with protein A coated beads with specific antibodies and subsequent PCR to detect *Salmonella enterica* in a variety of samples, such as vegetable salad, egg yolk and mince pork meat, among others. This combination overcomes the difficulties of complex matrices where usually the pathogen is found, and avoids the pre-processing before DNA extraction and the presence of inhibitors in the qPCR reaction. In that study, the LOD achieved was found 2 cfu/mL, considerably lower compared with that obtained with the ELISA or lateral flow chromatography (Vinayaka et al., 2019).

In another study, a new approach based on magnetic particles coated with recombinant bacteriophages was developed and applied successfully (Wang et al., 2016). With this method, viable *E. coli* cells were captured and detected by PCR in raw food products with a LOD of 10^2 cfu/mL. Engineered bacteriophages were biotinylated and coated to magnetic nanoparticles providing an efficient and specific separation.

Although many assays combining BMS and PCR have been developed, other procedures, such as fluorescence methods or biosensors can also be used in combination. Biosensors are a good promising tool for the detection of food microorganisms and many efforts have been made to develop different platforms. Thus, an impedance biosensor was developed for the detection of *Listeria monocytogenes* (Wang et al., 2017). For this purpose, specific antibodies against the microorganism and urease were used to coat gold nanoparticles. Bacterial cells were captured with high recovery values (94.7%-103.8%) and after washing, a solution containing urea was added, the catalyzed reaction allowed to measure bacterial concentration in the spiked sample. Furthermore, in another study (Wang et al., 2020), immunocapture was combined with a fluorescence biosensor based on quantum dots (QD) to detect *E. coli*

O157:H7. The addition of immunocapture increased considerably the sensitivity of this last assay.

Even though many strategies have been developed, the application of BMS to food science is still on a research stage. As pre-treatment is a very promising technique because it allows the concentration and enrichment of targeted bacteria from a great diversity of food samples. This is very important for detecting foodborne microorganisms considering their low concentration in raw samples. However, nanoparticles and microparticles are still an expensive option for many industries. Moreover, the different matrices where the microorganisms can be found may be a limitation for BMS application. In the future, cost reduction of MNPs production and discovery of new high affinity ligands will contribute to extend the application of BMS to food analysis.

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Hypothesis and research aims

Hypothesis:

Microbiological methods, such as MPN, are not suitable to detect *Clostridium tyrobutyricum* spores in raw milk because of the long time to obtain the results, which makes impossible to decide whether milk can be used to elaborate certain types of cheese. For this reason, fast and specific methods are needed for detecting *C. tyrobutyricum* spores in raw milk in a short time, no longer than one day.

Main objective:

Developing a new method to detect *C. tyrobutyricum* spores in raw milk based on Real-Time PCR (qPCR) and biomagnetic separation.

Specific objectives:

1. Development of a qPCR method to detect genomic DNA from vegetative cells and spores of *C. tyrobutyricum*.
2. Screening of different methods to disrupt *C. tyrobutyricum* spores and detect genomic DNA by qPCR.
3. Development of a procedure to detect *C. tyrobutyricum* spores in raw milk samples based on qPCR.
4. Detection of *C. tyrobutyricum* spores in raw milk samples from milk quality control laboratories.
5. Study of *Clostridium* species and other bacteria in raw milk samples.
6. Characterization of ligands with affinity for *C. tyrobutyricum* spores.
7. Development of a capture system for *C. tyrobutyricum* spores from raw milk based on magnetic particles coated with specific ligands and further detection by qPCR.

Chapter 1

Evaluation of methods for *Clostridium tyrobutyricum* spores disruption and detection by Real-Time PCR

1. Introduction

C. tyrobutyricum spores are resistance life forms difficult to disrupt. The first chapter of this thesis is focused on the screening of different disruption methods to optimize the extraction of genomic DNA from *C. tyrobutyricum* spores. The obtention of purified genomic DNA is critical to develop an efficient Real-Time PCR. Moreover, different sporulation conditions were tested to know if the sporulation process affected the spore disruption.

The calibration of Real-Time PCR (qPCR) was previously made with CTfla primers and genomic DNA from vegetative cells of *C. tyrobutyricum* CECT 4012 strain. After the calibration of qPCR, several treatments (chemical, enzymatic and mechanic) were evaluated to disrupt *C. tyrobutyricum* spores and the genomic DNA extracted in all conditions was amplified by qPCR. The selected disruption methods were applied to UHT milk spiked with known amounts of *C. tyrobutyricum* spores. A pre-treatment of milk based on subtilisin digestion was evaluated for spore detection in order to improve spore recovery. Finally, the methods selected as most efficient for spore disruption were applied to raw milk samples.

2. Material and methods

2. 1. Sporulation process

C. tyrobutyricum spores were obtained by two different methods to find the most efficient way to produce them: sporulation on agar plates and in liquid medium. *C. tyrobutyricum* CECT 4012 strain was obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). Bacteria were cultured from the CECT stock kept in cryovials at -80°C following the instructions of the supplier. One cryobead was inoculated in tubes with 10 mL of Reinforced Clostridial Medium (RCM) (Scharlau, Barcelona, Spain), a 2 cm layer of sterile liquid paraffin was added on the top of medium to create anaerobiosis and the tubes were incubated for 24 h at 37°C. Afterwards, 100 µL of bacterial suspension were spread uniformly over RCM agar plates. The plates were incubated inside Gaspak jars using the anaerobic gas generator AnaeroGen™ 2.5 L (Oxoid, Basingstoke, UK) and an anaerobic indicator (Oxoid) for 7 days at 37°C. The colonies were collected with a Digralsky spreader adding 4 mL of phosphate buffered saline (PBS) consisting of 140 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4. After collection of the colonies, the suspension of sporulated cells was centrifuged at 4000 x *g* for 15 min at 4°C and washed three times with sterile distilled water.

The sporulation in liquid medium was carried out from a 24 h *C. tyrobutyricum* culture in RCM medium, as described before (Lavilla et al., 2010). Briefly, 1 mL of the preculture obtained as explained above was inoculated into 50 mL of fresh RCM medium with a paraffin layer of 4 cm to create anaerobiosis. After 24 h, 50 mL of culture was inoculated into a molecular weight cut-off (MWCO) 12-14 kDa dialysis membrane (CelluSep, Membrane Filtration Products, Korea) immersed into Tryptone-Glucose-yeast Extract (TGE) medium (see **Table 2**). The membrane was activated previously, with distilled water for at least 30 min, changing the water several times during this period.

The dialysis membrane was introduced into a device that was composed of a glass tube (40 cm height x 7 cm diameter) closed with a stopper with three holes (**Figure 13A**). One of the tubes was used to inoculate the culture into the dialysis membrane, the second tube to pump in N_2 to create anaerobiosis (between 5 and 10 min of N_2 bubbling) and the third one to eliminate the residual gases originated from bacterial growth. The culture was incubated at least for 72 h and afterwards, the suspension of

sporulated cells was centrifuged at 4000 x *g* for 15 min at 4°C and washed three times with sterile distilled water.

Table 2. Tryptone-Glucose-Yeast Extract (TGE) medium based on Bergère & Hermier, (1970).

TGE medium (composition for 1 L)	
Tryptone	10 g
D-glucose	10 g
Yeast extract	1 g
Ascorbic acid	1 g
Sodium thioglycolate	0.5 g
$MgSO_4 \cdot 7H_2O$	200 mg
$MnSO_4 \cdot 7H_2O$	7 mg
$FeSO_4 \cdot 7H_2O$	10 mg
$CaCl_2 \cdot 12H_2O$	73.5 mg
Na_2HPO_4	2.3 g
NaH_2PO_4	1.7 g

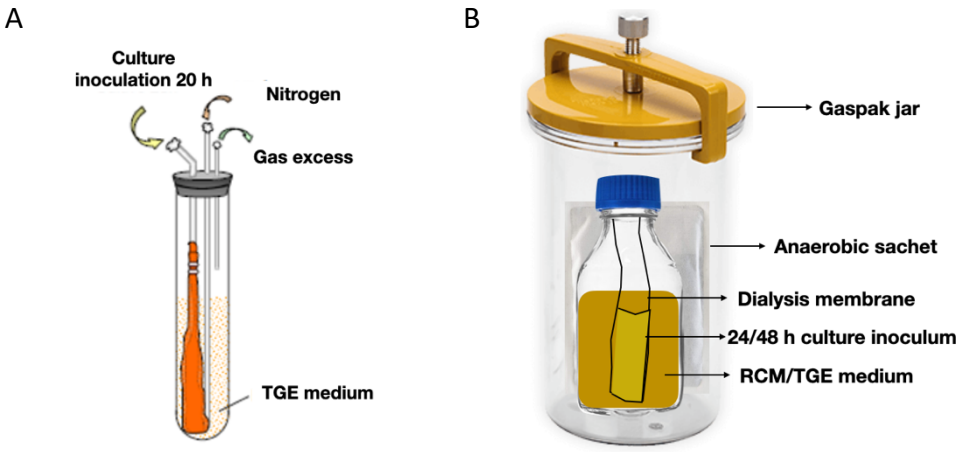

Figure 13. (A) Sporulation system in liquid medium adapted from Lavilla et al. (2008) and (B) sporulation system based on Bassi et al. (2013).

The sporulation in liquid medium was also performed as a variation of the system previously described by Bassi et al. (2013). The sporulation chamber was composed by a flask of 500 mL with 450 mL of RCM (for *C. tyrobutyricum* UZ01 strain) or TGE (for *C. tyrobutyricum* CECT 4012 strain) medium with a dialysis membrane of 10 kDa pore size and 30 cm length inside (CelluSep) previously activated as described before (see **Figure 13B**). After this step, the dialysis membrane was closed at one end with two knots. Then, 20 mL of non-sterile RCM/TGE medium was added into the dialysis membrane and all the system was sterilized with steam autoclave. A preculture of bacteria was prepared as described above and added into the dialysis membrane. Then, the flask was closed and put into a Gaspak chamber with an anerobic generator (AnaeroGen™ 2.5 L) and incubated for at least one month. After this time, the spores were collected as described in the previous paragraph.

2.2. Purification of spores

The bacterial cells obtained by the two sporulation methods were lysed with lysozyme from egg white (Sigma-Aldrich, St. Louis, MO, USA). The lysozyme was added to the suspension at a concentration of 0.4 mg/mL and the mixture was incubated for 24 h at 45°C in a total volume of 100 mL. Afterwards, the suspension was centrifuged for 15 min at 4000 x *g* and 4°C. The supernatant was discarded, and the precipitate was washed twice in the same conditions, with sterile distilled water to eliminate the cellular debris. The precipitate obtained was resuspended in 2 mL of sterile distilled water and applied to a Percoll® gradient (Sigma-Aldrich) to separate the spores from vegetative cells, as described before (Leuschner et al., 1999). The gradient was prepared with different concentrations of Percoll®: 1.13, 1.11, 1.09, 1.07 and 1.05 g/mL. The dilutions were prepared with the following equation:

$$V_0 = V\,(\,p - 0.1\,p_{10} - 0.9)/(\,p_0 - 1) \quad [mL]$$

Where:

V_0 = volume of pure Percoll®

V = volume of the desired solution

p = density of the desired solution

p_{10} = water density

p_0 = pure Percoll® density (1.13 g/mL at 20°C)

To create the gradient, 8 mL of each concentration of Percoll was added in a falcon tube, starting from the densest and ending with the less dense. Afterwards, 2 mL of the lysate was added on top of the gradient and it was centrifuged for 45 min at 4000 x *g* and 4°C. After centrifugation, the spores were collected from the bottom of the gradient, washed 5 times with sterile distilled water and recovered by centrifugation at 13000 x *g* for 10 min. The precipitate containing the spores was resuspended in 200 µL of PBS and the concentration was determined by counting them in a Thoma chamber under a phase contrast microscope ECLIPSE E400 (Nikon, Tokyo, Japan). Finally, the spore suspension was stored at -20°C until use.

2.3. Calibration curve of Real-Time PCR for *C. tyrobutyricum*

2.3.1. DNA extraction from C. tyrobutyricum vegetative cells

The calibration curve for qPCR with CTfla primers was done with 10-fold dilutions of genomic DNA from vegetative cells of *C. tyrobutyricum* CECT 4012. For genomic DNA obtention, vegetative cells were cultured in RCM broth under anaerobic conditions for 48 h. After that, 1.5 mL of the bacterial culture was collected and centrifuged at 13000 x *g* for 15 min. The DNA was obtained with GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Burlington, MA, USA) following the manufacturer instructions. First, lysozyme (Sigma-Aldrich) was added to the precipitate at a final concentration of 0.45 mg/mL and the mixture was incubated at 37°C for 30 min. After incubation, 20 µL of proteinase K at 20 mg/mL was added to the homogenized mixture and incubated at 55°C for 15 min. The lysate was diluted with ethanol and added to the silica column for DNA purification. The protocol was applied following manufacturer instructions. Genomic DNA was stored at -20°C until its use.

2.3.2. Real-Time PCR (qPCR)

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

The primers used were the CTfla (**Table 3**), which target the flagellin gen of *C. tyrobutyricum*, described in the study of López-Enríquez et al. (2007).

Table 3. CTfla primers sequence as described by López-Enríquez et al. (2007).

Primer	Sequence
CTflaF	5'-CAGTTACAATTACGAGAACACATGGA-3'
CTflaR	5'-TGTACCACCAACTAAAGCAACATCA-3'

The amplification process has an initial stage of 2 min at 50°C, where the enzyme uracil-DNA-glycosylase (UNG) is activated. The UNG removes the uracil present in the DNA cleaving the strand. This enzyme is used in qPCR to avoid carry-over contamination especially from previously amplified products. The following steps of the amplification process are common for both types of probes (SYBR Green and TaqMan), the first step being an initial denaturation of one cycle at 95°C during 10 min, then 50 cycles of amplification, with a denaturation at 95°C for 15 s, hybridization and elongation at 60°C during 1 min. The threshold cycle value (Ct) was obtained from the amplification curve and it was inversely proportional to the number of DNA copies. After 50 cycles of amplification, a melting curve process is performed that gives the melting temperature of the amplified DNA fragments (**Table 4**) .

Table 4. Conditions for qPCR amplification.

No. of cycles	UDG-activation		Previous denaturation		Denaturation		Annealing and polimerization	
	T (°C)	Time (min)	T (°C)	Time (min)	T (°C)	Time (s)	T (°C)	Time (min)
1	50	2 min	95	10	-	-	-	-
50	-	-			95	15	60	1

Each sample of genomic DNA was analyzed in duplicate by qPCR in three independent 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 with the following formula where x are the nanograms of DNA and N is the length of the genomic DNA (Prediger, 2013):

$$\text{Number of copies (molecules)} = \frac{\text{X ng} * 6.0221\text{x}10^{23}\ \text{molecules/mol}}{(\text{N} * 660\ \text{g/mol}) * 1 * 10^{9}\ \text{ng/g}}$$

In order to estimate the genomic length we considered the *C. tyrobutyricum* strain W428 chromosome, complete genome 3,011,209 bp circular DNA (GenBank accession number: CP016280.1.).

The efficiency was calculated according to "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR experiments". Robust and precise qPCR assays are usually correlated with high PCR efficiency (Bustin et al., 2009), which should be about 90-105% (Kralik & Ricchi, 2017). The linear dynamic range and the Limit of Detection (LOD) (Bustin et al., 2009) were also calculated.

The specificity of qPCR is given by the specificity of primers. In this study, the specificity was checked by an *in-silico* study in the National Center for Biotechnology Information (NCBI) by using the Basic Local Alignment Search Tool (Primer-BLAST).

The repeatability (intra-assay variation) was calculated as the coefficient of variation (CV) between the replicates for each point of the calibration curve in nanograms of DNA, in the same experiment. The CV was set up using the following formula CV = (SD*100)/ X, as SD is the standard deviation and X the mean Ct obtained (Broeders et al., 2014; Kralik & Ricchi, 2017).

The reproducibility (inter-assay variation) was calculated as the CV between runs with the following formula CV = (SD*100)/ X, as SD is the standard deviation and X the nanograms of DNA obtained for each point of the calibration curve. Reproducibility should have values of CV≤25% (Broeders et al., 2014; Kralik & Ricchi, 2017).

2.4. Disruption of spores: screening of different methods

Considering the difficulty to extract DNA from the spore core, it was necessary to test different disruption methods. The spores were resuspended in PBS after all the treatments. For each extraction, three independent experiments were carried out in three different days.

2.4.1. Microwave treatment

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

2.4.2. Bead beating treatment

The bead beating (BB) procedure was carried out following the method by Vandeventer et al. (2011) with some modifications. For spore lysis, 250 mg of 100 µm-diameter silica beads and 625 µL of 10^6 spores/mL suspension was added in tubes of 2 mL. The samples were bead beaten using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK, USA) at maximum speed for 1 min pulses, three times with an interval of 30 s, placing the tubes on ice between pulses.

2.4.3. DNA precipitation

The DNA precipitation protocol was followed according to Sambrook (2001). A volume of 10 µL of sodium acetate was added to a 100 µL suspension of *C. tyrobutyricum* spores at a concentration of 10^6 spores/mL, previously disrupted by MW or BB treatment. Then, 220 µL of 100% cold ethanol was added and incubated for 1 h at -80°C. Afterwards, the mixture was centrifuged for 15 min at 13000 x *g* (4°C). The supernatant was discarded, and the pellet was resuspended in 500 µL of 80% cold ethanol and centrifuged for 10 min at 13000 x *g* (4°C). Finally, the supernatant was discarded, and the pellet was air dried and resuspended in 100 µL of filtered Milli-Q water.

2.4.4. Proteinase K treatment

The enzyme proteinase K was added at a final concentration of 2 mg/mL to 100 µL of 10^6 spores/mL suspension and the mixture was incubated at 55°C for 30 min. Then, the proteinase was inactivated by incubating the mixture at 90°C for 10 min.

2.4.5. Heat treatment

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

2.4.6. Mercaptoethanol-lysozyme treatment

This treatment, described in the study of Torok (2003), required the addition to 100 µL of 10^6 spores/mL suspension, 90 µL of a buffer containing 3.6 M guanidine hydrochloride (pH 2.8) with 10% of 2-mercaptoethanol and 1 mg/mL lysozyme dissolved in 10 mM Tris-HCl buffer, pH 8, 1 mM EDTA and 5% Triton X-100. The solution was incubated for 1 h at 45°C.

2.4.7. Lysozyme-proteinase K-guanidine hydrochloride-Tween 20-Triton X-100 treatment

The method reported by López-Enríquez et al. (2007) for the quantification of *C. tyrobutyricum* using qPCR was followed with some modifications. First, 200 µL of 10 mM Tris-HCl, pH 8, with 50 mM EDTA, and 10 µL of 100 mg/mL lysozyme was added to 200 µL of a 10^6 spores/mL suspension. This mixture was incubated for 1 h at 37°C. Afterwards, one volume of 10 mM Tris-HCl, 1% SDS, 100 µg/mL of proteinase K was added to the suspension and the mixture was heated for 1 h at 37°C.

Then, one volume of 30 mM Tris-HCl buffer, pH 8, with 0.8 M guanidine hydrochloride, 7.5 mM EDTA, 5% Tween 20 and 0.5% of Triton X-100 was added to the suspension. After incubating at 65°C for 45 min, the mixture was centrifuged at 13000 x *g* for 10 min and the pellet was discarded. Afterwards, a phenol-chloroform extraction procedure was carried out for protein elimination, adding one volume of that mixture, and the resulting sample was transferred to a column of the kit GenElute™ Bacterial Genomic DNA for DNA purification.

2.4.8. DNA purification

DNA from vegetative cells was isolated with the DNA purification kit GenElute™ Bacterial Genomic DNA following the instructions of the manufacturer referred to Gram-positive bacteria and previously described in section 2.3.1.

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

2.5. Analysis of UHT milk spiked with *C. tyrobutyricum* spores by qPCR

A volume of 500 µL commercial UHT whole milk, with 3.6% fat and 3% protein, was spiked with a final concentration of 10^6 spores/mL of *C. tyrobutyricum* obtained by the liquid medium procedure, as previously described in section 2.1. Contaminated milk was treated with subtilisin, a non-specific protease used to treat milk samples for bacterial enumeration by BactoScan equipment (Foss, Hilleroed, Denmark), which was dissolved in the buffer provided by the manufacturer. A 500 µL volume of subtilisin solution (28 volumes of buffer:1 volume of subtilisin) was added to 500 µL of UHT milk spiked with spores. The mixture was heated at 60°C in a water bath for 1 h and centrifuged at 13000 x *g* for 30 min at room temperature. After centrifugation, milk fat and proteins were discarded, and the spores precipitated at the bottom of the vial were recovered. The spores were resuspended in 100 µL of PBS for MW treatment or in 625 µL for bead beating. After spore disruption, DNA was extracted with the GenElute™ Bacterial Genomic DNA kit. Two samples of milk were spiked with spores and analyzed by qPCR in duplicate. The experiment was repeated three times in different days.

2.6. Analysis of spore recovery in subtilisin-treated UHT milk

The efficiency of spore recovery with the subtilisin treatment was analyzed by colony counting. For this objective, 18 mL of UHT whole milk was spiked with *C. tyrobutyricum* spores to reach final concentrations of 10^7 or 5.55 x 10^5 spores/ml. Two samples of contaminated UHT milk were treated with subtilisin (18 ml of subtilisin and buffer) and other two samples were set up as controls without subtilisin. Samples were incubated for 1 h at 60°C followed by centrifugation at 13000 x *g* for 30 min.

After centrifugation, the milk fat layer and the precipitate with the spores were visible. The fat layer and the precipitate were recovered and each was resuspended in 10 mL of PBS. For the precipitate a 10^{-3} dilution in PBS was needed to count the colonies. For the fat layer of the untreated milk a 10^{-2} dilution was done and the fat layer of the subtilisin-treated milk was directly cultured. For each dilution or condition, 100 µL of suspension was spread on RCM agar plates. After 24 h of incubation at 37°C in anaerobic conditions the colonies were counted.

2.7. qPCR analysis of spores obtained by different disruption methods

In this analysis two probes were used for qPCR amplification, SYBR Green and TaqMan probe. In the case of SYBR Green reagent, the protocol applied was the same as described in section 2.3.

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

The primers used in both assays were the CTfla, which target the flagellin gen of *C. tyrobutyricum*, described in the study of López-Enríquez et al. (2007).

The amplification process, in the case of SYBR Green qPCR, has an initial stage at 50°C for 2 min, which activates the enzyme uracil-DNA-glycosylase (UNG), which is not present in the TaqMan probe procedure. The following steps of the amplification process are common for both types of qPCR and are described in section 2.3.

The amplification products were visualized in 1.5 % agarose gels stained with SYBR Safe® (Invitrogen). For this protocol, the agarose was dissolved in 0.089 M Tris-borate buffer (TBE), pH 8, with 2 mM EDTA. The loading buffer used to dilute the samples was composed by 0.25% (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanole FF and 15% (w/v) glycerol dissolved in sterile distilled water. The gel was run in a Sub-Cell GT cell (Biorad) for 45 min at 90 V. The bands were visualized in UV transilluminator (UVP BioDoc-It system, CA, USA).

2.7.1. Comparative analysis of DNA amplification from vegetative cells and spores

Genomic DNA from vegetative cells was extracted as described in section 2.3.1. Genomic DNA from spores were extracted from 10^7 spores/mL produced in liquid medium resuspended in 100 ul of PBS. The MW treatment and column purification was applied as described in section 2.4.1 and 2.4.8.

A step of ethanol precipitation in the presence of sodium acetate was also required to achieve a higher amount of DNA. The concentration of DNA extracted from spores and vegetative cells was measured using a NanoDrop 1000 (Thermo Fisher

Scientific, CA, USA) and diluted to achieve the following DNA concentrations: 30, 20, 10, 1, 0.1 and 0.01 ng/µL. A volume of 2 µL of each DNA solution was analyzed by qPCR by duplicate in three independent assays.

2.8. Applicability of the selected method: MW treatment and column purification

To study the applicability of MW and column purification method, selected as the best one, calibration curves were made in PBS and UHT milk. For this purpose, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 total spores of the CECT 4012 and UZ01 wild strains of *C. tyrobutyricum* were added in a final volume of 100 µL PBS and 500 µL UHT milk. The concentration of spores/mL was also calculated for each volume and is given in the results section.

To ensure the mixing of spore spiked UHT milk samples, the tubes were agitated for 30 min in a rotator at room temperature. After that, subtilisin was added and spores were recovered by centrifugation. Spores were disrupted by MW treatment followed by column purification, as previously described. The purified DNA was analyzed by qPCR, as described above.

For each condition, qPCR efficiency, linear range and LOD were calculated as previously described in section 2.3.2.

2.9. Analysis of raw milk samples by qPCR

Raw milk samples from Asociación Lechera de Aragón (AILA) were collected and aliquoted. Each sample was named with a code consisting of the letter A followed by the collection date, as an example A171021. These samples were routinely analyzed by AILA to ensure milk quality control, and came from farms that have occasionally LBD. A total number of 50 raw milk samples from AILA were analyzed by qPCR, 34 of them in a sample volume of 0.5 mL and 16 samples in a volume of 18 mL.

Previously to the analysis, milk samples were heated at 90°C for 10 min to inactivate vegetative cells. After heating, a volume of 500 µL or 18 mL was taken and subtilisin was added. Milk samples were treated as described in section 2.5. Then, DNA from milk samples was analyzed by qPCR as described in the same section.

2.10. Data analysis.

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

3. Results and discussion

3.1. Obtention and purification of spores

In **Figure 14A** a culture of *C. tyrobutyricum* vegetative cells grown for 48 h is shown. Although most of them are vegetative cells with the characteristic bacillus shape, there are also spores that can be easily differentiated by their brilliant and spheric shape. Some vegetative cells were seen with the refringent spore inside.

A B

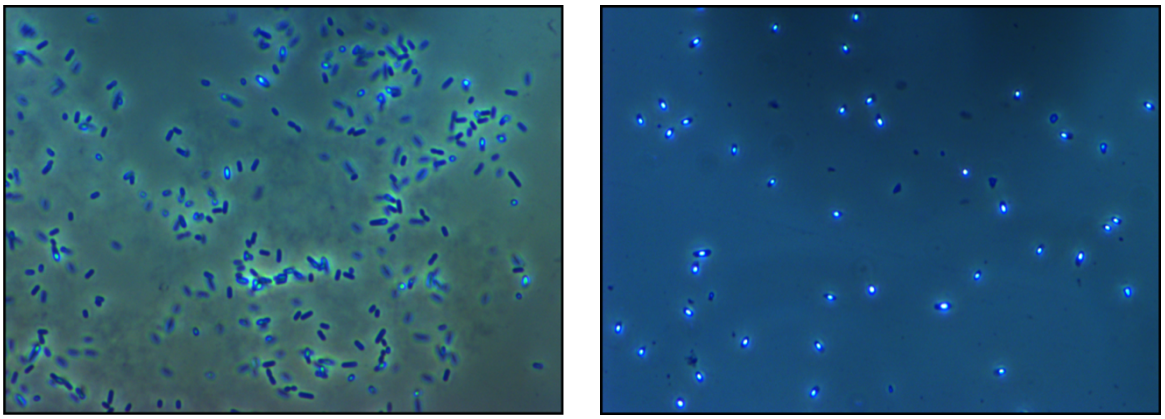

Figure 14. A: *C. tyrobutyricum* CECT 4012 vegetative cells grown for 48 h. B: suspension of *C. tyrobutyricum* CECT 4012 spores after purification by Percoll® gradient. Both preparations were visualized under optical microscope at 400x.

As it was demonstrated previously (Lavilla, 2008), the sporulation of *C. tyrobutyricum* in the dialysis membrane system allowed achieving 30-35% of spores in the suspension after 72 h of incubation. However, in the case of the sporulation procedure in agar plates, after 7 days of incubation higher percentages of spores were obtained compared to the liquid medium and the sporulation rate was found to be 50-60%. For this reason the sporulation on agar plate was considered as the best procedure to produce spores.

In **Figure 14B** a suspension of spores after being separated by the Percoll® gradient is shown. After this treatment, 95-100% of the cells found in the suspension were refringent spores.

3.2. Calibration curve of qPCR using CTfla primers

The calibration curve of qPCR with CTfla primers was done with 10-fold dilutions of DNA from vegetative cells of *C. tyrobutyricum* CECT 4012 (**Table 5**).

Table 5. Data obtained to build the calibration curve of qPCR with CTfla primers with 10-fold dilutions of genomic DNA from *C. tyrobutyricum* CECT 4012 vegetative cells. DNA was extracted using GenElute™ kit. Ct mean: cycle threshold mean; SD: standard deviation; Log (ng DNA): logarithm (nanograms DNA); GE: genome equivalent.

Total ng of DNA	Log (ng DNA)	Calculated GE	Ct mean	SD	Number of positive reactions
5	0.7	$1.5 \cdot 10^6$	19.34	0.24	6/6
$5 \cdot 10^{-1}$	-1.3	$1.5 \cdot 10^5$	22.89	0.12	6/6
$5 \cdot 10^{-2}$	-2.3	$1.5 \cdot 10^4$	26.22	0.15	6/6
$5 \cdot 10^{-3}$	-3.3	$1.5 \cdot 10^3$	29.78	0.19	6/6
$5 \cdot 10^{-4}$	-4.3	$1.5 \cdot 10^2$	32.95	0.12	6/6
$5 \cdot 10^{-5}$	-5.3	$1.5 \cdot 10^1$	35.57	0.53	6/6
$5 \cdot 10^{-6}$	-6.3	1.5	35.94	0.72	6/6
$5 \cdot 10^{-7}$	-7.3	0.15	37.42	0.95	6/6
		Linear equation:	**y= -3.307x + 21.785**	**R^2= 0.9978**	

According to "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR experiments" (Bustin et al., 2009), the efficiency of qPCR should be in the range of 90-105%. For CTfla primers the efficiency was set up in 98.79%. The linear range was analyzed between 5 ng of DNA and $5 \cdot 10^{-4}$ ng of DNA (150 genome equivalents). The sensitivity was expressed as the LOD (Bustin et al., 2009) and for the CTfla primer calibration curve was established in 10^{-7} ng of DNA.

The specificity of qPCR is given by the specificity of the primers, and those used in our study were checked previously by López-Enríquez et al. (2007). In their study different *Clostridium* species were tested, together with other non-clostridia bacteria, and the primers were found to be specific for *C. tyrobutyricum*. Moreover, we also

checked the specificity by an *in-silico* study in NCBI Primer-BLAST, and only one sequence was found with a length of 82 bp targeting *C. tyrobutyricum* (GenBank code: AJ242662). The melting curve was analyzed in each experiment and only one peak was obtained with Tm = 76.61 ± 0.10. Non-template controls were found negative in qPCR and no primer dimers were found. The repeatability (intra-assay variation) CV was found to be ≤5.8%, which is a good value, as is established that the CV should be ≤25% (Broeders et al., 2014; Kralik & Ricchi, 2017). The CV of reproducibility (recently named as intermediate precision) was calculated for each point of the calibration curve as presented in **Table 6**. The CV value is recommended to be ≤ 25% (Broeders et al., 2014; Kralik & Ricchi, 2017).

Table 6. Reproducibility analysis of each point of the calibration curve obtained by qPCR with CTfla primers for 10-fold dilutions of genomic DNA from *C. tyrobutyricum* CECT 4012 vegetative cells. DNA was extracted using GenElute™ kit. ng of DNA: nanograms of DNA; CV: coefficient of variation; SD: standard deviation (n=3).

ng of DNA	Calculated ng of DNA (mean)	SD	CV
10	11.10	$6.65 \cdot 10^{-1}$	5.99
5	5.60	$2.70 \cdot 10^{-1}$	4.82
$5 \cdot 10^{-1}$	$4.75 \cdot 10^{-1}$	$1.76 \cdot 10^{-2}$	3.71
$5 \cdot 10^{-2}$	$4.65 \cdot 10^{-2}$	$2.19 \cdot 10^{-3}$	4.72
$5 \cdot 10^{-3}$	$3.92 \cdot 10^{-3}$	$7.32 \cdot 10^{-4}$	18.66
$5 \cdot 10^{-4}$	$4.28 \cdot 10^{-4}$	$3.16 \cdot 10^{-5}$	7.39
$5 \cdot 10^{-5}$	$7.00 \cdot 10^{-5}$	$1.26 \cdot 10^{-5}$	17.94
$5 \cdot 10^{-6}$	$5.57 \cdot 10^{-5}$	$1.64 \cdot 10^{-5}$	29.35
$5 \cdot 10^{-7}$	$2.05 \cdot 10^{-6}$	$8.13 \cdot 10^{-6}$	39.68

The results show that a CV>25% was obtained for $5 \cdot 10^{-6}$ and $5 \cdot 10^{-7}$ ng of DNA, which corresponds respectively with 1.5 and less than 1 genome equivalent. This fact reveals that for less than 1.5 genome equivalents the assay is not reproducible. It is known that low DNA concentrations, usually below 10 or 100 DNA copies, produce high variability in the results due to a Poisson distribution (Thermofisher Scientific, 2022).

3.3. Evaluation of the spore disruption methods

The main aim of this study was to find an efficient and reliable method to obtain the highest amount of pure genomic DNA as possible from *C. tyrobutyricum* spores to be amplified and allow their subsequent detection. The results obtained by the disruption methods under qPCR evaluation are shown in **Table 7**. Two treatments proved to be clearly more effective for spore disruption than the others. The best results were obtained with MW heating followed by DNA purification with silica column, resulting in 100% of positive results with SYBR Green and 83.3% with the TaqMan probe. The second most efficient disruption method was BB followed by DNA purification, which gave an amplification percentage of 66.67% with SYBR Green and 50% with the TaqMan probe. The rest of the treatments analyzed were not considered in further analysis because of the low percentage of amplification obtained.

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

In all the assays performed, genomic DNA from *C. tyrobutyricum* vegetative cells was included as positive control. It was observed that for the same amount of genomic DNA, that obtained from vegetative cells amplified at lower number of cycles than that from spores. The Ct mean for the amplification of DNA from vegetative cells was 22.19

± 0.793 based on the mean of three independent experiments, where it was analyzed as a positive control. This fact will be described and discussed later in this section.

Table 7. Effect of different disruption methods on the detection 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 of three independent experiments with two replicates each (n=6). *Standard deviation is not included as only one sample amplified with this treatment.

Treatment	SYBR Green reagent		TaqMan probe	
	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

(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 8** correspond to the qPCR assays performed after treating liquid medium spores by MW and BB in comparison with those obtained on agar plates. Liquid medium spores amplified at 100% in both methods and with significantly lower Ct values (28.58 ± 1.57) in the case of MW treatment and column purification than that obtained for agar plate spores subjected to the same treatment (33.54 ± 2.05). Different results were obtained for agar plate spores with 66% of amplified samples after BB and column purification and with 100% after MW and column purification. For BB treatment and column purification no differences were found in Ct values between agar and liquid medium spores.

Table 8. Effect of MW and BB for disruption of *C. tyrobutyricum* spores (10^6 spores/mL) generated in liquid medium and on agar plate followed by DNA column purification, on its 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	Liquid medium spores		Agar plate spores	
	Ct value	% amplified samples	Ct value	% amplified samples
MW-DNA column purification	28.58 ± 1.57*	100	33.54 ± 2.05	100
BB-DNA column purification	33.23 ± 1.37	100	33.79 ± 3.08	66.67

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

3.4. Detection of spores obtained in solid or liquid media added to 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 in UHT milk instead of PBS, after spiking it with *C. tyrobutyricum* spores. The results obtained, shown in **Table 9**, are expressed as the percentage of amplification of genomic DNA recovered from spore spiked UHT milk. We observed the same behavior by using MW or BB as that described for PBS. Thus, 100% of amplification was found for DNA samples derived from spores produced in liquid medium extracted by both methods, and 33 and

58% for those from solid medium by BB and MW, respectively. The Ct values were lower in spores from liquid medium than in those corresponding to spores from solid medium. This fact should be considered in order to prepare the standards for a quantification method for *C. tyrobutyricum* spores in milk.

Table 9. Effect of MW and BB on disruption of spores produced in solid or liquid media added to UHT milk; in both methods a final step of column purification was used. qPCR was carried out with SYBR Green. The values represent the Ct mean ± 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	30.13 ± 0.97	100
BB-DNA column purification	38.20 ± 1.18	33	30.52 ± 1.33	100

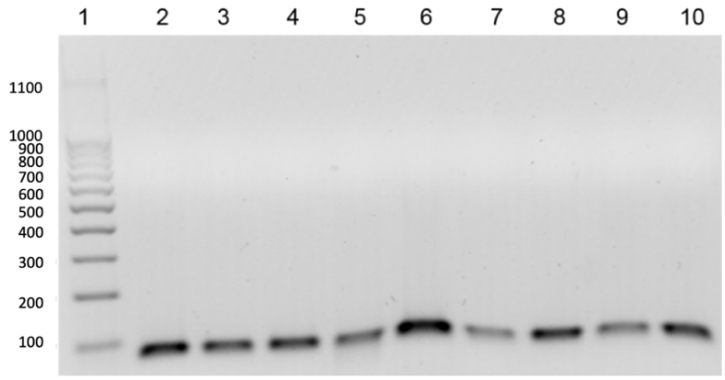

Figure 15. Analysis by agarose gel electrophoresis of qPCR products (82 bp) obtained in the amplification of DNA extracted from *C. tyrobutyricum* spores by different methods. 1: molecular weight DNA ladder; 2: *C. tyrobutyricum* vegetative cells as positive control; 3-6: DNA from spores in PBS , 3: agar plate spores treated by BB*, 4: liquid medium spores treated by BB*, 5: agar plate spores treated by MW*, 6: liquid medium spores treated by MW*; 7-10: DNA from spores in UHT milk treated with subtilisin, 7: agar plate spores treated by BB*, 8: liquid medium spores treated by BB*, 9: agar plate spores treated by MW*, 10: liquid medium spores treated by MW*. *With a final step of DNA column purification.

The qPCR products were analyzed by agarose gel electrophoresis to verify if the size of DNA amplicons was the same after applying the different disruption methods. The results obtained are shown in **Figure 15**, where it can be observed that the amplicons had the expected size of 82 bp, regardless the disruption method used.

Furthermore, we observed differences in the amount of DNA amplified that was higher for solid medium spores than for liquid medium spores. This result correlates well with the lower Ct found for solid medium spores

3.5. Comparative analysis of DNA amplification from vegetative cells and spores

DNA from vegetative cells was isolated by using the GenElute™ kit and that from liquid medium spores was extracted and purified by MW treatment and column purification. After purification, DNA was concentrated by the precipitation protocol described in section 2.4.3. Then, amplification of the same amount of DNA from each extraction was compared. As it can be observed in **Figure 16**, the results clearly show the differences in DNA amplification, as DNA from vegetative cells amplified at lower number of cycles than that from spores. Furthermore, the lowest quantity of DNA from vegetative cells that could be detected was 0.02 ng, while no less than 0.2 ng could be detected in the samples of DNA from spores. The standard curves obtained indicated that there was a difference of about 10 cycles between the Ct values of amplified DNA from vegetative cells and that from liquid medium spores; this difference was maintained for all the concentrations assayed. Consequently, the slope of the two regression lines is similar, indicating that the difference is not due to a random effect. The efficiency obtained in the qPCR of vegetative cells was 96.84%,

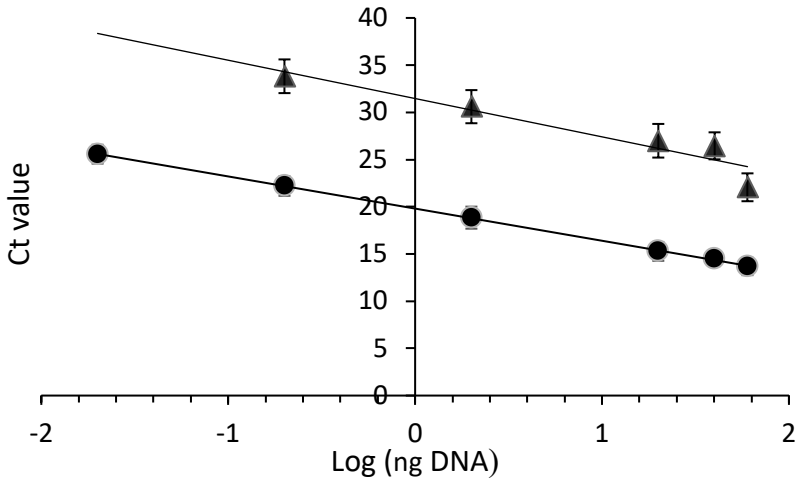

Figure 16. Standard curve obtained by qPCR for amplification of DNA from *C. tyrobutyricum* vegetative cells (●) and liquid medium spores (▲). The equation for the standard curve of vegetative cells is y = -3.406x + 18.779 with R^2= 0.9997 and for spores y = -4.0583x + 30.241 and R^2=0.8991.

