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Identification of *Clostridium beijerinckii, Cl. butyricum, Cl. sporogenes, Cl. tyrobutyricum* isolated from silage, raw milk and hard cheese by a multiplex PCR assay

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Late blowing, caused by the outgrowth of clostridial spores present in raw milk and originating from silage, can create considerable product loss, especially in the production of hard and semi-hard cheeses. The conventional method for the isolation of *Clostridium* spp. from cheeses with late-blowing symptoms is very complicated and the identification of isolates is problematic. The aim of this work was the development of a multiplex PCR method for the detection of the main dairy-related clostridia such as: *Cl. beijerinckii, Cl. butyricum, Cl. sporogenes, Cl. tyrobutyricum*. Samples derived from silage, raw milk and hard cheese were analysed by the most probable number (MPN) enumeration. Forty-four bacterial strains isolated from gas positive tubes were used to check the reliability of the multiplex PCR assay. The specificity of the primers was tested by individually analysing each primer pair and the primer pair combined in the multiplex PCR. It was interesting to note that the samples not identified by the multiplex PCR assay were amplified by V2–V3 16S rRNA primer pair and the sequencing revealed the aligned 16S rRNA sequences to be *Paenibacillus* and *Bacillus* spp. This new molecular assay provides a simple promising alternative to traditional microbiological methods for a rapid, sensitive detection of clostridia in dairy products.

Keywords: *Clostridium* spp., multiplex PCR, raw milk, silage, cheese.

Clostridia are gram-positive, spore-forming, anaerobic bacteria which are considered to be the principal organisms responsible for late blowing. Spores of *Clostridium* spp. (e.g. *Cl. tyrobutyricum, Cl. butyricum, Cl. sporogenes, Cl. beijerinckii*, but also *Cl. pasteurianum, Cl. tertium, Cl. perfringens*, and *Cl. tetanomorphum*) are able to survive the manufacture of processed cheese and may germinate and grow out in the product (Coulon et al. 1991; Guericke, 1993; Ingham et al. 1998; Le Bourhis et al. 2007).

Late blowing, caused by the outgrowth of clostridial spores present in raw milk and originating mainly from silage, can create considerable product loss, especially in the production of hard and semi-hard cheeses (Bergeres & Sivela, 1990; Coulon et al. 1991). This condition has been characterised by the formation of high amounts of CO_2 that causes the cheese-loaf to show a balloon-like expansion and could double in size. The clostridial species produce abundant gas and off-odours caused by the production of acetic acid, butyric acid, carbon dioxide, and hydrogen. Moreover, due to the large quantities of butyric acid produced, a rancid, distasteful consistence of the cheese is obtained (Innocente & Corradini, 1996) and for this reason the analytical determination of free volatile fatty acids, particularly of butyric acid, is a useful parameter for spotting anomalous fermentations in cheeses (Chavarri et al. 1997; Innocente et al. 2000).

One approach for presumptively enumerating *Clostridium* spp. endospores involves heat treatment of the sample to destroy vegetative cells, followed by a most probable number (MPN) enumeration, based on gas production in anaerobically incubated medium containing lactate as the fermentable organic compound (Cocolin et al. 2004). The MPN value can be confirmed by verifying the lactate fermentation ability of cells from gas positive tubes. This method enumerates all clostridia species capable of fermenting lactate, but further tests on pure cultures obtained from gas positive tubes, such as examination of endospores position, carbohydrate fermentation profiling, and gas chromatographic analysis of volatile and non-volatile organic acid by-products, are commonly performed to obtain the identification at the species level (Ingham et al. 1998).

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The conventional method for the isolation of *Clostridium* spp. from cheeses with late-blowing defect is very complicated and the identification of isolates is problematic since specific media to discriminate between the clostridial species do not exist and phenotypic discrimination is almost impossible (Bergères & Sivela, 1990; Klijn et al. 1995). For these reasons, more reliable and rapid methods for the isolation and identification of the causative agents of late blowing in cheese are needed.

In recent years PCR-based methods (Klijn et al. 1994; Pecoraro et al. 1999) and 16S rRNA probe-based hybridisation methods (Klijn et al. 1994; Knabel et al. 1997) have been developed with the disadvantage of being specific for a single species; so, for a complete study of *Clostridium* spp. in cheese a set of specific primers had to be available. More recently Cocolin et al. (2004) developed a PCR-denaturing gradient gel electrophoresis (DGGE) protocol to profile the microbial populations in blown cheeses while Le Bourhis et al. (2005) performed a PCR-TTGE approach to evaluate the evolution of the clostridial species during ripening. Moreover, Garde et al. (2011) identified by the amplification of 16S ribosomal DNA restriction analysis (ARDRA technique) the *Clostridium* spp. from Manchego cheese with late blowing defect.

