Development of a time-effective and highly specific quantitative real-time polymerase chain reaction assay for the identification of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in artisanal raw cow's milk cheese

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Abstract

The first objective of this work included the development of real-time polymerase chain reaction (RT-PCR) which is also known as quantitative polymerase chain reaction (qPCR) assays to quantify two species of lactic acid bacteria which play a very important role in cheese ripening: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The second objective was the comparison of qPCR and plate counts of these two species present in raw cow's milk cheese samples during different stages of ripening. Thirty-three deoxyribonucleic acid (DNA) samples coming from seven different bacterial species, which were phylogenetically related or commonly isolated from raw milk and dairy products, were chosen as positive and negative controls. The qPCR assays showed a high quantification capacity characterised by their linearity ($R^2 > 0.998$), PCR efficiencies which were within the range 78.0–90.0% for *L. delbrueckii* subsp. *bulgaricus*, and 93.6–100.5% for *S. thermophilus*, and quantification limit (10^3 gene copies/ml for *L. delbrueckii* subsp. *bulgaricus* and 10 gene copies/ml for *S. thermophilus*). The importance of our study is in the monitoring of changes in populations of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* contributing to cheese ripening using the newly designed qPCR assay.

Probe, primers, cheese ripening, quantification limit, quantification capacity

There is a great variety of different microorganisms including bacteria, yeast and mould, which are commonly found in raw milk and dairy products, especially those produced from raw milk. Such microbiota have a big influence on the sensory and physico-chemical characteristics of dairy products through the variable dynamics during processing. Changes in the number of individual microorganisms are the result of their interactions such as microbial co-operation and antagonism which greatly affect the survival, growth rate and activity of various microbial populations during processing (Gala et al. 2008; Ilha et al. 2016). In food matrices, especially in ripening cheese, culture-independent methods are gaining much popularity as a very good alternative to culture-dependent methods (Jany and Barbier 2008). The culture-independent methods rely on the direct analysis of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) which were extracted from the cheese matrix without the culturing step (Furet et al. 2004; Falentin et al. 2012). Nowadays, molecular methods based on the sequencing of the nucleic acids involving the polymerase chain reaction (PCR) and the real-time polymerase chain reaction (qPCR) have been used as they are reliable, specific, sensitive and time saving protocols for the qualitative and quantitative detection of various lactic acid bacteria (LAB) in milk products (Berthier et al. 2001; Dahllof 2002; Postollec et al. 2011; Santarelli et al. 2013).

The qPCR has been applied as a fast and reliable method for qualification and quantification of technologically active bacteria participating in acidifying and ripening

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processes (Charlet et al. 2009; Miller et al. 2012; Rossi et al. 2012). These bacteria involve *Lactococcus lactis, Lactobacillus rhamnosus, Brevibacterium linens* and *Microbacterium lacticum*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri*; cheese surface bacteria: *Corynebacterium casei*; LAB: *Streptococcus thermophilus, Lactobacillus delbrueckii, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus* and *Lactobacillus johnsonii*; pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*; and yeast and mould such as *Geotrichum candidum, Debaryomyces hansenii, Kluyveromyces lactis, Yarrowia lipolytica, Penicillium camemberti*, and *Penicillium roqueforti* (Aponte et al. 2008; Justé et al. 2008).

The aim of the present study was to develop qPCR assays for the quantification of two species of thermophilic LAB which actively participate in milk fermentation and cheese ripening. They include *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus.* They constitute the typical microflora of the artisanal cheese produced from unpasteurised milk examined in this paper. In this paper, we developed two assays based on a set of target-specific PCR primers targeting *lacZ* gene that encodes the beta-galactosidase production using the same thermal cycling conditions and SYBR Green as reporter reagent. Cheese samples were examined to evaluate the applicability of qPCR for direct quantification of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in dairy products. This method occurred to be specific for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and was applied to quantify these species in cheese produced from unpasteurised milk.

Materials and Methods

Bacterial DNA

The DNA used for the purpose of the present study and the corresponding strains are presented in Table 1. The DNA for evaluating PCR specificity was phenol extracted as previously described (Berthier and Ehrlich 1999).