The melting curves obtained for the amplification of DNA from liquid medium spores and vegetative cells showed Tm mean values very similar, of 76.18 ± 0.23 and 76.49 ± 0.07, respectively (see **Figure 17**).

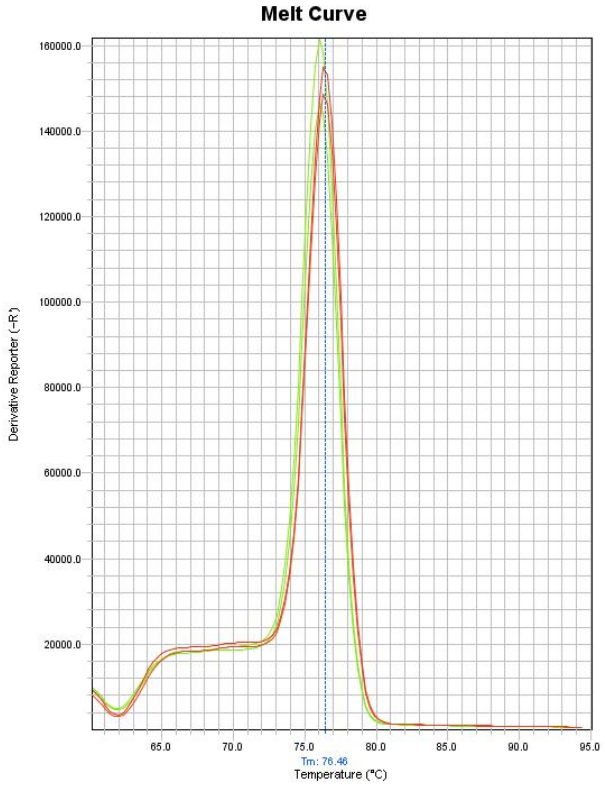

Figure 17. Melting curve obtained for amplification of DNA from vegetative cells (green) and spores (red) by qPCR with CTfla primers.

3.6. Spore recovery from milk treated with subtilisin and from untreated milk

The spore recovery was studied in UHT milk treated with subtilisin and in untreated milk with the aim of verifying that subtilisin treatment improved the procedure outcome compared to a simple centrifugation. The results obtained are shown in **Table 10**.

Table 10. Results obtained in the separation by centrifugation of 10^6 total spores from UHT milk treated with subtilisin and untreated milk. The experiment was done by duplicate in three different days, (n=6), *p value* < 0.05.

Milk	Spores in the fat layer (CFU/mL)
Treated with subtilisin	$5.15 \cdot 10^2 \pm 5.77 \cdot 10^2$
Untreated	$6.25 \cdot 10^4 \pm 4.37 \cdot 10^4$

Results show a higher number of cfu/ml corresponding to the spores lost in the fat layer of untreated milk compared with the milk digested with subtilisin and detergent. Although the standard deviation was high, significant differences were found between the number of spores lost in the fat of the two types of milk. These results prove that the application of subtilisin treatment allowed to recover 99.95% of spores in comparison with 94.3% of spores recovered in the precipitate of UHT milk untreated with subtilisin.

3.7. Applicability of the selected method: MW treatment and DNA column purification

To evaluate the MW treatment and DNA column purification as a routine protocol to detect *C. tyrobutyricum* spores by qPCR, the calibration curve was made in PBS and UHT milk spiked with different amounts of spores.

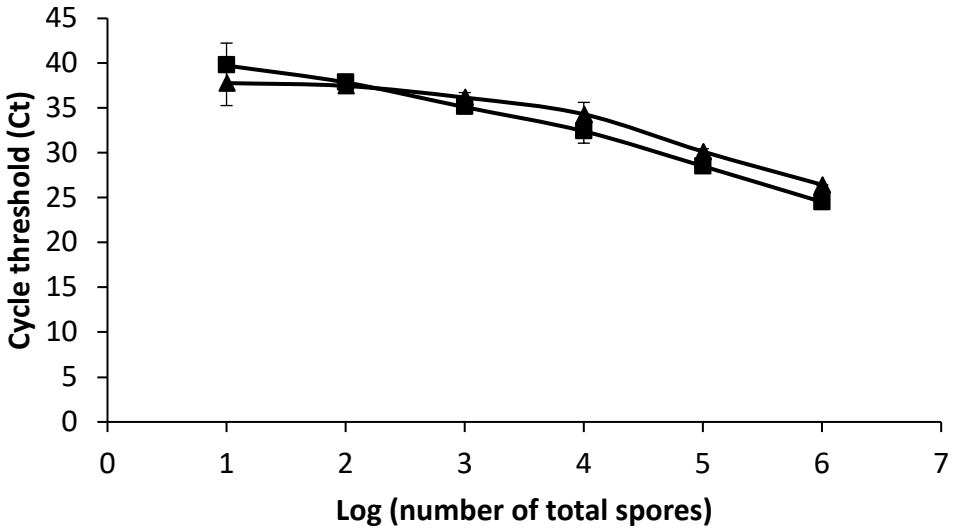

Figure 18. qPCR calibration curves for amplification of DNA from *C. tyrobutyricum* in PBS. Type strain CECT 4012 (▲) and UZ01 strain (■). DNA was extracted from spores by MW treatment and column purification (n=6).

The calibration curves obtained for the two strains of *C. tyrobutyricum* studied are presented in **Figure 18**. The LOD defined as the concentration that can be detected with reasonably certainty, being normally of 95%, was set up in 10^3 total spores for UZ01 strain suspended in PBS, one logarithmic higher than the obtained for CECT 4012 set up in 10^2 total spores (see **Table 11**). The linear range obtained for UZ01 strain was found between 10^2-10^6 spores whereas for CECT 4012 strain was between 10^3 and 10^6 spores.

Table 11. Detection and quantification of *C. tyrobutyricum* spores of CECT 4012 and UZ01 strains in 100 µL of PBS. DNA was extracted from spores by MW treatment and subjected to column purification. Ct: cycle threshold, LOD: limit of detection (n=6).

	Log (total spores)	Log (spores/mL)	Signal ratio	Linear range (total spores)	LOD (total spores)	qPCR efficiency (%)
UZ01 (PBS)	1	2	3/6	10^2-10^6	10^3	100.08%
	2	3	5/6			
	3	4	6/6			
	4	5	6/6			
	5	6	6/6			
	6	7	6/6			
	Linear equation: y =-3.32x + 44.98 R^2=0.99					

	Log (total spores)	Log (spores/mL)	Signal ratio	Linear range (total spores)	LOD (total spores)	qPCR efficiency (%)
CECT 4012 (PBS)	1	2	4/6	10^3-10^6	10^2	99.66%
	2	3	6/6			
	3	4	6/6			
	4	5	6/6			
	5	6	6/6			
	6	7	6/6			
	Linear equation: y=-3.33x + 46.76.R^2= 0.97					

The qPCR efficiency calculated for the linear range of each strain was found successful considering that good qPCR efficiencies have values in the range of 90-110%.

Although the results obtained showed that MW treatment and column purification is a protocol suitable to quantify spores in PBS, spores are normally found in milk. For this reason, the protocol was applied to UHT milk spiked with the same amounts of spores as described in PBS. The results are shown in **Figure 19** and **Table 12**.

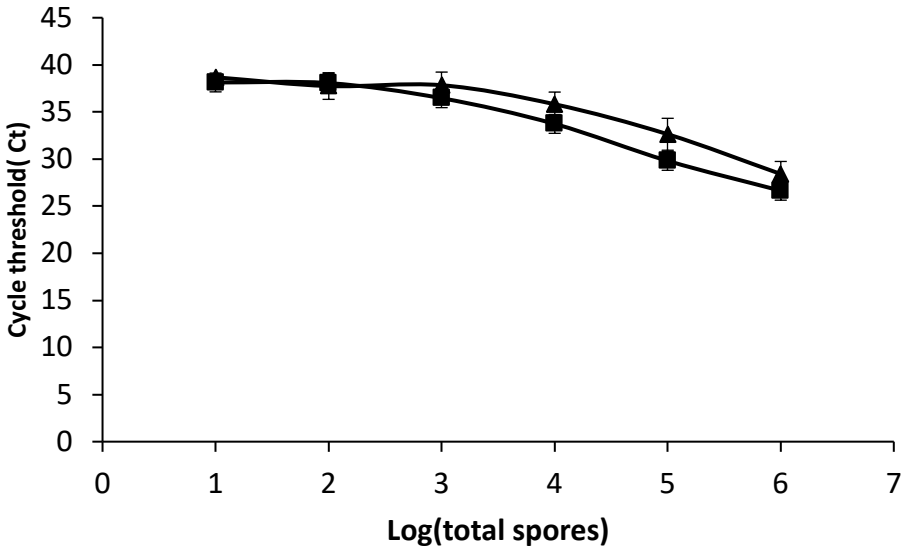

Figure 19. qPCR calibration curves for detection of *C. tyrobutyricum* spores of CECT 4012 and UZ01 strains in UHT milk. Type strains CECT 4012 (▲) and UZ01 (■). DNA was extracted from spores by MW treatment and subjected to column purification (n=6).

The LOD obtained in UHT milk for UZ01 strain was 10^2 spores, whereas was 10^3 spores for CECT 4012. The linear range for both strains was between 10^3-10^6 total spores in UHT milk condition. The Ct obtained for detection of UZ01 were lower than for CECT 4012 in UHT milk, suggesting that the wild strain could be more easily detected and/or the spore disruption was easier than for the type strain. Furthermore, an efficiency of 99.66% was obtained for detection of UZ01 by qPCR, which is a percentage within the acceptance criteria described before. However, the value of efficiency obtained for the detection of the type strain was found higher than the upper limit of the acceptance range set up in 105% according to recognized guidelines (Bustin et al., 2009).

These results indicate that UZ01 strain has lower LOD and lower Ct than the type strain in UHT milk. The calibration curve of the wild strain was used to quantify the spores in the milk samples analyzed.

Table 12. Detection and quantification of *C. tyrobutyricum* CECT 4012 and UZ01 strains in 0.5 mL of UHT milk treated with subtilisin. DNA from spores was extracted by MW treatment and column purification Ct: cycle threshold, LOD: limit of detection (n=6).

	Log (total spores)	Log(spores/mL)	Signal ratio	Linear range	LOD	qPCR efficiency (%)
UZ01 Treated UHT milk	**1**	**1.3**	5/6	10^3-10^6	10^2	99.66%
	2	**2.3**	6/6			
	3	**3.3**	6/6			
	4	**4.3**	6/6			
	5	**5.3**	6/6			
	6	**6.3**	6/6			
	Linear equation: y =-3.33x + 46.67 R^2=0.95					
	Log (total spores)	**Log(spores/mL)**	**Signal ratio**	**Linear range**	**LOD**	**qPCR efficiency (%)**
CECT 4012 Treated UHT milk	**1**	**1.3**	4/6	10^3-10^6	10^3	108.20%
	2	**2.3**	5/6			
	3	**3.3**	6/6			
	4	**4.3**	6/6			
	5	**5.3**	6/6			
	6	**6.3**	6/6			
	Linear equation: y=-3.14x + 47.82 R^2= 0.97					

3.8. Analysis of milk samples by qPCR

Raw milk samples from cow were analyzed to determine their contamination levels with *C. tyrobutyricum* spores by the selected method. The preliminary results are shown in **Figure 20** as the percentage of positive samples with only one or two positive replicates by qPCR. Samples were considered positive if Ct values were < 40 (Burns & Valdivia, 2008) and the melting curve was coincident with that of the positive control. From a total number of 34 milk samples, 44% were negative by qPCR and 56% were positive. From those that were positive, only 27% had a positive result in the two replicates. For this reason, we concluded that the precision of the method was low, suggesting that the Ct obtained was near the limit of detection of the qPCR technique and above Ct 30. Lower DNA levels, usually below 10 or 100 DNA copies, produce higher variability into the results due to a Poisson distribution (Thermofisher Scientific, 2022). Moreover, an inefficient lysis of spores could lead to low DNA levels in the final sample. For example, if an average of one target DNA molecule is dispensed per well, the Poisson distribution predicts that 37% of the wells will receive zero target copies, 37% will receive 1 copy, 18% will receive 2 copies, 6% will receive 3 copies, etc. (Thermofisher Scientific, 2022). As a consequence of the Poisson distribution or the sub-sampling error

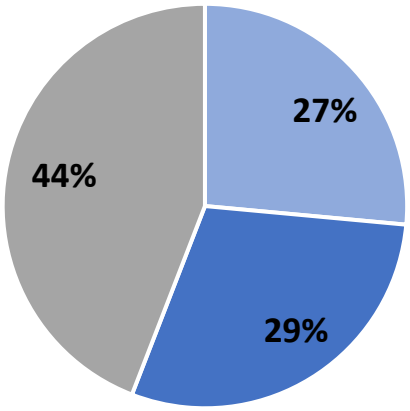

Figure 20. Percentage of positive and negative samples for detection of *C. tyrobutyricum* by qPCR in 0.5mL of raw cow's milk samples. Positive samples in two qPCR replicates (blue), positive samples in one qPCR replicate (dark blue) and negative samples in both qPCR replicates (grey). DNA was extracted by MW treatment and subjected to column purification (n=34).

and because in molecular biology usually a portion of the sample is tested, the results obtained in qPCR replicates are not so reproducible and may have higher CV (Taylor et al., 2019). Moreover, although the method proposed here was developed in UHT milk, the analysis is finally applied to raw milk samples and the complexity of this matrix can affect the final result.

The levels of *C. tyrobutyricum* spores found in cow's milk samples are summarized in **Table 13**. The spore levels are given in terms of total number of spores and the final concentration is expressed in spores/mL, considering the volume milk analyzed (0.5 mL). The data obtained demonstrate that an important number of milk samples had contamination levels in the ranges 18000-50000 spores/mL and 1000-4000 spores/mL.

Table 13. Levels of *C. tyrobutyricum* spores found in 0.5 mL raw cow's milk samples analyzed by qPCR and distribution of samples in each range. DNA from spores was extracted by MW treatment and subjected to column purification (n=34).

Range of total spores	Range of spores/mL	Number of samples
9000-25000	18000-50000	7
3000-8000	6000-16000	4
500-2000	1000-4000	6
100-400	200-800	2
Negative samples	Negative samples	15

The data of spore concentration in raw cow's milk samples obtained in this work are aligned with the data reported by Bassi et al. (2013). In the mentioned study, qPCR with specific primers for *C. tyrobutyricum* spores was performed in raw cow's milk samples obtaining values of spore concentration between 100 and 1000 spores/mL. The spore concentrations obtained in our study was higher than those reported in their mentioned study.

With the aim of checking if the lack of coincidence between replicates in some milk samples was due to having extracted a low amount of DNA from *C. tyrobutyricum* spores, the volume of milk was increased to 18 mL in the analysis of new fresh raw cow's milk samples.

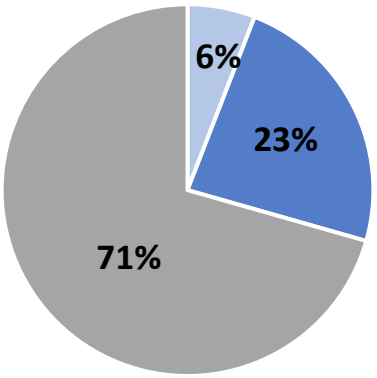

Figure 21. Percentage of positive and negative samples for detection of *C. tyrobutyricum* obtained in the qPCR analysis of 18 mL raw cow's milk samples. Positive samples in two qPCR replicates (blue), positive samples in one qPCR replicate (dark blue) and negative samples in both qPCR replicates (grey). DNA from spores was extracted by MW treatment and subjected to column purification (n=17).

From the analysis of 18 mL raw cow's milk samples by qPCR, only 6% of the total samples were positive for *C. tyrobutyricum* in the two replicates (**Figure 21**). Moreover, 23% of the samples were positive for one replicate and the remaining 71% were found negative. The concentration of spores in the samples analyzed is shown in **Table 14**.

Table 14. Levels of *C. tyrobutyricum* spores found in the analysis of 18 mL raw cow's milk samples by qPCR and distribution of samples in each range. DNA from spores was extracted by MW treatment and column purification (n=17).

Range of total spores	Range of spores/mL	Number of samples
1600-4000	8.8-222	3
300-400	16.6-22.2	2
Negative samples	Negative samples	12

The spore levels detected in this analysis were lower than those found in the previous analysis with 0.5 mL milk samples, being in the range 10^2-10^3. However, the qPCR analysis of 18 mL milk samples did not improve the repeatability in the qPCR replicates as, it was obtained a higher percentage of samples with only one positive replicate. The fact that the analysis of 18 and 0.5 mL raw milk samples were performed in different batches of milk could be a drawback, and therefore, it is difficult to conclude that increasing the volume of milk sample does not improve precision.

It is clear that more research is needed to solve the problems of the efficiency to break the spores and increase the purity of DNA obtained from raw samples. Therefore, other methods of DNA purification have been proposed with the application of new techniques. The next chapter of this thesis is focused on the development and validation of a method to detect *C. tyrobutyricum* spores in raw milk based on DNA purification with nanoparticles in a semi-automatic system.

4. Conclusions

Real-Time PCR is a powerful tool for the detection of a variety of microorganisms in food samples. In this study, different spore disruption methods were tested to evaluate DNA extraction from *C. tyrobutyricum* spores and amplification by qPCR using TaqMan® probes and SYBR Green® reagent. With the results obtained, it was concluded that two methods were efficient for disruption of *C. tyrobutyricum* spores, BB and MW followed by a step of silica column purification of DNA. Moreover, results obtained by qPCR with SYBR Green® showed lower Ct values and higher percentages of amplification compared to those obtained with TaqMan® probes.

The way to produce the spores in the laboratory could influence their detection, due to their different resistance. In this study, we have verified that spores obtained in agar are more resistant to disruption than those produced in liquid medium. It is assumed that spores with different resistance will be found in nature; however, in this work it was considered adequate to work with liquid medium spores, as it has been reported in many previous studies (Bassi et al., 2013; López-Enríquez et al., 2007). Furthermore, in this study we confirm that there are differences between the amplification by qPCR of DNA from vegetative cells and that from spores, and as a consequence, the vegetative cells can be detected more easily, with lower Ct and higher percentages of amplification. This must be taken into consideration to develop a detection and quantification method for *C. tyrobutyricum* spores based on qPCR.

In this work, raw milk samples from cow were treated with subtilisin and detergent, and the selected method based on spore disruption by MW (600 W, 15 min) and DNA purification with silica column was tested. Although the calibration curves obtained were within the acceptance criteria according to the international guidelines, lower precision was found for raw milk samples due to the non-coincidence between replicates. This problem could be due to the low quantity of DNA obtained from the samples and the sub-sampling error of the qPCR procedure. This fact was reported by other authors (Martínez et al., 2011) as spoilage microorganisms usually are present in low concentrations and the possible inhibitors present in samples, such as milk, could affect to qPCR performance. For this reason, the development of a method to detect *C. tyrobutyricum* spores in raw milk by qPCR is a great challenge.

We can conclude that the method proposed here has not proved as optimal as expected to be applied to raw milk samples although is simple and economic. Therefore, in the next chapter of this thesis the use of a novel DNA purification method based on nanoparticles in a semi-automatic system has been evaluated. The automatization of the method is really important to process a large number of samples in milk quality control laboratories.

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Chapter 2

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

1. Introduction

This chapter presents the optimization and validation of a method based on Real-Time PCR (qPCR) for the detection of *C. tyrobutyricum* spores in up to 1 mL of raw milk samples. The novel method evaluated here, extracts the DNA with magnetic nanoparticles in a semi-automatic system named as King Fisher Duo Prime system (KF). The applicability of the procedure developed has been tested in cow, ewe and goat raw milk samples naturally contaminated with butyric spores (n=202). Raw milk samples were kindly donated by milk quality control laboratories from three different geographical regions of Spain. Raw milk samples were treated with subtilisin then, DNA was recovered with KF system and finally, samples were analyzed by qPCR to test *C. tyrobutyricum* spores concentration. The results obtained showed levels of 10^2-10^3 *C. tyrobutyricum* spores per mL in the analyzed milks. The second main purpose of this part of the thesis was to check the presence of *C. tyrobutyricum* in raw milk samples by microbiological culture. For this purpose milk samples were cultured in a selective medium for butyric spores. Colonies were analyzed by multiplex PCR and 16S rDNA sequencing. The identification of the colonies revealed that other microorganisms such as *Lactobacillus* and *Paenibacillus* can grow in the selective medium. Furthermore, other *Clostridium* spp were found apart from *C. tyrobutyricum*.

2. Materials and methods

2.1. Strains and culture conditions

The strains summarized in **Table 15** were used for qPCR and multiplex PCR analysis. The sporulation and purification procedures to obtain the spores were carried out following the liquid medium protocol based on the work by Bassi et al. (2013), as it was previously explained (chapter 1, section 2.1. and 2.2).

Table 15. Strains of *Clostridium* species and broth media used for microbiological culture.

Microorganism	Strain	Source	Culture media
Clostridium tyrobutyricum	CECT 4012T	Spanish Type Culture Collection	RCM
Clostridium tyrobutyricum	UZ01	Isolated from AILA raw milk	RCM
Clostridium butyricum	CECT 361T	Spanish Type Culture Collection	RCM
Clostridium sporogenes	CECT 485T	Spanish Type Culture Collection	RCM
Clostridium beijerinckii	CECT 508T	Spanish Type Culture Collection	RCM

2.2. Enzymatic treatment of milk and spore recovery

UHT milk and raw milk were spiked with different concentrations of *C. tyrobutyricum* spores: 10^1-10^6 spores/mL in a final volume of 1 mL. For milk digestion, a solution containing subtilisin (Foss) was used as previously described in chapter 1, section 2.5. (Esteban et al., 2020). For this procedure, subtilisin was dissolved in a specific buffer containing a detergent (1:28, v/v) and added to milk in equal volumes (1:1). Then, the mixture was incubated at 60°C in a water bath for 30 min and after, centrifuged at 13000 x *g* for 30 min to obtain the spores in the precipitate. The same protocol was applied for field raw milk samples as is explained before.

2.3. Disruption of spores and DNA extraction

A novel method has been applied to simplify the DNA extraction from spores and improve the yield. The spore disruption and DNA purification was done with MagMax total nucleic acid isolation kit (Thermo Fisher) following the manufacturer instructions. This novel method is composed by a step of cell disruption using BB technique followed by the semi-automatic recovery of DNA with magnetic nanoparticles.

First, a step of BB was applied for spore disruption. For each subtilisin treated sample (as it was described in section 2.2), 232 µL of lysis binding buffer and 3 µL of carrier DNA were mixed and then added to the bead tube. Then, spores were disrupted by BB during 1 min at 6500 rpm in a Precellys 24 homogenizer (Bertin Technologies SAS, France).

Afterwards, a centrifugation at 13000 x *g* for 5 min was performed and 115 µL of the supernatant containing DNA was recovered and processed by KingFisher Duo Prime System (Thermo Fisher) herein after named as KF (**Figure 22A**).

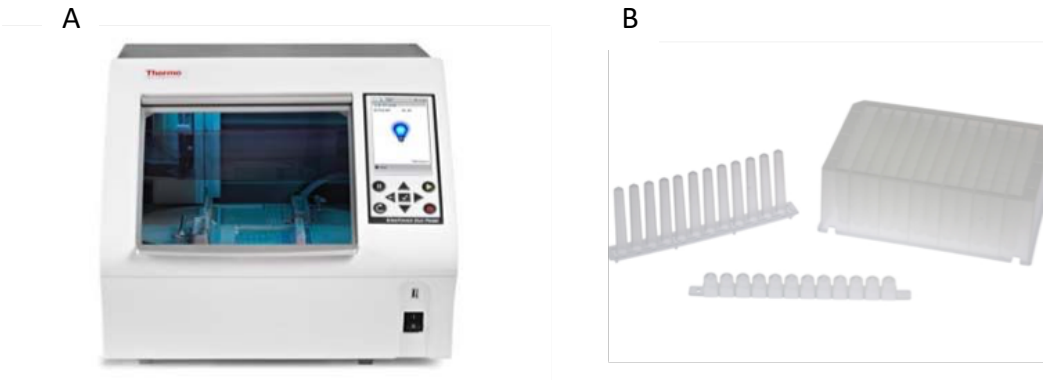

Figure 22. A: King Fisher Duo Prime System. B: processing plate and consumables.

For the analysis of samples in the KF, a specific plastic plate of 96 wells (see **Figure 22B**) was prepared following the instructions of the manufacturer. This plate must contain the DNA mixed with 65 µL of isopropanol and 20 µL of the bead mix containing the magnetic beads (line A) per well. Lines B and C are uploaded with 150 µL of wash solution 1. Lines D and E contain 150 µL of wash solution 2. Lines F and G must be empty and finally, line H must contain the tip where the magnetic row is set. An elution strip, separately from the 96-well plate, must be filled with 90 µL of elution solution.

For the automatic extraction of DNA, the program MagMax pathogen DNA/RNA was applied for 25 min. In the DNA purification process (**Figure 23**), first, the magnetic nanoparticles are mixed with the sample containing DNA. Then, the magnetic row recovers the complexes DNA-nanoparticles, which are transferred into the wash wells for washing. Finally, the DNA is eluted by heating and with the elution solution.

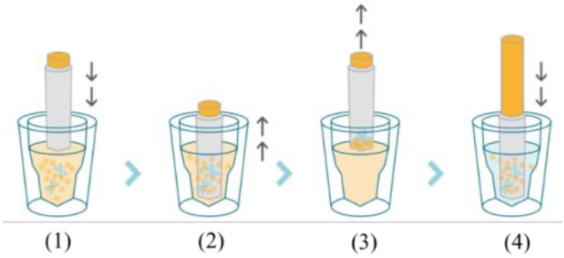

Figure 23. KingFisher Duo Prime System working for the purification of DNA. 1) First, the magnetic row (orange) is set into the tip comb (grey). 2) The DNA and the nanoparticles are well mixed. 3) The nanoparticles containing the DNA are recovered by the magnetic row and washed in the different wells. 4) Finally, the DNA is eluted and recovered.

Purified DNA was centrifuged 13000 x *g* for 3 min to eliminate the remnant nanoparticles. Finally, DNA was recovered and kept at -20°C until its use.

2.4. Raw milk samples

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

These samples were analyzed routinely by the laboratories to control milk quality and some of them derived from farms that had specific problems of milk contamination with butyric spores causing LBD.

Each sample was named with a first letter corresponding to A (AILA), L (Lekunberri) and C (Cataluña) followed by the year/month/day.

1 mL of raw milk samples was previously digested with subtilisin as previously described (section 2.2) and then DNA was extracted and further analyzed by qPCR (sections 2.3 and 2.5).

2.5. Real-Time PCR (qPCR)

Samples for the qPCR assay were prepared following the protocol previously described in chapter 1 (section 2.3.2). In **Table 16** the protocol for qPCR amplification is summarized.

Table 16. Summary of the conditions used for qPCR analysis to detect *C. tyrobutyricum* in raw cow, sheep and goat milk samples.

Reagent	Final amount per reaction
Mastermix SYBR® Green PowerUp	12.5 µL
CTfla forward primer	1.5 µL
CTfla reverse primer	1.5 µL
RNase-free water	4.5 µL
Purified genomic DNA	5 µL

The qPCR steps were the following: an initial stage at 50°C during 2 min to activate the uracyl DNA-glycosylase (UDG), an initial denaturation at 95°C for 10 min, 45 cycles of amplification, with a denaturation step at 95°C for 15 s, hybridization step at 60°C for 1 min and elongation step at 60°C for 1 min. The threshold cycle value (Ct), which is inversely proportional to the number of DNA copies, was obtained from the amplification curve. Each sample was tested in duplicate. Samples were considered negative when the 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 of the duplicates in the first qPCR run were analyzed in a second qPCR by duplicate. When at least one duplicate amplified in the second run, 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 validated previously to analyze milk samples as is described in section 2.8 of chapter 2.

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, being normally accepted a value of 95%.

2.6. Detection of butyric spores by microbiological culture

A protocol based on the method described by Jonsson (1990) for the detection of butyric spores in raw milk samples by microbiological analysis was applied. A volume of 500 µL of raw milk was previously heated at 80°C for 10 min to inactivate vegetative cells and then, it was added to 9 mL of RCM and incubated in anaerobiosis at 37°C for 48 h. Those cultures with turbidity, gas release and cheese smell were selected for the following evaluation (**Figure 24**).

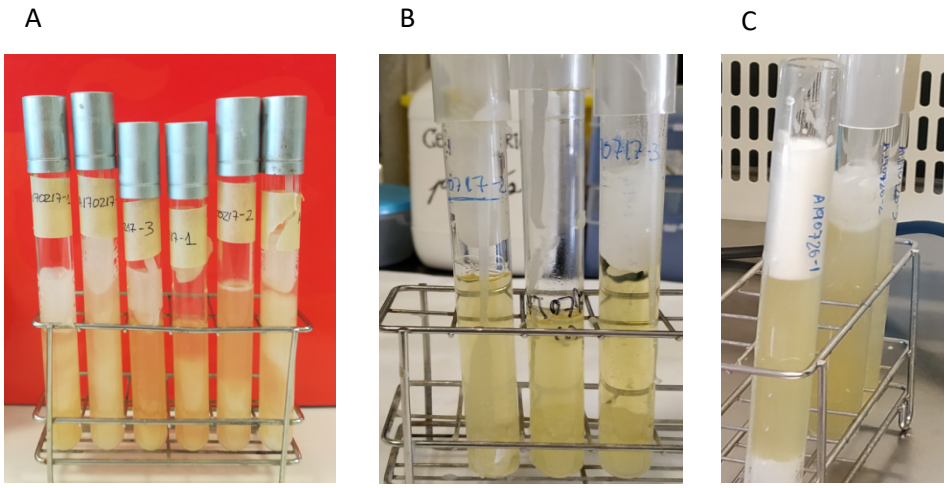

Figure 24. Pre-enrichment of raw cow milk samples in RCM media after 48 h of growth. Figures A and B show paraffin detachment in tubes as a consequence of gas production. The tubes in Figure C show visible gas production.

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

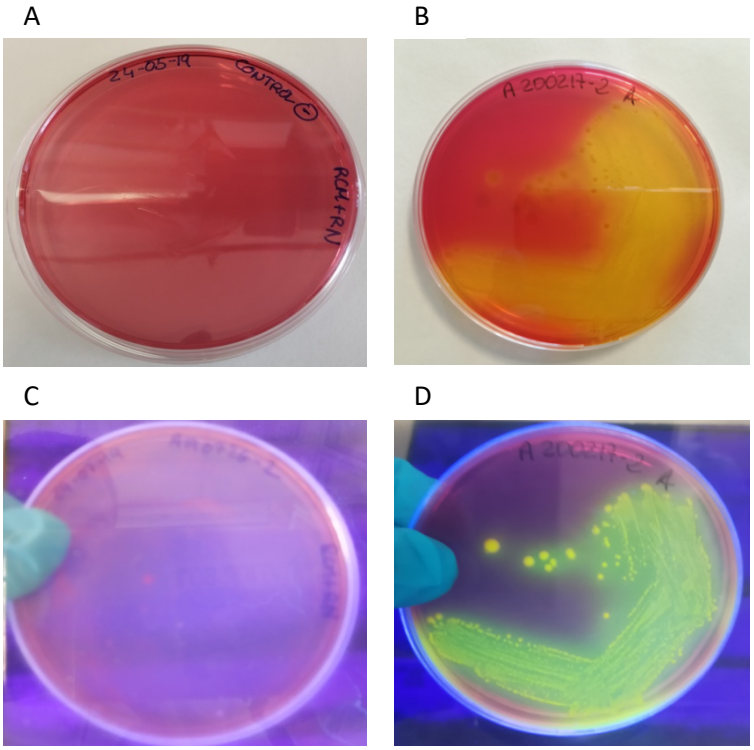

Figure 25. Pre-enriched raw cow milk samples cultured in RCM with neutral red and D-cycloserine after 4 days of incubation. A: negative sample; B: positive sample (yellow colonies); C: negative sample under UV; D: positive sample under UV (fluorescent yellow colonies).

Agar plates were incubated during 4 days at 37°C in Gaspak jars (Anaerocult, Merck Millipore, Burlington, MA, USA) adding an AnaeroGen sachet (Thermo Fisher) to create anaerobiosis. Colonies of butyric bacteria become yellow in this media and fluorescent under UV lamp. Raw cow milk samples were considered positive for butyric bacteria when the yellow and brilliant colonies grown on RCM agar were seen fluorescent under UV light (**Figure 25**).

2.7. Identification of bacterial colonies by multiplex PCR and 16S rDNA Sanger sequencing

Some individual colonies that were positive on RCM agar were subjected to multiplex PCR carried out as described before (Cremonesi et al., 2012), to identify the microorganisms. For this procedure, three positive colonies were collected and resuspended in 50 µL of sterile distilled water. The boiling method was applied to the colonies during 15 min for cell disruption. Then, samples were incubated directly at -20°C for 5 min and centrifuged at 10000 x *g* for 5 min to obtain a clean soluble phase

with DNA that was applied directly to the multiplex PCR. The primer sequences used are described in **Table 17**.

Table 17. Pair of primers used for the identification of *Clostridium* species grown in RCM selective medium. *ColA*: collagenase, *nifH:* nitrogenase iron protein, *hydA:* hydrogenase, *enr:* 2-eonato reductase. [µM]: final primer concentration.

Species/genera	Primer sequence	Amplicon length	Target gene	[µM]
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	Fw: 5'-GCGGCGTGCCTAATACATGC-3' Rv: 5'-CTACGGCTACCTTGTTACGA-3'	1000 bp	*16 S ribosome*	20

Samples for multiplex PCR were prepared by mixing 12.5 µL of PCR Master Mix 2x (Promega Biotech Ibérica, Madrid, Spain) with 0.5 µL of forward and reverse primers to detect four *Clostridium* species (*C. tyrobutyricum, C. butyricum, C. beijerinckii* and *C. sporogenes*) and 2 µL of DNA. Finally, the volume was completed with 6.5 µL of DNase free water (Invitrogen) to obtain a final volume of 25 µL. The PCR conditions are described in **Table 18**.

Table 18. Amplification conditions used for multiplex PCR to detect *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii* and *C. sporogenes*.

No. of cycles	**Denaturation**		**Annealing**		**Polymerization**		**Extension**	
	T (°C)	Time (min)	T (°C)	Time (min)	T (°C)	Time (min)	T (°C)	Time (min)
1	94	2	-	-	-	-	-	-
30	94	1	56	1	72	1	-	-
1	-	-	-	-	-	-	72	5

Colonies that were positive in RCM selective medium but negative by multiplex PCR were analyzed by 16S PCR and rDNA sequencing, to identify which microorganisms gave this particular result. For this purpose, a standard PCR was performed with primers targeting the 16S bacterial ribosome gene (Guerrieri et al., 2020). The PCR was performed at the same conditions as explained before for multiplex PCR, except the annealing temperature that was set up at 55°C.

The PCR products were sent to the Servicio General de Apoyo a la Investigación (SAI) at the University of Zaragoza (Zaragoza, Spain) where a Sanger sequencing and capillary electrophoresis were performed. The nucleotide sequence received by SAI was analyzed by Chromas 2.6.6 software (available in https://technelysium.com.au/wp/chromas/) and transformed to FASTA file. The FASTA sequence was introduced in Basic Local Alignment Search Tool (BLAST, available in https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information (NCBI) of the United States, which finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.

For amplicon visualization, a 3% agarose gel for multiplex PCR and 1.5% for the 16S PCR in Tris-Borate-EDTA buffer (TBE) composed of 90 mM Tris-base, 90 mM boric acid and 2 mM EDTA dihydrate, 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 included. The bands were visualized under UV lamp in a transilluminator.

2.8. Validation of qPCR for detection of *C. tyrobutyricum* spores

In-house validation of qPCR to detect *C. tyrobutyricum* spores was performed, following the same guidelines explained in section 2.3.2. of chapter 1. The efficiency of qPCR for each condition was calculated following the formula: $E = -1 + 10^{-1/slope}$. The linear range and LOD, described as the concentration that can be detected with reasonable certainty, being commonly accepted 95% (Bustin et al., 2009), were calculated.

The repeatability and reproducibility (also defined as intermediate precision) were reevaluated for the calibration curve of *C. tyrobutyricum* UZ01 strain (n=6) based on the spores/mL data. The calibration curve was performed with DNA obtained from raw cow milk samples spiked with different amounts of *C. tyrobutyricum* UZ01 spores, ranging from 10^1 to 10^6 spores/mL. DNA from different suspensions of spores was extracted three times in three different days and after, each calibration curve was analyzed by qPCR in duplicate. The repeatability (intra-assay variation) was calculated as the CV between duplicates for each qPCR run and each point of the curve, and the reproducibility (inter-assay variation) was calculated as the CV between qPCR runs with the following formula CV = (SD*100) / X, as SD the standard deviation and X the spores/mL mean obtained for each dilution.

The precision was also analyzed in raw milk samples naturally contaminated that were found positive for *C. tyrobutyricum* by qPCR, considering a positive sample when Ct value < 40 (Burns & Valdivia, 2008; Bustin et al., 2009). For this purpose, six positive samples (three from cow and three from ewe) were subjected, in duplicate, to a second and third qPCR. The percentage of amplification, spores/mL mean and SD were determined. The spores/mL value was calculated with the calibration curve previously obtained. Furthermore, six negative milk samples (three from cow, two from ewe and one from goat) were subjected to a second and third runs of qPCR to confirm the result obtained in the first qPCR.

The specificity of the qPCR is given by the specificity of the primers, which had been previously demonstrated by López-Enríquez et al. (2007). Furthermore, we also analyzed the target sequence by an *in silico* study in NCBI primer-BLAST and confirmed

its specificity for *C. tyrobutyricum*. In addition, DNA extracted from vegetative cells and from spores of *C. sporogenes*, *C. butyricum* and *C. beijerinckii* were analyzed by qPCR and were found negative with the primers used in this study.

The robustness of the qPCR was calculated following the matrix proposed by the FDA Foods Program Regulatory Science Steering Committee (RSSC) (2020) (**Table 19**).

Table 19. Matrix of the principal factors used to evaluate qPCR robustness for *C. tyrobutyricum* quantification. PCR equipment, StepOne 48 wells (A) and a StepOnePlus 96 wells (B); [Primers], primer concentration; DNA volume, volume of added DNA; MM volume, Master Mix volume; Annealing T, annealing temperature. NC: no change from optimized conditions.

Factor	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
PCR equipment	A	A	A	A	B	B	B	B
[Primers]	NC	-30%	NC	+30%	NC	-30%	NC	+30%
DNA volume	NC	-10%	+10%	NC	-10%	NC	NC	+10%
MM volume	-5%	-5%	+5%	+5%	+5%	+5%	-5%	-5%
Annealing T	+1°C	-1°C	+1°C	-1°C	-1°C	+1°C	-1°C	+1°C

For the robustness calculation, three points of the UZ01 calibration curve (10^2, 10^3 and 10^6) were analyzed by qPCR in triplicate for each condition. The qPCR for each condition was repeated twice (n=6). The spores/mL mean and standard deviation (SD) were calculated for each point of the robustness matrix with the calibration curve obtained.

The influence of individual factors on the robustness of the method was determined by statistical analysis of the differences (Di) between variables (reference versus modified) following the analysis of the Youden matrix (Youden & Steiner, 1975). The combined influence of all selected factors was calculated as the standard deviation of differences (SDi). The factors and their variations are summarized In **Table 20**.

Table 20. Variable factors for determination of qPCR robustness for *C. tyrobutyricum* quantification. Nine potentially variable factors were identified and their degree of variation established. Reference values are indicated in capital letters, while deviations are in lower case. MM: master mix for qPCR experiment.