In this study we developed a multiplex-PCR method in order to profile, with a rapid assay, *Cl. beijerinckii*, *Cl. butyricum, Cl. sporogenes, Cl. tyrobutyricum* in raw milk and cheese.

Materials and Methods

Bacterial strains and growth conditions

Cl. tyrobutyricum DSM#2637^T, *Cl. butyricum* DSM#10072^T, *Cl. beijerinckii* DSM#791^T, *Cl. baratii* DSM#601^T, *Cl. perfringens* DSM#756^T, *Cl. difficile* DSM#1296^T, *Bacillus cereus* DSM#31^T, *Escherichia coli* DSM#4064^T, *Lactobacillus helveticus* DSM#20075^T, *Lb. delbrueckii* subsp. *lactis* DSM#20072^T, *Lb. delbrueckii* subsp. *bulgaricus* DSM#20081^T, *Lb. fermentum* DSM#20052^T, *Enterococcus faecium* DSM#20477^T, *Ec. durans* DSM#20633^T, *Listeria monocytogenes* DSM BAA 679^T, *Salmonella enterica* DSM#17058^T, used in this study were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany); *Cl. sporogenes* ATCC 3584, *Staphylococcus aureus* ATCC 25923, *Ec. faecalis* ATCC 23655, *Streptococcus thermophilus* (BT63), *Lactococcus garvieae* (SV80), *Lc. lactis* subsp. *cremoris* (SV120), *Lc. lactis* subsp. *lactis* (SV131), *B. licheniformis* (IN2), and two *Paenibacillus polymyxa* (PA, PAP) were provided by the ISPA-CNR (Institute of Sciences of Food Production-Italian National Research Council, Milan, Italy) bacterial collection. The *Clostridium* strains were anaerobically incubated at 37 °C in Reinforced Clostridial Medium (RCM) (Oxoid, Milan, Italy) for 24 h. *Str. thermophilus* was cultured overnight at 37 °C in M17 broth (Scharlau Microbiology, Barcelona, Spain); *Lactobacillus* strains in MRS broth (Scharlau Microbiology) while *B. cereus* and *Esch. coli* were grown in Nutrient Broth (Difco, Milan, Italy) at 37 °C.

Samples and microbiological analysis

Most probable number (MPN) enumeration was performed on samples of silage ($n = 11$), raw milk ($n = 28$) and Grana Padano cheese ($n = 5$), without late blowing defect, with five tubes per dilution series and three dilutions. The culture medium used for MPN was prepared with milk supplemented with yeast extract (1·0%), sodium lactate (3·36%), sodium acetate (1·0%), cysteine (0·2%) with vaseline/paraffin (1:1, wt/wt) seals. The heat treatment applied to the inoculated milk medium was 80 °C for 10 min. The incubation period was 7 d at 37 °C. Forty-four bacterial strains isolated from gas positive tubes were used to check the reliability of the multiplex PCR assay (Table 1).

DNA extraction

For DNA extraction, 1 ml of overnight cultures (approximately $1–3 \times 10^9$ CFU/ml) for all the reference strains and for the silage, milk and cheese isolates with bacterial morphology similar to that of *Clostridium* spp. and presence of endospores, were analysed and the protocol described in Cremonesi et al. (2006) was used, without pre-treatment step.

PCR primer design

Based on published DNA sequences available in GenBank database, four primer pairs specific to the clostridia species *Cl. beijerinckii, Cl. butyricum, Cl. sporogenes, Cl. tyrobutyricum* were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3/, June 2011). All the primers used in this study, ranging from 20 to 22 mers, are shown in Table 2 and were designed in order to have similar melting temperatures and minimal interactions resulting in differentially sized products distinguishable in agarose gel electrophoresis. The target genes were chosen among the sequences available on the NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez, accessed June 2011). The primers were synthesised by Invitrogen (Minneapolis, MN, USA) and were resuspended to a final concentration of 100 pmol/µl in sterile double-distilled water.