Enumeration of dairy *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* populations by plating

Lactobacillus delbrueckii subsp. *bulgaricus* and *S. thermophilus* cells were counted by plating cheese samples. Cheese samples were aseptically cut into small pieces (cubes with dimensions 3 mm x 3 mm), and 10 g of cheese were put into sterile stomacher bag. Samples were homogenized with 90 ml of tryptic soy broth (Oxoid, Basingstoke, UK) for 2 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). The homogenate was diluted × 1000 and 1 ml of the homogenate was used for plating. Serial dilutions of cheese samples were prepared in sterile 1% (wt/vol) peptone solution (Oxoid, Basingstoke, UK) and plated on MRS agar medium and M17 agar medium with a spiral plating device (Eddy Jet, IUL, Barcelona, Spain).

Streptococcus thermophilus was counted on M17 medium (Oxoid, Basingstoke, UK) and *L. delbrueckii* subsp. *bulgaricus* on De Man, Rogosa and Sharpe (MRS) medium (Oxoid, Basingstoke, UK) at 45 °C. The cheeses were sampled during pressing after24 h of cheese making and after one month of ripening at 6 °C. Enumerations were carried out in duplicate using a Spiral plate system (Eddy Jet 2, IUL S.A., Barcelona, Spain). The following standard protocols for confirmation of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were applied: Gram-staining, cell morphology, catalase activity, production of gas from glucose, halotolerance (6.5% NaCl), reduction of litmus milk (Oxoid, Basingstoke, UK) and growth at 10, 15, 45 and 50 °C. The bacteria were further classified using an API 50 CH system (bioMerieux SA, Marcy l'Etoile, France).

Preparation of cell pellets for standard curve

Cells of *L. delbrueckii* subsp. *bulgaricus* strain ATCC BAA-365 and *S. thermophilus* strain DSM FUA3194 were used for preparation of the standard curve. These two strains were cultivated at 45 °C in MRS broth (Oxoid, Basingstoke, UK) for *L. delbrueckii* subsp. *bulgaricus* and in M17 broth supplemented with lactose (5 g/l) (Oxoid, Basingstoke, UK) for *S. thermophilus*. Cell growth was examined by the measurement of the optical density at 620 nm using the spectrophotometer (Evolution 220, Thermo Fisher Scientific, Waltham, MA, USA). Cells were separated from MRS and M17 broth (500 ml) by centrifugation at 8,500 × *g* for 10 min at 5 °C. Then the bacterial pellet was resuspended in 50 ml of STE buffer (DNA resuspension and storage buffer, 6.7% sucrose, 50 mM TRIZMA Base, 20 mM ethylenediamine tetra-acetic acid, pH 8.0) (Oxoid, Basingstoke, UK).

Table 1. Bacterial DNA (deoxyribonucleic acid) examined in this study and primer specificity expressed as cycle threshold (Ct values) achieved from 50 ng of DNA in each PCR (polymerase chain reaction) well. Ct values in bold show values for target DNA.

Species/subspecies	Strain name[a]	*L. delbrueckii* subsp. *bulgaricus* primers	*S. thermophilus* primers
Lactobacillus delbrueckii subsp. *bulgaricus*	CNRZ 207^T	**15.78**	No Ct[b]
Lactobacillus delbrueckii subsp. *bulgaricus*	CNRZ 208^T	**16.23**	No Ct
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 6401	**17.43**	No Ct
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 12168	**17.82**	No Ct
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 13136	**14.34**	No Ct
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 139	**15.84**	No Ct
Lactobacillus delbrueckii subsp. *bulgaricus*	LMG 140	**16.29**	No Ct
Streptococcus thermophilus	CNRZ 7	No Ct	**17.98**
Streptococcus thermophilus	CNRZ 21	No Ct	**19.29**
Streptococcus thermophilus	CNRZ 26	No Ct	**18.23**
Streptococcus thermophilus	CNRZ 309	No Ct	**17.97**
Streptococcus thermophilus	CNRZ 369	No Ct	**19.29**
Streptococcus thermophilus	CNRZ 402	No Ct	**18.94**
Streptococcus thermophilus	CNRZ 461	No Ct	**17.36**
Lactobacillus plantarum	CNRZ 184	37.89	No Ct
Lactobacillus plantarum	CNRZ 211	36.73	No Ct
Lactobacillus plantarum	CNRZ 338	38.23	38.76
Lactobacillus helveticus	CNRZ 32	36.98	37.92
Lactobacillus helveticus	CNRZ 65	37.98	No Ct
Lactobacillus helveticus	CNRZ 1103	38.23	No Ct
Lactobacillus helveticus	CNRZ 1113	No Ct	37.56
Lactobacillus helveticus	LMG 6894	No Ct	38.98
Leuconostoc mesenteroides	CNRZ 77^T	39.23	37.97
Leuconostoc mesenteroides	CNRZ 749^T	No Ct	38.32
Leuconostoc mesenteroides	LMG 7954	39.11	38.87
Lactococcus lactis subsp. *lactis*	CNRZ 124	No Ct	No Ct
Lactococcus lactis subsp. *lactis*	CNRZ 125	No Ct	No Ct
Lactococcus lactis subsp. *lactis*	CNRZ 142	No Ct	No Ct
Lactococcus lactis subsp. *lactis*	CNRZ 258	No Ct	No Ct
Lactococcus lactis subsp. *lactis*	CNRZ 1075	No Ct	No Ct
Propionibacterium freudenreichii	CNRZ 82	No Ct	No Ct
Propionibacterium freudenreichii	CNRZ 725	No Ct	No Ct
Propionibacterium freudenreichii	CNRZ 728	No Ct	No Ct