Variable factor	Reference	Deviation
PCR equipment	A (StepOne 48 wells)	a (Step one 96 wells)
[Primers] -	B (600 nM)	b (400 nM)
DNA volume -	C (5 µL)	c (4.5 µL)
MM volume -	D (12 µL)	d (11.88 µL)
Annealing T -	E (60°C)	e (59°C)
[Primers] +	F (600 nM)	f (800 nM)
DNA volume +	G (5 µL)	g (5.5 µL)
MM volume +	H (12 µL)	h (13.13 µL)
Annealing T +	I (60°C)	i (61°C)

2.9. Data analysis

The Ct values were obtained using the qPCR software Step One™ 2.3. version (Life Technologies, Carlsbad, CA, USA). The mean and standard deviations of Ct values were calculated using the Microsoft® Excel version 16.44 (Microsoft Spain, Madrid). Sequence analysis of 16S bacterial ribosome were analyzed and export 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 spores. On the one hand, the release of spores from milk components is essential. For this purpose, the use of a solution containing a detergent and a protease has been proved as the most effective method in this study (chapter 1). On the other hand, a novel approach not applied before for extraction and purification of DNA from butyric spores has been evaluated. This method consisted in breaking the spores by BB and isolating their DNA by magnetic beads. This method is proposed because it shows two main advantages compared to the method selected in the first part of this study (MW treatment and column purification): automation and improvement of the DNA yield. On this basis, the main objective of our study was to validate the whole method, the DNA extraction together with the qPCR, to detect *C. tyrobutyricum* spores.

qPCR efficiency, linear range and LOD values were calculated from the calibration curve obtained for CECT 4012T and UZ01 (**Table 21**). Efficiency for CECT 4012 was 93.12%, whereas 96.50% was found for UZ01 strain. Values for qPCR efficiency should be in the range of 90-100% (Bustin et al., 2009). The LOD achieved for CECT 4012 strain was set up in 10^4 spores/mL, whereas that obtained for UZ01 was considerably lower, being of 10^2 spores/mL. The calibration curve initially obtained for *C. tyrobutyricum* strain type CECT 4012 was also compared with that for the wild strain UZ01 isolated from raw cow milk (**Figure 26**). Lower Ct cycles were obtained for UZ01 in comparison with those for CECT 4012. The linear range obtained for UZ01 was from 10^2 to 10^6 spores/mL, while the range obtained for CECT 4012 was from 10^3 to 10^6 spores/mL. These results showed that the wild strain was detected more easily than the type one. The UZ01 calibration curve was used for the quantification of *C. tyrobutyricum* spores in naturally contaminated milk samples because lower LOD was achieved with this strain. However, with the results obtained in this study, we cannot give an explanation for the differences found between both strains. Even if more research would be needed to establish the cause of such differences, it is clear that the resistance of spores might be a decisive factor influencing spore breakage and DNA recovery.

Table 21. Detection and quantification of spores from two strains of *C. tyrobutyricum* in raw milk (1 mL spiked samples) treated with subtilisin. DNA was extracted from spores by KF method. Ct: cycle threshold. R^2: regression coefficient. LOD: limit of detection, SD: standard deviation (n=6).

Sample	Log (spores/mL)	Signal ratio	Linear range	LOD	qPCR efficiency %
Raw milk (subtilisin treated) UZ01	1	4/6	10^6-10^2	10^2	96.53
	2	6/6			
	3	6/6			
	4	6/6			
	5	6/6			
	6	6/6			
	Linear equation: y=-3.40x + 41.04 R^2 = 0.99				
	Log (spores/mL)	**Signal ratio**	**Linear range**	**LOD**	**qPCR efficiency %**
Raw milk (subtilisin treated) CECT 4012	1	1/6	10^6-10^3	10^4	93.12
	2	2/6			
	3	5/6			
	4	6/6			
	5	6/6			
	6	6/6			
	Linear equation: y=-3.49x + 47.50 R^2 = 1				

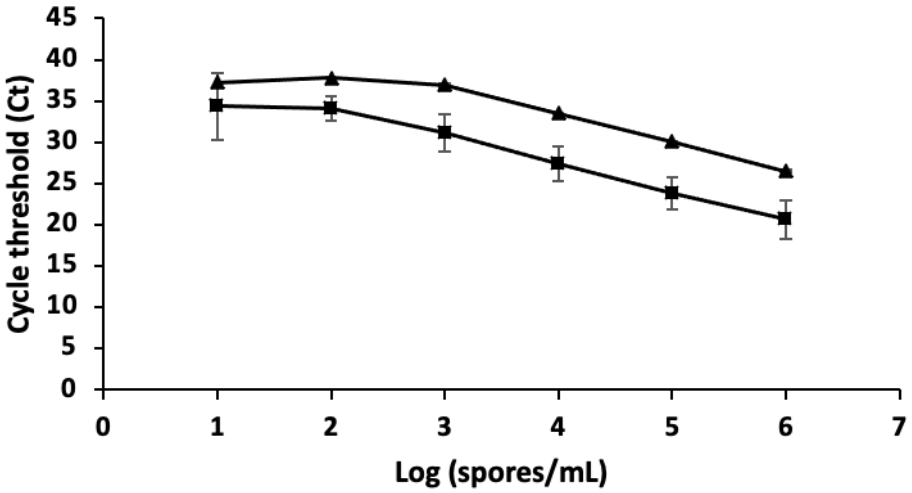

Figure 26. qPCR calibration curves for detection and quantification of *C. tyrobutyricum* spores in spiked raw milk samples. DNA was extracted from spores by the KF method. Type strain CECT 4012 (■) and UZ01 strain (▲)

In comparison with the MW treatment followed by column purification developed in chapter 1, lower Ct cycles were obtained for UZ01 wild strain applying the KF method with a LOD of 10^2 spores/mL. However, better linear range for UZ01 strain was obtained with the KF method ranging from 10^7 to 10^2 spores/mL, whereas applying MW the linear range was from 10^3 to 10^7 spores in 0.5 mL of UHT milk for the same strain. An improvement in linear range and Ct cycles was obtained for UZ01 in raw milk, which is the natural media of butyric spores.

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

Once the performance of the KF and qPCR method was evaluated according to recognized guidelines, raw milk samples from cow, ewe and goat were analyzed to verify the presence of *C. tyrobutyricum* spores in real samples.

In this study, a total number 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 also cultured in RCM selective media to compare the results with those obtained by qPCR.

As is shown in **Table 22**, from the total number of milk samples analyzed for each species, 28% and 43% of cow and ewe milk samples, respectively, were positive by qPCR, while only 10% goat milk samples were found positive.

Table 22. Percentage of positive and negative samples of raw milk samples from cow (n=68), ewe (n=86) and goat (n=48) by qPCR.

	Positive samples (%)	Negative samples (%)
Cow	28	72
Ewe	43	57
Goat	10	90

As it has been previously indicated, samples were considered positive for *C. tyrobutyricum* by qPCR when Ct values were <40. The melting curve was obtained to check out the melting temperature of the selected amplicon and was coincident in 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 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 estimated concentration of spores was between 4 and 214 spores/mL. These results indicate that the levels of *C. tyrobutyricum* spores in some samples were lower than the limit of detection set to 10^2 spores/mL in the proposed method. Of the total qPCR positive cow milk samples, only 26% could be quantified because the rest of the samples presented Ct values 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 faeces, 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 100-1000 CFU/mL, in the same order to that found in our study. However, the percentage of positive samples for *C. tyrobutyricum* spores found in our study was higher than that reported by Arnaboldi et al. (2021) that was 15.28% of the 144 total samples analyzed from Emilia Romagna and Lombardia (Italy), where samples were

collected for spore enumeration by MPN method. The levels of spores reported in that study were in the range of 363 and 1508 spores/L.

A high number of positive samples for *C. tyrobutyricum* spores were found in ewe milks analyzed by qPCR, 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 presented values below the limit of detection ($<10^2$ spores/mL). The mean concentration of spores obtained in positive ewe milk samples was 3689 ± 3412, much higher than the values obtained in cow milk samples.

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

The intermediate precision was found to be low in those milks that amplified only in one duplicate (50%). These milk samples were subjected to a second qPCR in the same conditions as 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 of spore levels below the LOD of the method and/or due to the presence of butyric spores with high resistance to breakage, which can make difficult the extraction of DNA.

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 1.60 and 4.40 log spores/L in cow milk in the Netherlands and Turchi et al. (2016) found concentrations from 360 to more than 110,000 spores/L in ewe milk in Italy. The variability found in the levels of *C. tyrobutyricum* spores in different studies can be due to differences in the type of animal feeding and animal management.

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

3.3. Validation of qPCR

The reproducibility (inter-assay variation or recently described as intermediate precision) has been calculated for each point of the calibration curve. The results are presented in **Table 23**. According to international guidelines, repeatability and intermediate precision should not exceed 25% over the whole dynamic range of the assay (FDA Foods Program Regulatory Science Steering Committee, RSSC, 2020). All the CV values found were lower than 25% except for 10^3 and 10^1 spores/mL level. The experiment was performed using three different spore preparations from three independent extractions. The CV values obtained below 10^3 spores/mL highlight the difficulty of extracting DNA, as it was described before (Esteban et al. 2020).

Table 23. Determination of the reproducibility in cow milk spiked with different concentrations of *C. tyrobutyricum* UZ01 spores analyzed by qPCR in three independent experiments. DNA was extracted from spores by KF method. CV: coefficient of variation; Ct: cycle threshold; SD: standard deviation. (n=6).

Log [spores/mL]	Ct Mean	Ct SD	CV (%)	Spores/mL	CV (%)
1	34.32	4.09	11.93	144.90	99.88
2	34.07	1.49	4.38	112.34	19.3
3	31.08	2.26	7.29	856	29.6
4	27.39	2.05	7.49	10076	5.5
5	23.81	1.94	8.17	114729	15.4
6	20.67	2.31	11.20	947490	2.2

The results obtained for the evaluation of robustness are presented in **Table 24**. The CV was calculated for each condition of the ruggedness matrix. The assay is considered robust if the SDi of each point of the calibration curve is lower than the standard deviation (SD) under reproducibility conditions. In this study, higher SDi was obtained in comparison with SD for each point of the calibration curve analyzed (**Table 25)**. For this reason, it is concluded that the analyzed factors affect considerably the results of the qPCR assay.

Table 24. SDi and SD for each condition evaluated in the robustness assay performed to analyze cow milk samples spiked with 10^2, 10^3 and 10^6 spores/mL. DNA from spores was extracted by KF method. SDi: standard deviations of differences, SD: standard deviation under conditions of reproducibility (n=6).

Spores/mL	SDi	SD	Factors affecting qPCR
10^2	44.69	19.3	A, B, E and F
10^3	195.56	29.6	A, B, E and H
10^6	$2.29 \cdot 10^{10}$	$2.14 \cdot 10^5$	A, B, E and H

Furthermore, three main factors affected considerably the results of qPCR: the PCR equipment (A), the reduction in the primer concentration (B), and the decrease in the annealing temperature (E). The increase in the primer concentration (F) affected only the detection of 10^2 spores/mL, while the increase in the master mix volume (H) induced changes in the detection of 10^3 and 10^6 spores/mL.

In this study, samples with a positive result only in one replicate were subjected to a second qPCR. If the Ct of one of the replicates in the second qPCR was below 40, the sample was considered positive. After this verification step, 84% of the total cow milk positive samples were positive in the first analysis. A similar situation was obtained for ewe milks with 94.60% positive samples in the first run. However, for goat milks only 14% of positive samples were positive in the first qPCR run. As it was shown in chapter 1, the DNA extraction from spores in raw milk samples, by MW treatment and column purification, reported lower qPCR precision values. In fact, 51.80% of the positive samples treated with MW and column purification needed a second PCR to confirm the result. With the method of BB and KingFisher Duo Prime System for extraction of spore DNA better qPCR precision was obtained. These results manifest that is necessary a second qPCR to verify positive results when only one replicate is positive in the first run.

For the intermediate precision analysis, positive raw milk samples were subjected to three different runs of qPCR. As it is shown in **Table 25**, 66.6% of the samples amplified in all the qPCR runs performed. However, one of them only amplified at 50% and another at 83.3%. This could be explained by a low DNA concentration in

those samples or due to a low efficiency in DNA extraction. Moreover, high values of standard deviation for Ct were obtained for raw milk samples analyzed by qPCR (**Table 25**). It has been reported that samples with Ct>30 have lower precision and intermediate precision due to the Poisson distribution and the sub-sampling error (Taylor et al., 2019). Moreover, the values of spore concentration obtained were below the limit of detection of the validated method.

The repetition of the analysis of negative samples by qPCR, revealed that most of them, 66.6%, did not amplify in a second and third runs of qPCR. Although some of them amplified at cycles above 40, we considered them as negative. We consider important that the two duplicates amplify in the same qPCR run to consider the sample as positive. The intermediate precision of the samples with only one duplicate as positive is low and could be due to a false positive.

Table 25. Determination of the precision in the analysis of positive milk samples for *C. tyrobutyricum* by qPCR between runs (n=6). Ct: cycle threshold; SD: standard deviation.

Samples (positive)	**qPCR 1**			**qPCR 2**			**qPCR 3**			**Mean Ct**	**SD**	**Signal ratio**	**Mean spores/ mL**	**SD spores/ mL**
	Mean Ct	Signal ratio	Spores/ mL	Mean Ct	Signal ratio	Spores/ mL	Mean Ct	Signal ratio	Spores/ mL					
1	38.79	2/2	4.59	34.03	2/2	115.28	36.81	2/2	17.54	36.54	2.39	6/6	45.81	60.52
2	35.45	2/2	44.08	30.15	2/2	1595.66	30.93	2/2	940.87	32.18	2.86	6/6	860.20	778.93
3	32.99	2/2	233.16	31.46	2/2	657.12	32.94	2/2	241.19	32.46	0.71	6/6	377.16	242.50
4	35.68	2/2	37.71	-	0/2	-	34.3	1/2	96.02	34.99	0.98	3/6	66.86	41.23
5	34.51	2/2	83.29	32.96	2/2	237.94	35.11	2/2	55.49	33.74	1.10	6/6	125.57	98.30
6	34.81	2/2	67.97	33.51	1/2	163.95	38.70	2/2	4.87	35.67	2.70	5/6	78.93	80.10

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

For the study of *Clostridium* spp. in raw milk samples they were cultured in selective medium after being subjected to heat treatment to inactivate vegetative cells, as described in section 2.6. 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 **Figure 27**. 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 of clostridia (Jonsson, 1990). For ewe milks, the percentage of samples giving positive colonies was 33%. Contrary to what we expected, the percentage of goat milk samples with positive colonies was higher than in the other species, being of 65%.

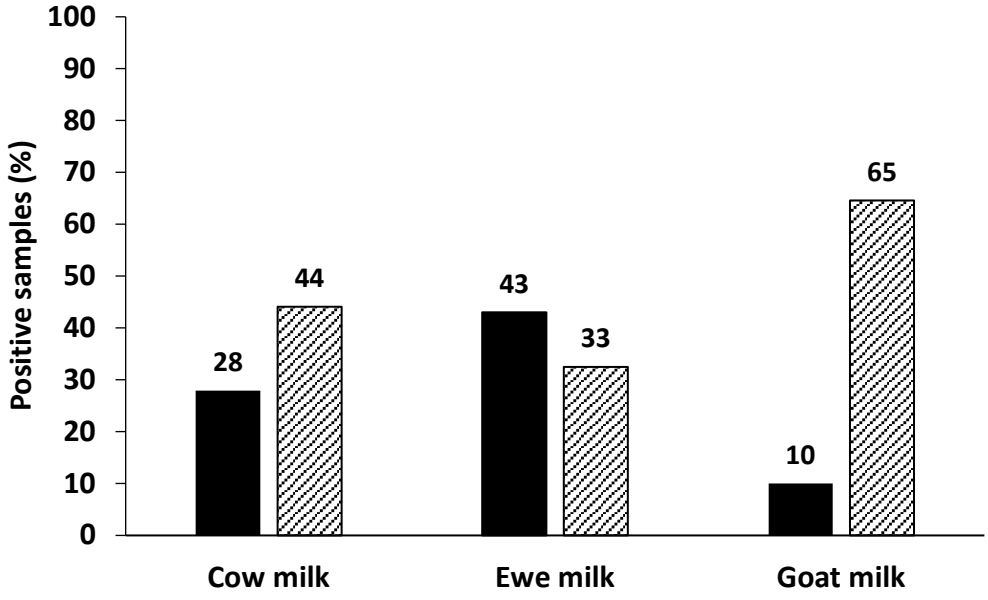

Figure 27. Percentage of positive samples for *C. tyrobutyricum*, by qPCR considering Ct <40 (■) and positive by culture in RCM selective medium (▨). DNA from spores was extracted by KF method. Raw milk samples analyzed were from cow (n=68), ewe (n=86) and goat (n=48).

The coincidence between the results obtained by both methods, microbiological culture and qPCR, was 14.7% 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 in comparison with the total number of samples analyzed (**see Annex 1**). Thus, an important number of samples displayed different results depending on the type of analysis. It is important to remark 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 by MPN, being in the former 15.28% and 85.41% in the latter. Therefore*,* our results suggest the presence of bacteria belonging to other genera in the milk samples analyzed.

Multiplex PCR and 16S rDNA sequencing were performed to elucidate whether some bacteria from other genera than *Clostridium* had grown in the selective media, which would allow us to better understand the results obtained.

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

To evaluate the presence of other *Clostridium* species, a multiplex PCR was performed in 22 cow milk samples that were positive in the selective culture medium. The multiplex PCR used was developed by Cremonesi et al. (2012) and four different species of clostridia were analyzed in the same PCR reaction: *C. tyrobutyricum, C. sporogenes, C. beijerinckii* and *C. butyricum.* In **Figure 28**, a DNA electrophoresis of products obtained in a multiplex PCR of colonies isolated from cow raw milk samples is shown. Samples 1 and 2 were positive for *C. sporogenes* (549 bp) and sample 4 was positive for *C. tyrobutyricum* (210 bp).

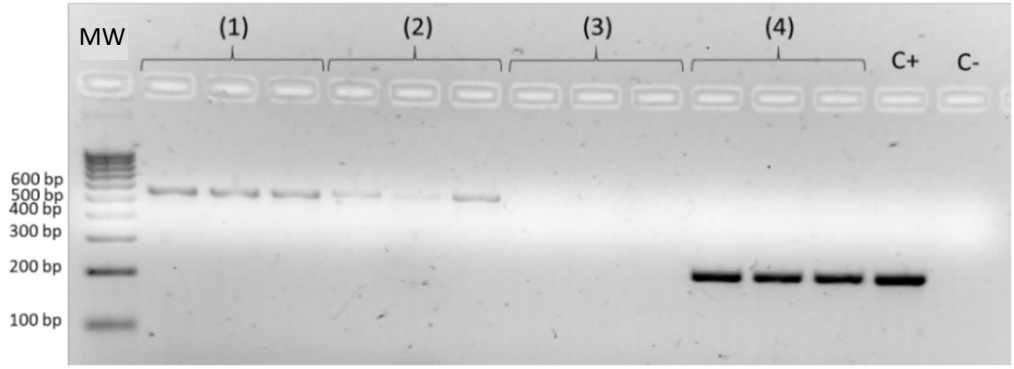

Figure 28. DNA electrophoresis of multiplex PCR products of isolated colonies from cow raw milk samples cultured in RCM selective medium. DNA was extracted from colonies by boiling method. MW: molecular weight marker; 1: A171116-11; 2: A171116-12; 3: A171215-4; 4: A190717-2. C+: positive control for *C. tyrobutyricum*; C-: negative control.

The results obtained applying multiplex PCR showed 72% negative samples of the total analyzed. However, with this method we could identify 28% of the samples analyzed, corresponding 14% to *C. tyrobutyricum* and 14% to *C. sporogenes.* These results indicate that other species different from those analyzed in the multiplex PCR, were isolated in the selective agar medium.

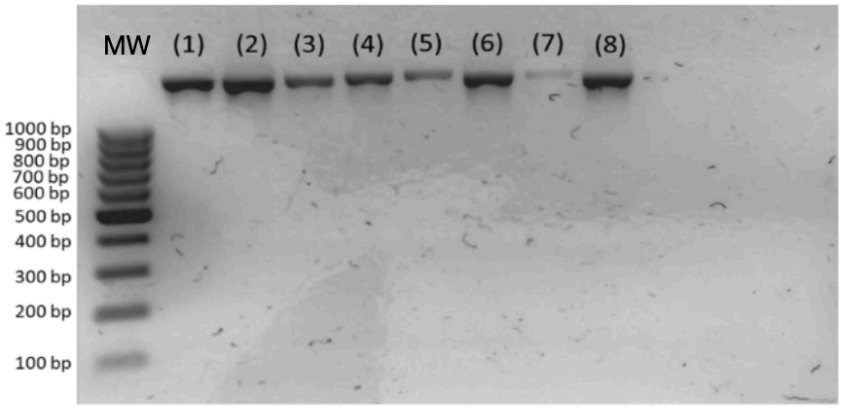

Figure 29. DNA electrophoresis of the 16S bacterial rDNA PCR products of colonies isolated from the cultures of cow and goat milk samples in RCM selective medium. DNA was extracted from colonies by boiling method. MW: Molecular weight marker. 1: A200618-1; 2: L200612-8; 3: L200612-16; 4: L200612-17; 5: L200612-19; 6: L200612-20; 7: L200612-22; 8: L200612-23.

In order to identify positive bacteria in microbiological culture that did not correspond to the species analyzed by multiplex PCR, a standard PCR with specific primers for 16S rDNA gene was performed. The sequencing of this gene allowed to identify the genus and species of the bacteria obtained from the colonies isolated in the

selective medium. The band around 1100 bp corresponding to the amplified 16S bacterial rDNA fragment is shown in **Figure 29**. A total number of 62 raw milk samples (25 from cow, 11 from ewe and 26 from goat) identified as positive in RCM selective media were analyzed by 16S rDNA. The results at the genus level are shown in **Figure 30**.

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 milk samples only *Clostridium* and *Lactobacillus* were found. As it could be expected, *Clostridium* was found as the predominant genus in cow (48%) and ewe milks (55%). *Lactobacillus* was identified in 88% of the analyzed colonies from goat milks. These results demonstrate that the selective agar medium normally used to isolate *Clostridium* spp. is not completely specific and other microorganisms can grow on it, probably displacing *Clostridium* and avoiding the germination of the spores and the subsequent proliferation of vegetative cells.

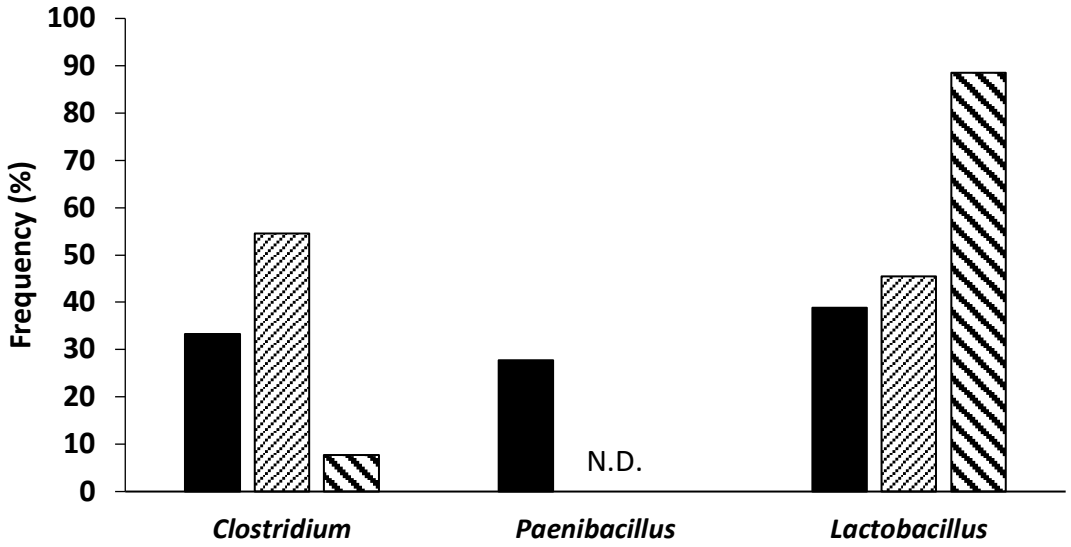

Figure 30. Frequency and distribution of *Clostridium*, *Paenibacillus* and *Lactobacillus* found in cow (■), ewe (▨) and goat (◪) milk samples cultured in RCM selective medium and identified by 16S rDNA sequencing and multiplex PCR (n=55). N.D.: not detected.

Jonsson (1990) described the *Bacillus* colonies grown in the selective medium with a faint yellow orange colour that could be distinguished easily from the appearance of the *Clostridium* colonies. However, the differentiation between the two genera was not so easy in our study. Many of the *Lactobacillus* colonies cultured were not as brilliant as *Clostridium*, as it can be observed in **Figure 31B**. However, some of them had an orange-yellow colour under the UV (see **Figure 31C**) and were brilliant too. For this reason, we consider essential to identify the colonies by PCR or by 16S rDNA sequencing.

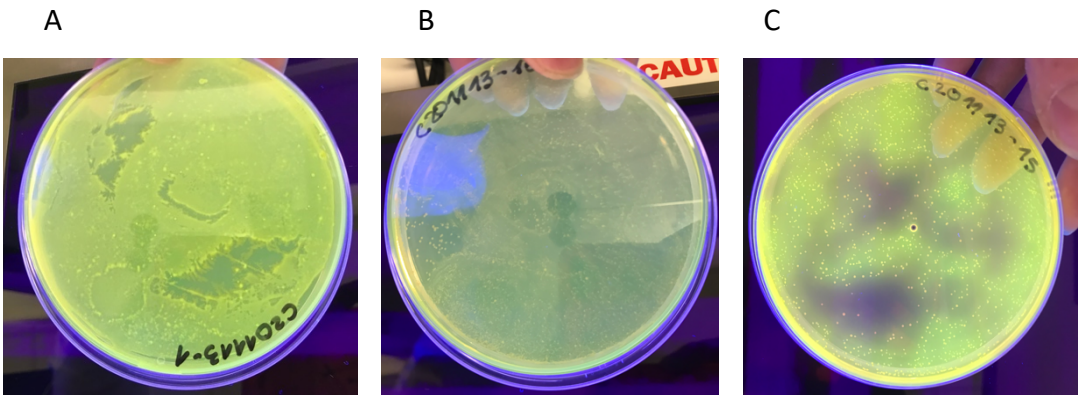

Figure 31. Differentiation of *Lactobacillus* and *Clostridium* colonies found in the culture of goat milk samples under UV light. The species identified by 16S rDNA sequencing in the cultures were A: *Clostridium sporogenes*; B: *Lacticaseibacillus paracasei*; ; C: *Lentilactobacillus parabuchneri.*

Moreover, some milk samples in which *Lactobacillus* was identified presented detachment of the paraffin and gas production in the pre-culture. Therefore, it was complicated to determine the final result of the microbiological culture, as a mixed population could be present and besides, some *Lactobacillus* species are heterofermentative. This fact supports the results found in our study and those reported in the study performed by Arnaboldi et al. (2021), in which they compared the results obtained by qPCR with those by MPN method, not finding much correlation between them.

An important percentage of the colonies isolated from raw cow milk samples (24%) were identified as *Paenibacillus* suggesting that these bacteria were present in a relevant proportion and grew in the 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 to milk pasteurization, what explains its resistance to the heat treatment applied in this study to milk samples before culture.

Lactobacillus is a genus of Gram positive, non-spore forming, aero-tolerant or anaerobic bacteria and is the largest genus of the acid lactic bacteria group. In our study, *Lactobacillus* have been found as the predominant bacteria in cow and goat milk, in the last one constituting 92% of the colonies selected for identification. *Lactobacillus* have been reported as resistant microorganisms, surviving after ewe milk pasteurization (Salmerón et al., 2002). The contamination of milk with *Lactobacillus* may occur similarly to that with *Clostridium* spores. The udder can be contaminated with bacteria during the milking process from soil, manure, grass, silage or other feeds. The contamination may also take place during milk handling and storage (De Angelis & Gobbetti, 2016). In addition, *Lactobacillus* has been reported as the main genus of goat milk microbiota (Hernández-Saldaña et al., 2016; Pisano et al., 2019) and goat cheese, in which the majority of the isolates were identified as *Lactobacillus* species (Nikolic, 2008). The bacteria that have been reported in bovine milk microbiota belong to many different genera, such as *Ralstonia*, *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, *Psychrobacter*, *Bradyrhizobium*, *Corynebacterium*, *Pelomonas*, and *Staphylococcus* (Addis et al., 2016; Taponen et al., 2019). However, although *Lactobacillus* can be present in bovine raw milk, is not the predominant genus (Zhang et al., 2015). It has been reported by different authors that ewe's milk microbiota depends on breed. *Lactobacillus* was found as the second most important genus in Assaf ewe's milk (Esteban-Blanco et al., 2020a); however, in the milk of Churra ewe breed *Lactobacillus* was not present in a relevant proportion (Esteban-Blanco et al., 2020b). The differences in the milk microbiota of the three species analyzed could explain the results obtained in our study. *Lactobacillus* spp. identified in this study were *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*), as the most frequent, and *Lacticaseibacillus casei* (formerly *Lactobacillus casei*). Other species isolated, though with lower frequency, were *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*), *Lentilactobacillus parabuchneri* (formerly *Lactobacillus parabuchneri*), *Lactobacillus gallinarum* and *Lacticaseibacillus zeae* (formerly *Lactobacillus zeae*).

In addition, it is important to take into account that *Paenibacillus* and *Lactobacillus* are heterofermentative lactic acid bacteria, and consequently they are gas producers, and as we have found in our study, they can grow in selective media for *Clostridium* in anaerobic conditions. Therefore, the presence of those bacteria in milk can give false positive results in the MPN method for butyric bacteria.

Based on the results obtained, the microbiological culture used in this study was found rather non-specific for butyric bacteria due to the growth of bacteria from two main genera: *Paenibacillus* and *Lactobacillus.* Other specific media should be considered for further studies, as that recently developed by Brändle et al. (2018).

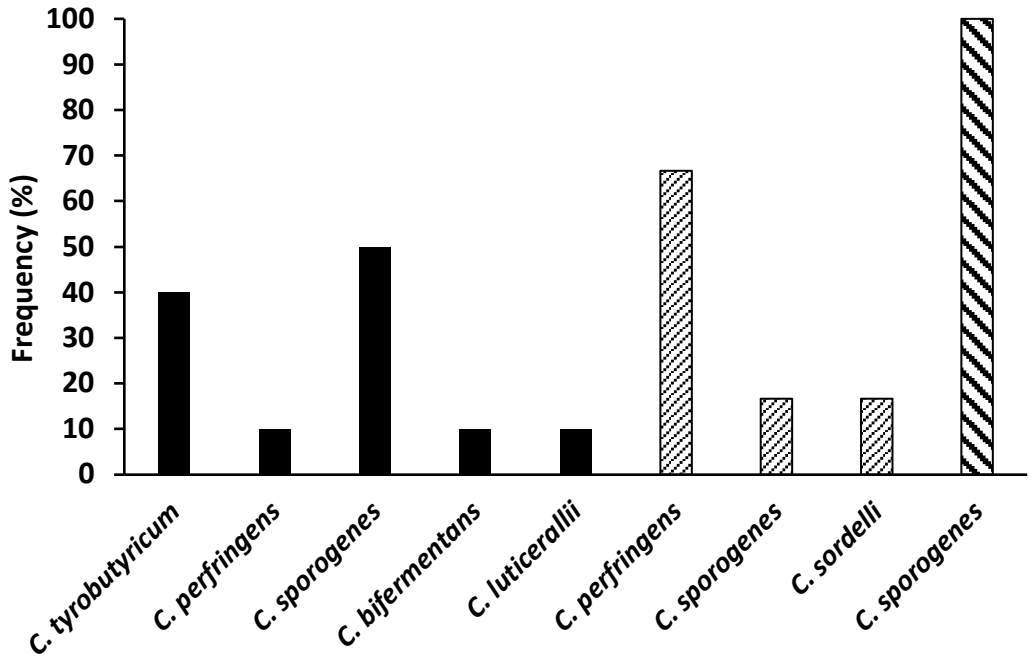

Figure 32. Frequency and distribution of *Clostridium* spp. identified in colonies isolated from cow (■), ewe (▨) and goat (▧) milk samples cultured in RCM selective medium and analyze by 16S rDNA sequencing and multiplex PCR (n=20).

As shown in **Figure 32**, several *Clostridium* spp. were identified from the colonies isolated in RCM with D-cycloserine and neutral red. The most predominant *Clostridium* species found 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 *Clostridium* species were found in our study, such as

C. perfringens, *C. luticeralli* and *C. bifermentans*. These 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. sporogene*s and *C. sordellii* were also isolated but in 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* in ewe milk samples. Additionally, Arias et al. (2013) and Garde et al. (2011) found a relevant presence of *C. sporogenes* in ovine milk. However, *C. sordellii* was not found by other authors in a relevant proportion in bovine, ovine or goat milk. In this study, only *C. sporogenes* was found in goat milk.

In our study, *C. sporogenes* has been found as one of the main species (40%) when considering all types 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 MPN technique and multiplex PCR. They also found that positive samples for *C. tyrobutyricum* by qPCR were also positive for *C. sporogenes* in multiplex PCR. Moreover, in our study the second species enumerated was *C. perfringens* with a frequency of 25% considering the samples of all species. At present, the contribution of *C. perfringens* to LBD is not clear 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 it has not been isolated 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 predominant species of *Clostridium* considering all the types of milk analyzed. This fact reveals that the species of *Clostridium* present in milk used for cheese manufacture can be different depending on the geographical origin of milk, on the type of milk analyzed and on the feed used for the lactating animals, as many authors have previously reported (Brändle et al., 2018; Reindl et al., 2014; Turchi et al., 2016). This fact must be taken into account to develop a detection method based on qPCR. The presence of different *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 the detection of butyric spores.

The aim of this study was to develop a fast and efficient method to detect *C. tyrobutyricum* spores in raw milk samples. As described previously, raw milk was digested with a solution containing detergent and subtilisin, which facilitates the recovery of the spores after centrifugation. The combination of BB with a semi-automatic method that extracts the DNA with magnetic beads allows purifying spore DNA for qPCR in routine analysis. The entire protocol described here allows determining the levels of *C. tyrobutyricum* spores in 1 mL of raw milk in no more than 5 h. As described previously, processing time of analysis is critical to know *C. tyrobutyricum* spore levels to decide the final destination of raw milk. The first qPCR developed to detect *C. tyrobutyricum* spores in raw milk needed multiple enzymatic steps and a final purification step to obtain pure DNA, which took at least 4 h besides the time for qPCR (López-Enríquez et al., 2007). The most recent study to detect *C. tyrobutyricum* spores in raw milk samples extracts the DNA with an enzymatic protocol that needs also 4 h of incubation before qPCR (Arnaboldi et al., 2021). Although this last protocol was found successful to detect and quantify *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 supposes a clear improvement for future implementation in the analysis of high number of samples. Moreover, this is the first study that analyzes *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 species depending on location and type of milk.

Recently, a new promising approach has been developed based on a Loop-Mediated Isothermal Amplification (LAMP) (Cecere et al., 2021). In this study, 20 mL of raw milk samples were digested to recover the spores, and DNA was extracted by heating. After DNA extraction, isothermal amplification was done and the result was revealed by colorimetric change (naked eye). The entire protocol is simple and allows to

have a result in at least 3 h. However, the study lacks an evaluation in field raw milk samples, which should be essential in order to validate the method.

Furthermore, in the present study several *Clostridium* species were also identified in milk samples, based on RCM used as selective media and 16S rDNA sequencing, to verify qPCR results. However, we have checked that it is very difficult to establish a correlation between the results of the microbiological culture and qPCR, because there is not a medium really selective for 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 analysing milk samples by MPN.

4. Conclusions

The novel approach developed in this study to detect *C. tyrobutyricum* spores is based on milk digestion, magnetic separation and purification of DNA followed by qPCR. This method has a LOD of 10^2 spores/mL for a *C. tyrobutyricum* wild strain in raw milk spiked samples. This improvement has allowed reducing considerably the time for detection and quantification of *C. tyrobutyricum,* being able to carry out the whole analysis in no more than 5 h, which is relatively short in comparison with the microbiological methods normally used. This method could be also applied for the identification and enumeration of other microorganisms in milk or other foods.

In comparison with the results obtained by MW treatment and DNA column purification shown in chapter 1, the new method by KF improved considerably the qPCR precision. The purification of spore DNA with magnetic microparticles in the KF method, could be more efficient and recover higher amounts of DNA because lower Ct values were obtained for the same amount of spores, in comparison with MW treatment. Furthermore, the protocol followed here was applied in 1 mL of raw milk in comparison with the previous done in UHT milk achieving a LOD of 10^2 spores/mL. The calibration curve made in raw milk is closer to the real situation because the analysis of *Clostridium* spores will be done in raw milk samples.

The analysis of raw milk samples from cow, ewe and goat has revealed that the concentration of *C. tyrobutyricum* is on the range of 10^2-10^3 spores/mL. The LOD of qPCR might be a limitation of the method because low counts of spores in raw milk can lead to the appearance of LBD in cheese.

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

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Chapter 3

Study of affinity ligands for capture and detection of *C. tyrobutyricum* spores

1. Introduction

This chapter is focused on the development of magnetic particles coated with affine ligands to capture *C. tyrobutyricum* spores in milk. The final purpose is to combine this capture system with the qPCR previously developed, as a fast and highly sensitive method of detection. An immunocapture method for *C. tyrobutyricum* spores was previously developed by Lavilla et al. (2012). In that work, it was reported that specific peptides obtained by Phage Display could be a real alternative to the use of antibodies from animals. For this reason, the search for synthetic ligands is addressed in this part of the thesis.

In this chapter, we describe the study of two main ligands that could be good candidates for magnetic particle functionalization. Firstly, a peptide (pCZS1) selected by the Phage Display technique against *C. tyrobutyricum* spores was evaluated. Secondly, with the aim of improving spore affinity for the peptide during magnetic separation we evaluated the combination of the soluble part of G3P protein (tG3P), which is the phage protein where the peptides of the library are attached, with pCZS1. The affinity of both ligands against *C. tyrobutyricum* spores was studied by isothermal titration calorimetry (ITC) and flow cytometry. After this step, micro and nanoparticles were coated with pCZS1 and tG3P, and capture efficiency (CE) for the spores was determined. Considering the results obtained from these experiments, another approach was assayed, which consisted in obtaining a fusion protein composed by the complete protein G3P (cG3P) with the pCZS1 peptide and evaluating its affinity for *C. tyrobutyricum* spores.

2. Materials and methods

2.1. Selection of the affine peptide (pCZS1)

The affine peptide pCZS1 for *C. tyrobutyricum* spores was selected by Phage Display technique, performed by our research group in collaboration with ZEULAB S.L. (Zaragoza, Spain). The positive selection of pCZS1 was done against a mix of butyric spores: *C. tyrobutyricum*, *C. butyricum* and *C. acetobutylicum.* Furthermore, the negative selection was done against *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Escherichia coli* and *Geobacillus stearothermophilus*. The pCZS1 peptide had originally a sequence of 12 amino acids and showed similar characteristics to that obtained previously by this research group (Lavilla et al., 2009). In comparison with the previous peptide developed, the new one was selected including a negative selection against *C. tyrobutyricum* vegetative cells.

After pCZS1 selection, the synthesis was carried out by the Peptide Synthesis Core Facility at the Universitat Pompeu Fabra (Barcelona, Spain), adding a chain of amino acids as spacer and a molecule of fluorescein isothiocyanate (FITC) in one of the amino acids of the sequence. The final size of pCZS1 was 18 amino acids. The whole sequence is not disclosed as is patentable.

2.2. Recombinant expression of pCZS1-G3P protein.

2.2.1. Design of pET28A pCZS1-G3P protein constructs.

Two additional affine ligands were designed as fusion proteins composed of two forms of G3P protein (whole and truncated) from the M13 phage used in the Phage Display technique, and the pCZS1 peptide. The Phage Display library consists of a variety of recombinant bacteriophages which express peptides attached to their coat proteins. Usually, the peptides are displayed at the G3P protein of the phage, which is an attachment protein enrolled in bacterial infection by the phage (Ledsgaard et al., 2018). G3P protein was selected as a spacer arm because in Phage Display technique the pCZS1 peptide was first presented to the our target displayed in the protein. In **Figure 33** an example of the M13 virus structure and phagemid in the Phage Display library is presented (Kügler et al., 2013). In this example, the oligopeptide (green) is bound to G3P

protein by an histidine tail. In our experiments, the histidine tail was added after the peptide selection.