DNA amplification

The multiplex PCR was performed using ~ 40 ng of the DNA template, 1·25 U of GoTaq DNA Polymerase (Promega), 10 µl of 5 × Colorless GoTaq Reaction Buffer (Promega), 6 µl of $MgCl_2$ 25 mM, 1 µl of dNTPs Mix (10 mM each), the primer pairs amount described in Table 2 and double-distilled water to the final volume of 50 µl. Reactions were carried out in a GeneAmp PCR System 2700 thermal cycler (Applied

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Table 1. Samples used for multiplex-PCR validation

Samples	Source	Multiplex PCR	Sequencing with V2–V3 16S rRNA primer
1	Milk	*Cl. sporogenes*	*Cl. sporogenes*
2	Milk	*Cl. sporogenes*	*Cl. sporogenes*
3	Milk	*Cl. sporogenes*	*Cl. sporogenes*
4	Milk	*Cl. sporogenes*	*Cl. sporogenes*
5	Milk	*Cl. sporogenes*	*Cl. sporogenes*
6	Milk	*Cl. sporogenes*	*Cl. sporogenes*
7	Silage	*Cl. sporogenes*	*Cl. sporogenes*
8	Milk		*Paenibacillus polymyxa*
9	Milk		*Paenibacillus polymyxa*
10	Milk		*Paenibacillus polymyxa*
11	Silage	*Cl. sporogenes*	*Cl. sporogenes*
12	Milk		*Clostridium* spp.
13	Silage	*Cl. sporogenes*	*Cl. sporogenes*
14	Cheese		*Paenibacillus polymyxa*
15	Cheese	*Cl. sporogenes*	*Cl. sporogenes*
16	Cheese	*Cl. sporogenes*	*Cl. sporogenes*
17	Silage	*Cl. sporogenes*	*Cl. sporogenes*
18	Silage	*Cl. sporogenes*	*Cl. sporogenes*
19	Silage	*Cl. sporogenes*	*Cl. sporogenes*
20	Silage	*Cl. sporogenes*	*Cl. sporogenes*
21	Silage	*Cl. sporogenes*	*Cl. sporogenes*
22	Silage		*Cl. sporogenes* or *Cl. botulinum*
23	Milk		*Paenibacillus polymyxa*
24	Milk		*Paenibacillus polymyxa*
25	Milk		*Paenibacillus polymyxa*
26	Milk		*Paenibacillus polymyxa*
27	Milk		*Staphylococcus lugdunensis*
28	Milk	*Cl. sporogenes*	*Cl. sporogenes*
29	Milk		*Clostridium* spp.
30	Milk		*Clostridium* spp.
31	Cheese	*Cl. beijerinckii*	*Cl. Beijerinckii*
32	Cheese		*Staphylococcus* spp.
33	Milk		*Bacillus* spp.
34	Milk		Uncultured
35	Milk		*Bacillus licheniformis*
36	Milk		*Bacillus licheniformis*
37	Milk		*Bacillus licheniformis*
38	Milk		*Bacillus licheniformis*
39	Milk		Uncultured
40	Milk		*Bacillus licheniformis*
41	Milk		*Bacillus licheniformis*
42	Milk		*Bacillus licheniformis*
43	Silage	*Cl. sporogenes*	*Cl. sporogenes*
44	Silage	*Cl. sporogenes*	*Cl. sporogenes*

Biosystems, Foster City, CA, USA) with the following conditions: initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min. Final extension was carried out at 72 °C for 5 min. The amplified PCR products were distinguished by gel electrophoresis in a 3% agarose gel (GellyPhor, Euroclone, Milan, Italy), stained with ethidium bromide (0·05 mg/ml; Sigma Aldrich, Milan, Italy), and visualised by UV transilluminator (BioView Ltd, Nes Ziona, Israel). A 100 bp DNA ladder (Finnzymes, Espoo, Finland) was included in each gel electrophoresis.

Specificity and sensitivity test

The specificity of the assay was tested using the DNA of target (artificial balance mixes composed by three out of four DNA of typed strains) and non-target reference strains. Serial dilutions of a genomic DNA pool obtained from the *Clostridium* target reference strains, starting from 200 to 0·39 ng were used to test the sensitivity of the assay. The PCR products were quantified using the Agilent BioAnalyser 2100 applied to the DNA 7500 LabChip kit (Agilent Technologies, Palo, Alto, CA, USA).