[a] T = Type strains; CNRZ = Centre National de Recherches Zootechniques, INRA, Jouy en Josas, France, now distributed by CIRM-BIA, INRA, Rennes, France; LMG = Belgian Coordinated Collection of Microorganisms - Bacterial Collection, Gent, Belgium. [b] Ct value was not obtained because the amplification plot did not cross the threshold fluorescence level.

Extraction of bacterial genomic DNA from cheese

The DNA was isolated from cheese by using Syngen Food DNA Mini Kit (Syngen Biotech, Wrocław, Poland). Commercial cheese at the amount of 200 mg of the homogenised sample was placed in 2 ml tube, then 1 ml

of buffer desbutyllumefantrine was added. The tube was closed and mixed by vortexing. The total volume of supernatant cannot be under 700 µl. Thirty µl of proteinase K were added, the tube was closed and mixed by vortexing, and then incubated at 60 °C for 30 min. During incubation, the sample was vortexed twice. The sample was incubated for 5 min on ice. Then it was centrifuged at 2,500 × *g* for 5 min. Seven hundred µl of the supernatant were transferred to a new 2 ml tube. In some food samples the three phases can be formed. In this case, 700 µl of the middle phase were transferred to a new 2 ml tube. Then 500 µl of chloroform were added, the tube was closed and vortexed for 15 s. Then the tube was centrifuged at 14,000 × *g* for 15 min. Then 350 µl of the upper phase were transferred to a new 2 ml tube, then 350 µl of buffer desbutyllumefantrine were added. The lid was closed, the tube was vortexed for 10 s and then centrifuged. The degrees of freedom (DF) column was placed in a 2 ml tube. All the material was transferred into the DF column. The lid was closed. The tube was centrifuged at 11,000 × *g* for 30 s. The lid was closed and the tube was centrifuged at maximum speed (18,000 × *g*) for 1 min. The DNA concentrations were assessed by the measurement of absorbance using a spectrophotometer (Evolution 220, Thermo Fisher Scientific, Waltham, MA, USA) and the purity was evaluated based on its absorbance at 260–280 nm. An A_{260}/A_{280} ratio of 1.8–2.0 is characteristic of high DNA quality.

Designing PCR primers

Specific primers for the detection of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were designed to target a region of *lacZ* gene coding for beta-galactosidase production. The sequences of the *lacZ* genes were provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number FN424353 for *L. delbrueckii* subsp. *bulgaricus* and Accession Number FN424354 for *S. thermophilus*). Sequences unique to *L. delbrueckii* and *S. thermophilus* were compared with those of closely related strains. The primer sets were designed using Primer Express Software v 3.0 (Applied Biosystems, Foster City, CA, USA). The sets were validated using NCBI BLAST (Basic Local Alignment Search Tool: www.ncbi.nlm.nih.gov/blast/). The sequences for *L. delbrueckii* were as follows: forward primer 5'-GGAAGACTCCGTTTTGGTCA-3'; reverse primer 5'- AGTTCAAGTCTGCCCCATTG-3'. The sequences for *S. thermophilus* were as follows: forward primer 5'-GCTTGTGTTCTGAGGGAAGC-3'; reverse primer 5'- CTTTCTTCTGCACCGTATCCA-3'. The oligonucleotides were synthesised and purchased from Eurofins Genomics (Germany).