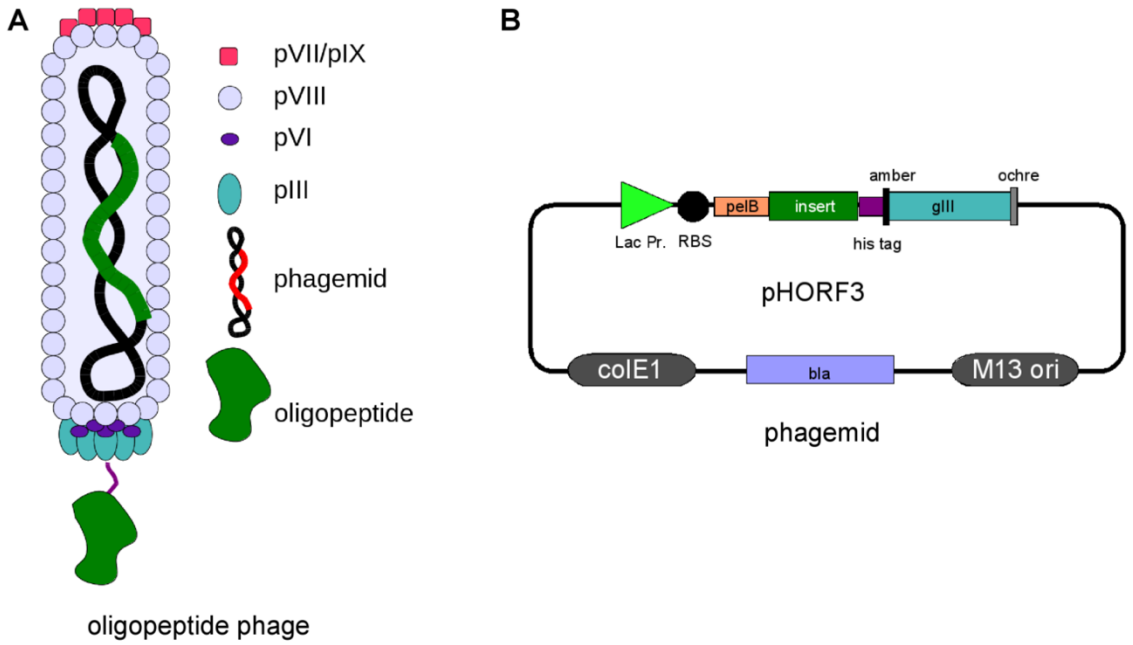

Figure 33. A) Structure of M13 phage and B) sequence of phagemid. pIII: phage protein III or G3P. Abbreviations: lacPr promoter: promoter of the bacterial lac operon; RBS: ribosome binding site; pelB: signal peptide sequence of bacterial pectate lyase *Erwinia caratovora*, mediating secretion into the periplasmic space; gIII: gene coding for the phage protein III; amber: amber stop codon; his tag: six histidine residues; ochre: ochre stop codon. The elements of the inserts are not drawn to scale (Kügler et al., 2013).

The selection of affine peptides by Phage Display technique is known as biopanning. For the biopanning procedure the selected target is usually immobilized in 96 wells microtiter plates. The unbound target molecules are washed away and then the remaining binding sites are blocked with detergents or unrelated proteins. After this step, the phage library is added and unbound phages are removed by stringent washing. Afterwards, the peptide/phage that specifically binds to target is eluted with reduced pH or proteolysis step. The selected phages are amplified by infection of *E. coli* cells. Usually several rounds of biopanning are needed to achieve a significant enrichment in peptides (Pande et al., 2010).

In this work, G3P protein was used as a spacer arm to improve the binding of the spores to the peptide attached to magnetic particles. With this approach the

environment is similar to that of the biopanning step, when the peptide was first selected against the spores.

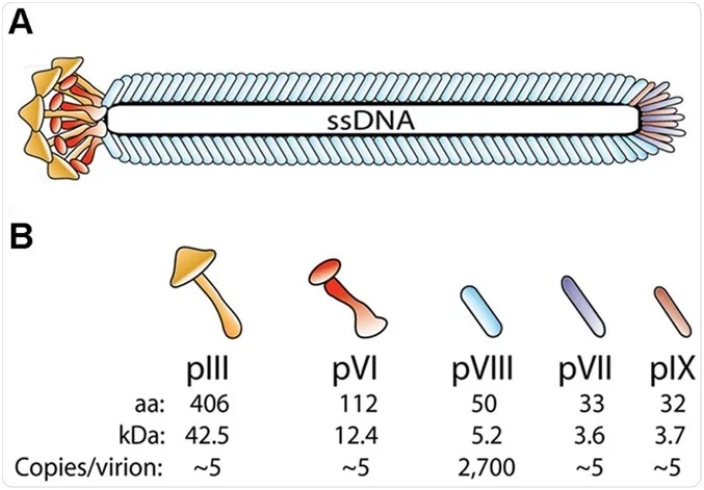

Figure 34. A) Schematic drawing of bacteriophage structure. The virus structure is composed by five proteins that involve the ssDNA. B) pIII is the protein G3P of the M13 phage. Adapted from Løset et al. (2011).

In **Figure 34** the main proteins that compose the M13 phage used in the Phage Display library are represented. G3P protein has a molecular weight of 42.5 kDa and is a transmembrane protein. Considering that transmembrane proteins usually are unstable and can lead to inclusion bodies during their expression in *E. coli* (Zoonens & Miroux, 2010), we designed two ligands: the truncated G3P protein expressing only the soluble domains (D1-D2) with the pCZS1 peptide attached, henceforward named tG3P; and the entire G3P protein with pCZS1 peptide attached, hereinafter named cG3P.

The commercial plasmid pET28A as illustrated in **Figure 35** was selected for the expression of both proteins, cG3P and tG3P. The recombinant protein encoding sequence is expressed by the T7 promoter induced by IPTG (isopropyl β-d-1-thiogalactopyranoside). The sequence of the affine peptide is located at the N-terminal of the soluble domains (D1-D2) followed by the transmembrane domain. In both constructs, a polyhistidine-tag was added to the C-terminal region to assist the purification of the proteins.

Moreover, a kanamycin resistance sequence is incorporated into the plasmid to allow the selection of transformant colonies. These plasmids were provided by GenScript (Piscataway, NJ, USA).

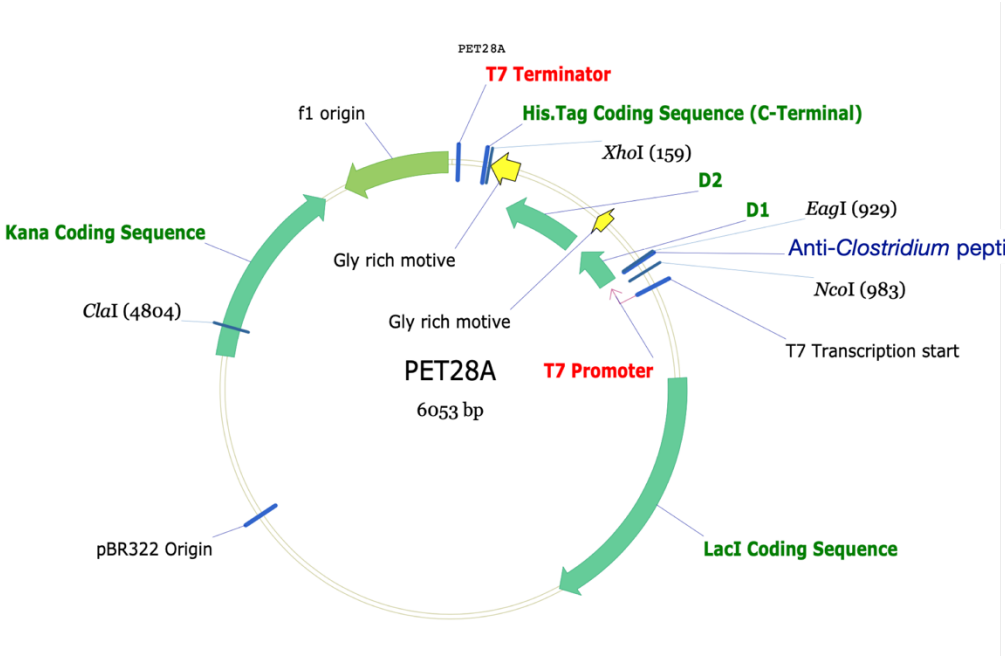

Figure 35. Plasmid for pCZS1-G3P expression complex in *Escherichia coli*. D1 and D2 correspond to domain 1 and 2 of G3P, respectively.

2.2.2. Obtaining BL21 competent E. coli cells

An inoculum of BL21 *E. coli* cells (Merck) kept in glycerol at -20°C was cultured in 50 mL of Luria-Bertani medium (LB , Scharlau, Barcelona, Spain) at 150 rpm and 37°C. After 24 h, 5 mL of culture were sub-cultured in 500 mL of LB medium and were grown in the same conditions during 24 h. Then, the culture was introduced in ice for 30 min to reduce the metabolism of bacteria.

Afterwards, cells were harvested in 50 mL tubes by centrifugation at 3000 x *g* for 15 min at 4°C. The supernatant was discarded and the precipitate was washed with 50 mL of ice-cold 50 mM $MgCl_2$. Then, the resuspended precipitate was centrifuged at 2000 x *g* for 15 min at 4°C. The supernatant was also discarded and the precipitate was resuspended in 50 mL of cold 50 mM $CaCl_2$.

The mixture was incubated on ice for 20 min. Then, cells were recovered by centrifugation at 2000 x *g* for 15 min at 4°C. Finally, the precipitate was resuspended in

4 mL of cold 85 mM $CaCl_2$ with 15% glycerol (v/v) and fractions of 50 µl were kept at -80°C until its use.

2.2.3. Transformation of BL21 E. coli cells

Different conditions were tested for bacterial transformation to select kanamycin-resistant colonies. First, 2, 5, and 10 µL of the stock plasmid (49.5 ng/µL) were added to a 50 µL fraction of *E. coli* competent cells. Each condition was carried out in duplicate. Two samples of competent cells were subjected to the same treatment without adding plasmid, as a negative control, to test the viability of competent cells. The plasmid was added to the cells and the mixture was incubated for 30 min on ice. Then, cells were subjected to heat shock treatment induced in a water bath at 37°C for 45 s and the tubes were immediately returned to ice for 2 min. Finally, 450 µL of LB medium were added and cells were incubated for 1 h at 200 rpm and 37°C.

Afterwards, the suspension was subjected to centrifugation at 5000 x *g* for 5 min to precipitate the bacteria. The bacterial precipitate was spread on LB agar plates with 25 µg/mL of kanamycin and incubated at 37°C for 24 h.

2.2.4. Induction of protein expression

Kanamycin resistant colonies grown on LB agar plates were picked with an inoculation loop and resuspended in 10 mL of LB medium supplemented with 25 µg/mL of kanamycin, in 50 mL tubes. The inoculum was incubated in different conditions to select the optimal one. These conditions were: IPTG induction at room temperature (RT) for 6 h and IPTG induction at RT overnight. Both conditions were tested under agitation at 150 rpm. After incubation, 5 mL of culture was added to 495 mL of fresh LB medium supplemented with 25 µg/mL of kanamycin in flask bottles of 1 L. The culture was incubated at 37°C and 200 rpm for 3-4 h until the absorbance at 600 nm reached the target value of 0.5-0.6. Once the culture reached the required absorbance, 5 mL of a 50 mM solution of IPTG was added to induce the expression of the protein, and the culture was incubated overnight at 37°C with orbital shaking at 150 rpm. Finally, the culture was centrifuged at 5000 x *g* during 10 min to obtain the bacterial paste that was kept at -20°C until protein purification.

For cG3P protein induction, kanamycin was not added to the media during the expression. The experimental results showed an incompatibility between kanamycin and the expression of the protein resulting in a lack of over-expressed protein.

To check the induction of cG3P and tG3P expression, a sample from the overnight culture without IPTG and with IPTG were taken to be analyzed by SDS-PAGE (section 2.4 of chapter 3).

2.3. Purification of cG3P and tG3P

2.3.1. Immobilized metal affinity chromatography

The purification process of cG3P and tG3P was performed using a HiTrap™ chelating column provided by Cytiva (Global Life Sciences Solutions USA, Marlborough, MA, USA) following the instructions of the manufacturer. The automated system for protein purification ÄKTA™ start system (Cytiva) was used. The target protein is expected to bind to Ni^{2+} ions attached to HiTrap™ chelating column by the histidine tag. For cG3P and tG3P purification, a HiTrap™ chelating column of 1 mL (Cytiva) was used. The applied protocol was specific for proteins that normally form inclusion bodies and are difficult to be solubilized from transformant *E. coli* precipitate. In our case, the difficulty in obtaining the protein could be due to the fact that G3P is a transmembrane protein highly hydrophobic and because of the histidine tag addition, which enhances the formation of inclusion bodies in *E. coli* (Zhu et al., 2013). The main steps for the isolation of tG3P and cG3P proteins are summarized in **Figure 36**.

The first step is to isolate the inclusion bodies which are aggregates of insoluble protein in the bacterial precipitate. For this procedure, 4 mL of Tris-HCl, pH 8, was added to the bacterial cell precipitate (section 2.2.4 chapter 3). Then, the sample was sonicated for 4 x 10 s at 90% amplitude on ice with 10 s of interval between sonications. A centrifugation at 13000 x *g* for 10 min was performed to remove cellular debris. Afterwards, the precipitate was resuspended in 3 mL of cold solution consisting of 20 mM Tris-HCl, pH 8, with 2 M urea, 0.5 M NaCl and 2% Triton X-100, and it was sonicated in the same conditions as described above.

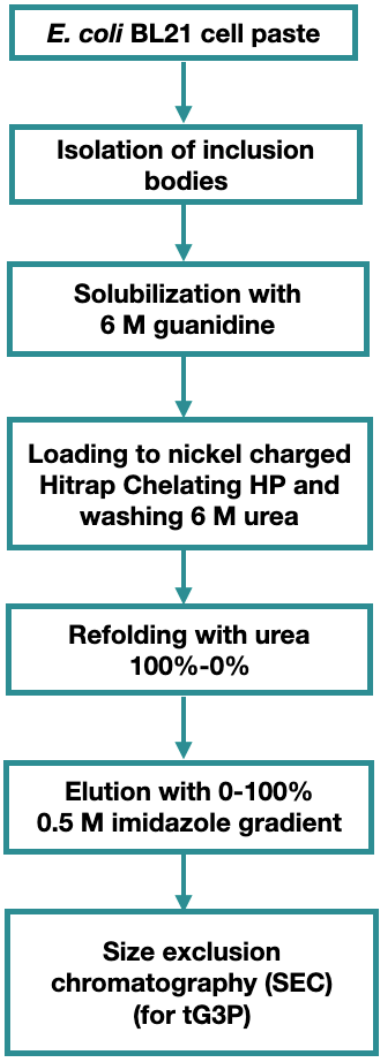

Figure 36. General procedure for tG3P and cG3P isolation from transformed *E. coli* cell paste.

The second step was to solubilize the protein from the inclusion bodies through a denaturation step of the precipitated proteins. Thus, the precipitate was solubilized in 5 mL of 20 mM Tris-HCl pH 8, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, and 2.5 mM mercaptoethanol (binding buffer). The mixture was dissolved in agitation for 30 min at RT. After that, centrifugation at 13000 x *g* for 15 min at 4°C was performed. Then, the supernatant was recovered and filtrated by 0.2 µm before being loaded on the HiTrap™ chelating column. All the steps of the chromatography were made at a 0.5 mL/min flow rate.

The HiTrap™ column must be equilibrated before sample application with at least 10 mL of binding buffer. Afterwards, the sample was applied to the HiTrap™ column of 1 mL followed by a washing with 10 mL of binding buffer. Then, the column

was washed with 10 mL of 20 mM Tris-HCl, pH 8, with 0.5 M NaCl, 5 mM imidazole, 6 M urea and 2.5 mM mercaptoethanol (washing buffer).

After washing the column, a linear gradient was applied with 20 mM Tris-HCl, pH 8, with 0.5 M NaCl, 5 mM imidazole and 2.5 mM mercaptoethanol (refolding buffer), to refold the protein attached to the matrix of the affinity column. Gradient of urea was performed from 100% to 0% with a volume of 30 mL and 0.5 mL/min flow rate starting with a 6 M concentration of urea and finishing with buffer lacking urea to promote the refolding.

The elution of the protein was made with a linear gradient of imidazole from 0 to 100% composed of a buffer containing 20 mM Tris-HCl, pH 8, with 0.5 M NaCl, 500 mM imidazole and 2.5 mM mercaptoethanol (elution buffer). The fractions eluted were collected in 1 mL eppendorf vials.

After the chromatography, the column was washed with 10 mL of distilled water and regenerated with 10 mL of 0.1 M $NiSO_4$ (regeneration solution). To eliminate the excess of $NiSO_4$ a final washing with 10 mL of distilled water was performed. Finally, a solution of 20% ethanol was applied to the column for its storage at 4°C.

2.3.2. Size exclusion chromatography

The fractions obtained in the metal affinity chromatography were subjected to size exclusion chromatography with Sephacryl S-200 high resolution (Cytiva) to eliminate the contaminant bands appearing together with tG3P. This purification step was not necessary for cG3P regarding the results obtained in SDS-PAGE.

First, 180 mL of Sephacryl 200 HR (S-200) gel (Cytiva) was diluted with 20 mM sodium phosphate buffer pH 7.5, 150 mM NaCl (equilibration and packing buffer). The mixture was degassed with a vacuum water pump. Afterwards, the matrix was added to a XK16/70 column (Cytiva) with a height of 60 cm and a total column volume (CV) of 120.6 mL. After matrix sedimentation by gravity, an equilibration step was performed with 1.5 CV of equilibration buffer.

A volume of 1 mL of the sample obtained in the metal affinity chromatography (section 2.3.1 of chapter 3) was loaded into the column. Then, 180 mL of the

equilibration buffer was passed through the column at 0.5 mL/min with a peristaltic pump (GE Amershan Pharmacia Biotech). Eluted fractions of 3 mL were collected by an automatic fraction collector (GE Amershan Pharmacia Biotech). These fractions were analyzed by spectrophotometry at 280 nm. Finally, according to the chromatographic profile obtained, the peak was collected based on the chromatogram absorbance. Fractions were selected and concentrated in Amicon® ultra centrifugal filter units (Merck Millipore, Burlington, MA, USA) of 10 kDa MWCO (molecular weight cut off) and 4 mL volume. Then, concentrated samples were kept at -20°C until its use.

2.4. Electrophoretic techniques and protein quantification

2.4.1. Preparation of polyacrylamide gels

Electrophoresis gels of 12% polyacrylamide were used for analysis of tG3P and cG3P fusion proteins isolated by chromatography. First, the resolving gel was made by mixing 4 mL of 30% of acrylamide-bis-acrylamide (Bio-Rad) (v/v), 2.5 mL of 1.5 M Tris-HCl, pH 8.8, 100 µl of 10% SDS (sodium dodecyl sulphate) (VWR International Eurolab, Llinars del Vallès, Spain) (w/v) and 3.4 mL of Milli-Q water. Before pouring the gel into the Mini PROTEAN casting chambers (Bio-Rad), 10 µl of tetramethylethylenediamine (TEMED) (Sigma- Aldrich) and 50 µl of 10% ammonium persulfate (APS) (Sigma -Aldrich) was added to the mixture for catalyzing the polymerization of the gel. A final volume of 5 mL was poured into the glasses, covering the gel with a thin layer of 2-butanol to avoid its dehydration. Polymerization was left at RT at least for 1 h.

Table 26. Composition of resolving and stacking gel for SDS-PAGE electrophoresis. Gel buffer for resolving gel was composed of 1.5 M Tris-HCl, pH 8.8. Gel buffer for stacking gel was composed of 0.5 M Tris-HCl, pH 6.8.

	Distilled water (mL)	30% of acrylamide (mL)	Gel buffer (mL)	10% SDS (w/v) (mL)
Resolving gel	3.4	4.0	2.50	0.10
Stacking gel	2.2	1.5	1.25	0.05

Once resolving gel was solidified, the layer of 2-butanol was removed with Milli-Q water and the gel surface was dried with Whatman 3MM paper (Cytiva). After this

step, the stacking gel of 9% polyacrylamide was added to the cast to allow proteins to be aligned before entering into the resolving gel. The stacking gel was prepared by mixing 1.5 mL of 30% of acrylamide-bis-acrylamide solution, 1.25 mL of 0.5 M Tris-HCl pH 6.8, 50 µl of 10% SDS (w/v) and 2.2 mL of Milli-Q water. For gel polymerization, 5 µl of TEMED and 25 µl of 10% of ammonium persulfate solution (Sigma-Aldrich) were added. After the application of the gel into the glasses of the caster, the comb with the wells to charge the samples was inserted immediately. The gel was polymerized after 1 h of incubation, and then, it was kept between papers impregnated with a solution consisting of 10 mL of 1.5 M Tris-HCl, pH 8.8, 2 mL of 10% SDS (w/v), and 150 ml of Milli-Q water at 4°C until its use.

2.4.2. Electrophoresis in polyacrylamide gels and staining

The electrophoresis in polyacrylamide gel with SDS (SDS-PAGE) was carried out following the method described previously (Laemmli, 1970). Samples were diluted 1:1 with 126 mM Tris-HCl, pH 6.8, with 20% glycerol, 4% SDS and 0.02% bromophenol blue (Laemmli buffer). Furthermore, to break the disulfide bonds of the proteins, 10% of mercaptoethanol (v/v) was added to Laemmli buffer.

Then, the samples were boiled in a water bath during 5 min to denature proteins and confer them the same charge density, thus allowing their separation by their molecular weight. After this, the electrophoresis chamber Mini PROTEAN Tetra Cell (Bio-Rad) was filled with Tris-glycine buffer composed of 25 mM Tris base, 192 mM glycine, 0.1% SDS (w/v), pH 8.3, and a 10-12 µL volume of samples was charged into the gel wells. The electrophoresis was run for 45 min at 180 V.

After that, the gel was stained with a solution composed of 0.065% Coomassie blue R dissolved in 30% methanol, 10% acetic acid and 10% glycerol. After incubation for 1 h with Coomassie blue solution in gentle shaking, the gel was washed out for 1 h with a destaining solution composed of 25% methanol, 8% acetic acid and 2% glycerol dissolved in distilled water.

2.4.3. Protein quantification by BCA and Micro BCA assay

Protein content of the samples obtained by different procedures was quantified by the bicinchoninic acid assay (BCA) provided by Pierce (Rockford, IL, USA). This method allows the detection and quantification of total protein by a colorimetric reaction which

involves two steps. First, the Biuret reaction takes place, whose faint blue color results from the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium. Second, the chelation of BCA with the cuprous ion occurs, resulting in intense purple color. The BCA/copper complex is water-soluble and exhibits a linear correlation between absorbance at 562 nm and protein concentration.

Standards for the calibration curve were prepared by dissolving bovine serum albumin (BSA) (Sigma-Aldrich) in the specific buffer of the protein that is going to be analyzed, from 0.05 mg/mL to 2 mg/mL. After that, 25 µl of each standard solution or sample and 200 µl of BCA reaction solution were mixed into a 96 well plate. The BCA reaction solution called working reagent was previously prepared following the kit instructions.

The plate was shaken for 30 s and incubated for 30 min at 37°C on a heat block, and after, it was read using a plate reader Multiskan MS from Labsystem (Helsinki, Finlandia) at 560 nm. The absorbance of the samples was interpolated in the calibration curve obtained with the standards by representing the absorbance on the y-axis and the standard concentrations on the x-axis.

To know the total protein bound to magnetic particles, a BCA assay variant named Micro BCA (Pierce) was used. The quantification range of this assay for the microplate procedure is 2-40 µg/mL. The BSA standards were prepared according to the linear range of Micro BCA. A volume of 150 µL of each standard solution or sample was added in a 96 well plate followed by addition of 150 µL of Micro BCA working reagent previously prepared according to the instructions of manufacturer. The plate was shaken for 30 s and incubated for 2 h at 37°C on a heat block. After the incubation, absorbance was read at 560 nm.

2.5. Western blotting

Western blotting technique consisted of two different steps. First, an SDS-PAGE electrophoresis was made following the protocol described in the previous section 2.4. of this chapter. Second, proteins separated in the electrophoresis gel were transferred to a nitrocellulose membrane (Bio-Rad). The protein transference from the gel to the nitrocellulose membrane was carried out in a transference system TE 70 Semi-Dry Transfer unit of Amersham Biosciences (Piscataway, NJ, USA). The components are

placed in the order that is described below, starting from the appliance base of the device, where is located the positive pole, and ending with the cover of the device, where is the negative pole (Licor, 2016) (**Figure 37**):

1. One Whatman filter 3MM paper moistened with 0.3 M Tris, pH 10.4, with 10% methanol.
2. Two Whatman filter 3MM papers moistened with 0.025 M Tris, pH 10.4, with 10% methanol.
3. The nitrocellulose membrane was previously activated immersing it for 10 s in 0.025 M Tris, pH 10.4, with 10% methanol.
4. Electrophoresis gel.
5. Three Whatman filter 3MM papers moistened with 0.025 M Tris-HCl, pH 9.4, 40 mM glycine, with 0.1% SDS and 20% methanol.

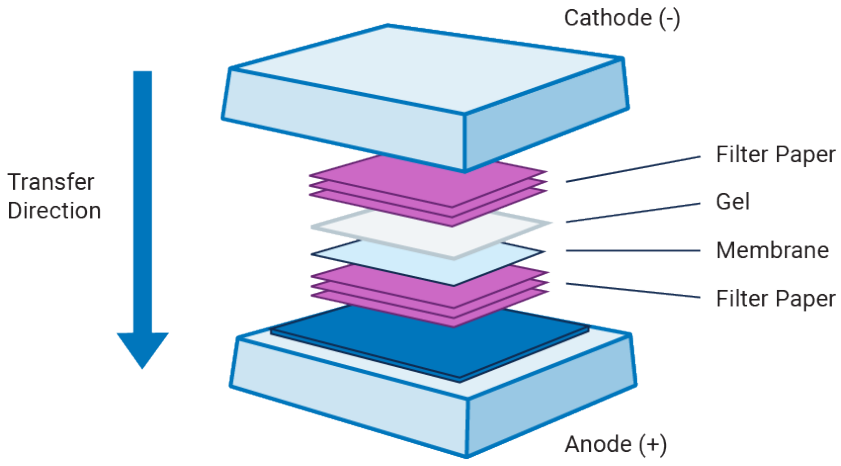

Figure 37. General procedure for Western blotting assay in a Semi-Dry Transfer Unit

The transference was performed for 1 h by applying an electric field of 80 mA. After the transference, the membrane was blocked with TBS (0.05 M Tris-HCl, pH 7.4, 0.5 M NaCl, 2% Tween-20) for 3 min. Afterwards, the membrane was washed three times for 5 min with TBS with 0.1% Tween 20 (TBS-T). After this step, the membrane was incubated with the first antibody diluted in TBS-T for 1 h at RT (see **Table 27**).

After the transference, the target proteins were detected with their specific antibodies. Thus, the membrane was incubated with the first antibody diluted in TBS-T for 1 h at RT. After the incubation with the first antibody, the membrane was washed

with TBS-T three times during 5 min each washing with gentle agitation. Then, the second antibody diluted in TBS-T was added to the membrane. After 1 h of incubation a washing step was performed as described before. The origin an dilutions of the antibodies are described in Table 26. Finally, the result was revealed adding the substrate solution containing 0.06% 4-cloro-1-naftol (w/v), 20% methanol and 0.1% hydrogen peroxide (v/v) dissolved in PBS. The enzymatic reaction was stopped with distilled water.

Table 27. Antibodies and dilutions used for Western blotting analysis. POX: horseradish peroxidase

Antibody	Dilution	Manufacturer
First antibody: Anti-histag	1/2500	(Sigma-Aldrich)
Second antibody: Anti-mouse POX	1/1000	(Sigma-Aldrich)

2.6. Determination of cG3P and tG3P affinity for butyric spores

2.6.1. Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry (ITC) is used to determine thermodynamic parameters of interactions between molecules in solution (Saboury, 2006). In this study, this technique has been applied to analyze the affinity of the ligands developed to capture *Clostridium* spores. The analysis was carried out at constant temperature to obtain the heat exchange of the reaction and to determine the affinity of ligands. ITC experiments provided information about the number of binding sites (n), equilibrium constant (K_d), enthalpy of binding (ΔH), Gibbs free energy of the binding process (ΔG) and entropy of binding (ΔS) corresponding to the interaction between a biomolecule and a ligand (Velazquez-Campoy & Freire, 2006).

The ITC system consists of a sample cell in continuous stirring and an injection controller, which injects the ligand into the cell containing the biomolecule. The calorimeter has two cells: one is the sample cell into which the ligand is titrated using an injection syringe and the other acts as a reference cell which contains water or the specific buffer. After each addition, the heat released or absorbed as a result of the interaction is monitored by the isothermal titration calorimeter obtaining a peak of

power against time for each injection. The heat corresponding to each injection can be calculated from the surface area under the peak (Saboury, 2006).

The instrument used was an isothermal titration calorimeter VP-ITC (MicroCal, Malvern Instruments, UK) in which the sample cell had a volume of 1.409 mL and the total volume injected was 274 µl, with a first injection of 4 µl and the following 27 injections with a volume of 10 µl applied every 10 s. During the experiment, the injection controller, where the solutions of the pCZS1 peptide or tG3P or cG3P proteins were located, was continuously stirred at 469 rpm to mix the proteins added to the spore suspension contained in the cell sample. The assay was made with 10^6 spores/mL ($7.6 \cdot 10^{-12}$ mM) suspension and a solution of 0.086 mM pCZS1 or G3P proteins.

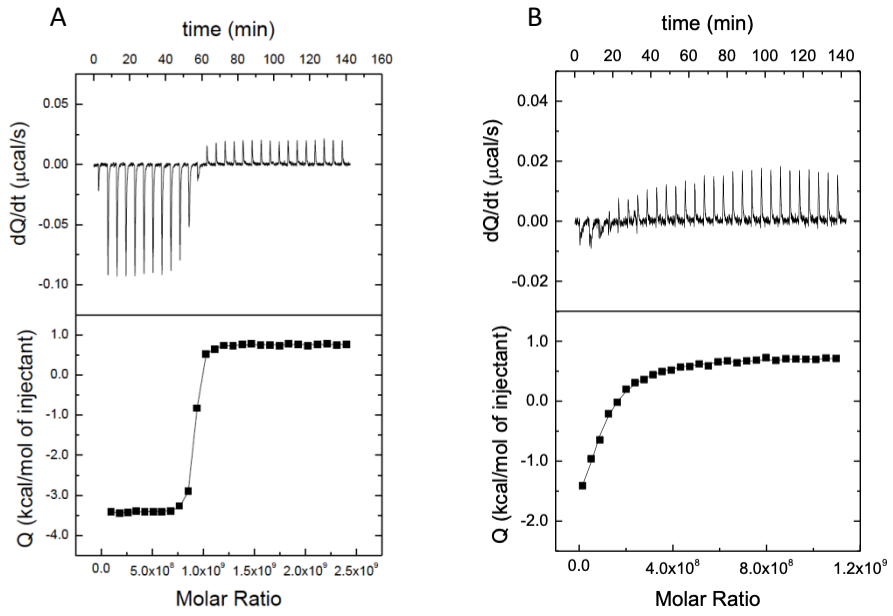

Figure 38. ITC determination of the binding thermodynamics of pCZS1 (A) and tG3P (B) interaction with *C. tyrobutyricum* spores. A) Titration of pCZS1 (0.086 mM) into a suspension of *C. tyrobutyricum* spores (7.6×10^{-12} mM), B) Titration of tG3P (0.086 mM) into a suspension of *C. tyrobutyricum* spores (7.6×10^{-12} mM). The assay was performed in PBS (pH 7.4).

Figure 38 represents the thermogram of the ITC assay for pCZS1 (**A**) and tG3P (**B**). The upper plots show the thermogram, which represents the thermal power required to maintain a minimum temperature difference between a sample and a reference cell as a function of time (Velazquez-Campoy & Freire, 2006). The lower plots show the binding isotherm (titrant-normalized heat effects per injection as a function of

the molar ratio, the quotient between the titrant and titrand concentrations in the sample cell). From the thermogram, binding sites (*n*), equilibrium constant (K_d), enthalpy of binding (Δ*H*), Gibbs free energy of the binding process (Δ*G*) and entropy of binding (Δ*S*) were calculated.

A total of five species of butyric acid Clostridia were evaluated for pCZS1 binding: *C. tyrobutyricum, C. sporogenes, C. acetobutylicum, C. butyricum* and *C. beijerinckii* and *Geobacillus stearothermophilus* as negative control. However, for both G3P proteins, only *C. tyrobutyricum* was evaluated.

These experiments were made in collaboration with the research group of Dr. Adrián Velázquez Campoy at the Instituto de Biocomputación y Física de Sistemas Complejos (BIFI), following his protocol and providing assistance in the data analysis (Velazquez-Campoy & Freire, 2006).

2.6.2. Labelling of tG3P protein with Alexa™ 488

The tG3P protein was labeled with Alexa™ 488 TFP ester (Invitrogen) for the affinity study by flow cytometry. The protocol for the labelling was followed according to manufacturer's instructions. First, tG3P stock solution at 1 mg/mL was concentrated to 10 mg/mL in a final volume of 100 µl with 2 mL Amicon® ultra centrifugal filter unit of 10000 kDa pore size (Merck Millipore). The amine-reactive dye was dissolved in 10 µl of dimethyl sulfoxide (DMSO) at a final concentration of 10 mg/mL. After this step, while stirring the protein solution, the total volume of the reactive dye was added slowly. The mixture was incubated for 1 h in continuous stirring, in darkness at RT.

The labeled protein was separated from the unbound dye in a Sephadex G-25 PD10 desalting column (Cytiva). Before loading the tG3P protein, a solution of 1 mg/mL BSA dissolved in PBS was added to the Sephadex G-25 PD10 to avoid non-specific interactions. Before the addition of the labeled protein, the column was washed with 10 mL of PBS until the absorbance of the eluate at 280 nm was established below 0.02. At this point, the labeled protein was added to the column, and it was recovered in 1 mL fractions by eluting with 10 mL of PBS.

Finally, the absorbance of fractions was measured at 280 nm and they were concentrated to a final volume of 4 mL by using Amicon® ultra centrifugal filter units

10000 kDa pore size (Merck Millipore). The concentrated solution was kept at -20°C until its use.

2.6.3. Flow cytometry

Flow cytometry can be defined as automated microscopy where cells are passed one by one through a laser that records cell characteristics. Flow cytometers can analyze thousands of cells per second. As cells pass through the laser light, three parameters are recorded: forward scatter (FSC), which is proportional to cell size, side scatter (SSC), which is proportional to cell complexity, and finally, fluorescence (Veal et al., 2000). The autofluorescence of cells comes from cell components, such as chlorophyll or flavin nucleotides. Cells can be distinguished by their autofluorescence and shape characteristics. However, the detection and discrimination between cells are achieved using fluorescent probes, which enhance the detection (Veal et al., 2000). To obtain reliable and specific cell counts, dyes that label DNA or membrane receptors are added.

From the beginning, flow cytometry has been applied for the study and detection of mammalian cells. However, in the last years, flow cytometry has emerged as a tool for microbial detection and characterization. The main limitation of detecting bacterial cells by flow cytometry is their size. Bacterial cells are approximately one thousandth the volume of a mammalian cell. Moreover, bacterial spores are smaller than vegetative cells (Veal et al., 2000). For this reason, it is a real challenge to apply flow cytometry to microbiology. However, different assays have been performed to detect viable and non-viable cells and for bacterial counting. For the detection, dyes are added to discriminate between bacterial cells and cellular debris or "noise", considering that samples where bacteria are usually found, are complex (Life Technologies, 2011).

The flow cytometry assays were performed with the equipment Guava® EasyCyte™ Single Sample Flow Cytometer (Merck Millipore). The main advantage of this cytometer is that a small sample volume of 200 µl is used instead of 1-2 mL minimum volume needed for conventional cytometers.

The acquisition and sample analysis was made using the InCyte tool of GuavaSoft™ software 3.1 (Merck Millipore). The maximum events for the sample acquisition were set up in 5000, following the manufacturer's instructions.

2.6.4. Flow cytometer calibration

For the flow cytometer calibration, a suspension of 10^7 *C. tyrobutyricum* spores in 100 µL of sterile PBS was analyzed as positive control and a sample of PBS as negative control. All the solutions used for flow cytometry analysis were previously filtered through 0.22 µm to reduce the background noise as much as possible. The side scatter (SSC) and forward scatter (FSC) parameters were set up using the points of the dot plot corresponding to the spore suspension and the negative control. In this case, the gate of spores was established to differentiate the background noise of cellular debris presented in the sample.

Considering that spores (1 µm size) are smaller than mammalian cells or bacteria, using only light dispersion parameters makes difficult to differentiate properly the spores from the debris. For this reason, labeled ligands pCZS1-FITC and tG3P-Alexa 488 were used to specifically detect the spores and study the interaction between them.

From a stock of 10^9 spores/mL a suspension of 10^7 *C. tyrobutyricum* spores in 100 µl of PBS was prepared. Then, centrifugation was performed at 13000 x *g* for 10 min and the precipitated spores were resuspended in 100 µL of PBS with 0.1% BSA (w/v) and 0.2% Tween 20 (v/v) (blocking solution). Then, 10 µl of pCZS1 from a 2 mg/mL stock solution were added. The mixture was incubated for 1 h at RT in agitation and then, centrifugation was made in the same conditions as described before, followed by two washes with the blocking solution to remove the unbound ligand. Finally, the precipitate of spores was resuspended in 200 µl of PBS. The same procedure was made with PBS without spores, as negative control. To test the optimal concentration of pCZS1 to detect 10^7 *C. tyrobutyricum* spores, the following concentrations of pCZS1 were tested 0.02, 0.2, 2, 10, 20, and 200 µg/mL.

The same protocol was applied for *C. tyrobutyricum* spores detection with tG3P labeled with Alexa 488. To test the optimal concentration of tG3P the following concentrations were tested: 1, 10 and 100 µg/mL. Spores from *Geobacillus stearothermophilus* were used as negative control in flow cytometry assays. This sporulated bacteria was selected based on previous publications which demonstrate that spores of *G. stearothermophilus* can contaminate milk during the milking process

(Ávila & Carvalho, 2020). Moreover, *G. stearothermophilus* spores are ubiquitous and can be found in a variety of environments.

The spores were fitted into the gate (R1) considering SSC and FSC parameters. For both ligands, a green fluorescence histogram was recorded on a logarithmic scale against counts. The threshold of the green fluorescence in a positive sample was established as the maximum fluorescence achieved by the PBS stained with pCZS1 and the autofluorescence of spores without labelling. Below this signal, all the samples would be considered negative.

To sum up, one sample was considered as positive when the dot plot fitted into the gate (R1) of spores and also when the fluorescence histogram (fluorescence) was positive. To analyze the results, the mean fluorescence intensity (MFI) gated by spores and the percentage of spores with fluorescence were calculated.

For the affinity study of both ligands, flow cytometry assays were performed with different strains of *C. tyrobutyricum,* taking as negative control *G. stearothermophilus.* Moreover, five additional species of *Clostridium* involved in LBD, *C. sporogenes, C. butyricum, C. acetobutylicum,* and *C. beijerinckii*, were analyzed to evaluate the cross reactivity with pCZS1. MFI and the percentage of spores with fluorescence were determined for each condition.

2.7. Functionalization of magnetic microparticles with pCZS1

The functionalization of microparticles with pCZS1 was performed by Nanoimmunotech S.L. (Zaragoza, Spain). The selected microparticles of 1 µm size were BcMag™ long-arm Thiol-activated and BcMag™ long-arm Iodoacetyl-activated (Bioclone). The coated particles were tested without blocking agent and blocked with L-cysteine (L-Cys) or polyethylene glycol (PEG) polymers to avoid unspecific interactions. The efficiency of coating was monitored by the emission of fluorescence as pCZS1 was labeled with FITC.

The capacity of microparticles to capture *C. tyrobutyricum* spores was evaluated using suspensions in PBS and UHT milk. For the analysis in PBS, 10^3 spores in 100 µl of PBS were mixed with 100 µl of Iodo or Thiol microparticles at a concentration of 2 x 10^9 particles/mL. The mixture was incubated at 4°C for 2 h in continuous gentle rotation in

a rotator mixer (Lab Logistics Group, GmbH, Meckenheim, Germany). After this period, the microparticles were separated from the solution by using DynaMag™-2 magnet (Thermo Fisher) and resuspended in 100 µl of PBS. The supernatant was kept for further analysis.

For the analysis in UHT milk, 500 µl of commercial UHT whole milk were spiked with 10^3 and 10^2 total spores, and then 500 µl of subtilisin was added. The mixture was incubated for 30 min at 60 °C and after, centrifuged at 13000 x *g* for 30 min to recover the spores. Then, 100 µl of sodium acetate, pH 3.4, was added to neutralize the spore precipitate. After this step, 100 µL of coated particles were added and incubated at 4°C for 2 h. After the incubation, particles were recovered with a DynaMag™-2 magnet and finally, resuspended in 100 µL of PBS.