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Table 2. Primers and target genes used in this study

Primer name	Target gene	Accession number	Primer sequence (5′–3′)	Amount (μM)	Expected size (bp)
Cl-SPOR-F3031	*col*A	AB090330	TTGGGATTTTGGGGATAACA	30	549
Cl-SPOR-R3579			TCCGTATCGTTGTCGTCTTG	30	
Cl-BEIJ-F363	*nif*H	AY649323	TGACACGATTTTTCATTCTCCA	20	448
Cl-BEIJ-R820			TCCATTGCCTTAATGACAGGT	20	
Cl-BUTY-F1329	*hyd*A	EU366290	ATGGGTTAGGCAAGCAGAAA	15	312
Cl-BUTY-R1640			GCTGGATCTGCCTTCTCATC	15	
Cl-TYRO-F1253	*enr*	Y09960	TGGTGTTCCACAAGAAGCTG	15	210
Cl-TYRO-R1462			GCAGCTGGATTTACTGCACA	15	

*col*A, collagenase; *nif*H, nitrogenase iron protein; *hyd*A, hydrogenase; *enr*, 2-enoate reductase

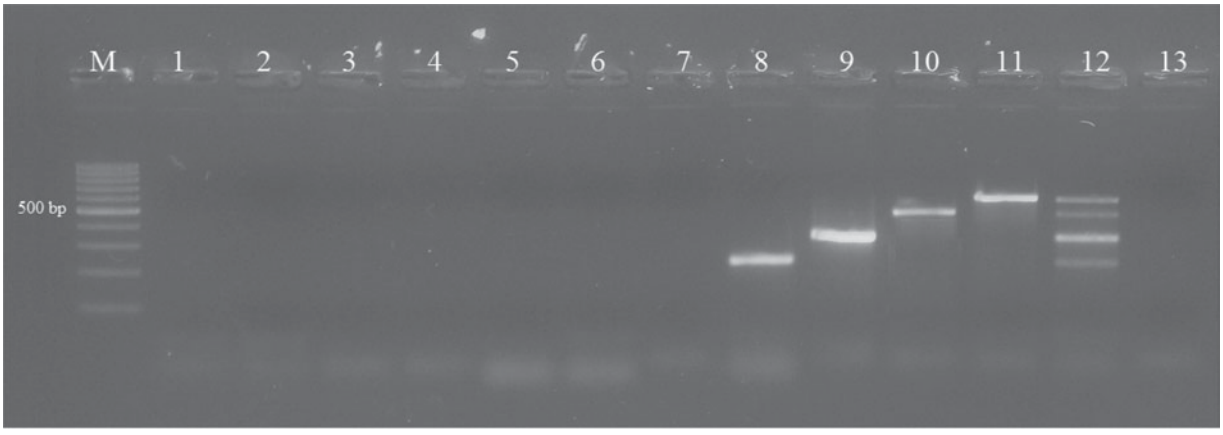

Fig. 1. Examples of multiplex PCR results obtained using target (DNA of typed strains) and non-target reference strains. From lane 1 to lane 7 *Cl. difficile, Cl. perfringens, L. delbrueckii* subsp. *lactis, Str. thermophilus, L. monocytogenes, Cl. baratii, St. aureus,* respectively; lane 8, *Cl. tyrobutyricum* (210 bp); lane 9, *Cl. butyricum* (312 bp); lane 10, *Cl. beijerinckii* (448 bp); lane 11, *Cl. sporogenes* (549 bp); lane 12, *Cl. tyrobutyricum* (210 bp), *Cl. butyricum* (312 bp), *Cl. beijerinckii* (448 bp) and *Cl. sporogenes* (549 bp); lane 13: negative control (PCR master mix without DNA). M: 100 bp DNA ladder (Finnzymes).

Sequencing

Forty-four isolates from silage, raw milk and cheeses (Table 1) were used to check the reliability of the multiplex PCR assay. PCR with universal primers HDA1 and HDA2 amplifying the V2-V3 16S rRNA gene region was performed as described by Walter et al. (2000) and sequences were analysed by Primm s.r.l. (Milan, Italy). The resulting chromatograms were analysed by the software Bioedit and a similarity search with the nucleotide sequence database at NCBI by BLASTN.