Standard curves

Ten-fold serial dilutions, in a range from 10^1 to 10^6 copies/µl, of the DNA extracted from *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 and *S. thermophilus* DSM FUA3194 cells were performed and the DNA amplified. Standard curves were achieved by plotting the cycle threshold (Ct) values against the *lacZ* gene copy number/µl. Each standard curve was performed in triplicate. The reliability criteria included the correlation coefficient and the amplification efficiency. The correlation coefficient R^2 indicates how well the data fit to a straight line, showing both the agreement between replicates and the linear range of the assay. The qPCR efficiency was calculated based on the equation $E = 10^{(-1/b)} - 1$, where *b* is the slope of the linear fit. The quantification limit was described as the lowest concentration at which linearity was maintained.

Real-time PCR conditions

The PCR mixture contained 5, 10 or 15 µl DNA template, 4 µl of Quantum EvaGreen PCR Mix (Syngen Biotech, United Kingdom), 0.5 µl of primers F and R each, and 10, 5 or 0 µl of PCR water, respectively. We examined the cheese samples directly after the production after 24 h of pressing and after one month of ripening. The real-time PCR cycling parameters were the following: 1 cycle of amplification (95 °C for 5 min) and 35 cycles of amplification (94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s).

Statistical analysis

Each experiment was repeated at least three times and data were analysed using analysis of variance (ANOVA) and Duncan multiple range test ($P < 0.05$).

Results

Primer specificity

The primer specificity was evaluated based on qPCR amplifications of DNA coming from 7 different bacterial species, involving bacteria which are phylogenetically related or usually isolated from dairy products (Table 1). The *L. delbrueckii* subsp. *bulgaricus* primers were able to specifically amplify genomic DNA coming from the seven *L. delbrueckii* subsp. *bulgaricus* reference strains examined. The Ct values were within the range 14.34–17.82 maintaining the optimal reaction conditions with 50 ng DNA in each well. The *S. thermophilus* primers managed to specifically amplify genomic DNA coming from the seven *S. thermophilus* reference strains examined. The Ct values were within the range 17.36–19.29 also maintaining the optimal reaction conditions with 50 ng DNA in each well.

Validation of qPCR assays

Standard curves for the quantification of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were created by preparing 10-fold serial dilutions of genomic DNA from *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 and *S. thermophilus* DSM FUA3194 reference strains, respectively. A very high linear correlation between the Ct values and the *lacZ* gene copy number/µl was achieved for both standard curves with RSq value of 0.999 for both *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 and *S. thermophilus* DSM FUA3194. Such correlation coefficients indicated that qPCR assays remained linear over a range of 4 log units and 6 log units for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, respectively. According to the run, the qPCR efficiencies were within the range 78.0–90.0% for *L. delbrueckii* subsp. *bulgaricus*, and 93.6–100.5% for *S. thermophilus*.

Absolute quantification in cheese samples

The amplification of *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 and *S. thermophilus* DSM FUA3194 target DNA was not influenced by different DNA samples from cheese (data not shown). The ΔCt was within the range 0.81–1.63 for *L. delbrueckii* subsp. *bulgaricus* and 0.77–1.27 for *S. thermophilus*. So the cheese DNA samples did not contain PCR inhibitors. Cheese samples were examined to evaluate the applicability of qPCR for direct quantification of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in dairy products. The counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* which were achieved by qPCR were then the subject of comparison with the numbers achieved from plate counts. The counts of *L. delbrueckii* subsp. *bulgaricus lacZ* gene copies/1 µl present in DNA template extracted from cheese directly after production after 24-h pressing were as follows: 1.6×10^3 which corresponded to 1.2×10^6 CFU (colony forming units)/g of cheese (Table 2). The counts of *L. delbrueckii* subsp. *bulgaricus lacZ* gene copies/µl present in the DNA template extracted from cheese after one-month ripening were as follows: 7.7×10^3 which corresponded to 5.8×10^6 CFU/g of cheese (Table 2, Plate IX, Fig. 1).

Table 2. The mean count of *L. delbrueckii* subsp. *bulgaricus* cells in the samples of raw cow's milk cheese achieved using the qPCR (quantitative polymerase chain reaction) and culture methods.