To determine the percentage of spore recovery, 100 µl of the supernatant and the whole microparticle suspension were cultured on RCM agar plates, separately. The solution was spread onto the plates by streaking technique and the incubation was performed in anaerobic jars at 37°C for 48 h.

The colonies grown on plates seeded with the supernatant and the microparticles were counted to establish the percentage of spore recovery by applying the following formula:

$$\text{CE \% (Capture efficiency)} = \frac{n_p}{n_s + n_p}$$

n_p = number of spores bound to microparticle suspension

n_s= number of spores free in the supernatant

2.8. Functionalization of magnetic micro and nanoparticles with tG3P

Magnetic micro and nanoparticles were also coated with truncated G3P protein (tG3P) to test the capture efficiency of *C. tyrobutyricum* spores.

The magnetic microparticles used were Dynabeads™ His-Tag Isolation and Pulldown (Invitrogen) of 1 µm size and 40 mg/mL concentration. These microparticles can strongly bind proteins with a histidine tag through the Co-NTA complex. The other

type of particles used was Nanomag®-D Ni-NTA of 250 nm particle size, with the same type of binding than that of Dynabeads.

Different concentrations of particles and protein were tested to evaluate the optimal conditions for tG3P binding to the particles. First, 50 µg of Dynabeads microparticles were washed twice with washing/binding buffer in the conditions described in **Table 28**. After that, the microparticles were resuspended in 600 µl of binding buffer, and the protein was added at the corresponding concentration to complete a volume of 700 µl. Different amounts of protein were tested to find the optimal one corresponding to 25, 50, 100 and 200 µg of tG3P. After this step, particles and protein were incubated under orbital agitation for 1 h at RT to ensure the binding. The supernatant obtained after the functionalization was measured by Micro BCA to know the amount of the protein bound to the particles. In the case of Nanomag®-D nanoparticles, 1 mg of particles were washed twice with washing buffer to remove the remnant azide. After that, 50 µg of tG3P protein were added to a final volume of 1 mL. Then, the mixture was incubated for 1 h at RT under orbital agitation in a rotator mixer (Lab Logistics Group). The supernatant after the functionalization was measured by Micro BCA to know the amount of the protein bound to the particles.

Table 28. Characteristics of micro and nanoparticles and conditions used for the experiment of magnetic bioseparation. NS: not specified by manufacturer.

Type of particle (size, nm)	Particles/mg	Concentration	Wash/binding buffer	Pull&down buffer
Dynabeads™ His-Tag Co-NTA (1000)	NS	40 mg/mL	50 mM sodium phosphate, pH 8, 300 mM NaCl, 0.01% Tween®-20	3.25 mM sodium phosphate, pH 8, 70 mM NaCl, 0.01% Tween®-20
Nanomag®-D Ni-NTA (250)	$4.9 \cdot 10^{10}$	10 mg/mL	PBS 0.02% Tween®-20	PBS 0.02% Tween®-20

Once the particles were coated with the target protein, the bioseparation assays of *Clostridium* spores were carried out and the capture efficiency was determined by microbiological culture as explained before in section 2.7. First, 10^3 spores were incubated with 50 µg of Dynabeads coated with tG3P in a final volume of 1 mL of P&D buffer or UHT milk. The mixture was incubated under orbital agitation for 1 h. After this step, the microparticles were recovered by using DynaMag™-2 magnet. The supernatant and microparticles were cultured separately in RCM agar plates as previously described.

For Nanomag®-D particles a similar protocol than the one described for Dynabeads was performed. 100 mg of Nanomag®-D particles coated with tG3P protein were incubated with 10^3 spores in a final volume of 1 mL of PBS 0.02% Tween®-20 or UHT milk. Then, the mixture was incubated for 1 h under orbital agitation and the nanoparticles were recovered by using a DynaMag™-2 magnet. The supernatant and microparticles were cultured separately in RCM agar plates as previously described.

The indirect assay was also evaluated with tG3P protein and Dynabeads microparticles. First, 10^3 spores were incubated with 50 µg of tG3P in a final volume of 700 µL of P&D buffer and the mixture was incubated under orbital agitation for 1 h. After this step, 50 µg of Dynabeads microparticles, previously washed, was added and incubated for 1 h under orbital agitation to recover the spore-tG3P complexes. Finally, the microparticles were recovered by using DynaMag™-2 magnet. The supernatant and microparticles were cultured separately in RCM agar plates as previously described.

3. Results and discussion

3.1. Obtention of transformant *E. coli*

Results of *E. coli* cell transformation with pET28A-tG3P plasmid are shown in **Figure 39**. Positive control consisted of *E. coli* cultured without plasmid in LB agar without kanamycin, to assure the viability of the bacteria after the transformation treatment. Negative control was done with *E. coli* cells without plasmid cultured in LB agar supplemented with 25 µg/mL of kanamycin, to show antibiotic activity. Increasing quantities of plasmid, from 2 to 10 µL, were added to competent cells and all of them were successful. Considering these results, 2 µL of plasmid solution with tG3P or cG3P was used to obtain transformant colonies of *E. coli*.

Once the transformation process was optimized, protein expression in bacteria was tested to produce the maximum protein yield.

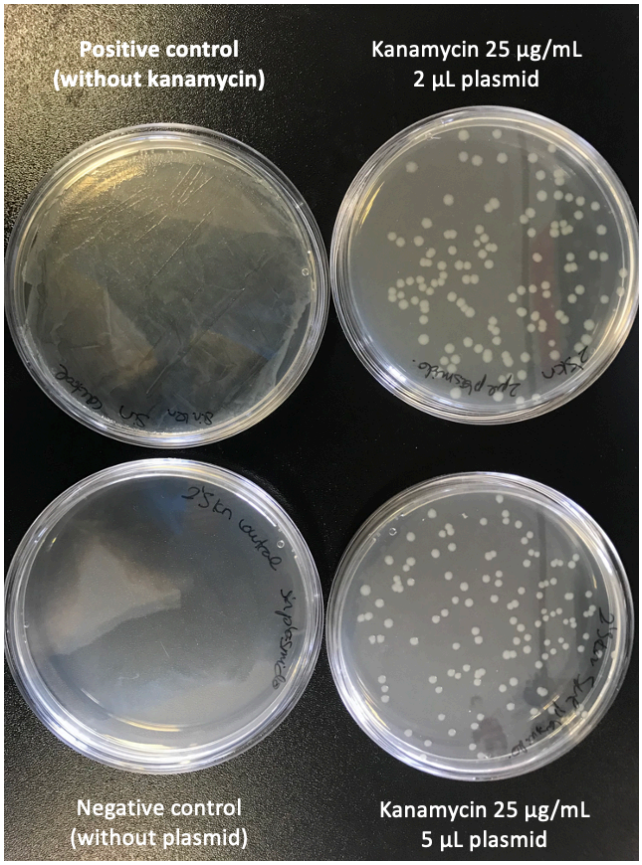

Figure 39. Colonies obtained from *E. coli* transformation with tG3P plasmid. Positive control, negative control and two different conditions of transformation (2 µL and 5 µL of plasmid solution).

3.2. Expression of tG3P and cG3Pproteins in BL21 *E. coli* cells

Optimal conditions for protein expression were evaluated for tG3P. Among five different conditions tested for induced protein over-expression in BL21 *E. coli* cells, the results obtained by SDS-PAGE electrophoresis revealed that after IPTG addition to the culture, the overnight incubation at 37°C showed the highest yield of protein expression (**Figure 40**). A strong protein band appears at the level of 42.5 kDa, being the expected molecular weight of the protein (Sidhu, 2001). Results highlighted temperature and time as key points to improve the yield of protein expression. Moreover, no tG3P protein was found in the supernatant of the culture, suggesting that the protein is expressed inside the *E.coli* cells. The optimal conditions obtained for tG3P protein expression were applied for cG3P protein, since tG3P was produced and isolated before cG3P.

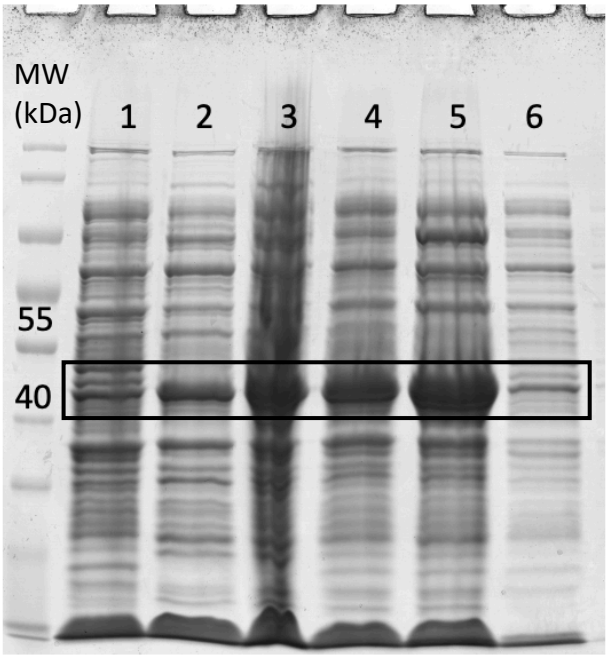

Figure 40. SDS-PAGE in 4-20% polyacrylamide gel of the precipitates of BL21 *E. coli* cells at different conditions of tG3P expression, stained with Coomassie blue. MW: Molecular weight marker. 1: *E. coli* cells without IPTG, 2: *E. coli* cells with IPTG induction at RT for 6 h, 3: *E. coli* cells with IPTG induction at RT overnight, 4: *E. coli* cells with IPTG induction at 37°C for 6 h., 5: *E. coli* cells with IPTG induction at 37°C overnight, 6: supernatant of the cell culture after the induction of the expression obtained in condition 5.

However, although tG3P and cG3P are similar proteins, different results were obtained when the optimal conditions described for tG3P expression were applied for the expression of the complete protein. The addition of kanamycin during IPTG induction affected protein expression, as it is shown in **Figure 41**. After revising the purification conditions, we found that the overexpression only took place when kanamycin was removed from the culture media. The overnight induction at 37°C produced a strong protein band in the range 55-70 kDa molecular weight. Due to these results, during the induction of cG3P, kanamycin was not added.

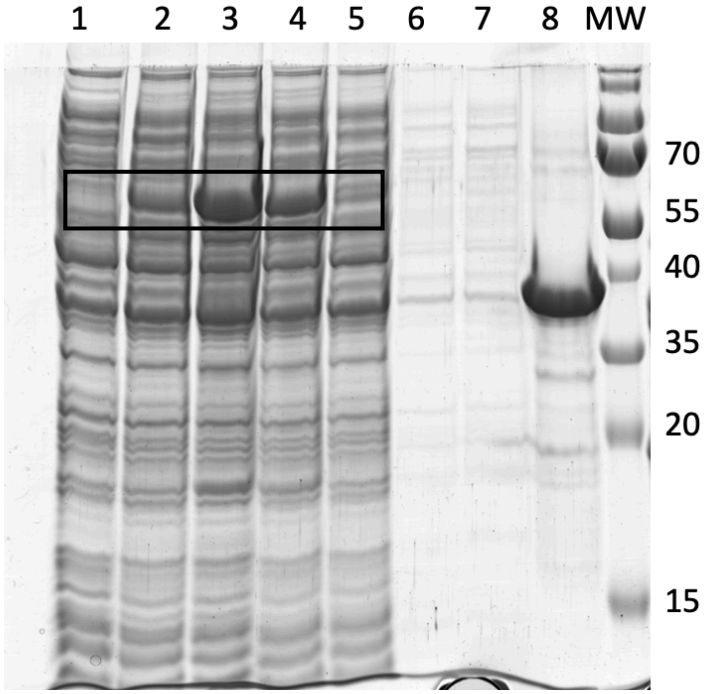

Figure 41. SDS-PAGE in 11% polyacrylamide gel of the precipitates of BL21 *E. coli* cells at different conditions of cG3P expression.1: *E. coli* without adding IPTG, 2: culture 1 of *E. coli* induction with IPTG with 25 µg/mL kanamycin, 3: culture 1 of *E. coli* induction with IPTG without kanamycin, 4: culture 2 of *E. coli* induction with IPTG without kanamycin, 5: culture 2 of *E. coli* induction with IPTG with 25 µg/mL kanamycin, 6: supernatant from culture 1, 7: supernatant from culture 2, 8: tG3P isolated in HiTrap chelating column. Culture 1 and 2 are replicates.

3.3. Purification of tG3P and cG3P by affinity and size exclusion chromatographies

After protein overexpression, the precipitate of BL21 cells containing tG3P or cG3P was subjected to a solubilization treatment to isolate inclusion bodies where the protein is found. Several efforts were made to solubilize the protein by standard methods, such as sonication with a simple buffer (Tris-HCl, PBS with detergents, acidic treatment), but the protein still remained entrapped in the bacterial precipitate. For this reason, a protocol for solubilization of inclusion bodies was tested for extracting the protein with high yield (Cytiva Life Sciences, 2020). For each chromatography, 100 mL of the post-induction culture were centrifuged to obtain the bacterial precipitate. After solubilizing the precipitate, affinity chromatography was performed in the AKTA® start system to isolate tG3P and the chromatogram obtained is shown in **Figure 42**. The volume corresponding to the unbound proteins was recorded by the UV 280 change from 10 to 40 mL.

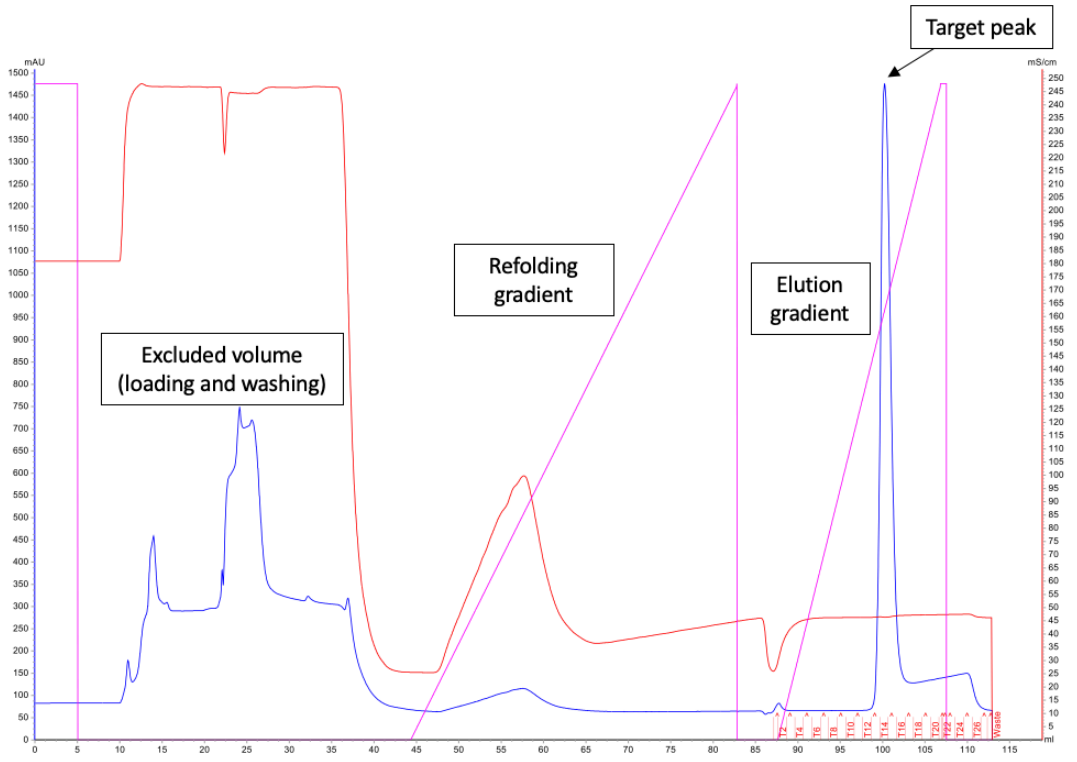

Figure 42. Chromatogram obtained from tG3P isolation by affinity chromatography. Blue line records UV280, red line records conductivity and pink line records gradient concentration (first refolding gradient and second elution gradient).

The volume corresponding to unbound proteins is named as excluded volume and is obtained in the loading and washing step. Then, the refolding gradient was performed to ensure the re-naturalization of the protein attached to the affinity column and corresponded to the volume from 40 to 83 mL. After that, the linear elution gradient started with the addition of imidazole, which applied from the 87 mL volume. The protein was eluted when the buffer contained approximately 60-70% imidazole (volume 99 to 102), meaning a strong affinity of the protein to the column. All the elution volume was collected in 1 mL fractions and showed a maximum absorbance of 1450 mAU**.** The selected fractions containing the main peak were pooled and concentrated by Amicon® ultra centrifugal filter (MWCO 10 kDa), reporting a concentration of 5 mg/mL of protein in 1 mL.

When the protein purity was analyzed by SDS-PAGE, contaminant proteins were found (**Figure 43A**). Consequently, a size exclusion chromatography on Sephacryl S-200 was performed to eliminate the contaminant proteins. The chromatogram obtained for this chromatography is shown in **Figure 43B.**

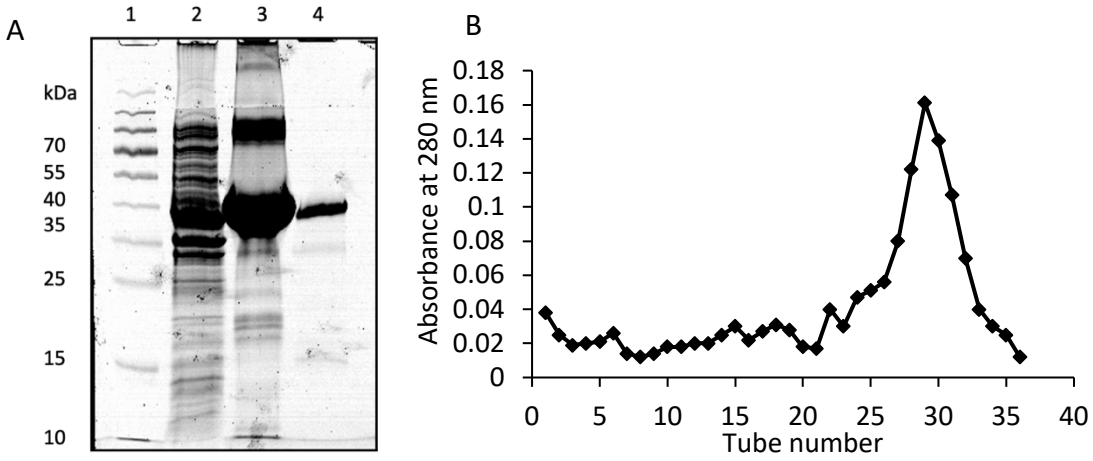

Figure 43. A: SDS-PAGE in 11 % polyacrylamide gel stained with Coomassie blue. 1: molecular weight pattern, 2: precipitate of BL21 *E. coli* cells, 3: fraction obtained from affinity chromatography, 4: tG3P protein concentrated at 1.8 mg/mL after Sephacryl S-200. B: Chromatogram obtained from the purification of tG3P protein in Sephacryl S-200 column. Fraction size of 3 mL.

The first peak obtained in the chromatography (fractions 5-20) corresponded to the contaminant proteins that were isolated together with tG3P in the affinity chromatography. The second peak, from 26 to 33 fractions, corresponded to the tG3P

protein as it can be seen in lane 4 of the SDS-PAGE (**Figure 43A)**. The protein concentration after the second chromatography was measured by BCA achieving a concentration of 1.8 mg/mL in 1 mL, obtained by mixing the fractions corresponding to the second peak. Although contaminant bands were successfully eliminated, the yield after the exclusion size chromatography was of 36%, a significant reduction compared to the protein amount obtained by affinity chromatography.

A Western blotting analysis with anti-histidine antibody was performed to ensure that the isolated protein included the poly-histidine tag in its structure confirming that the isolated protein corresponded with the target recombinant protein. A strong band was revealed in the Western blotting around 40 kDa, as shown in **Figure 44,** which is the expected size of the tG3P protein confirming that the purification was successful.

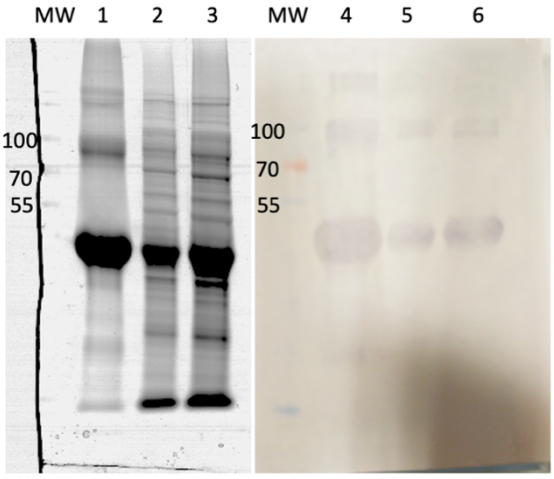

Figure 44. Western blotting of tG3P revealed with antibodies anti-histidine. On the left, SDS-PAGE in 11% polyacrylamide gel and on the right the Western blotting obtained after transference of the gel. 1: tG3P isolated by affinity chromatography, 2: bacterial precipitate diluted 1/2, 3: bacterial precipitate diluted 1/10, 4: tG3P isolated by affinity chromatography, 5: bacterial precipitate diluted 1/2, 6: bacterial precipitate diluted 1/10.

Considering the results obtained for tG3P, the same purification procedure was applied for cG3P. The chromatogram obtained for cG3P isolation by HiTrap™ chelating column shows a different peak from that obtained for tG3P with a maximum absorbance of 350 mAU (see **Figure 45**) instead of the 1450 mAU recorded for tG3P protein. Fractions 13-26 (black box in the chromatogram) obtained in the affinity chromatography were analyzed by SDS-PAGE (**Figure 46**). The results from the chromatogram and electrophoresis showed less quantity of cG3P in comparison with

tG3P. This fact could be due to a lower amount of protein solubilized from bacterial precipitate or to protein aggregation into the HiTrap™ chelating column.

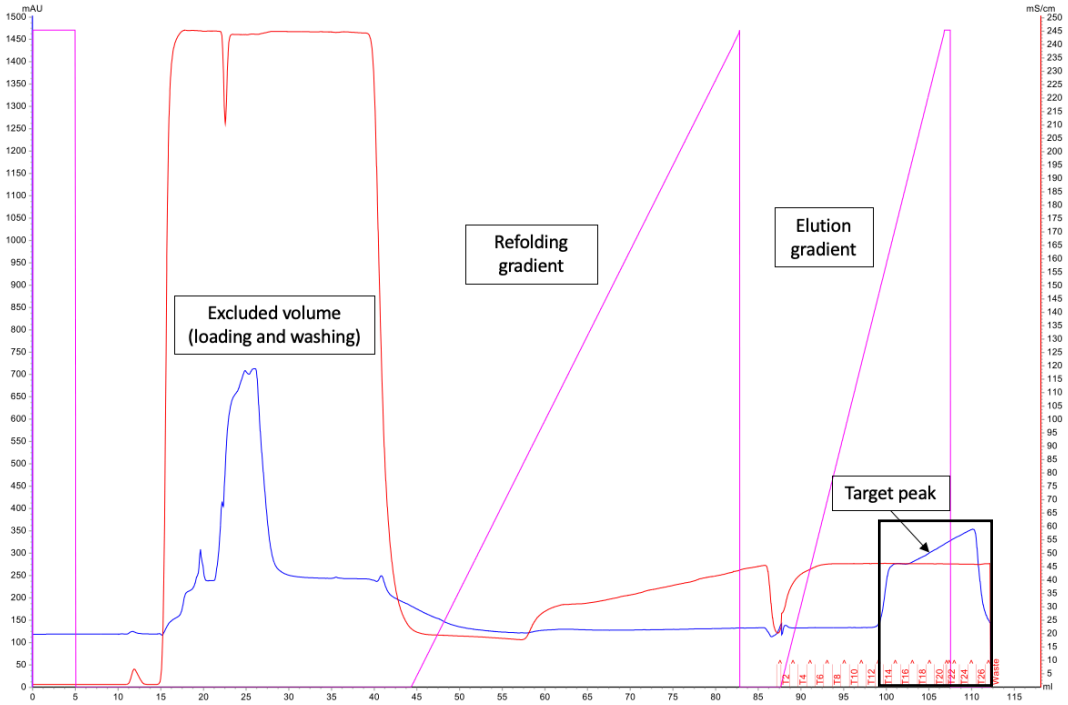

Figure 45. Chromatogram obtained from cG3P isolation by affinity chromatography. Blue line records UV280, red line records conductivity and pink line records the gradient concentration (first refolding gradient and second elution gradient).

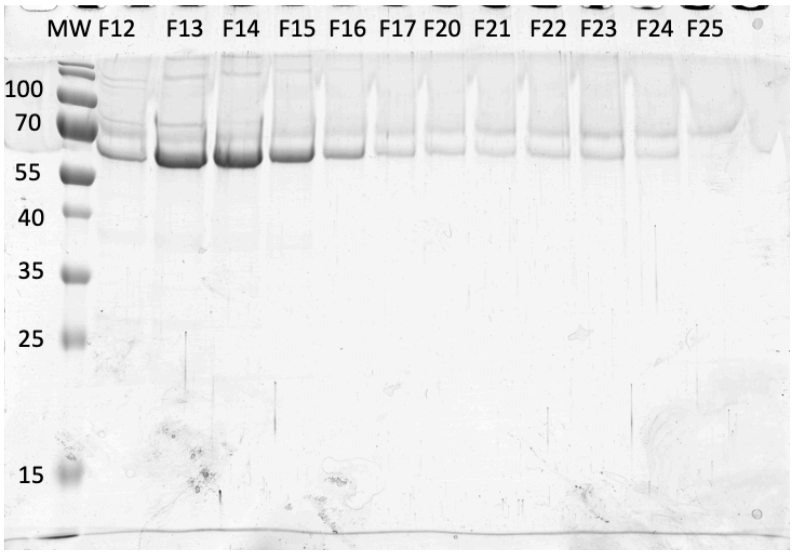

Figure 46. SDS-PAGE in 11% polyacrylamide gel of fractions obtained from the affinity chromatography of cG3P, stained with Coomassie blue. F: corresponds to individual fractions.

After analyzing the fractions by electrophoresis, those from 13 to 26 were recovered and concentrated. For the concentration, a dialysis membrane with MWCO 10 kDa was used instead of Amicon® ultra centrifugal filter (MWCO 10 kDa), as we found that the protein precipitated in this device. The final concentration of the isolated protein was 200 µg/mL in 1 mL volume. The yield obtained for cG3P after size exclusion chromatography was 11%, which was significantly lower than that obtained for tG3P.

Purified tG3P was kept at -20°C until its use. However, cG3P was unstable during the freezing and thawing cycles, possibly due to the transmembrane nature of the protein (Garber Cohen et al., 2010). For this reason, it was kept at 4°C in PBS with 0.01% sodium azide to avoid microbial contamination.

3.4. Evaluation of pCZS1, tG3P and cG3P affinity for butyric spores by isothermal titration calorimetry (ITC)

3.4.1. Evaluation of pCZS1 affinity for spores by ITC

The affinity of pCZS1 for butyric spores was evaluated by ITC. The results obtained from these analyses are summarized in **Table 29**.

Table 29. Results obtained from the analysis of pCZS1 affinity for spores of different *Clostridium* species and *Geobacillus stearothermophilus* by ITC. T: temperature; K: association constant for the peptide-binding site interaction; K_d: dissociation constant for the peptide-binding site interaction; ΔH: enthalpy for the peptide-binding site interaction; N: number of peptide binding sites on the surface of the spores.

Spore species	T (°C)	K (M^{-1})	K_d (µM)	ΔH (kcal/mol)	N
C. tyrobutyricum	15	$1.7 \cdot 10^{8}$	$5.9 \cdot 10^{-3}$	-4.2	$4.0 \cdot 10^{8}$
C. sporogenes	15	$1.5 \cdot 10^{5}$	6.7	-5.9	$6.6 \cdot 10^{7}$
C. butyricum	25	$7.4 \cdot 10^{5}$	1.4	3.2	$8.7 \cdot 10^{7}$
C. acetobutylicum	25	$2.5 \cdot 10^{5}$	4.0	-7.0	$9.5 \cdot 10^{7}$
C. beijerinckii	25	$6.5 \cdot 10^{5}$	1.5	-2.6	$8.2 \cdot 10^{7}$
G. stearothermophilus	15	$3.4 \cdot 10^{7}$	$2.9\ 10^{-2}$	-3.7	$1.3 \cdot 10^{8}$

The affinity of pCZS1 was evaluated against five species of butyric acid Clostridia involved in LBD. From all the species analyzed, *C. tyrobutyricum* showed the lowest K_d meaning that the peptide presents the highest affinity for this species. Usually, for antibodies, K_d values are around the low micromolar (10^{-6}) and nanomolar (10^{-9}) values. However, high-affinity antibodies are in the low nanomolar range (10^{-9}) and very high-affinity antibodies in the picomolar (10^{-12}) range (Abcam, 2022). For *C. tyrobutyricum* spores, a K_d in the low nanomolar range (5.9 nM) was found, which means that pCZS1 has a high affinity for the spores of this species. The rest of butyric species were found in the low micromolar range showing less affinity for the peptide.

Contrary to what was expected, spores from *G. stearothermophilus* analyzed by ITC as negative control, reported lower K_d than that of *Clostridium* species other than *C. tyrobutyricum*. Based on these results, it was concluded that spores from *G. stearothermophilus* have a high affinity for pCZS1. Although a negative selection was performed during the Phage Display selection against the spores of this bacterium, it could not have been as effective as desired. This fact could be explained by the presence of common surface affine regions between the spores of both species. Nevertheless, the K_d value for *C. tyrobutyricum* spores was five times higher compared to *G. stearothermophilus.*

3.4.2. Evaluation of tG3P and cG3P affinity for spores by ITC

Truncated G3P (tG3P) and complete G3P (cG3P) previously isolated and purified were evaluated by ITC to study the affinity for *C. tyrobutyricum* spores. For these proteins, no evaluation was made against other *Clostridium* species.

Table 30. Results obtained from the analysis of tG3P and cG3P affinity for *C. tyrobutyricum* spores by ITC. T: temperature; K: association constant for the protein-binding site interaction; K_d: dissociation constant for the peptide-binding site interaction; ΔH: enthalpy for the peptide-binding site interaction; N: number of protein binding sites on the surface of the spores.

Target protein	T (°C)	K (M^{-1})	K_d (μM)	ΔH (kcal/mol)	N
tG3P	15	$5.7\ 10^5$	1.8	-5.8	$6.7\cdot 10^7$
cG3P	15	$6.2\ 10^5$	1.6	6.8	$5.5\cdot 10^8$

As shown in **Table 30**, K_d values obtained for tG3P and cG3P were similar for both proteins. Moreover, cG3P revealed higher number of protein binding sites in comparison with tG3P. The K_d obtained for tG3P and cG3P were considerably higher than that obtained for pCZS1, suggesting that these proteins have lower affinity than the peptide for *C. tyrobutyricum* spores.

3.5. Evaluation of pCZS1 and tG3P affinity for butyric spores by flow cytometry

3.5.1. Parameters and optimal concentration of pCZS1 and tG3P for C. tyrobutyricum spores detection

The detection of bacterial spores by flow cytometry has been also proposed by different authors. Lavilla et al. (2010) developed a method for the detection of *C. tyrobutyricum* spores in raw milk by flow cytometry, achieving a LOD of 10^3 spores/100 mL of milk, using polyclonal antibodies stained with Alexa 488. Other published studies have used flow cytometry to study *Bacillus* spores germination after applying high-pressure (HP) treatments. The flow cytometer protocol developed provided information about different populations found during the germination step after HP treatments (Zhang et al., 2020). However, considering the low concentration of spoilage microorganisms in raw samples (Martínez et al., 2011) and of *C. tyrobutyricum* spores in milk, which could be less than 10^2 spores/mL, as previously demonstrated in this thesis, it is a real challenge to develop a detection method based on flow cytometry. The studies recently published that detect spores by flow cytometry use high concentrations, around 10^{11} spores/mL (Genovese et al., 2021), which is far from the real concentration of spores in raw samples. For this reason, flow cytometry has been used in this work to study the affinity for different ligands to butyric spores for further functionalization of magnetic particles.

pCZS1 and tG3P were evaluated by flow cytometry to test their capacity to detect *C. tyrobutyricum* spores. The aim of these assays was also to determine the affinity of the different ligands for butyric spores.

In the first place, the gate in which the spores were going to be identified without fluorescence labelling was set considering the side scatter (SSC) and forward scatter (FSC) detector. It was necessary to adjust both parameters to a logarithmic scale because of the small size of the spores (around 1 µm). Moreover, the results were compared with that obtained for PBS to test the background. The region defined by SSC and FSC was named as gate R1. The number of events acquired was set up in 5000 following the instructions of the manufacturer **(Figure 47A)**.

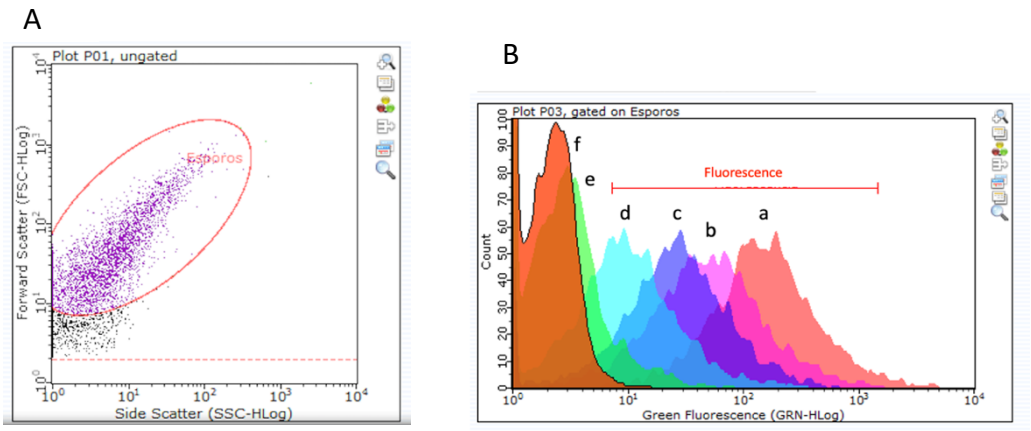

Figure 47. A) Selected gate for a suspension of 10^7 *C. tyrobutyricum* spores by SSC and FSC detectors. B) Fluorescence histogram gated on spores. Each histogram represents one concentration of pCZS1 incubated with a 10^7 suspension of *C. tyrobutyricum* spores/mL. a) 200 µg/mL, b) 20 µg/mL, c) 10 µg/mL, d) 2 µg/mL, e) 0.2 µg/mL and f) 0.02 µg/mL.

For the fluorescence histogram, the red horizontal bar drawn in **Figure 47B** represents the fluorescence range in which a sample is considered as positive. The red horizontal bar was adjusted to the intrinsic fluorescence of a solution of 10^7 *C. tyrobutyricum* spores without staining with pCZS1. Moreover, a sample with PBS without spores was stained and treated with the same protocol as the samples with spores with the aim of determining the fluorescence background. Therefore, the fluorescence threshold was set up according to those two parameters. The green fluorescence axis was adjusted also to a logarithmic scale. In **Figure 47B** the number of events and the green fluorescence of a suspension of 10^7 spores incubated with different concentrations of pCZS1 are represented. After the identification of spores, the optimal concentration of pCZS1 to detect them was evaluated.

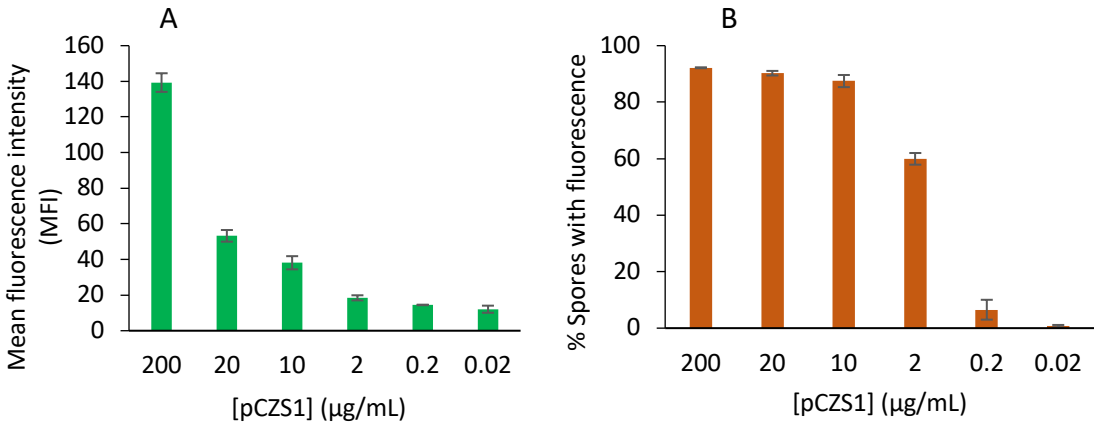

Figure 48. Detection of 10^7 *C. tyrobutyricum* spores by flow cytometry with pCZS1. A) Mean fluorescence intensity (MFI) of *C. tyrobutyricum* spores incubated with different concentrations of pCZS1. B) Percentage of *C. tyrobutyricum* spores detected in the gate of spores using different concentrations of pCZS1. Values are given as the mean and vertical bars indicate standard deviation, determined from three different experiments, carried out by duplicate (n=6).

In **Figure 48A** the MFI for each concentration of pCZS1 is shown and **Figure 48B** represents the percentage of spores detected at the different concentrations evaluated. Among all conditions tested, pCZS1 at 200 µg/m Lprovided the highest MFI value. Regarding the percentage of spores detected, the highest values were achieved between 10 and 200 ug/mL of pCZS1. Considering that a MFI value of 38 was high enough to detect fluorescent spores the concentration of 10 µg/mL pCZS1 was selected to carry out the following experiments.

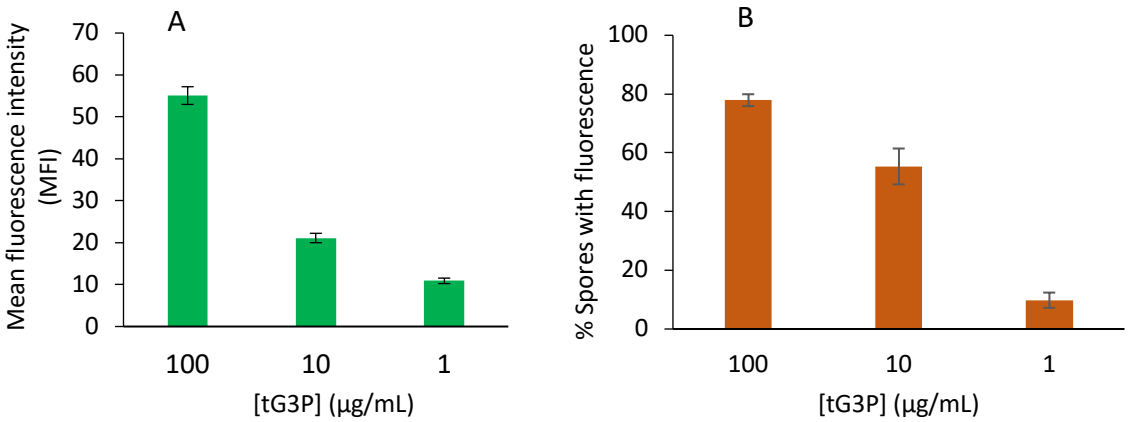

Figure 49. Detection of 10^7 *C. tyrobutyricum* spores by flow cytometry with tG3P labeled with Alexa 488. Mean fluorescence intensity (MFI) (A) and percentage of spores with fluorescence (B) using different concentrations of tG3P. Values are given as the mean and vertical bars indicate standard deviation, determined from two different experiments, carried out by duplicate (n=4).

Three concentrations of Alexa 488 labeled tG39 were evaluated to detect *C. tyrobutyricum* spores: 1, 10, and 100 µg/mL. Results of this assay are shown in **Figure 49**. The highest concentration of tG3P, 100 µg/mL, was able to detect 77.90% of spores with 55.08 of MFI. The MFI value obtained was higher than that obtained with 10 µg/mL of pCZS1, although the percentage of spores detected with tG3P was lower.

Considering that concentrations of tG3P under 100 µg/mL resulted in low levels of spore detection, these conditions were discarded. Results showed that it was necessary a concentration of tG3P ten times higher than that of pCZS1 to reach good values of percentage of spores detected and MFI. This can be explained by the different molecular weight of both ligands since there are more molecules of fluorescein associated to pCZS1 than for tG3P for the same amount of ligand. These results are in line with those obtained by ITC analysis, demonstrating that tG3P protein has lower affinity for *C. tyrobutyricum* spores and fewer binding sites than pCZS1 (see **Table 29** and **Table 30**).