Results

Multiplex PCR optimisation

The multiplex PCR method provides an approach enabling a simultaneous and specific detection of more than one *Clostridium* spp. The primers designed in this study had the same melting temperature of 59–60 °C but different amplification products of 549, 448, 312 and 210 bp for *Cl. sporogenes, Cl. bejierinkii, Cl. butyricum* and *Cl. tyrobutyricum* respectively, easily interpretable during the agarose gel electrophoresis. Each primer pairs of the multiplex PCR, tested individually by using reference strains with known genotype and artificial balance mixes, showed good specificity, amplifying PCR products of the expected size with no additional or non-specific products. The concentration of each primer set was adjusted and PCR conditions were modified obtaining equal yields for all the amplification products. Strains belonging to *Lactobacillus, Streptococcus*, and *Enterococcus* genera, such as *Lb. helveticus, Lb. delbrueckii* subsp. *lactis, Lb. delbrueckii* subsp. *bulgaricus, Lb. fermentum, Ec. faecalis, Ec. faecium, Ec. durans, Str. thermophilus, Lc. garvieae, Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis, Cl. baratii, Cl. perfringens, Cl. difficile, B. cereus, B. licheniformis, P. polymyxa, Staph. aureus, Esch. coli, List. monocytogenes, Sal. enterica*, which can coexist with our target, were used as negative controls and did not generate false-positive results (examples in Fig. 1).

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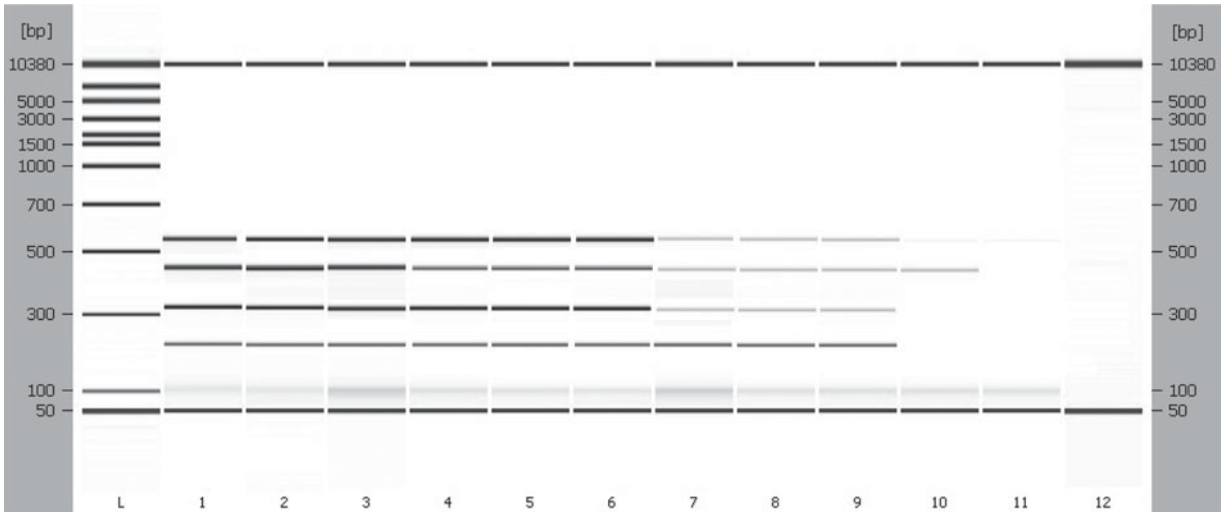

Fig. 2. Sensitivity of the multiplex PCR: analyses of genomic DNA pool obtained from the *Cl. sporogenes* (549 bp), *Cl. beijerinckii* (448 bp), *Cl. butyricum* (312 bp) and *Cl. tyrobutyricum* (210 bp) reference strains. The concentration of each DNA fragment was calculated using the Agilent 2100 Bioanalyser software. The 'gel-like image' provided by the instrument shows the multiplex PCR results obtained using 200 ng (lane 1), 100 ng (lane 2), 50 ng (lane 3), 25 ng (lane 4), 12·5 ng (lane 5), 6·25 ng (lane 6), 3·125 ng (lane 7), 1·56 ng (lane 8), 0·78 ng (lane 9), 0·39 ng (lane 10), 0·195 ng (lane 11) of the genomic DNA pool from the reference strains. Lane 12: negative control (PCR master mix without DNA). Lane L: DNA 7500 ladder.