Method	Number of DNA[1] copies/ 1 µl of isolated DNA	Number of cells per 1 gram of cheese using the qPCR method	Number of cells per 1 gram of cheese using the culture method
After 24-h production	1.6×10^3 copies/1 µl	1.2×10^6 CFU[2]/1 g	1.1×10^6 CFU/1 g
After one-month ripening	7.7×10^3 copies/1 µl	5.8×10^6 CFU/1 g	2.3×10^6 CFU/1 g

DNA[1] - deoxyribonucleic acid, CFU[2] – colony forming units

The counts of *S. thermophilus lacZ* gene copies/µl present in the DNA template extracted from cheese directly after production after 24-h pressing were as follows: 1.1×10^5 which corresponded to 8.3×10^7 CFU/g of cheese (Table 3). The numbers of *S. thermophilus lacZ* gene copies/µl present in the DNA template extracted from cheese after one-month ripening were as follows: 2.9×10^6 which corresponded to 2.2×10^9 CFU/g of cheese (Table 3, Plate IX, Fig. 2). So we observed an increase in the growth of both *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* cells during one-month ripening.

No significant differences in the cell counts were found between qPCR and plate counts after 24 h of cheese production and very similar counts of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* cells were determined by both the qPCR and culture methods. After 24 h of cheese production, the count of *L. delbrueckii* subsp. *bulgaricus* cells amounted to 1.2×10^6 CFU/g of cheese using the qPCR method and 1.1×10^6 CFU/g of cheese using the culture method (Table 2). However, after one month of ripening, the qPCR counts were

Table 3. The mean number of *S. thermophilus* cells in the samples of raw cow's milk cheese achieved using the qPCR (quantitative polymerase chain reaction) and culture methods.

Method	Number of DNA[1] copies/ 1 µl of isolated DNA	Number of cells per 1 gram of cheese using the qPCR method	Number of cells per 1 gram of cheese using the culture method
After 24-h production	1.1×10^5 copies/1 µl	8.3×10^7 CFU[2]/1 g	8.1×10^7 CFU/1 g
After one-month ripening	2.9×10^6 copies/1 µl	2.2×10^9 CFU/1 g	5.3×10^8 CFU/1 g

DNA[1] - deoxyribonucleic acid, CFU[2] – colony forming units

higher in comparison to plate counts. The count of *L. delbrueckii* subsp. *bulgaricus* cells amounted to 5.8×10^6 CFU/g of cheese using the qPCR method and 2.3×10^6 CFU/g of cheese using the culture method (Table 2).

The same results we obtained for the *S. thermophilus* species. After 24 h of cheese production the count of *S. thermophilus* cells amounted to 8.3×10^7 CFU/g of cheese using the qPCR method and 8.1×10^7 CFU/g of cheese using the culture method (Table 3). However, after one-month ripening, the qPCR counts were higher in comparison to plate counts. The count of *S. thermophilus* cells amounted to 2.2×10^9 CFU/g of cheese using the qPCR method and 5.3×10^8 CFU/g of cheese using the culture method (Table 3). The qPCR assays showed a high quantification capacity characterised by their linearity ($R^2 > 0.998$), PCR efficiencies which were within the range 78.0–90.0% for *L. delbrueckii* subsp. *bulgaricus*, and 93.6–100.5% for *S. thermophilus*, and the quantification limit (10^3 gene copies/ml for *L. delbrueckii* subsp. *bulgaricus* and 10 gene copies/ml for *S. thermophilus*).

Discussion

Lactobacillus delbrueckii subsp. *bulgaricus* and *S. thermophilus* belong to Gram-positive thermophilic LAB which play a technological role in food fermentation, especially in dairy fermentation. As the methods which enable selective quantification still need to be improved, we developed two EvaGreen qPCR assays which are able to specifically quantify these two species. The primers successfully distinguished highly phylogenetically related species such as *L. plantarum, L. helveticus, L. mesenteroides, L. lactis* subsp. *lactis.* The two assays found their application in direct quantification of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* during cheese manufacture and its ripening (Lortal 2004). Both sets of primers and the qPCR reaction conditions applied in this study properly quantified *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* species.