3.5.2. Evaluation of selectivity and cross-reactivity of ligands for spores by flow cytometry

Once the flow cytometry technique was set up to detect *C. tyrobutyricum* spores, the affinity of pCZS1 and tG3P for different strains of *C. tyrobutyricum* and for *G. stearothermophilus* was analyzed.

In **Figure 50**, MFI and the percentage of spores detected with fluorescence for each strain of *C. tyrobutyricum*. The amount of spores detected for three strains of *C. tyrobutyricum* was in the range of 84-87%. Moreover, the MFI value was 31 and 33 for *C. tyrobutyricum* UZ01 (wild strain) and CECT 4012 (type strain), respectively. *C. tyrobutyricum* UC7086 (Italian wild strain) showed an MFI value of 43, slightly higher than those of the other strains tested.

As previously done by ITC, *G. stearothermophilus* spores were analyzed showing a significantly lower percentage of spores with fluorescence, only 38% could be detected with pCZS1, suggesting that the peptide had low affinity for the spores of this bacterium by flow citometry. Furthermore, the MFI value for *G. stearothermophilus* spores was 13, considerably lower than the value obtained for the spores of *C. tyrobutyricum* strains.

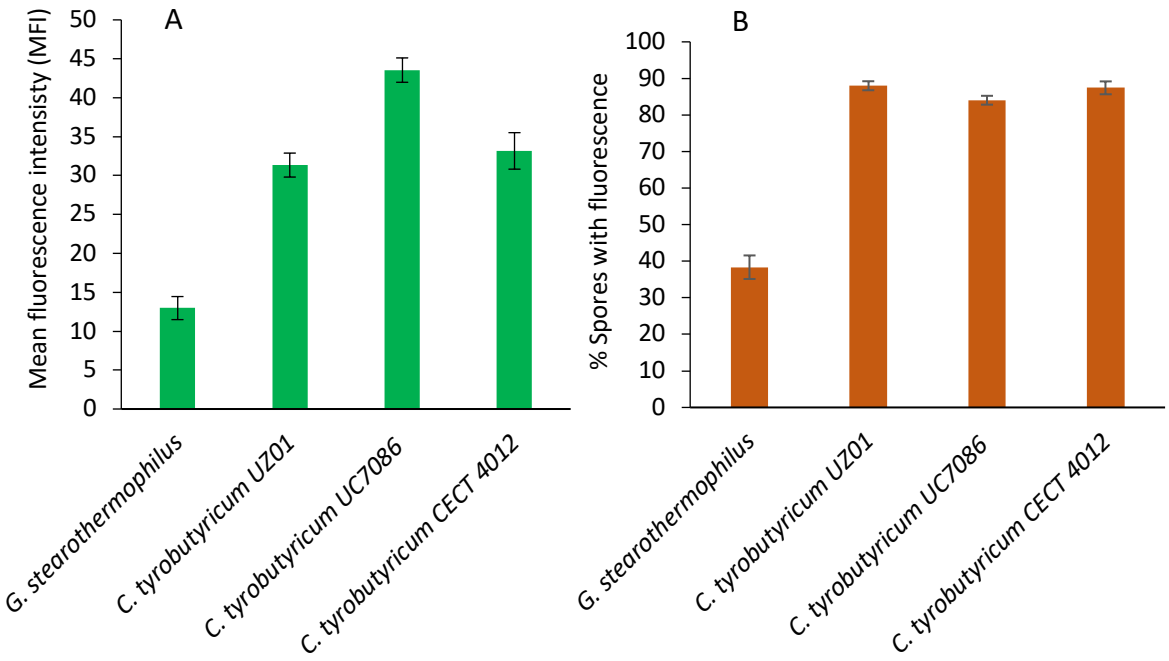

Figure 50. Interaction of pCZS1 with 10^7 spores of different strains of *C. tyrobutyricum* and *G. stearothermophilus* by flow cytometry. (A) Mean fluorescence intensity (MFI) and (B) percentage of spores with fluorescence are represented. Values are given as the mean and vertical bars indicate standard deviation, determined from three different experiments, carried out by duplicate (n=6).

In addition, the detection of other four *Clostridium* species commonly involved in LBD was evaluated, as previously done by ITC. The species evaluated by flow cytometry with pCZS1 were *C. sporogenes, C. butyricum, C. acetobutylicum,* and *C. beijerinckii*. Regarding the results shown in **Figure 51**, the highest MFI and percentage of fluorescent spores values were achieved for *C. tyrobutyricum* CECT 4012. The four species tested different from *C. tyrobutyricum* had similar values of MFI varying from 13.75 (*C. beijerinckii*) to 15.43 (*C. sporogenes*), taking into account that the MFI reported for *C. tyrobutyricum* CECT 4012 was 31.95. The comparison of the percentage of spores detected with fluorescence revealed that these four species had close values, between 58.42% and 70%, which demonstrated that pCZS1 could bind to other *Clostridium* species with similar affinity. Nevertheless, *C. tyrobutyricum* was the species with the highest percentage of fluorescent spores (84.84%). These observations correlate with the results obtained by ITC, where K_d values obtained for *C. sporogenes, C. butyricum,*

C. acetobutylicum and *C. beijerinckii* were higher than that for *C. tyrobutyricum* which means lower affinity.

In conclusion, pCZS1 has a high affinity for *C. tyrobutyricum* spores considering the data obtained by ITC and flow cytometry, and these results make this ligand a good candidate to functionalize microparticles for spore bioseparation. Moreover, considering the K_d and MFI data obtained, pCZS1 could have a certain affinity for other *Clostridium* species. As the peptide was first selected against *C. tyrobutyricum* with the Phage Display technique, this finding makes sense. However, as the spore coat may have common proteins or regions among different bacterial strains and species, there may be some undesirable cross-reactions in the binding of pCZS1 to other spores. Further studies about the affine regions and proteins of the spores would be needed to elucidate the binding of pCZS1 to other bacteria.

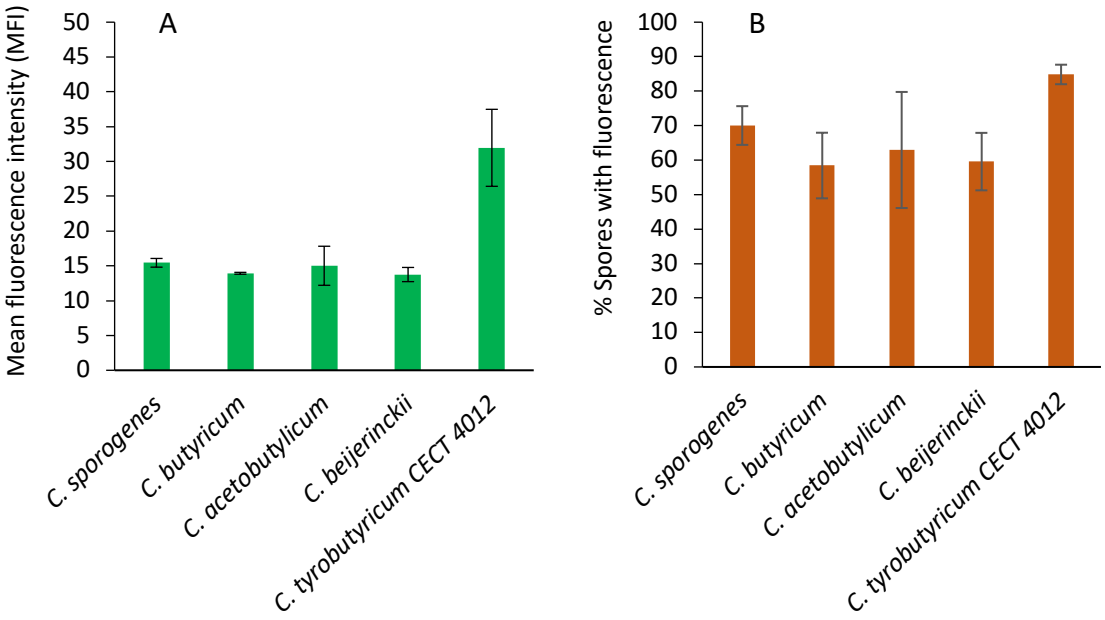

Figure 51. Detection of spores of five *Clostridium* species involved in LBD by flow cytometry with pCZS1. All the assays were done with 10^7spore suspensions. (A) Mean fluorescence intensity (MFI) and (B) percentage of spores with fluorescence are represented for each species. Vertical bars indicate standard deviation, determined from two different experiments, carried out by duplicate (n=4).

Finally, the affinity of tG3P labeled with Alexa 488 was evaluated by flow cytometry for different strains of *C. tyrobutyricum*. In this assay two wild strains from Italy were included, *C. tyrobutyricum* UC9036 and UC7086. The results showed similar values of MFI and percentage of spores for the two *C. tyrobutyricum* strains as shown in **Figure 52**. For *G. stearothermophilus*, used as negative control, 55% of the spores were detected with fluorescence, but with high standard deviation and the MFI value was 22, being half of the value recorded for *C. tyrobutyricum*.

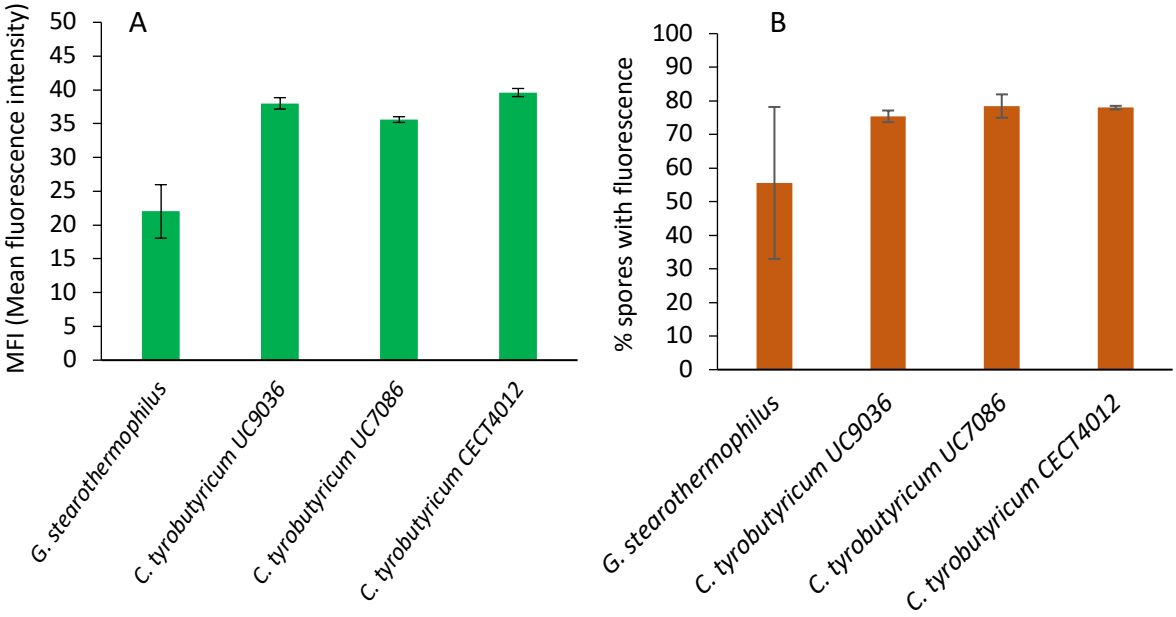

Figure 52. Detection of spores of three *C. tyrobutyricum* strains involved in LBD and of *G. stearothermophilus* with tG3P by flow cytometry. All the assays were done with 10^7 spore suspensions. (A) Mean fluorescence intensity (MFI) and (B) the percentage of spores with fluorescence are represented for each strain. Vertical bars indicate standard deviation, determined from one experiment, carried out by duplicate (n=2).

The data obtained by flow cytometry showed affinity of tG3P for *C. tyrobutyricum,* which agrees with ITC results, but the assay needed to be performed with a concentration of tG3P ten times higher than that used with pCZS1. These results are in agreement with the fact that for the same quantity of ligand there are more labeled molecules of pCZS1 than of G3P protein.

3.6. Capture of *C. tyrobutyricum* spores by magnetic beads coated with pCZS1

3.6.1. Characterization of microparticles coated with pCZS1

In this section, the binding of pCZS1 to magnetic particles was evaluated to develop an efficient system to capture *C. tyrobutyricum* spores in milk. Two types of microparticles were functionalized: BcMag™ long-arm Thiol-activated (Bioclone) and BcMag™ long-arm Iodoacetyl-activated (Bioclone), both particles of 1 µm size.

First, an initial characterization by UV-visible and Ellman assay revealed that only 50% of the thiol groups of the peptide were available for their binding to microparticles. For this reason, before microparticle coating a complete reduction of pCZS1 was done. The characterization showed that the molar ratio between the thiol groups and peptide concentration was around 1, which means that almost all the thiol groups were available on the peptide after its reduction. The coating with the reduced pCZS1 was done and the quantity of bound peptide for each particle was determined by the UV visible spectrum at 497 nm. The characteristics of coated microparticles and stability were measured by Dynamic Light Scattering (DLS) after incubation in UHT milk.

Table 31. Characteristics of Thiol and Iodoacetyl-activated microparticles coated with pCZS1 peptide by DLS.

	Peptide molecules bound per particle	**Size (nm)**	**Polydispersity index**
BcMag™ long-arm Thiol-activated	12 x 10^6	1162 ± 111	0.25
BcMag™ long-arm Iodoacetyl-activated	18 x 10^6	1112 ± 88	0.23

The main characteristics of microparticles coated with pCZS1 peptide are summarized in **Table 31**. DLS measurement reported a similar value of size to that specified by the manufacturer that is around 1000 nm. Moreover, the polydispersity index is considered inside the acceptance criteria if the value is lower than 0.3 (Mudalige et al., 2019; Nanoimmunotech S.L., 2018).

Fluorescence was measured for both types of particles before and after the incubation in UHT milk. As presented in **Figure 53**, both particles showed a similar spectrum in the two conditions, and considering DLS results this means that coated particles are stable in milk and that no aggregation occurred. The results obtained from the characterization of microparticles allowed to design the assays in order to test the capacity of the coated particles to capture *C. tyrobutyricum* spores.

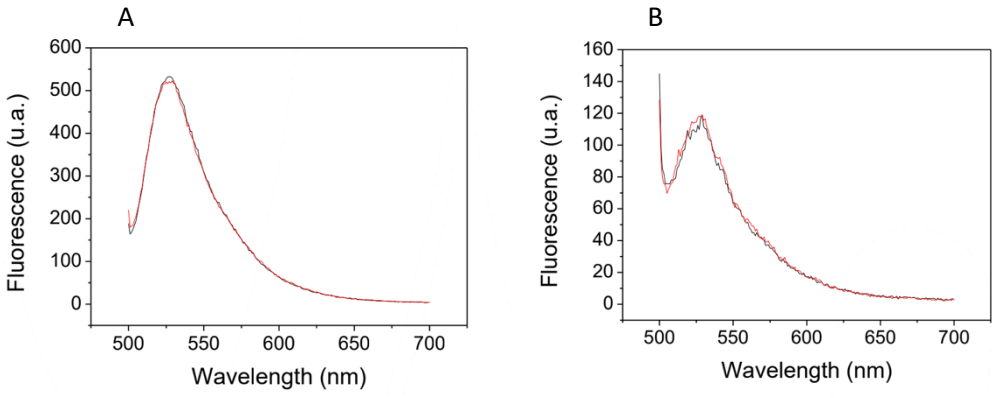

Figure 53. Fluorescence emission spectrum for (A) BcMag™ long-arm Thiol-activated and (B) BcMag™ long-arm Iodoacetyl-activated coated with pCZS1 in PBS pH 7.3 (black line) and after incubation in UHT milk (red line).

3.6.2. Assays of spore capture by microparticles coated with pCZS1

To determine the optimal conditions for capturing *C. tyrobutyricum* spores, preliminary assays were done with different conditions. First, the CE was evaluated in PBS and UHT milk. The results of CE obtained for each type of microparticle and different conditions in this preliminary assay are summarized in **Table 32**. Both particles allow recovering around 90% of *C. tyrobutyricum* spores in PBS, as it was found in a previous study (Lavilla et al., 2012), where similar CE values were obtained in PBS using affine peptides bound to micro and nanoparticles, for *C. tyrobutyricum* spores. However, our results showed that the CE decreased in UHT milk and only around 10-14% of the spores were recovered.

Table 32. Capture efficiency (CE) (%) of microparticles coated with pCZS1 for 10^3 *C. tyrobutyricum* spores in PBS, UHT milk and subtilisin-treated UHT milk.

	PBS	**UHT milk**	**Subtilisin-treated UHT milk**
BcMag™ long-arm Thiol-activated	89.6	10.3	6.3
BcMag™ long-arm Iodoacetyl-activated	90.4	13.7	22.5

Considering the results obtained in UHT milk, an assay was done treating milk with subtilisin, though still low CE was obtained for both particles. The results revealed that coated microparticles recovered *C. tyrobutyricum* spores in an aqueous and simple buffer as PBS, but in a complex matrix like UHT milk, the recovery was less efficient. Although long-arm particles were selected to allow, as much as possible, the availability of the peptide to capture spores, low CE was reported in UHT milk.

The results obtained in the preliminary assays allowed us to conclude that the functionalized particles were not able to recover *C. tyrobutyricum* spores in milk. To solve this problem, BcMag™ long-arm Thiol-activated and BcMag™ long-arm Iodoacetyl-activated were coated with pCZS1 and blocked with polyethilenglycol (PEG) or cysteine (Cys) to avoid unspecific binding of milk proteins to the sites of the particle surface non covered by the peptide, which could reduce CE.

The preliminary assay to evaluate the spore capture with microparticles coated with pCZS1 and blocked with PEG provided similar results than those previously obtained without blocking (**Figure 54**). Results showed that particles could recover spores with high CE from PBS, but lower values were obtained when the assay was performed in UHT milk. Considering the blocking compounds, the best one when using BcMag™ long-arm Thiol-activated particles was PEG and for BcMag™ long-arm Iodoacetyl-activated particles was cysteine.

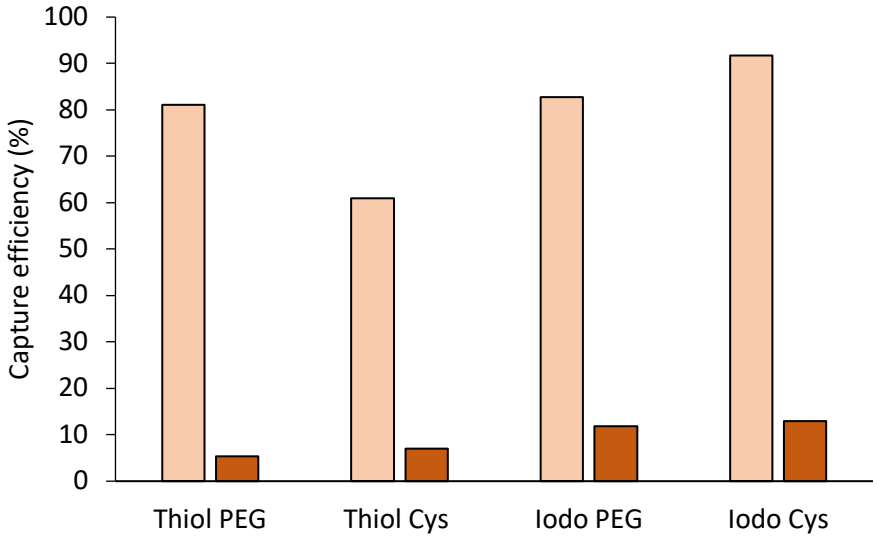

Figure 54. Capture efficiency (CE) of BcMag™ long-arm Thiol-activated particles and BcMag™ long-arm Iodoacetyl-activated particles coated with pCZS1 and blocked with PEG or cysteine in PBS (□) and UHT milk (■) for capturing 10^3 *C. tyrobutyricum* spores.

A last assay was done to capture 10^2 and 10^3 *C. tyrobutyricum* spores from 1 mL UHT milk treated with subtilisin by adding BcMag™ long-arm Iodoacetyl-activated and Thiol-activated without blocking. First, UHT milk was spiked with the desired amount of spores, and then subtilisin was added. Milk was incubated and centrifuged to obtain a precipitate with spores. The precipitate was resuspended in sodium acetate buffer, pH 3.5, to neutralize the solution. After this step, microparticles were added to perform the bioseparation.

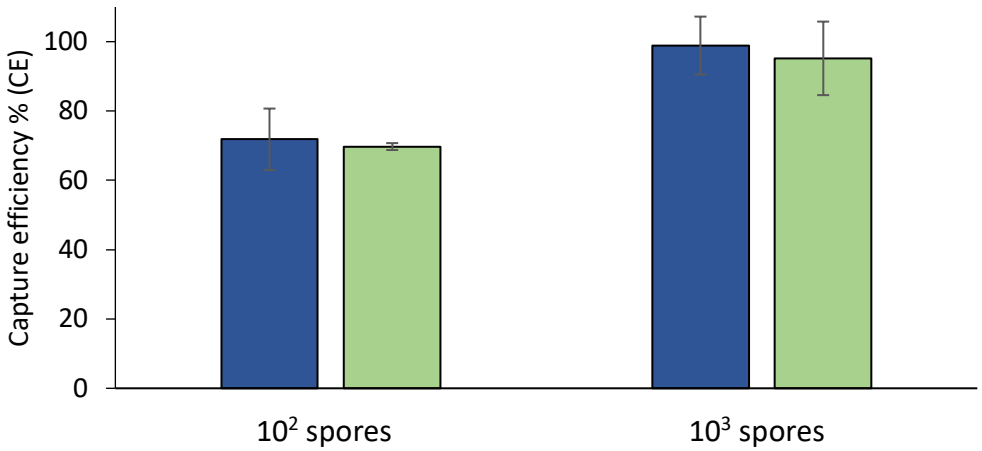

Figure 55. Capture efficiency (CE) of microparticles coated with pCZS1 in UHT milk treated with subtilisin for 10^2 and 10^3 *C. tyrobutyricum* spores. BcMag™ long-arm Thiol-activated particles. (■) and BcMag™ long-arm Iodoacetyl-activated particles (■). Values are given as the mean and vertical bars indicate standard deviation, determined from three different experiments (n=3).

Results obtained are summarized in **Figure 55**, where it can be observed that high CE of about 90% was obtained for 10^3 spores and 70% for 10^2 spores. These differences could be due to the difficulty in capturing a small number of spores in milk because the different macromolecular components of milk make more difficult the interactions between microparticles and spores.

The results obtained in the assays of spore capture in milk revealed that an efficient separation could be only performed when milk was previously treated with subtilisin. It was concluded that the system developed worked in a simple aqueous buffer as PBS, but the bioseparation in UHT milk required an additional treatment, probably due to the complex composition of milk and the low size of the peptide. Although CE values were successful for 10^3 spores, another approach was designed with the aim of simplifying the recovery of spores in milk without any treatment. For this reason, the microparticles were functionalized with tG3P protein expressed including pCZS1. In this format the G3P protein is expected to act as a spacer-arm on the particle, emulating the environment of the peptide during the biopanning process in which the peptide was first selected.

3.7. Capture of *C. tyrobutyricum* spores using micro and nanoparticles coated with tG3P

3.7.1. Capture of spores using Dynabeads™ His-Tag coated with tG3P

Dynabeads™ His-Tag (1 µm size) coated with tG3P were analyzed to capture *C. tyrobutyricum* spores. These microparticles were selected because of the easy way to bind the protein in a correct orientation to capture the spores. The histidine tag is located in the C-terminal region of the tG3P protein while the peptide is in the N-terminal region. Therefore, the complex formation of the microparticle and the protein by the histidine tag ensures a proper exposition of the peptide to allow the capture of *C. tyrobutyricum* spores. Moreover, the binding through the histidine tag provides high specificity. With this purpose and following the instructions of the manufacturer, 1 mg of Dynabeads™ His-Tag were incubated with different concentrations of tG3P.

Results showed that 50 µg of tG3P was bound to around 80% microparticles (**Figure 56**). This result is similar to that reported by the manufacturer who established a binding capacity of 40 µg of a 28 kDa histidine-tagged protein/mg beads. Based on these results, this ratio was used for the following experiments.

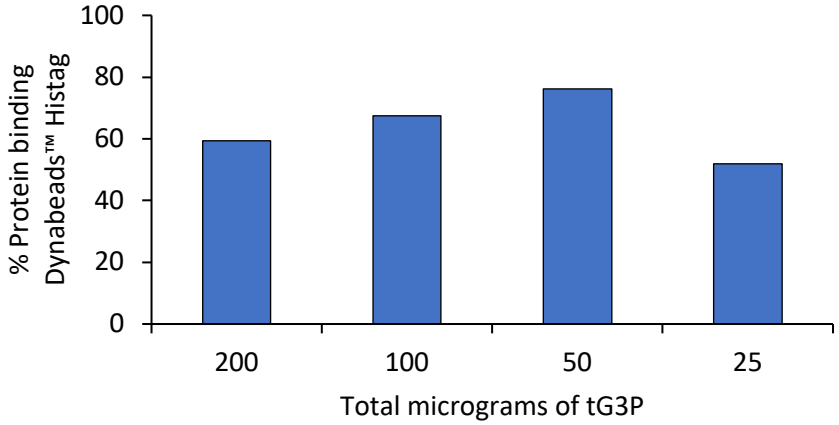

Figure 56. Percentage of tG3P binding to 1 mg of of Dynabeads™ His-Tag. The coating was done in pull&down (P&D) buffer.

The DLS and polydispersity values of tG3P-Dynabeads™ were also evaluated to confirm that the capture assays can be done in pull&down (P&D) buffer provided by manufacturer. Measurements were carried out with the microparticles diluted in binding & wash (B&W) buffer provided for the coating process and in P&D buffer, recommended for further experiments, such as his-tag protein purification.

Table 33. Measurement of tG3P-Dynabeads™ size (nm) and polydispersity index by DLS in P&D and B&W buffer.

	DLS measurement (nm)	**Polydispersity index**
B&W buffer	4138 ± 626	0.28
P&D buffer	1294 ± 121	0.15

The results obtained (**Table 33**) showed that the value of particle size were closer to 1 µm for the P&D buffer condition. For the B&W buffer condition, the polydispersity index was higher than the one expected. After this characterization, capture assays were done with *C. tyrobutyricum* spores in P&D buffer and UHT milk.

Table 34. Capture efficiency (CE) obtained for the capture of 10^3 *C. tyrobutyricum* spores in 1 mL of P&D buffer or UHT milk with tG3P-Dynabeads™. The values are expressed as the mean ± standard deviation (n=3).

	P&D buffer	**UHT milk**
Direct assay	2.63 ± 1.70	1.72 ± 0.70
Indirect assay	24.20 ± 0.17	N/A

Table 34 summarizes the data obtained in the spore capture assays with tG3P-Dynabeads™. In P&D buffer and UHT milk, low values of CE were obtained. The indirect assay provided better results but with low CE, far from acceptance criteria.

The low values of CE were not due to the low stability of tG3P-Dynabeads™ in milk because no precipitation or aggregation was seen when the assay was performed in UHT milk or P&D buffer. Moreover, a previous study (Conzuelo et al., 2014) showed that coated Dynabeads™ with an histidine recombinant protein for detecting antibiotics could be used in milk diluted 1:1 with PBS-T. However, in the mentioned study, the target molecule was an antibiotic in solution, which is quite different from capturing a bacterial spore in suspension.

Based on the results obtained, different strategies were applied to enhance the CE by blocking the coated particles with BSA, performing the capture assay in other buffers, or increasing the incubation time. Neither of these conditions achieved an improvement in CE. Furthermore, it was supposed that tG3P protein was oriented as expected based on Dynabeads™ coating and chemistry. Previous results obtained for immunocapture of *C. tyrobutyricum* spores with magnetic particles proved that the spore capture was influenced by particle size (Lavilla et al., 2012). From that study, it was concluded that small nanoparticles (200 nm) separated the spores with higher CE rates in comparison to magnetic microparticles (1 µm). For this reason, Nanomag®-D Ni-NTA of 250 nm nanoparticles were evaluated for spore capture.

3.7.2. Capture of spores using magnetic particles Nanomag®-D Ni-NTA coated with tG3P

Nanomag®-D Ni-NTA magnetic particles were selected because the chemistry and coating with tG3P protein were the same as described for Dynabeads™ allowing us to test the particle size effect.

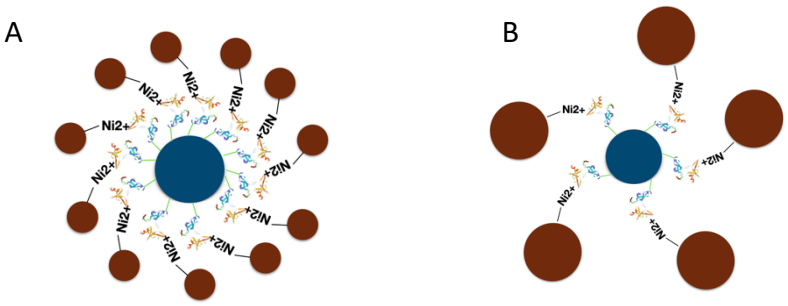

Figure 57. Simulation of *C. tyrobutyricum* spore capture by (A) Nanomag®-D Ni-NTA of 250 nm size and (B) Dynabeads™ His-Tag of 1000 nm size. Blue spheres represents the spore and brown spheres the particles.

Nanomag®-D Ni-NTA (250 nm) are smaller than Dynabeads™ His-Tag and consequently it is probable that one spore binds a high number of particles (**Figure 57A**). This assumption might explain that Nanomag®-D Ni-NTA could recover the spores from a complex matrix as UHT milk more efficiently. On the other side, although Dynabeads™ His-Tag (1 µm) can perform stronger magnetic separation than Nanomag®-D Ni-NTA , steric problems to capture the spores might appear as their size is the same as that of the particles (see **Figure 57B**). For this reason, our strategy was using Nanomag®-D Ni-NTA for spore separation.

First, the protein binding to Nanomag®-D Ni-NTA was evaluated by measurement of protein content in the supernatant, using micro BCA, after coating the particles with tG3P. A total amount of protein around 10-11 µg was bound to 1 mg of microparticles. After this assay, capture experiments were done in PBS-T and UHT milk to test the CE of microparticles to recover *C. tyrobutyricum* spores.

The results obtained in the capture assays are presented in **Table 35**. Different amounts of microparticles were applied to recover the same quantity of spores. The CE values obtained were low in PBS-T and UHT milk, indicating that microparticles were not able to recover efficiently *C. tyrobutyricum* spores. Moreover, increasing the amount of microparticles did not improve the CE value.

Table 35. Capture efficiency (CE) of 10^3 *C. tyrobutyricum* spores in PBS-T or UHT milk for different amounts of Nanomag®-D Ni-NTA microparticles coated with tG3P protein. CE: capture efficiency, SD: standard deviation, N/A: not analyzed. CE values correspond to three different experiments (n=3).

	PBS-T buffer	**UHT milk**
Amount of microparticles	**CE (mean ± SD)**	**CE (mean ± SD)**
1 mg	30.86 ± 7.70	3.43 ± 2.10
2 mg	20.12 ± 4.80	N/A
3 mg	29.19 ± 7.20	N/A

Based on the results obtained with Dynabeads™ and Nanomag®-D Ni-NTA we concluded that tG3P protein was not an efficient ligand to recover *C. tyrobutyricum* spores from a simple buffer as PBS or PBS-T.

The purification process of tG3P is aggressive because high amounts of urea and guanidine hydrochloride are used to solubilize the bacterial precipitate containing the tG3P protein. Although a refolding step is done during the purification process, the protein conformation might be affected. This hypothesis is supported by the K_d values obtained from ITC, where the affinity of the tG3P protein was found lower than that reported for pCZS1. Moreover, a third ligand, cG3P, was evaluated by ITC showing similar results to those found for tG3P. Taking into account that cG3P characteristics were not better than those of tG3P and cG3P was quite unstable, probably because of its transmembrane nature, magnetic particles were not coated with cG3P.

The characterization of pCZS1 and the CE results obtained with coated particles with it showed that this peptide could be useful to recover *C. tyrobutyricum* spores from an aqueous and simple buffer. However, in this work particles coated with pCZS1 did not work efficiently in milk, which is the natural media where *C. tyrobutyricum* spores are pretended to be detected. For this reason, in the next chapter of this thesis the results of a final attempt by coating magnetic particles with antibodies against *C. tyrobutyricum* spores was performed to capture them from milk.

4. Conclusions

In this chapter synthetic ligands were evaluated as alternatives to antibodies obtained from animals. First, pCZS1 peptide obtained by Phage Display technique demonstrated high affinity for *C. tyrobutyricum* spores based on the results found by ITC and flow cytometry assays. The dissociation constant (K_d) obtained for pCZS1 was comparable to that of high-affine antibodies. However, microparticles coated with pCZS1 could only recover *C. tyrobutyricum* from PBS and UHT milk treated with subtilisin, but were unable to recover spores from UHT milk without treatment. However, pCZS1 could be a useful ligand for the detection of spores in a system like flow cytometry.

The other ligands studied were truncated G3P and complete G3P expressed as a fusion protein with pCZS1 in *E. coli*. The expression was successful for both ligands although inclusion bodies were formed in the host cell. As a consequence, a complex protocol was applied for protein solubilization, which needed complete denaturation of the bacterial pellet, though the yield was quite good. The purification of complete G3P provided lower yield than that obtained for tG3P, probably because the transmembrane domain affected to protein isolation. Moreover, cG3P showed stability problems that complicates its use.

Both recombinant proteins reported similar K_d in ITC assay, being higher than that obtained for pCZS1, revealing that the peptide has more affinity for the spores than tG3P and cG3P. Nevertheless, the results obtained by flow cytometry allowed to conclude that tG3P has affinity for *Clostridium* spores.

Magnetic particles of two different sizes (1 µm and 250 nm) were coated with tG3P protein to test capture efficiency (CE) for *C. tyrobutyricum* spores. Although many efforts were done to find optimal conditions, the CE values were far from the acceptance criteria for an aqueous buffer and UHT milk. For this reason, although the affinity of tG3P was checked by ITC and flow cytometry, it was concluded that this ligand was not suitable to develop a capture system for spores.

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Chapter 4

Immunocapture of *C. tyrobutyricum* spores from milk

1. Introduction

Based on the previous results obtained with magnetic particles coated with protein G3P and the peptide pCZS1, as described in chapter 3, immunocapture of *C. tyrobutyricum* spores from UHT and raw milk using magnetic particles functionalized with specific rabbit polyclonal antibodies was explored.

In this chapter, the spore recovery from UHT milk was evaluated using Protein A and Protein G particles of 250 nm and 1 µm size, respectively, coated with specific antibodies. Once the capture method was set up, a qPCR for detecting *C. tyrobutyricum* spores was applied after separation of spores from raw milk with the coated particles. This research work represents the first successful approach to recover *C. tyrobutyricum* spores in raw milk followed by their detection using qPCR.

2. Material and methods

2.1. Production of antiserum anti-*C. tyrobutyricum* spores

The antisera used for the experiments described in this chapter was obtained previously in the research group by immunization of female White Zealand's rabbits. This protocol was performed as described by Lavilla et al. (2008). The antiserum was produced in rabbits by inoculating a first dose with 0.5 mL of a 10^9 suspension of *C. tyrobutyricum* spores/mL in 0.5 M NaCl, 10 mM K_2HPO_4/KH_2PO_4 buffer, pH 7.4, with 0.5 mL of complete Freund's adjuvant (Sigma). After the first dose, rabbits were monthly boosted with an equivalent suspension of *C. tyrobutyricum* spores prepared in incomplete Freund's adjuvant (Sigma). After immunization, rabbits were bled by marginal ear vein every 15 days. Blood was coagulated spontaneously at room temperature and the serum was pooled and stored at -20°C until its use.

2.2. Immunodotting

The immunodotting technique was used to evaluate the reactivity of different rabbit antisera with *C. tyrobutyricum* spores.

The spore suspensions were immobilized on a nitrocellulose membrane by adding 2 µL of each solution. BSA was added as a negative control from a 1 mg/mL stock. Samples were left to dry at RT. After that, a solution of PBS with 3% skimmed milk was added to block the non-covered binding sites of the nitrocellulose membrane. An incubation of 1 h at RT in continuous agitation was performed. After this step, the membrane was washed three times during 2 min with PBS. After the washes, dilutions of antisera or isolated antibodies were performed in 1% skimmed milk diluted in PBS. Each membrane was treated separately and incubated with the proper antibody dilution for 1 h at RT in continuous stirring and afterwards, each membrane was washed three times for 2 min with PBS. Later, antibodies anti-rabbit IgG labeled with peroxidase were diluted 1/1000 in 1% skimmed milk and incubated for 1 h at RT in agitation. Each membrane was washed three times and the resulted signal was developed by incubating the membrane with the peroxidase substrate containing 0.6 mg/mL of 4-chloro 1-naphthol (Sigma-Aldrich) diluted in PBS with 20% of methanol and 0.09% of H_2O_2. The

reaction was stopped with distilled water when the dots of grey-purple color were visible.

2.3. Purification of anti-*C. tyrobutyricum* spore antibodies

2.3.1. Immobilization of C. tyrobutyricum spores on CNBr-activated Sepharose 4B

CNBr-activated Sepharose 4B was used to prepare the immunosorbent for purification of specific antibodies against *C. tyrobutyricum* spores. CNBr-activated Sepharose 4B is a pre-activated medium for immobilization of ligands containing primary amines. Usually, the immobilized ligands are proteins, peptides, or nucleic acids but in this case, *C. tyrobutyricum* spores were used based on a previous experience of spores insolubilized with glutaraldehyde (Lavilla et al., 2010). The protocol was carried out as follows.

One gram of CNBr-activated Sepharose 4B was dissolved in 3.5 mL of 1 mM HCl and washed for 15 min with 200 mL of the same solution in a sintered glass filter. A total volume of 300 µL of a 10^8 spores/mL *C. tyrobutyricum* CECT 4012 suspension was added to 5 mL of 0.1 M $NaHCO_3$ pH 8.3. The spore suspension was added to the activated Sepharose in a falcon tube and the mixture was incubated overnight at 4°C in continuous orbital agitation.

After incubation, the excess of ligand was washed with 5 CV of 0.1 M $NaHCO_3$, pH 8.3. The remaining active groups of Sepharose were blocked with 0.1 M Tris-HCl buffer, pH 8, for 2 h at RT. After blocking, the Sepharose was washed with at least three cycles of alternating pH. The washes were done with 5 CV of each buffer. Thus, each cycle consisted of a wash with 0.1 M sodium acetate, 0.5 M NaCl, pH 4, followed by a wash with 0.1 M Tris-HCl, 0.5 M NaCl, pH 8. After the washes, the gel was degassed and introduced in a 10 mL syringe. The total volume of the Sepharose gel with the immobilized spores was 3 mL.

2.3.2. Isolation of specific antibodies against C. tyrobutyricum spores by affinity chromatography

First, 10 mL of rabbit serum were passed through the Sepharose 4B with bound *C. tyrobutyricum* spores, at a flow rate of 1 mL/min, using a peristaltic pump (Amershan Biosciences, United Kingdom), in continuous recirculation for 2 h. This step ensured the

binding of antibodies to the immobilized spores in the Sepharose gel. After the incubation, the column was washed with 25 mM K_2HPO_4, pH 8, with 0.5 M NaCl, until the absorbance of the excluded fractions at 280 nm was lower than 0.01. The specific antibodies were eluted with 0.1 M glycine-HCl, pH 2.8, and collected in 1 mL fractions. These fractions were immediately neutralized by adding in the collection tubes 44 µL of 0.5 M Tris-HCl, pH 8.5.

Selected fractions were pooled, concentrated and dialyzed against PBS in Amicon® ultra centrifugal filter units (Merck Millipore) of 10 kDa and 4 mL volume. Finally, the isolated antibodies were kept at -20°C until its use.

2.3.3. Purification of antibodies against C. tyrobutyricum spores by Protein A affinity chromatography

Based on the results obtained by electrophoretic analysis of the fractions obtained by Sepharose 4B affinity chromatography, an additional purification step was needed to remove contaminant proteins. For this purpose, a HiTrap protein A HP 1 mL volume (Cytiva) was used. The purification was performed in an ÄKTA start system at a flow rate of 1 mL/min.