Sensitivity

The analysis of serial dilutions, made from genomic DNA pool obtained from reference strains of the target species, gave correct amplification from 200 ng down to 0·78 ng of DNA (Fig. 2), corresponding approximately to 10^2 CFU/ml. For *Cl. beijerinckii* (448 bp) a signal was obtained down to 0·39 ng (Fig. 2). A detection limit of 100 CFU/g available for *Clostridium* sensitivity in cheese samples was established by Le Bourhis et al. (2005) with the development of a TTGE protocol.

Multiplex PCR validation

Forty-four bacterial strains isolated from gas positive tubes were used to check the reliability of the multiplex PCR assay (Table 1). For 20 samples out of 44 analysed, the results obtained by multiplex PCR were confirmed by sequencing of V2-V3 16S rRNA region. For the remaining 24 samples, the sequencing revealed the aligned 16S rRNA sequences to be *Paenibacillus polymyxa* (8 samples), *Clostridium* spp. (4 samples), *Bacillus* spp. (*B. licheniformis*, 8 samples), *Staphylococcus* spp. (2 samples) and uncultured (2 samples). Like clostridia, *P. polymyxa* and *B. licheniformis* are spore-forming, able to produce gas and their original habitat is soil.

Discussion

In the last decade, different molecular methods have been applied for a better understanding of the late blowing spoilage process. Previously developed protocols (Klijn et al. 1994; Pecoraro et al. 1999) have the main disadvantage of being specific for a single species, which means that for a complete study of the *Clostridium* spp. ecology in raw milk or cheese with late-blowing defect, a set of specific probes must be available to identify all the agents responsible for the alteration.

The routine diagnostic methods used by analytical laboratory for detecting *Clostridium* spp. require several days for a visible response. For this reason it is difficult to satisfy the requirements of cheese-makers as the milks from various sources have already been mixed and passed onto the production process by the time a positive result is obtained.

The protocol was first optimised by using reference strains to determine the best experimental conditions for the multiplex assay. Also strains belonging to *Lactobacillus, Streptococcus*, and *Enterococcus* genera were tested since they are present in milk and cheese environment, where it can be necessary to perform a detection of *Clostridia* species. The optimised protocol can distinguish the four target *Clostridia* species and no amplifications were obtained among other *Clostridium* spp. (*Cl. difficile, Cl. perfringens*) or non-target species. For this assay, target genes such as *col*A (*Cl. sporogenes*), a collagenase that catalyses the degradation of collagen, *nif*H (*Cl. beijerinckii*), an enzyme responsible for nitrogen fixation, *hyd*A (*Cl. butyricum*), an enzyme that catalyse the reversible oxidation of hydrogen, and *enr* (*Cl. tyrobutyricum*), an enzyme that participates in phenylalanine metabolism, have been chosen among the sequence available on the NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez); these genes, less conserved than 16S rRNA, have been shown to be useful for the discrimination of closely related bacterial species.

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As previously described (Ingham et al. 1998; Le Bourhis et al. 2007), the analysed samples revealed the presence of *Clostridium* spp. in silage, raw milk and cheese. The prevalent species detected with multiplex PCR was *Cl. sporogenes* isolated from silage (10 samples), raw milk (7 samples) and hard cheese (3 samples). Similar results were also obtained from Lycken & Borch (2006) who isolated 124 Clostridia from 42 cheeses and 33% were identified as *Cl. sporogenes*. More recently Garde et al. (2011), using the ARDRA technique, analysed 57 Manchego cheeses and out of 223 isolates from spoiled cheeses, 78·9% were identified as *Cl. sporogenes* while 10·3, 9·0 and 1·8% were *Cl. bejierinckii, Cl. tyrobutyricum* and *Cl. butyricum* respectively.

This is the first study which allows the detection and differentiation of four clostridial species with a single test. It is noteworthy that clostridia such as *Cl. butyricum* and *Cl. beijerinckii* cannot easily be identified by normal culture and biochemical techniques but can readily be differentiated by this multiplex PCR. Furthermore, similar to *Cl. tyrobutyricum, P. polymyxa,* isolated from 7 milk and 1 cheese samples, is spore-forming, is able to produce gas but not to make milk coagulation. This multiplex PCR, joined with an efficient extraction protocol, provides a simple promising alternative to traditional microbiological methods for a rapid, sensitive detection of four different species of *Clostridia, Cl. tyrobutyricum, Cl. sporogenes, Cl. butyricum* and *Cl. beijerinckii*, in animal feeds and in dairy products for monitoring the products from 'farm to table'.

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