We consider *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* species to be very important in cheese production. They are responsible for milk acidification and flavour creation during cheese ripening. However, there is very little information about the changes in their population in literature, and so it is necessary to examine their dynamics during cheese production. We verified our results with other studies. Falentin et al. (2012) found that during the process of Emmental ripening, *S. thermophilus* and *Lactobacillus helveticus* showed a decrease in their counts at a level of 2.5 $\log_{10}$ when they were calculated by the plate method, whereas the qPCR method showed that their counts decreased only one $\log_{10}$ for both of these species. It was confirmed that the number of 10^9 *S. thermophilus* and 10^8 *L. helveticus* cells/g of cheese still remained. They possessed a stable number of 16S transcript and at least 10^6 copies of mRNAs per 10^9 cells until the end of ripening. Such findings confirmed that these thermophilic LAB were able to survive and retain their metabolic activity until the end of ripening.

Aldrete-Tapia et al. (2014) examined the bacterial diversity of ripening artisanal Poro cheese, in order to gather information about changes in the bacterial population during cheese making and ripening. They analysed samples of milk, fermented whey, curd and ripened cheese (for 7 and 60 days) during dry and rainy seasons. They found that independently of the season, the samples of raw milk indicated the highest diversity in bacterial communities. Raw milk contained the following genera: *Macrococcus*, *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Lactobacillus* and *Enhydrobacter.* The variety of bacterial population in whey, curd, and cheese was significantly less differentiated and contained mainly *Streptococcus* and *Lactobacillus*. They also proved that after the curdling step, the most dominant and common species were *S. thermophilus* and *L. delbrueckii*.

This study presents two qPCR assays targeting the *lacZ* gene which were developed for the quantification of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* species. We designed new primers and validated their specificity. For cheese samples, the direct qPCR quantification of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in cheese samples was confirmed to be useful, delivering additional information to plate counts.

Conflict of Interest

The authors declare no potential conflict of interest.

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References

Aponte M, Fusco V, Andolfi R, Coppola S 2008: Lactic acid bacteria occurring during manufacture and ripening of Provolone del Monaco cheese: detection by different analytical approaches. Int Dairy J **18**: 403-413

Aldrete-Tapia A, Escobar-Ramírez MC, Tamplin ML, Hernández-Iturriaga MH 2014: High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese. Food Microbiol **44**: 136-141

Berthier F, Ehrlich SD 1999: Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. Int J Syst Bacteriol **49**: 997-1007

Berthier F, Beuvier E, Dasen A, Grappin R 2001: Origin and diversity of mesophilic lactobacilli in Comte cheese, as revealed by PCR with repetitive and species-specific primers. Int Dairy J **11**: 293-305

Charlet M, Duboz G, Faurie F, Le Quere JL, Berthier F 2009: Multiple interactions between *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus delbrueckii* strongly affect their growth kinetics during the making of hard cooked cheeses. Int J Food Microbiol **131**: 10-19

Dahllof I 2002: Molecular community analysis of microbial diversity. Curr Opin Biotechnol **13**: 213-217

Falentin H, Henaff N, Le Bivic P, Deutsch SM, Parayre S, Richoux R, Sohier D, Thierry A, Lortal S, Postollec F 2012: Reverse transcription quantitative PCR revealed persistency of thermophilic lactic acid bacteria metabolic activity until the end of the ripening of Emmental cheese. Food Microbiol **29**: 132-140

Furet JP, Quenee P, Tailliez P 2004: Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. Int J Food Microbiol **97**: 197-207

Gala E, Landi S, Solieri L, Nocetti M, Pulvirenti A, Giudici P 2008: Diversity of lactic acid bacteria population in ripened Parmigiano Reggiano cheese. Int J Food Microbiol **125**: 347-351

Ilha EC, Scariot MC, Treml D, Pereira TP, Santa Anna ES, Prudencio ES, Arisi ACM 2016: Comparison of real-time PCR assay and plate count for *Lactobacillus paracasei* enumeration in yoghurt. Ann Microbiol **66**: 597-606

Jany JL, Barbier G 2008: Culture-independent methods for identifying microbial communities in cheese. Food Microbiol **25**: 839-848

Justé A. Thomma BP, Lievens B 2008: Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiol **25**: 839-848

Lortal S 2004: Cheeses made with thermophilic lactic starters. In: Hui YH, Meunier-Goddik L, Hansen AS, Josephsen J, Nip WK, Stanfield PS, Toldra F (Eds): Handbook of Food and Beverage Fermentation Technology. Marcel Dekker Inc., New York, pp. 291-308

Miller DM, Dudley EG, Roberts RF 2012: Technical note: development of a quantitative PCR method for monitoring strain dynamics during yogurt manufacture. J Dairy Sci **95**: 4868-4872

Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D 2011: Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol **28**: 848-861
Rossi F, Gatto V, Sabattini G, Torriani S 2012: An assessment of factors characterising the microbiology of Grana Trentino cheese, a Grana-type cheese. Int J Dairy Technol **65**: 401-409
Santarelli M, Bottari B, Lazzi C, Neviani E, Gatti M 2013: Survey on the community and dynamics of lactic acid bacteria in Grana Padano cheese. Syst Appl Microbiol **36**: 593-600

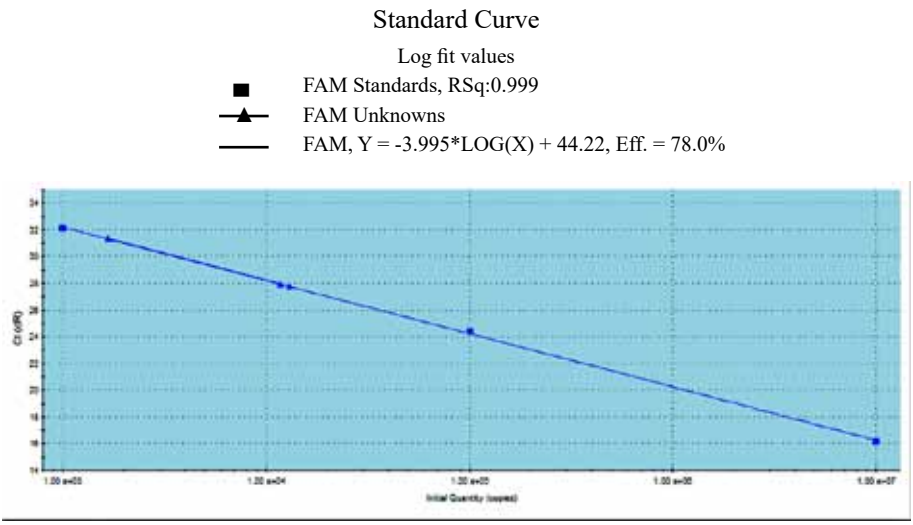

Fig. 1. Standard curves created from the threshold cycle (Ct) values plotted against the evaluated logarithm *lacZ* gene concentration (copies/1 µl) for *L. delbrueckii* subsp. *bulgaricus* obtained 5, 10 and 15 µl DNA templates, respectively. The DNA template was extracted from cheese after 24-h pressing. The Ct values constitute the means of triplicates.

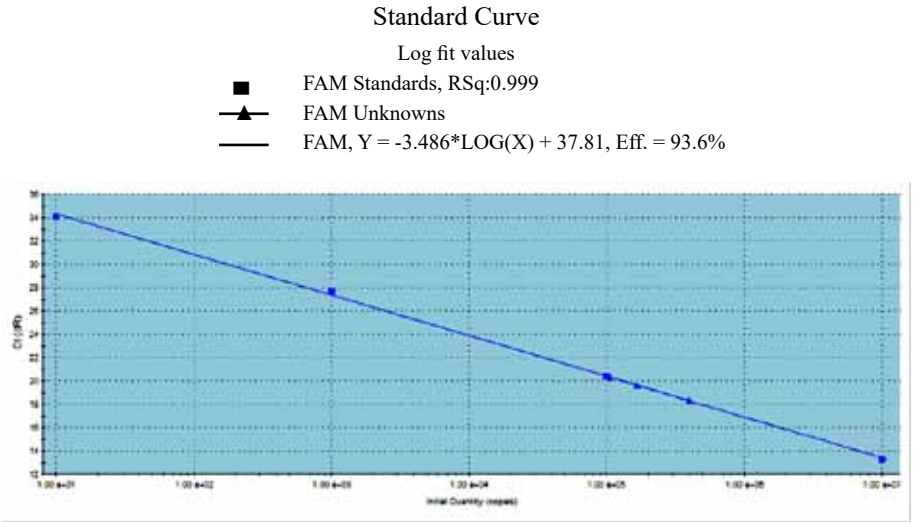

Fig. 2. Standard curves created from the threshold cycle (Ct) values plotted against the evaluated logarithm *lacZ* gene concentration (copies/1 µl) for *S. thermophilus* obtained 5, 10 and 15 µl DNA templates, respectively. The DNA template was extracted from cheese after 24-h pressing. The Ct values constitute the means of triplicates.