For the purification, the column was equilibrated with the following binding buffer: 5 mL of 20 mM sodium phosphate, pH 7. Then, the sample was applied containing 980 µg of protein in 5 mL of PBS. The applied sample was composed of a pool of purified antibodies from Sepharose 4B. After that, the column was washed with 5-10 mL of binding buffer until the UV 280 signal reached the baseline. Then, the antibodies were eluted with 5 mL of 0.1 M glycine-HCl, pH 2.8. The elution volume was collected in 1 mL fractions and to avoid the denaturation of antibodies, 60 µL of 1 M Tris-HCl, pH 9, was added to each tube before collection. After the elution, the column was equilibrated with 10 mL of binding buffer and finally, it was stored in 20% ethanol at 4°C. All buffers and sample were previously filtrated by low protein binding 0.45 and 0.22 µm filters.

Based on the chromatogram obtained, fractions were concentrated and diafiltrated in Amicon® ultra centrifugal filter units of 10 kDa and 4 mL volume by using PBS as final buffer (Merck Millipore). The concentrated antibodies were stored at -20 °C until use.

2.4. Polyacrylamide gel electrophoresis with SDS (SDS-PAGE) and protein quantification

SDS-PAGE was performed as previously described in section 2.4.2 of chapter 3. Reducing conditions with mercaptoethanol were used to differentiate heavy and light chains of antibodies. The protein concentration of antibody solution was measured by BCA assay following the protocol described in section 2.4.3 of chapter 3.

2.5. Micro and nanoparticle coating with antibodies

For spore immunocapture, Dynabeads™ protein G microparticles of 1 µm size (Thermo Fisher) and Nanomag®-D protein A nanoparticles of 250 nm (Micromod, Rostock, Germany) were evaluated. The main characteristics of particles are summarized in **Table 36**.

Table 36. Characteristics of Dynabeads™ protein G and Nanomag®-D protein A used for immunocapture of *C. tyrobutyricum* spores. NS: not specified by manufacturer.

Type of particle (size nm)	Particles per mg	Concentration	Binding buffer	Binding capacity	Protein per particle
Dynabeads™ protein G (1000)	NS	30 mg/mL	PBS + 0.02% Tween®-20	8 µg human IgG/mg particles	NS
Nanomag®-D protein A (250)	$4.9 \cdot 10^{10}$	10 mg/mL	PBS + 0.02% Tween®-20	NS	1.5-2 µg protein A/mg particles

For the coating with antibodies, 1 mg of Dynabeads™ protein G microparticles was washed twice with PBS and 0.02 % Tween®-20 (PBS-T) before the addition of antibodies and resuspended in 990 µL of PBS-T. After this step, 10 µg of specific antibodies of a 155 µg/mL solution in PBS was added to reach a final volume of 1 mL.

The mixture was incubated in a rotator for 1 h at RT. Then, the particles were separated with a magnet DynaMag™-2 (Thermo Fisher). The supernatant was recovered and particles were resuspended in 100 µL of PBS-T.

Nanomag®-D protein A beads were coated using the procedure recommended by the manufacturer. Thus, 1 mg of washed particles in 990 µL of PBS-T were coated with 10 µg of specific antibodies from a 155 µg/mL stock solution in PBS in a final volume of 1 mL and incubated in a rotator for 1 h at RT. After the coating of both particles, the protein content of supernatants was determined by Micro BCA assay to establish the real amount of antibody bound to the particles. Coated particles were immediately used in the immunocapture assays or kept at 4°C until their use.

2.6. Immunocapture *of C. tyrobutyricum* spores in milk and detection by qPCR

2.6.1. Immunocapture of C. tyrobutyricum spores with protein A and protein G particles

The immunocapture of *C. tyrobutyricum* spores was first evaluated in PBS-T and then in UHT and raw milk. For the assays, 10^3 total spores of *C. tyrobutyricum* CECT 4012 were used in the different matrices proposed.

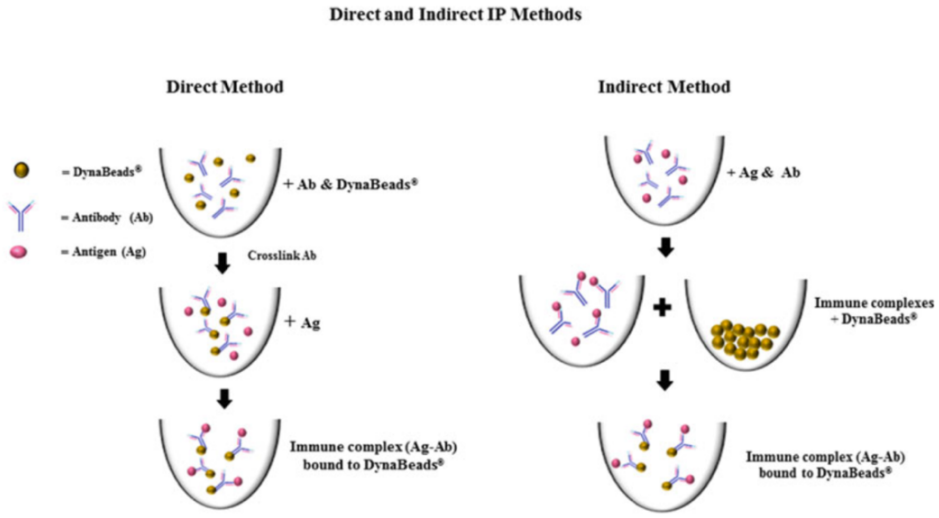

Figure 58. Immunocapture of *C. tyrobutyricum* spores with Dynabeads™ following direct and indirect method. In the direct method Dynabeads™ coated with antibodies were incubated with spores. In the indirect method the antibody was incubated with spores and then Dynabeads™ were added to form immune complexes. Adapted from Williamson et al. (2018).

The Dynabeads™ protein G microparticles were used in an indirect assay (see **Figure 58**) to capture *C. tyrobutyricum* spores. In this assay, 10 µg of specific antibodies

were added to 10^3 spores in 1 mL of PBS. For the assays done in UHT milk, 10^3 spores were spiked in 1 mL, 5 mL and 10 mL. The mixture was incubated in a wheel rotator for 1 h at RT to ensure the binding of antibodies to the spore. After this step, 1 mg of Dynabeads™ protein G were added to the mixture and incubated for 1 h at RT to promote the capture of the spore-antibody complex by the particles. Particles were recovered using a DynaMag™-2 magnet which allowed to separate them from the supernatant. After the separation, particles were resuspended in 100 µL of PBS and seeded on RCM agar plates by uniformly spreading the suspension on the agar. The plates were incubated in Gaspak™ jars in anaerobiosis at 37°C for 48 h. The recovered supernatant was also cultured in the same conditions by seeding 100 µL on the agar plates. After 48 h of incubation, the colonies were counted and CE was determined for each condition as previously described in chapter 3.

$$\text{CE \% (Capture efficiency)} = \frac{n_p}{n_s + n_p}$$

n_p = number of spores bound to microparticle suspension

n_s= number of free spores in the supernatant

For the immunocapture assay with Nanomag®-D protein A beads, a direct assay was selected. Nanoparticles coated with the antibodies were added to PBS or UHT milk artificially spiked with 10^3 spores of *C. tyrobutyricum.* For the assay in PBS a total volume of 1 mL was used, for UHT milk 1 mL, 5 mL, and 10 mL were evaluated. The mixture was incubated for 1 h at RT in a wheel rotator. After the incubation, particles were recovered by using a DynaMag™-2 magnet. The supernatant was kept for further analysis and protein A particles were washed three times with PBS-T to remove contaminants from UHT milk. Then, microparticles were resuspended in 100 µL of PBS and cultured on RCM agar plates as described previously. Moreover, 100 µL of supernatant were also cultured to determine CE at each condition.

To establish the CE for *G. stearothermophilus* spores kindly donated by ZEULAB S.L., the same protocol as described for *C. tyrobutyricum* spores was performed. After the capture step, the spores were cultured in LB agar an incubated for 24 h at 55°C. The negative control of non-sporulated bacteria used in this study was *Cronobacter sakazakii*

CECT 858 supplied by the Spanish Type Culture Collection. For the reference stock, the bacteria were fixed to porous rings and stored in cryovials at -80°C. One ring was transferred into a tube with 10 mL of trypticase soy broth (TSB) (Merck, Darmstad, Germany) supplemented with 0.6% (w/v) yeast extract (YE) (Oxoid, Basingstoke, UK) and incubated for 24 h at 37 °C in aerobiosis. After the incubation visible growth was seen and bacteria was counted with Thoma chamber. Based on the calculated concentration dilutions were done to achieve 10^3 total CFU. After this, the bioseparation protocol described before was applied.

2.6.2. Immunocapture of C. tyrobutyricum spores in UHT and raw milk and detection by qPCR

The final purpose of this part of the study was to establish an efficient method to separate *C. tyrobutyricum* spores from milk and them subsequent detection by qPCR. The assay was done in 5 mL and 10 mL of UHT or raw milk spiked with 10^3 *C. tyrobutyricum* spores. Raw milk from cow previously treated at 90°C for 15 min to destroy vegetative cells was used. In order to test the specificity of qPCR a sample of raw milk without spores was included to ensure that non-specific amplification was found. As explained in the previous section, Dynabeads™ protein G microparticles and Nanomag®-D protein A beads were added to the samples at different conditions and incubated. After immunocapture, particles were recovered and washed three times in the case of protein A nanoparticles. Protein G microparticles were not washed, as it was checked that a relevant percentage of spores was lost during washes. Finally, particles were resuspended in 100 µL of PBS.

After the immunocapture assay, particles from protein A and protein G were subjected to MW treatment at 600 W for 15 min as it was described in chapter 1 section 2.4.1 and 2.4.8. This step allows efficiently breaking of *C. tyrobutyricum* spores and release of their DNA for further processing. Then, particles were separated using the DynaMag™-2 magnet and the supernatant was processed to purify the genomic DNA using the GenElute Bacterial DNA kit (Sigma-Aldrich), as previously described. The genomic DNA was eluted in a final volume of 100 µL.

The protocol applied for qPCR analysis and the amplification process were previously described in section 2.3.2 of chapter 1 (see **Table 37**).

Table 37. Reagents and quantities used for qPCR amplification after immunocapture with particles coated with polyclonal antibodies.

Reagent	Final amount per reaction
Master Mix SYBR® Green PowerUp	12.5 µL
CTfla primer forward	1.5 µL
CTfla primer reverse	1.5 µL
RNase-free water	7.5 µL
Purified genomic DNA	2 µL

3. Results and discussion

3.1. Immunodotting of rabbit antiserum

First of all, several rabbit antisera against *C. tyrobutyricum* spores were evaluated to select the optimal one to purify specific antibodies. The results obtained by immunodotting assay are presented in **Figure 59**.

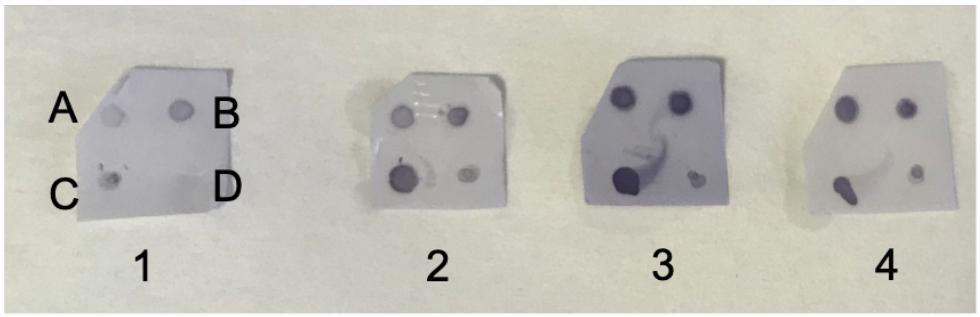

Figure 59. Immunodotting of different rabbit antisera against Clostridial spores. 1: Pre-immune serum, 2: Antiserum from immunized rabbit with a mix of *Clostridium* species, 3: Antiserum from immunized rabbit 1 with *C. tyrobutyricum* spores CECT 4012 (1), 4: Antiserum from immunized rabbit 2 with *C. tyrobutyricum* spores CECT 4012 (2). A: *C. tyrobutyricum* CECT 4012 spores, B: *C. tyrobutyricum* UZ01 spores, C: *C. sporogenes* spores, D: *G. stearothermophilus* spores.

The membrane of condition 1 was incubated with pre-immune serum, corresponding to serum extracted before the immunization with the target antigen. The aim of this condition was to test non-specific interactions between the antibodies of the serum and the target antigen. The result obtained revealed a slight non-specific reaction with *C. tyrobutyricum* spores UZ01 and *C. sporogenes*. The immunodotting of condition 2 was incubated with an antiserum against a mix of *Clostridium* spores (*C. tyrobutyricum*, *C. sporogenes*, *C. beijerinckii* and *C. butyricum),* exhibiting a positive reaction for all the bacteria tested. However, taking into account the result of the immunodotting incubated with the antiserum obtained by immunization only with *C. tyrobutyricum* spores, a higher signal was obtained than that obtained with the antiserum against the mix of spores (conditions 3 and 4). Anyhow, a positive signal was achieved with the antiserum of condition 3 for all the *Clostridium* spp analyzed which can be useful for detecting them. However, a non-desirable positive reaction of the specific antiserum against *G. stearothermophillus* was also observed. This cross-reaction would be probably due to the presence of common epitopes on the spore coat.

3.2. Purification of specific antibodies against *C. tyrobutyricum* spores

Specific antibodies against *C. tyrobutyricum* spores were purified by affinity chromatography as described before. The chromatogram obtained is presented in **Figure 60A**.

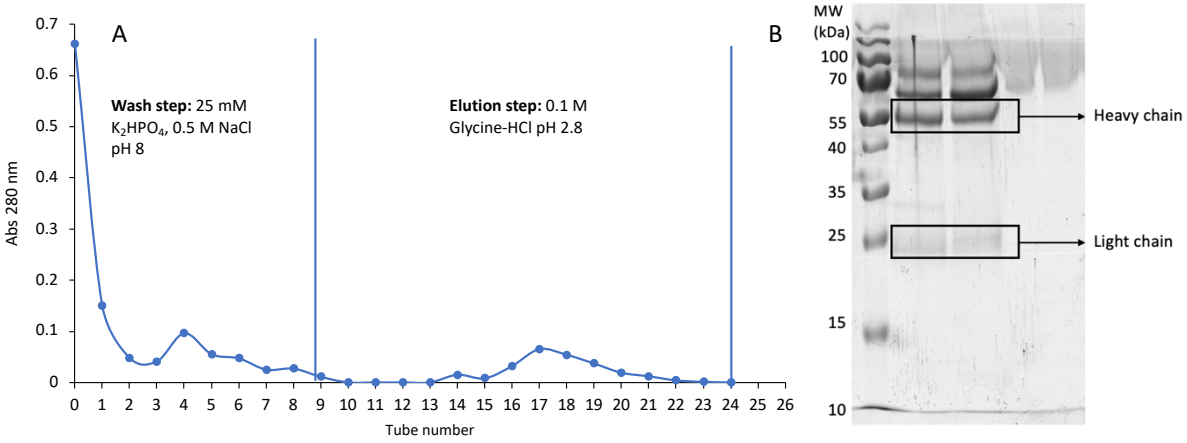

Figure 60. A) Chromatogram obtained from the affinity chromatography to obtain antibodies against *C. tyrobutyricum* spores. B) SDS-PAGE electrophoresis of the peak corresponding to the eluted volume. The electrophoresis was done in 11% polyacrylamide gel in reducing conditions and stained with Coomassie blue.

The peak obtained by the elution with 0.1 M glycine-HCl, pH 2.8, reached a maximum absorbance at 280 nm of 0.065 (tube number 17). The tubes from 15 to 21 were mixed, concentrated and diafiltrated with PBS to 1 mL. The final concentration of specific antibodies determined by the BCA assay was 200 µg/mL.

Figure 60B shows the SDS-PAGE performed in reducing conditions. The common rabbit IgG isotype has a molecular weight of 150 kDa with two heavy chains of 50 kDa each and other two light chains of 25 kDa each (Johnson, 2013). In reducing conditions heavy and light chains are splitted and visible independently. In **Figure 60B**, the light chain is slightly visible, at the same level as the 25 kDa band of the molecular weight marker, and the heavy chains next to the 55 kDa MW band of the marker. However, two bands also appeared around 66 and 80 kDa, which probably correspond to serum albumin and transferrin, respectively, as both proteins are present in high levels in rabbit serum (Majorek et al., 2012).

With the aim of removing contaminant proteins, the antibodies obtained were finally purified with a protein A affinity column. After performing five isolations of specific antibodies in Sepharose 4B a volume of 1 mL with 1.012 UV_{280} absorbance was obtained and subjected to Protein A affinity chromatography. **Figure 61A** shows the chromatogram obtained from protein A purification, in which a peak is seen after elution. The fractions corresponding to that peak were pooled and concentrated until obtaining 1 mL of 155 µg/mL, considering that the extinction coefficient ($E^{1\%}_{280}$) of IgG is 1.38 mL/cm/g.

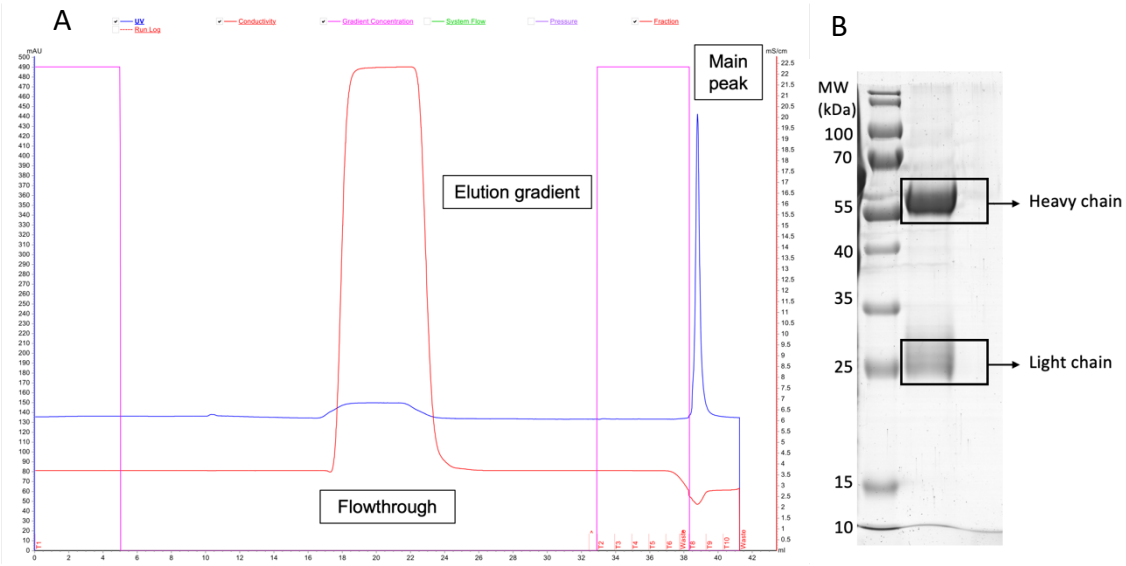

Figure 61. A) Chromatogram obtained from the purification of antibodies by Protein A affinity chromatography. Blue line records UV280 signal, red line the conductivity and pink line the gradient concentration B) Reducing SDS-PAGE in an 11% polyacrylamide gel of the concentrated fractions obtained by affinity chromatography.

The SDS-PAGE in reducing conditions (**Figure 61B**) shows only two bands after purification of specific antibodies by Protein A chromatography, at around 50 and 25 kDa as expected. After this purification step, antibodies against *C. tyrobutyricum* spores were ready to be bound to Protein A and Protein G particles. The specific antibodies were tested by immunodoting assay against *C. tyrobutyricum* CECT 4012 and UZ01 spores, *C. sporogenes* and *G. stearothermophilus* spores. Visible signal was seen for all the conditions tested, even for the negative control *G. stearothermophilus* (data not shown).

3.3. Immunocapture of *C. tyrobutyricum* spores in PBS-T and UHT milk

3.3.1. Immunocapture of spores in 1 mL sample of PBS-T and UHT milk

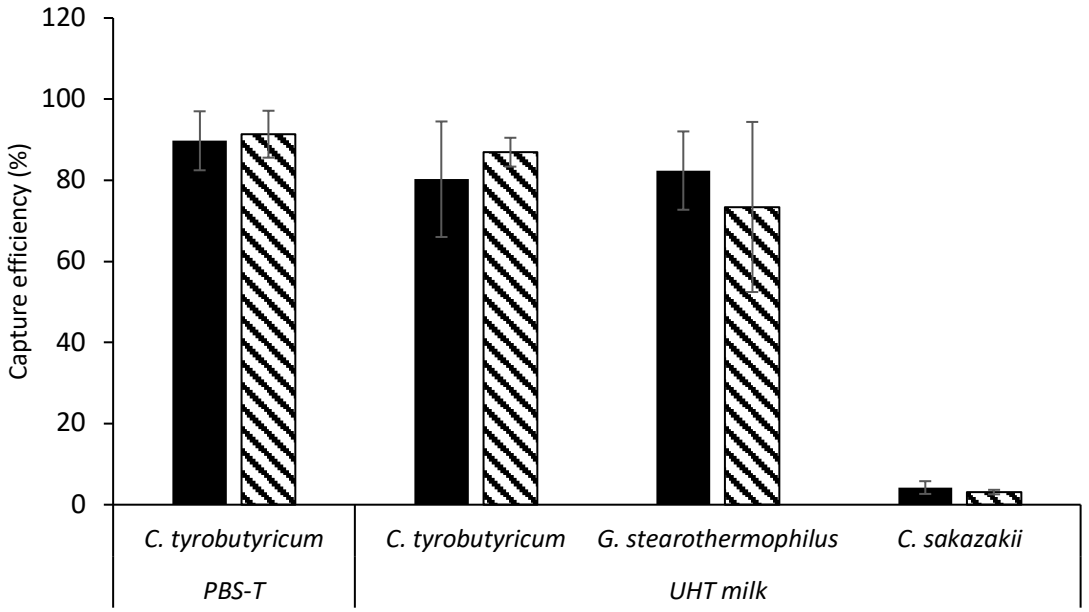

Figure 62. Capture efficiency (CE) for 10^3 *C. tyrobutyricum* spores in PBS-T and UHT milk with protein A (■) and G (▨) particles. *Geobacillus stearothermophilus* spores and *Cronobacter sakazakii* vegetative cells were evaluated in UHT milk as negative controls. The CE was obtained by microbiological culture. The values indicate the mean ± standard deviation, determined from three different experiments (n=3).

The results obtained in the immunocapture assays with Protein A (250 nm) and Protein G (1000 nm) particles are shown in **Figure 62**. Although for protein G particles only the indirect assay was found successful, for Protein A particles direct assay worked under the expected in PBS-T and UHT milk. These two strategies achieved high values of CE, 80% for Protein A and 86% for protein G, in the capture of 10^3 *C. tyrobutyricum* spores in 1 mL of UHT milk. Moreover, *G. stearothermophilus* spores were also recovered with high rates, being 82% for Protein A particles and 73% for Protein G particles in UHT milk, indicating that the antibodies have cross-reaction with *G. stearothermophilus* spores, probably due to common surface antigens between spores. However, vegetative cells from *C. sakazakii,* a Gram-negative bacterium, were not captured with either of the two particles. No statistically differences were found between Protein A and G particles in UHT milk and PBS-T conditions for the bacteria assayed by using unpaired t-test with p value < 0.05.

In a previous work, it was reported that 200 nm magnetic nanoparticles coated with specific antibodies against *C. tyrobutyricum* spores, captured them with higher values of CE (85-95%) than the 1 µm microparticles (65% CE) (Lavilla et al., 2012). In the present study, only the magnetic particles of 250 nm were able to capture *C. tyrobutyricum* spores from milk applying the direct assay. As it was suggested previously by Lavilla et al. (2012), the spore recovery depends on the particle size. Considering that *C. tyrobutyricum* spores have approximately 1 µm size, and Protein A nanoparticles 250 nm size, these particles can form more easily the spore-bead complex than the protein G particles of 1 µm when using the direct assay. As a consequence, a higher number of Protein A particles can surround the spore, thus promoting a better separation under the direct assay condition. Although CE values are similar for both types of particles and no significant differences were reported, it would be more useful to apply a direct assay because of the reduction of incubation times.

Dynabeads™ protein G particles were evaluated for re-using them in subsequent assays with the aim of optimizing the costs. This procedure was not performed in Protein A particles because those were unstable and aggregate in acidic conditions. First, new Protein G particles were used and spores were eluted with 20 µL of elution buffer (50 mM glycine, pH 2.8), and then, the eluted spores were cultured to calculate the CE. Afterwards, a second experiment was performed with the recovered particles following the same protocol. At the end, the spores were eluted with elution buffer in the same conditions as described before. The assays were performed in PBS-T and UHT milk.

In **Figure 63** are presented the results obtained in the assay to evaluate the possibility of reusing the Dynabeads™ protein G particles. The CE obtained for *C. tyrobutyricum* spores in UHT and PBS-T with new particles was in the range 86-91% as previously obtained (**Figure 62**). However, only 76% CE was obtained in PBS-T and 50% CE in UHT milk with reused microparticles. Significant differences were found in CE values obtained by using new and re-used microparticles when the assay was performed in UHT milk. The low values of CE and the high standard deviation obtained with reused particles in UHT milk could be due to the more complex composition of milk that can block some binding sites of the particle surface, thus decreasing their binding capacity after the first use. No significant differences were found on CE values obtained in PBS-T

when re-using protein G microparticles suggesting that milk could be a limitation to re-use Protein G particles.

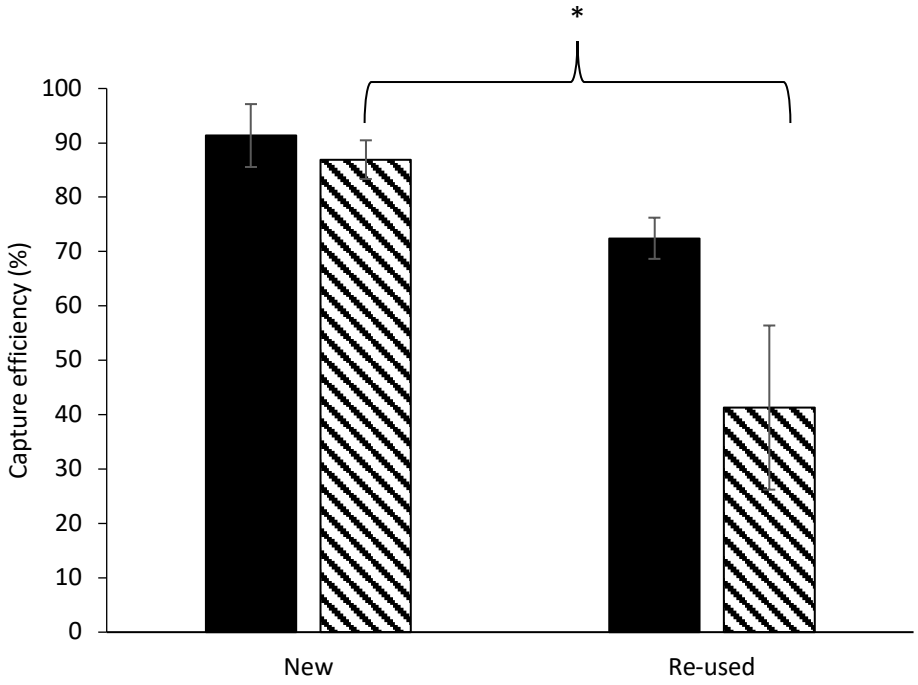

Figure 63. Capture efficiency (CE) of 10^3 *C. tyrobutyricum* spores in PBS-T (■) and UHT milk (▨) with new and reused Dynabeads™ protein G particles. CE was calculated by microbiological culture in RCM medium. The values indicate the mean ± standard deviation, determined from three different experiments (n=3). *p *value* < *0.05* compared with the control (new microparticles).

Moreover, it was observed that one washing with PBS-T after the immunocapture to remove the milk contaminants eluted 63-74-% of the spores attached to the Protein G particles. A second wash removed 23% of the remnant spores suggesting that the binding of spores or antibody to Protein G particles was not strong. The washing effect was also evaluated in Protein A particles after the immunocapture, and only around 5-6% of the spores were lost with the washes, a considerably low percentage in comparison with that obtained for Protein G particles. Based on the results obtained, it was assumed that no washes could be done in Protein G particles due to the low stability of spore binding to them. For Protein A, three washes of 500 µL were performed to remove milk contaminants as it was demonstrated that a low percentage of spores were lost with this step.

3.3.2. Immunocapture of spores in 5 mL and 10 mL sample of UHT milk

The results obtained in 1 mL samples allowed to test functionalized Protein A and Protein G particles in 5 and 10 mL of UHT milk. The level of butyric bacteria spores in milk is usually low, as it has been shown in this thesis. Therefore, increasing the volume of milk sample could be a way to improve spore detection. Considering that in the qPCR method developed the sample volume is of 1 mL, an immunocapture step will allow working with a sample of higher volume.

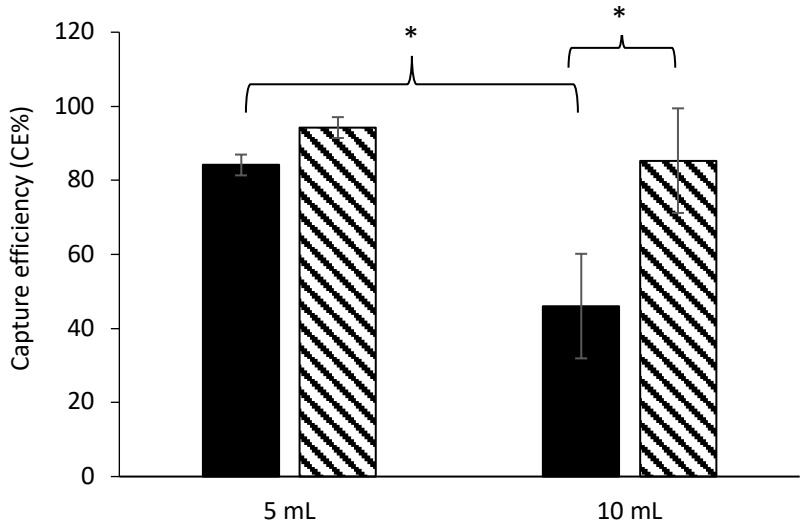

Figure 64. Capture efficiency (CE) for 10^3 *C. tyrobutyricum* spores in 5 and 10 mL of UHT milk using Protein A (■) and Protein G milk (▨) magnetic particles. CE was calculated by microbiological culture in RCM medium. The values indicate the mean ± standard deviation, determined from three different experiments (n=3). **p value < 0.05.*

The CE results obtained for *C. tyrobutyricum* spores in 5 and 10 mL of UHT milk are summarized in **Figure 64**. Results showed that CE values were high for both types of particles in 5 mL of UHT milk, being 94% for Protein G and 84% for Protein A. However, the same protocol applied to 10 mL of UHT milk, revealed an important and statistically significant decrease in CE for Protein A nanoparticles, from 84% in 5 mL to 46% in 10 mL. However, Protein G particles were able to recover 84% of the spores in 10 mL of UHT milk, a similar value to that obtained in 5 mL. The differences in CE values obtained between both particles could be explained by the lower size of Protein A particles (250 nm) in comparison to Protein G particles (1 µm) and the volume of UHT milk used (10 mL). UHT milk is a complex matrix in contrast with PBS-T and the mobility and

dispersibility of Protein A particles can be affected. However, as Protein G particles have higher size they seem to be able to recover almost all the spores with a similar CE value that that reported in PBS-T.

Although the CE values of particles coated with pCZS1 were in the range of the acceptance criteria, this assay could only be performed in UHT milk treated with subtilisin or in PBS. However, the use of synthetic ligands as pCZS1 provides a reliable assay omitting the differences on the production of polyclonal antibodies between animals, avoiding the immunization and purification protocols and also reducing the costs. On the other side, the main advantage of the particles coated with antibodies is that the assay can be applied to milk directly without any treatment, which reduces the time of analysis. As a conclusion, both ligands could be useful to develop an immunocapture system to recover spores from milk providing high CE values.

3.4. Immunocapture of *C. tyrobutyricum* spores in UHT and raw milk and qPCR detection

The final objective was to combine the immunocapture with the qPCR to obtain an easy method for detecting *C. tyrobutyricum* spores in milk. For this purpose, immunocapture assays followed by qPCR detection were performed to test the viability of combining both techniques. Results obtained in the immunocapture of 10^3 C. *tyrobutyricum* spores in UHT milk with Protein A and Protein G particles and the following detection by qPCR are presented in **Table 38**.

Table 38. Ct and values of spores/mL obtained in the immunocapture of 10^3 *C. tyrobutyricum* spores in UHT milk and detection by qPCR. SD: standard deviation. Mean was obtained from three different experiments analyzed by duplicate in qPCR (n=6).

	Protein A (250 nm)			Protein G (1 µm)		
	Ct mean	**SD**	**Amplification rate**	**Ct mean**	**SD**	**Amplification rate**
5 mL	35.50	0.08	6/6	36.81	1.93	3/6
10 mL	36.96	2.08	6/6	38.38	0.49	4/6

Immunocapture with Protein A particles revealed lower Ct cycles and a higher amplification rate for 5 and 10 mL compared with Protein G particles. In addition, the use of protein G particles showed low amplification rate probably because no washes could be performed with these particles due to the loss of spores. The qPCR was considered qualitative indicating only the presence or absence of *C. tyrobutyricum* spores.

Based on the results obtained, protein A nanoparticles were selected for immunocapture and qPCR detection of *C. tyrobutyricum* spores in raw milk. The results obtained in the immunocapture and qPCR detection assay for *C. tyrobutyricum* spores in raw milk are summarized in **Table 39** .

Table 39. Ct mean obtained in the immunocapture of 10^3 *C. tyrobutyricum* spores in raw milk and detection by qPCR. SD: standard deviation. Mean was obtained from three different experiments analyzed by duplicate in qPCR (n=6).

	Protein A (250 nm)		
	Ct mean	**SD**	**Amplification rate**
5 mL	33.28	0.16	6/6
10 mL	34.04	0.19	6/6

The Ct obtained revealed that Protein A nanoparticles were able to recover the spores in all the experiments done, with an amplification rate of 100%. Similar Ct values were obtained for 5 mL and 10 mL of raw milk. In all the assays a negative control was included, consisting of Protein A particles coated with antibodies incubated in spore free raw milk thermally treated (80°C 10 min) to test the specificity of the qPCR in a complex matrix as raw milk. All the negative control tested did not amplify, demonstrating that the immunocapture followed by qPCR detection was specific for *C. tyrobutyricum* spores in raw milk.

Although the microbiological culture showed that only half of the spores could be recovered from 10 mL of UHT milk with Protein A nanoparticles, this result was not observed when performing qPCR analysis, reporting similar Ct values in 5 and 10 ml of raw milk.

In comparison with the results obtained with protein G3P and pCZS1 described previously in chapter 3, those obtained with specific antibodies were very satisfactory, as were able to capture around 90% of *C. tyrobutyricum* spores directly in milk. Antibodies are the molecules of choice to detect or capture ligands and have been used in different platforms, such as ELISA, flow cytometry, lateral flow, or immunocapture with magnetic particles. Antibodies are proteins highly specialized in the recognition of antigens, and the use of Protein G/A particles in this thesis provided the correct orientation because antibodies are bound to the particles by the Fc fraction, allowing high rates of bioseparation.

Affine peptides against *C. tyrobutyricum* were found as an alternative to antibodies for biomagnetic separation in the research group in a previous study (Lavilla et al., 2012). In this thesis, pCZS1 peptide was found successful to recover *C. tyrobutyricum* spores in UHT milk treated with subtilisin. However, the strategies developed to improve the sequence of the original peptide and avoid the limitation of its short size, have not enhanced the spore recovery directly in milk. However, synthetic peptides have many advantages in comparison with antibodies as it is no necessary the use of experimental animals and the application of purification steps. The results obtained allow to conclude that an immunocapture method based on antibodies or synthetic peptides can be the basis for a method to detect *C. tyrobutyricum* spores in raw milk in a short time. In this thesis the combination of immunocapture of *C. tyrobutyricum* spores followed by qPCR detection was found as a promising tool to enhance the detection of spores introducing a step of concentration in milk.

4. Conclusions

In this chapter, results of the evaluation of an immunocapture method based on specific antibodies against *C. tyrobutyricum* spores have been shown. First, antiserum from rabbits immunized with spores of *C. tyrobutyricum* CECT 4012 was selected based on immunodotting results. Antibodies were isolated using insolubilized *C. tyrobutyricum* spores, followed by purification with affinity chromatography.

Two types of magnetic particles were evaluated: Protein A nanoparticles (250 nm) and Protein G microparticles (1 µm) for immunocapture of spores. Protein A particles worked well in a direct assay and Protein G particles only as indirect assay. The assays performed in 1 mL of PBS-T and UHT milk demonstrated for both particles similar CE values. Moreover, the binding of the antibody and/or the spore to Protein G was not very strong and did not allow the washing steps because produced a significant loss of spores. This effect was not seen in Protein A particles.

The immunocapture of spores in 5 mL of UHT milk reported high CE values for both types of particles. However, a reduction to half was seen in CE values for Protein A particles in 10 mL of UHT milk. This effect could be due to the smaller particle size compared to Protein G particles, which recovered almost all the spores in 10 mL of UHT milk.

The application of qPCR analysis in 5 and 10 mL of UHT milk allowed detecting the spores with 100% amplification rates for Protein A particles. Protein G particles showed lower amplification rates and higher Ct probably due to the impossibility to perform the washes and the presence of milk contaminants non-specifically bound to the particles. The immunocapture and qPCR detection of spores in raw milk was finally done only with Protein A particles and the results showed similar Ct in both volumes tested (5 and 10 mL) and amplification rates of 100%.

Based on the results reported in this chapter, a capture system based on nanoparticles (250 nm size) functionalized with specific antibodies is the optimal option to recover *C. tyrobutyricum* spores from raw milk and to detect them by qPCR. Moreover, the direct assay is the more commonly used format in commercial kits because it requires shorter incubation times than the indirect assay. For this purpose,

10^3 spores were detected in 10 mL of raw milk (10^2 spores/mL) in up to 5 h based on an easy protocol. Further research would be needed to evaluate this method in large volumes of raw milk as 20 or 30 mL for recovering *C. tyrobutyricum* spores considering the low spore counts that usually are present in raw milk samples. However, in this work, the main basis is established for the development of an immunocapture method followed by qPCR detection, which can be effective in raw milk.

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General discussion

Butyric bacteria are responsible for late blowing defect (LBD) in semi-hard and hard cheeses as a consequence of the fermentation that produce during the ripening stage. The resistance life forms of these microorganisms are the spores, which can survive and resist to different technological treatments. The resistance of spores makes very difficult to remove or inactivate them and neither of the technological treatments applied to milk, such as heating, microfiltration or bactofugation, assure their complete elimination. Although many species of butyric bacteria can be involved in LBD, *Clostridium tyrobutyricum* is considered the principal causative agent by the scientific community. Consequently, this work has been focused on the development of a rapid and specific method to detect *C. tyrobutyricum* spores in milk.

Nowadays, Most Probable Number (MPN) analysis is used for the detection and enumeration of butyric spores in milk. However, this microbiological method needs at least between two and seven days to achieve the final result. It would be necessary to reduce the time of the detection assay to know the concentration of butyric spores before milk processing, to avoid using contaminated milk with them in the manufacture of semi-hard and hard cheeses, which require long maturation. This thesis proposed a method based on qPCR to detect *C. tyrobutyricum* spores in raw milk samples. The qPCR assay is faster and more specific than the MPN method.

The qPCR developed in this thesis was first validated with DNA from *C. tyrobutyricum* vegetative cells, analyzing efficiency, linear range and reproducibility. Once the qPCR was validated according to recognized guidelines (Bustin et al., 2009) it was optimized to detect *C. tyrobutyricum* spores.

The extraction of enough genomic DNA from the original sample is essential for developing an efficient qPCR (Martínez et al., 2011). Hence, the first point of this thesis focused on the screening of different disruption methods to lyse *C. tyrobutyricum* spores. Vegetative cells are easily disrupted with an enzymatic treatment based on proteinase K and lysozyme or mechanical treatment as bead beating (BB) with a lysis buffer. However, spores have more resistance to enzymatic and mechanical treatments than vegetative cells. This characteristic represents a great challenge in finding the

optimal disruption method for *C. tyrobutyricum* spores, as it has been reported for other types of bacterial spores (Setlow, 2014; Torok, 2003). The results of the screening of several disruption methods led us to conclude that the combination of BB and microwaves (MW) , followed by a step of DNA purification on a silica-column, was the optimal choice to disrupt *C. tyrobutyricum* spores in PBS and obtain pure DNA (Esteban et al., 2020). Moreover, two types of reagents were tested for qPCR amplification, being the TaqMan probe and the SYBR Green reagent. SYBR Green provided lower cycles and higher amplification rates than the TaqMan probe, contrary to what might be expected, as the latter is considered more specific.

The sporulation process of *C. tyrobutyricum* influences the characteristics of spores and the final qPCR result, as it has been demonstrated in this thesis. DNA from spores produced in agar amplified later in qPCR than that from spores obtained in liquid medium, suggesting that the former are more resistant than the latter, as some authors had previously reported for other bacterial spores (Abhyankar et al., 2016; Rose et al., 2007). It is assumed that different types of spores with different resistance will be found in nature and in the silages that contaminate dairy farms. Therefore, the differences in the spore resistance must be considered to develop a detection method because the results can be variable depending on the disruption method.

It is well known that spores interact with milk components, especially with fat, interfering with their detection. In order to recover *C. tyrobutyricum* spores from milk, a solution with subtilisin and a detergent was used to treat milk samples, followed by a centrifugation step. Milk digestion with subtilisin recovered almost all the spores in comparison with a simple centrifugation, as it was checked experimentally (Esteban et al., 2020). After the enzymatic treatment, BB and MW were applied to the precipitate containing the spores. We found that MW treatment provided better results, in terms of Ct values and amplification rates, than BB. Finally, MW treatment followed by column purification was selected to extract DNA from spores for further analysis.

The calibration curve for qPCR was made in PBS and UHT milk spiked with different amounts of *C. tyrobutyricum* spores, treating the precipitate containing the recovered spores with MW and column purification. qPCR efficiency, linear range, limit of detection (LOD) and amplification rates were evaluated, obtaining results within the

acceptance criteria (Bustin et al., 2009). We found different LOD for the wild strain UZ01 (10^2 spores) compared with the type strain CECT 4012 (10^3 spores) in UHT milk. Further research would be needed to explain the differences found in the parameters of qPCR analysis for detecting both strains. However, these results demonstrate differences in the spore disruption of both strains and this affects to the detection by qPCR.

Considering the good results previously obtained, MW treatment and column purification of DNA, were applied to raw cow milk samples provided by laboratories of milk quality control. However, by applying that DNA extraction method, lower precision between qPCR replicates was observed, probably due to the low level of spores in milk. Other authors describe that despite the sensitivity of qPCR, the concentration of spoilage microorganisms is usually below the LOD of qPCR in food samples (Martínez et al., 2011). Furthermore, an inefficient breakage of spores can contribute to this limitation, taking into account that the spores of natural origin may be heterogeneous in terms of their resistance to being broken. An enrichment step could be introduced to avoid this problem as it has been previously proposed (Malorny et al., 2004), but this type of procedure would require the germination and growing of the bacteria, which would mean long incubation times. The main objective of this thesis was to establish the basis for a detection method that could be performed in one day.

By applying MW treatment and column purification to the precipitate of spores recovered after subtilisin treatment in raw milk samples, the qPCR precision achieved was not completely satisfactory. Although this method was selected as successful, the low precision found when applied to raw milk samples could be due to low DNA recovery. In order to improve DNA recovery and qPCR precision, a novel extraction method was proposed, as described in chapter 2, based on BB disruption of spores and DNA purification with magnetic particles. The novelty of this method in comparison with MW treatment and column purification is the use of a commercial kit and automated system called King Fisher (KF) Duo Prime System, which is being implemented in the biopharmaceutical and biotechnological industry for many applications, such as DNA purification or protein digestion for peptide mapping (Buchanan et al., 2020).

The qPCR calibration curve was made in raw milk spiked with different amounts of spores by extracting the DNA with this new protocol proposed. Raw milk was treated

with subtilisin, and then the precipitate of spores was disrupted and DNA extracted by the KF method. Similar parameters were obtained regarding linear range and qPCR efficiency compared to those obtained applying the MW treatment. However, lower Ct values were achieved for *C. tyrobutyricum* UZ01 strain, which means that the DNA recovery was better with the KF method, considering that the calibration curve for KF was made in raw milk, compared to MW treatment and column purification performed in UHT milk. This would be an advantage of KF method, since the final objective is to analyze raw milk samples susceptible of being contaminated with butyric spores.

Once the qPCR calibration was made on spore spiked raw cow's milk, a total number of 202 raw milk samples from cow (68), ewe (86) and goat (48) from farmers of three different geographical regions of Spain, were analyzed to test *C. tyrobutyricum* levels of contamination. To our knowledge, this is the second study that analyzes an important number of raw milk samples after that of Arnaboldi et al. (2021). Our study revealed that a great number of samples gave positive Ct values, but below the LOD. Consequently, the majority of milk samples could not be quantified for *C. tyrobutyricum* spores. A low percentage of samples were quantified successfully, reporting levels between 10^2 and 10^3 spores/mL. These values are in agreement with those reported by other authors (Bassi et al., 2013; Arnaboldi et al., 2021) by qPCR. The qPCR precision between replicates was successfully improved with the new method for DNA extraction, in comparison with MW treatment and column purification.

The extraction of DNA from *C. tyrobutyricum* spores by the KF method followed by qPCR provided results in about five hours, which is a shorter time than the used in other methods described by authors working in the same research field (Arnaboldi et al., 2021; López-Enríquez et al., 2007). Furthermore, in comparison with MW treatment, the application of KF method for DNA extraction and further amplification by qPCR gave better precision and provided more reliable results. Although KF gave successful results for raw milk, MW treatment followed by column purification can be considered a good choice when the KF equipment is not available for spore disruption in an aqueous buffer or simple matrix as PBS because the calibration curve provides results inside the acceptance criteria.

From the total number of samples (202) analyzed only 26% from cow, 11% from ewe and 80% from goat could be quantified because the rest were out of the linear range. For this reason, it was concluded that the proposed method could be considered qualitative, regarding the levels of spores obtained in the analyzed samples. A step of concentration using a higher volume of raw milk could be a solution to have more spores in the final sample. Furthermore, several assays were made to validate the developed Real-Time PCR considering precision, reproducibility and also robustness, among other parameters. The variations introduced in the method for analysing the robustness, demonstrated that the method could be sensitive to some of the variations. Moreover, the reproducibility of raw milk samples was low, probably due to the high Ct values that were obtained, which are on the limit to consider a qPCR as quantitative. It is known that Ct>30 have high variability compared to lower Ct cycles (Thermofisher Scientific, 2022) and because of the sub-sampling error when low DNA amounts are analyzed. The use of greater amount of milk sample and/or a step of concentration of spores could be introduced to solve this problem as spore capture with magnetic beads. This point is discussed in the last chapter of this thesis.

To verify the presence of *C. tyrobutyricum* and other species of *Clostridium* in raw milk samples, they were cultured in RCM selective media with D-cycloserine and neutral red, a medium that was previously described as specific for butyric bacteria (Jonsson, 1990). The colonies were analyzed by multiplex PCR and 16S rDNA sequencing to identify the bacterial species. The predominant bacteria that were identified belonged to *Paenibacillus* and *Lactobacillus* genera*,* in higher or lower proportion depending on the type of milk analyzed. These results allowed us to conclude that the culture media was not completely specific for butyric bacteria, and it should be complemented with other analysis as the 16S rDNA sequencing, which provides more information.

As regards butyric species, we found that *C. sporogenes* was the predominant one in cow and ewe, followed by *C. tyrobutyricum* which was only isolated in cow milk. Other species, such as *C. perfringens* were also isolated, being the predominant one in ewe's milk. This finding is aligned with other studies (Arnaboldi et al., 2021; Turchi et al., 2016) in which *C. sporogenes* was reported as the main species isolated from raw cow

milk, and revealed the presence of other butyric species. The data obtained in this study allow us to conclude that a variety of *Clostridium* species are present in raw milk samples that could contribute to LBD, although more research would be needed to demonstrate that they can trigger LBD in cheese. Furthermore, in this work, differences were found in the predominant species depending on the type of milk analyzed (ewe, cow or goat), in agreement with the studies made by other authors in milk from goat (Reindl et al., 2014), ewe (Turchi et al., 2016) and cow (Zhang et al., 2015). This fact must be taken into account to develop a qPCR method, which would need to include primers targeting the main butyric species. First, it would be necessary to confirm which butyric species are usually present in the target raw milk to be analyzed.

The other main objective of this thesis was to develop a capture system for *C. tyrobutyricum* spores. The main purpose was to recover them from raw milk as a previous concentration step, which can be critical because low levels of butyric spores in raw milk can produce LBD in mature cheese. Therefore, it would be necessary to improve the LOD we have achieved in the first developed method based on qPCR assay, and the use of higher volumes of raw milk (10 or 100 mL) by introducing a concentration step with magnetic particles might be a way. A step of concentration could allow to recover a higher amount of DNA reporting giving values of Ct within the LOD, which would make the method quantitative for samples with low levels of spores.

In the last few years, specific peptides selected by the Phage Display technique have been proposed as an alternative to polyclonal antibodies obtained in animals (Lavilla et al., 2012). In this work, we proposed two synthetic ligands to use them to functionalize magnetic particles to capture *C. tyrobutyricum* spores.

The peptide presented in this work (pCZS1) was found to be a good candidate as a detection and capture molecule for *C. tyrobutyricum* spores. Based on the results obtained, the affinity of pCZS1 for *C. tyrobutyricum* spores was demonstrated in our study by ITC and flow cytometry. The peptide bound to magnetic particles could recover *C. tyrobutyricum* spores from PBS and from UHT milk by applying a treatment with subtilisin and detergent. Although, our main purpose was to develop a capture system based on magnetic particles to recover spores directly from milk, the results obtained prove that applying a simple treatment to milk, the recovery is possible achieving CE

values within the acceptance criteria. Moreover, the introduction of synthetic peptides instead of antibodies in capture systems, reduce the use of animals and the subsequent purification steps which leads to less costs. We consider a positive advantage that the peptide can be easily synthetized and used to functionalize magnetic particles. The way of synthesis, avoids some problems related with differences between animals that could lead to less affinity of antibodies for spores and lower yields. As a consequence, it was concluded that pCZS1 could be a valuable ligand for a capture system for spores in subtilisin milk treated. Furthermore, our results show that pCZS1 could be useful for detecting *C. tyrobutyricum* spores by flow cytometry or other systems as lateral flow, although more research is needed to achieve this goal.

One of the reasons that could explain the weak recovery of spores with magnetic particles coated with pCZS1 directly in milk, is the small size of the peptide although, some amino acids were added to the original sequence of the peptide in its synthesis to increase its length and enhance spore recovery. Since incorporating this chain of amino acids did not seem to facilitate the capture of spores when the peptide was bound to particles, another strategy was assayed. This new attempt consisted in using as ligand the G3P protein from M13 phage by expressing it with pCZS1 attached to one extreme of the protein. G3P is a structural protein of the M13 phage, where the peptides of the library are displayed as fusion proteins (Pande et al., 2010). Taking into account that the selection of pCZS1 was made being attached to G3P protein in the Phage Display technique, it seemed to be a good strategy. The main aim of this approach was to reproduce the environment where the peptide was first presented and selected against *C. tyrobutyricum* spores. Furthermore, considering that probably the small size of pCZS1 is probably a drawback for the capture system efficiency, the G3P protein could be helpful as a spacer-arm, and provide an optimal orientation of the peptide.

As a first approach, the soluble domain of G3P was expressed in *E. coli* with pCZS1 producing what we called tG3P protein. The purification process of tG3P required multiple steps because it formed inclusion bodies in *E. coli*. However, high yields of a purified protein were obtained by applying the solubilization of inclusion bodies with urea and guanidine hydrochloride and the renaturation of the protein into the Hitrap™ column (Cytiva Life Sciences, 2020). After optimization of the purification process, the

affinity of tG3P for *C. tyrobutyricum* spores was measured by ITC and flow cytometry. The results showed that higher K_d was obtained for tG3P in comparison with pCZS1. After this step, tG3P was bound to magnetic particles designed to bind his-tag proteins and unfortunately, low spore recoveries were obtained in an aqueous buffer and milk. One of the reasons that could explain this fact is that the purification process of tG3P required the denaturation of the protein and which could affect to the tridimensional structure resulting in an incorrect orientation of the peptide. The orientation is critical for the correct recovery of spores from the matrix.

Based on the last results, a third ligand composed by the whole molecule of G3P with pCZS1 (named cG3P) was expressed and purified. Regarding the purification process of cG3P low yield was obtained in comparison with tG3P, probably because cG3P includes the transmembrane part of the protein, which may affect to the purification process. Moreover, the protein precipitated during storage, which made its handling very difficult. The affinity of cG3P for *C. tyrobutyricum* spores was measured by ITC and the K_d obtained was very similar to that of tG3P. Taking into account the results obtained, cG3P was not used to coat magnetic particles. We concluded that tG3P and cG3P did not provide any significant improvement respect to pCZS1 as ligands to functionalize magnetic particles to capture *C. tyrobutyricum* spores.

The CE results obtained in this thesis are aligned with the results obtained in the study by Lavilla et al. (2012), in which a specific peptide selected by Phage Display and affine for *C. tyrobutyricum* spores was evaluated and bound to magnetic particles as a capture system. In the mentioned study, capture efficiencies near 90% were obtained for spore capture in PBS. However, that method was not finally applied to capture spores in milk.

Taking into account that synthetic ligands as pCZS1, could be only able to recover spores from subtilisin treated milk. The last part of this thesis was focused on the study of the application of rabbit polyclonal antibodies against *C. tyrobutyricum* spores as ligands bound to magnetic particles for spore recovery. There are many studies in which antibodies are used to functionalize magnetic particles for bacterial immunocapture (Fisher et al., 2009; Wang et al., 2020). Antibodies have been conventionally the first choice to develop a detection system for different targets, including microorganisms,

because they have a complementary and complex structure for their capture and detection. However, in the last decades other ligands have been proposed to reduce the use of experimental animals, though not always with good results.

The use of magnetic particles coated with polyclonal antibodies recovered *C. tyrobutyricum* spores from milk with rates near 90%. Moreover, after immunocapture of spores from raw milk, they could be detected successfully by qPCR, setting a LOD of 10^2 spores/mL. A method combining immunocapture of *C. tyrobutyricum* spores from raw milk with qPCR detection is a novel approach that could be used in raw milk samples. Furthermore, among all the particles assayed, the small magnetic nanoparticles with protein A (250 nm) provided the best results in terms of qPCR detection after immunocapture, as it has been reported by other authors (Chen & Park, 2018; Lavilla et al., 2012).

Although more research would be needed, in this work, it has been proven that only antibodies could recover spores directly from milk efficiently. Synthetic ligands such as pCZS1 and tG3P could be an excellent alternative to antibodies produced in animals; however, only pCZS1 could recover spores from milk digested with subtilisin with rates near 90%. From the results obtained, it is concluded that pCZS1 worked well in a detection system such as flow cytometry, where the spore capture is not required, and only the detection is done. This fact could be explained because of the lack of wide affine regions in synthetic ligands such as pCZS1 in comparison with polyclonal antibodies, which have a complementary structure for bacterial recognition and capture.

To sum up, several efforts were made in this thesis to find an optimal synthetic ligand that allowed spore capture from milk, though the results obtained show that peptides could be a valuable choice, considering that a previous step is needed to achieve good CE rates. However, based on the results obtained, polyclonal antibodies were found able to recover successfully spores in untreated milk. It is expected that in a near future, new techniques will provide synthetic ligands that could be comparable to polyclonal antibodies.

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Final conclusions

Conclusions:

1. Several methods have been tested to disrupt *Clostridium tyrobutyricum* spores and extract their DNA to be amplified by qPCR. Bead beating and microwaves followed by silica-column DNA purification were the most efficient methods in PBS. However, microwaves provided better results when UHT milk spiked with spores were analysed.
2. The conditions of sporulation clearly influence the spore resistance. Spores of *C. tyrobutyricum* produced in liquid medium were better detected by qPCR than those produced in agar medium.
3. The calibration of qPCR with known amounts of *C. tyrobutyricum* spores in PBS and UHT milk, extracting DNA with microwaves and column purification, complies with the main acceptance criteria of recognized guidelines (efficiency, linear range, LOD). However, the application of this method in field raw milk samples revealed low precision of the Ct values obtained by qPCR, probably due to the presence of spores with higher resistance to disruption and/or low levels of spores in the samples.
4. The introduction of King Fisher Duo Prime system, a novel method to break spores and isolate their DNA, as a previous step to qPCR analysis, improved Ct values and qPCR precision in comparison with microwaves treatment.
5. The qPCR analysis of 202 raw milk samples from cow, ewe and goat from three different geographical regions of Spain, applying the King Fisher Duo Prime system for spore disruption and DNA purification, also improved the precision of the method compared to the microwaves and column purification.
6. The method proposed, based on qPCR, 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.
7. The selective media for species of *Clostridium* genus based on RCM agar with D-cycloserine and neutral red was not proved to be that specific as it also allowed

the growth of other bacteria of *Paenibacillus* and *Lactobacillus* genera, which were identified by 16S rDNA gene sequencing. The number of positive samples by qPCR did not correlate with the number of samples positive by microbiological culture.

8. Although an important percentage of milk samples were positive by qPCR for *C. tyrobutyricum*, this microorganism was only isolated in cow samples. The presence of other *Clostridium* spp in raw milk samples from cow, ewe and goat as *C. sporogenes* and *C. perfringens,* was demonstrated. The predominant species are variable depending of the milk origin (cow, ewe or goat). This fact must be considered in order to develop a method based on qPCR to detect butyric spores.
9. The affine peptide pCZS1 selected by Phage Display had a K_d for the interaction with *C. tyrobutyricum* spores comparable to established values for specific antibodies. Magnetic particles coated with this peptide were able to recover spores from milk by applying a previous treatment with subtilisin.
10. The designed recombinant proteins tG3P and cG3P, which included pCZS1 in their sequence, had similar K_d values for their interaction with *C. tyrobutyricum* spores, but higher than that of pCZS1. Moreover, capture efficiency values of magnetic particles coated with tG3P for the spores were not satisfactory neither in PBS nor in UHT milk.
11. Polyclonal antibodies against *C. tyrobutyricum* spores were bound to protein A and G magnetic particles. The values of spore recovery with these particles were near 90% in PBS and in UHT milk.
12. The immunocapture of *C. tyrobutyricum* spores in raw milk was done with Protein A magnetic particles followed by qPCR detection obtaining 100% amplification rates and high reproducibility in Ct values between assays. The amount of spores achieved for the immunocapture followed by qPCR detection was 10^2 spores/mL in 10 mL of raw milk.

Conclusiones finales

Conclusiones:

1. Se han ensayado diferentes métodos para la ruptura de esporos de *Clostridium tyrobutyricum* y la extracción de su ADN para ser amplificado mediante qPCR. Los métodos de *bead beating* y microondas combinados con la purificación mediante columna de sílica, fueron los más eficientes en PBS. Sin embargo, el tratamiento de microondas proporcionó mejores resultados en leche UHT contaminada artificialmente con esporos.
2. Las condiciones de esporulación afectan claramente a la resistencia de los esporos. Los esporos de *C. tyrobutyricum* producidos en medio líquido fueron detectados mejor mediante qPCR que los producidos en medio agar sólido.
3. La calibración de la qPCR con cantidades conocidas de esporos de *C. tyrobutyricum* en PBS y leche UHT, extrayendo el ADN mediante microondas y purificación en columna, cumple con los criterios de aceptación de las guías reconocidas (eficiencia, rango lineal, límite de detección). Sin embargo, la aplicación de este método a la leche cruda proporcionó una baja precisión de la qPCR, probablemente debido a la presencia de esporos con alta resistencia a la ruptura o bajos niveles de esporos en las muestras analizadas.
4. La introducción del Sistema King Fisher Duo Prime System, un método novedoso para romper los esporos y liberar su ADN, como paso previo al análisis mediante qPCR, mejoró los valores de Ct y la precisión de la qPCR, en comparación con el tratamiento de microondas.
5. El análisis por qPCR de 202 muestras de leche cruda de vaca, oveja y cabra procedentes de tres zonas geográficas españolas diferentes, aplicando el método del King Fisher Duo Prime System para la ruptura de los esporos y la posterior purificación del ADN, también mejoró la precisión del método en comparación con el tratamiento de microondas y la purificación en columna.
6. El método propuesto, basado en qPCR, debería ser considerado cualitativo, aunque, en una primera aproximación fue concebido como cuantitativo. Para valores bajos de concentración de esporos, particularmente valores $<10^2$esporos/mL, nuestro método solo puede distinguir entre muestras positivas y negativas. Sin embargo, el procedimiento es cuantitativo para muestras de

leche contaminadas artificialmente con esporos; para muestras de leche cruda cuya concentración es desconocida, sólo sería cuantitativo para aquellas altamente contaminadas.

7. El medio selectivo para clostridios basado en agar RCM suplementado con D-cicloserina y rojo neutro, no fue tan selectivo como se esperaba, dado que permitió el crecimiento de otras bacterias del género *Paenibacillus* y *Lactobacillus*, que fueron identificadas mediante secuenciación del 16S rADN bacteriano. El número de muestras positivas por qPCR no se correlacionó con el número de muestras positivas por cultivo microbiológico.
8. Aunque se obtuvo un alto número de muestras de leche cruda positivas por qPCR frente a *C. tyrobutyricum,* esta bacteria solo fue aislada en leches de vaca. Se han encontrado otros clostridios en leches crudas de vaca, oveja y cabra, como *C. sporogenes* y *C. perfringens*. Además, la especie predominante en las leches es dependiente de su origen (vaca, oveja o cabra). Este hecho debe ser considerado para elaborar un método de detección de los esporos butíricos basado en qPCR.
9. El péptido afín pCZS1, seleccionado mediante Phage Display, tuvo un valor de K_d para la interacción con los esporos de *C. tyrobutyricum* comparable a valores establecidos para anticuerpos específicos. Las partículas magnéticas funcionalizadas con el péptido fueron capaces de recuperar los esporos de la leche aplicando un tratamiento previo con subtilisina.
10. Las proteínas recombinantes diseñadas, tG3P y cG3P, que incluían en su secuencia el péptido pCZS1, tuvieron valores similares de K_d para su interacción con esporos de *C. tyrobutyricum*, pero mayores que los obtenidos para el péptido pCZS1. Además, se obtuvieron valores poco satisfactorios para la eficiencia de captura de los esporos con partículas magnéticas funcionalizadas con la proteína tG3P, tanto en PBS como en leche UHT.
11. Los anticuerpos policlonales frente a *C. tyrobutyricum* se unieron a partículas magnéticas con proteína A y proteína G. Los valores de recuperación de esporos con estas partículas fueron cercanos al 90% tanto en PBS como en leche UHT.
12. La inmunocaptura de esporos de *C. tyrobutyricum* en leche cruda se realizó con partículas magnéticas con proteína A y su posterior detección mediante qPCR. Se obtuvieron porcentajes de amplificación del 100% y una alta reproducibilidad

en los valores de Ct entre ensayos. La cantidad de esporos detectada mediante la combinación de la inmunocaptura y la qPCR fue de 10^2 esporos/mL en 10 mL de leche cruda.

Annex 1: Supplementary information for chapter 2

Supporting data from qPCR and microbiological culture (RCM selective medium)

In this paragraph it is presented the results in qPCR and RCM selective medium.

Table 40. Summary of cow milk samples analysis by qPCR and RCM selective medium followed by 16 rDNA identification. n=68 considering that 19 samples were negative in both methods. NA: not amplified.

Summary of samples cow milk n=68		
Positive in selective medium	**Positive in qPCR**	**16S rDNA sequencing**
10	POS	*1 Clostridium perfringens*
		1 Clostridium tyrobutyricum
		1 Clostridium bifermentans
		2 Lacticaseibacillus paracasei
		1 Clostridium luticerallii
		1 Paenibacillus macerans
		3 samples were not identified in 16S rDNA sequencing analysis
20	NA	*5 Paenibacillus*
		3 Clostridium tyrobutyricum
		5 Clostridium sporogenes
		2 Lacticaseibacillus rhamnosus
		2 Lacticaseibacillus paracasei
		1 Lacticaseibacillus casei
		2 samples were not identified in 16S rDNA sequencing analysis
NEG	9	*Not identified by 16s rDNA*

Table 41. Summary of ewe milk samples analysis by qPCR and RCM selective medium followed by 16 rDNA sequencing. n=86 considering that 21 samples were negative in both methods. NA: not amplified.

Summary of samples ewe milk n=86		
Positive in selective medium	**Positive in qPCR**	**16S rDNA sequencing**
6	POS	*4 Clostridium perfringens*
		2 could not be identified by 16S rDNA sequencing
22	NA	*5 Lacticaseibacillus paracasei*
		1 Clostridium sordellii
		1 Clostridium sporogenes
		15 were not identified by 16S rDNA
NEG	31	*N/A*

Table 42. Summary of goat milk samples analysis by qPCR and RCM selective medium followed by 16 rDNA sequencing. n=48 considering that 18 samples were negative in both methods. NA: not amplified.

Summary of samples goat milk n=48		
Positive in selective medium	**Positive in qPCR**	**16S rDNA sequencing**
4	POS	*1 Lacticaseibacillus paracasei*
		1 Lentilactobacillus parabuchneri
		1 Lacticaseibacillus rhamnosus
		1 not identified by 16S rDNA sequencing
27	NA	*3 Lacticaseibacillus casei*
		11 Lacticaseibacillus paracasei
		1 Lactobacillus gallinarum
		3 Lacticaseibacillus rhamnomsus
		1 Lacticaseibacillus zeae
		2 Clostridium sporogenes
		6 not identified by 16S rDNA sequencing
NEG	3	*Not identified*

Annex 2: Research articles published

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

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Milk

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.

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

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treatment. Consequently, the spores can germinate and cause the late blowing defect during the ripening stage, when the anaerobic conditions inside cheese are favourable (Heyndrickx, 2011). This problem affects mainly hard and semi-hard cheeses, among them, Grana Padano, Parmigiano Reggiano, and Manchego cheeses (Garde et al., 2011).

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

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

In the recent years, the efforts in developing alternative and fast methods for the detection of *C. tyrobutyricum* have increased. Lavilla et al. (2010) published an article describing a detection method based on flow cytometry using a fluorophore-labelled polyclonal antibody against *C. tyrobutyricum* spores. In that study, the detection limit was established in 10^3 spores/100 mL. The use of magnetic nanoparticles with specific ligands, such as antibodies or affine peptides, were also proposed by Lavilla et al. (2012) as novel methods for the detection of *C. tyrobutyricum* spores. Although those methods are faster and highly specific, their introduction into the routine analysis is not easy due to the high cost of the materials and equipment required.

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

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

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

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

The first aim of this study was to evaluate several methods for the disruption of *Clostridium tyrobutyricum* spores to achieve pure DNA to be amplified by real time PCR. The second objective of this study was to compare the disruption of spores produced on agar plates with that of spores induced in liquid medium.

2. Material and methods

2.1. Sporulation process

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

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

2.2. Purification of spores

The bacterial cells were lysed with lysozyme from egg white (Sigma-Aldrich, St. Louis, MO, USA) added to the suspension at a concentration of 0.4 mg/mL and incubated for 24 h at 45 °C in a total volume of 100 mL. Afterwards, the suspension was centrifuged for 15 min at 4000 ×*g* and 4 °C. The supernatant was discarded, and the precipitate was washed twice in the same conditions using sterile distilled water to eliminate the cellular debris. The precipitate obtained was resuspended in 2 mL of sterile distilled water and applied to a Percoll® gradient

(Sigma-Aldrich) to separate the spores from vegetative cells, as described before (Leuschner et al., 1999). The gradient was prepared with different concentrations of Percoll®: 1.13, 1.11, 1.09, 1.07 and 1.05 g/mL. The different solutions were placed in falcon tubes, adding 10 mL of each concentration, starting from the densest and ending with the less dense. Afterwards, 2 mL of the lysate was added on top of the gradient and it was centrifuged for 45 min at 4000 ×*g* and 4 °C. After the centrifugation, the spores were collected from the bottom of the gradient, washed 5 times with sterile distilled water and recovered by centrifugation at 13000 ×*g* for 10 min. The precipitate containing the spores was resuspended in 200 µL of PBS and the concentration was determined by counting in a Thoma chamber under a phase contrast microscope ECLIPSE E400 (Nikon, Tokyo, Japan). Finally, the spore suspension was stored at −20 °C until use.

2.3. Disruption of spores

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

2.3.1. Microwave treatment

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

2.3.2. Bead beating treatment

The bead beating procedure was carried out following the method by Vandeventer et al. (2011), with some modifications. For the spore lysis, 250 mg of 100 µm-diameter silica beads and 625 µL of 10^6 spores/mL suspension were added in tubes of 2 mL. The samples were bead beaten using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK, USA) at maximum speed for 1 min pulses, three times, with an interval of 30 s, placing the tubes in ice between each pulse.

2.3.3. DNA precipitation

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

2.3.4. Proteinase K treatment

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

2.3.5. Heat treatment

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

2.3.6. 2-Mercaptoethanol-lysozyme treatment

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

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

The method reported by López-Enríquez et al. (2007) for the quantification of *C. tyrobutyricum* using qPCR was followed with some modifications. To begin with, 200 µL of 10 mM Tris-HCl, pH 8, with 50 mM EDTA, and 10 µL of 100 mg/mL lysozyme were added to 200 µL of a suspension containing 10^6 spores/mL. This mixture was incubated for 1 h at 37 °C. Afterwards, one volume of 10 mM Tris-HCl, 1% SDS, 100 µg/mL of proteinase K was added and the mixture was heated for 1 h at 37 °C.

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

2.3.8. DNA purification

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

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

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

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

2.5. Quantitative real-time PCR (qPCR)

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

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

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

The amplification process, in the case of the SYBR Green qPCR, has

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

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

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

2.6. Analysis of DNA amplification from vegetative cells and spores

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

For extraction of DNA, 10^7 spores/mL produced in liquid medium were resuspended in 100 L of PBS and subjected to microwave treatment and column purification. The step of ethanol precipitation in the presence of sodium acetate was also needed, to achieve a high amount of DNA.

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

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

2.7. Data analysis

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

3. Results

3.1. Evaluation of the spore disruption methods

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

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

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

The effectiveness of disruption methods on spores generated on agar plates and in liquid medium was compared. The spores generated on

Table 1
Effect of different disruption methods on the amplification by qPCR of *C. tyrobutyricum* spores (10^6 spores/mL) generated on agar plates. Method based on López-Enríquez et al. (2007).

Treatment	SYBR Green reagent		TaqMan probe	
	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

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. (1) Method based on López-Enríquez et al. (2007), as it appears in the original manuscript, because only the last method of the table (marked with (1)) is based on the study by López-Enríquez et al.

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.

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

The values represent the Ct mean ± standard deviation from three independent experiments with two replicates each ($n = 6$).
* Significant differences for $p^{<}0.05$ between agar plate and liquid medium spores.

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.

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.

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

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

The qPCR products were analyzed by agarose gel electrophoresis to verify that the size of DNA amplicons was the same after applying the different disruption methods. The results obtained are shown in Fig. 1, where it can be observed the expected size of the amplicons, around 90–100 bp, regardless the disruption method used. Furthermore, we

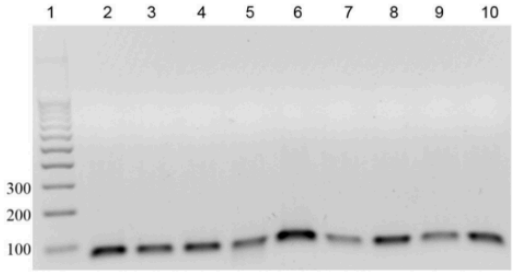

Fig. 1. Analysis by agarose gel electrophoresis of qPCR products (82 bp) obtained from the amplification of DNA extracted from *C. tyrobutyricum* spores by different methods. Lane 1, molecular weight base pair; Lane 2, vegetative cells; Lanes 3–6, PBS spiked with spores: Lane 3, DNA from solid medium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

observed differences in the amount of DNA amplified from solid medium spores, compared to liquid medium spores, which correlates well with the lower Ct found for solid medium spores.

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

DNA from vegetative cells and liquid medium spores was extracted and purified by the same method, and the amplification of the same amount of DNA from each extraction was compared. As it can be observed in Fig. 2, the results clearly show the differences in DNA amplification, as the DNA from vegetative cells amplified at lower number of cycles than that from spores. Furthermore, the lowest quantity of DNA from vegetative cells that could be detected was 0.02 ng, while no less than 0.2 ng could be detected from spores. The standard curves obtained indicate that there is a difference of about 10 cycles in the Ct values between DNA amplification from vegetative cells and that from liquid medium spores, and this difference is maintained for all the concentrations assayed. Consequently, the slope of the two regression

Table 3
Effect of the most efficient disruption methods on spores produced in solid or liquid media added to UHT milk; in both methods a final step of column purification was used. qPCR was carried out with SYBR Green.

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

The values represent the Ct mean ± standard deviation from three independent experiments ($n = 6$).

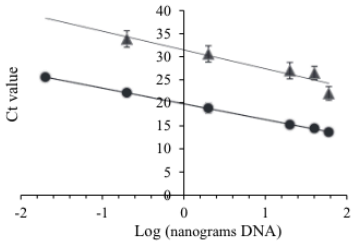

Fig. 2. Standard curve obtained by qPCR for amplification of DNA from *C. tyrobutyricum* vegetative cells (●) and liquid medium spores (▲). The equation for standard curve of vegetative cells is $y = -3.406\times + 18.779$ with $R^2 = 0.9997$ and for spores $y = -4.0583\times + 30.241$ and $R^2 = 0.8991$.

lines is similar, indicating that the difference is not due to a random effect. The qPCR efficiency obtained for vegetative cells was 96.84%, whereas for spores it was only of 59.23%.

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

4. Discussion

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

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

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

We also studied the extraction of DNA from *C. tyrobutyricum* spores in UHT milk for qPCR analysis, with the aim of mimicking the real conditions. The main challenge to achieve an efficient method for detecting *C. tyrobutyricum* spores in milk is to find a simple and reliable method to separate and concentrate the spores that are distributed in milk phases. We propose an effective method for milk digestion with subtilisin enzyme. This non-specific protease is routinely used to count the total bacteria present in milk with the Bactoscan equipment (Foss Electric) in the Milk Quality Control Laboratories. Our results show that this method is a good approach to develop a fast procedure for *C. tyrobutyricum* spore detection. The treatment of UHT milk with subtilisin allows its effective digestion, thus obtaining a clean pellet of spores, compared to the non-treated milk, which can be subjected to disruption by different methods, as we have tested in the present study. This is a novel approach, considering that other digestion procedures previously reported have higher number of steps (López-Enríquez et al., 2007) or are based in a simple centrifugation (Morandi et al., 2015), which presents the problem that a percentage of spores can be retained in the fat layer. As far as we have tested in UHT and raw milk, subtilisin treatment allows recovering almost all the spores in the pellet, as we have checked by plate count (Supplementary material).

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

In the current study, the lowest amount of DNA from liquid medium spores being detected was 0.2 ng, with a Ct mean value of 33.82, which corresponds to approximately 10^5 genome equivalents, while for the same amount of DNA from vegetative cells the amplification presented Ct mean values of 25.57. The lowest amount of DNA detected for vegetative cells was set up in 10^{-6} nanograms of genomic DNA equivalent to approximately 1 genome. These results indicate that higher amounts of DNA have to be extracted to detect the spores than to detect the vegetative cells. This fact increases considerably the limit of detection for *Clostridium* spores, which are more resistant and consequently more difficult to be disrupted than vegetative cells. Moreover, the slope of the regression line obtained for amplification of vegetative cell DNA by using the *CTfla* primers was −3.406, very similar to the value reported in the original study, of −3.443 (López-Enríquez et al., 2007), with an excellent linearity in both studies. However, the linearity of the regression line obtained for DNA spore amplification ($R^2 = 0.8991$) in our study differed from the linearity obtained in that by López-Enríquez et al. (2007), probably due to the experimental differences between the procedures.

The results reported in this study have revealed the difficulty of detecting DNA of clostridial spores by qPCR when vegetative cells are present, since vegetative cells amplified at lower cycles than spores.

Therefore, in a mixture of DNA from clostridial spores and vegetative cells, the DNA from vegetative cells would amplify earlier by qPCR due to its nature, thus hiding the presence of clostridial spores. Due to this fact it is very important to inactivate the vegetative cells that could be present in raw milk in a first step, with a heat treatment of 90 °C for 10 min. It could be also adequate to treat the samples with DNase in order to detect only the DNA from spores after disruption (Morandi et al., 2015). In our experimental conditions, the Percoll gradient used for spore purification separated very well the vegetative cell debris from the spores. In addition, the subtilisin treatment applied in our method washes off the possible contamination of DNA from vegetative cells, as it migrates to the soluble phase that is eliminated.

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

5. Conclusion

The subtilisin digestion of milk combined with microwave treatment followed by DNA column purification has been proved as the best procedure for detecting *C. tyrobutyricum* spores by qPCR in milk among all the methods tested. Furthermore, in this study we have proved that the way the spore is produced affects its detection by qPCR. We found that spores from liquid medium amplified earlier than those produced on agar plates. Differences between the amplification of genomic DNA from vegetative cells and from spores were also found, the second one showing higher Ct values. The DNA of spores might be highly condensed due to SASP, which might limit the action of Taq polymerase during the amplification reaction. This problem does not occur with the vegetative cells, because their DNA is more accessible.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105818.

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

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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.

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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: $E = -1 + 10^{-1/\text{slope}}$. 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%			

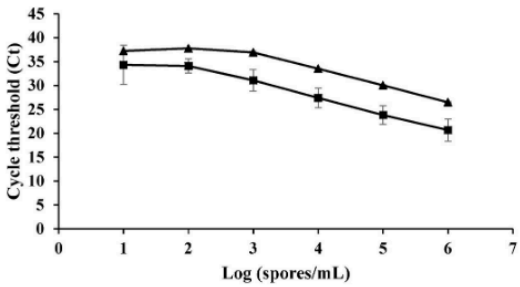

Fig. 1. qPCR calibration curves for amplification of *C. tyrobutyricum* in cow raw milk samples spiked with spores. Type strain CECT 4012 (▲) and UZ01 strain (■).

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

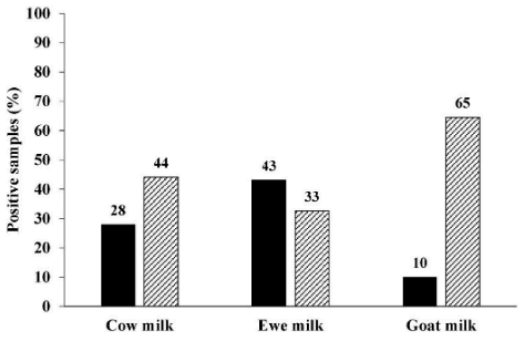

Fig. 2. Percentage of positive samples for *C. tyrobutyricum* by qPCR considering Ct < 40 (■) and positive by culture in RCM selective medium (▨). The raw milk samples analyzed were from cow (n = 68), ewe (n = 86) and goat (n = 48).

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

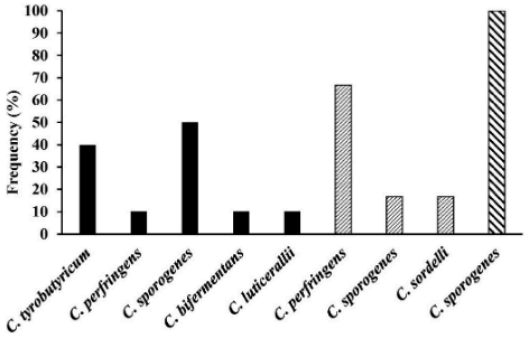

Fig. 3. Frequency and distribution of *Clostridium* spp. in colonies isolated from cow (■), ewe (▨) and goat (▧) milk samples cultured in RCM selective medium and identified by 16S rDNA sequencing and multiplex PCR. N = 20.

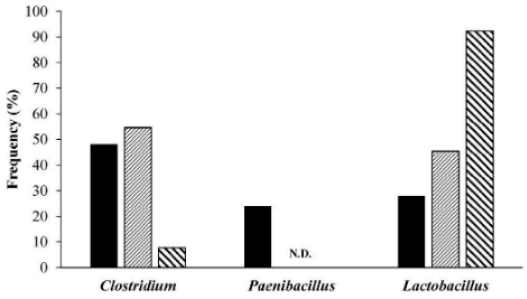

Fig. 4. Frequency and distribution of *Clostridium*, *Paenibacillus* and *Lactobacillus* found in cow (■), ewe (▨) and goat (▧) milk cultured in RCM selective medium and identified by 16S rDNA sequencing and multiplex PCR. N = 62. N.D.: not detected.

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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