Polish Journal of Microbiology
2017, Vol. 66, No 4, 491–499

ORIGINAL PAPER

Identification of *Lactobacillus delbrueckii* and *Streptococcus thermophilus* Strains Present in Artisanal Raw Cow Milk Cheese Using Real-time PCR and Classic Plate Count Methods

MILENA A. STACHELSKA*

Institute of Food Technology and Gastronomy, Lomza State University of Applied Sciences, Lomza, Poland

Submitted 28 March 2017, revised and accepted 23 May 2017

Abstract

The aim of this paper was to detect *Lactobacillus delbrueckii* and *Streptococcus thermophilus* using real-time quantitative PCR assay in 7-day ripening cheese produced from unpasteurised milk. Real-time quantitative PCR assays were designed to identify and enumerate the chosen species of lactic acid bacteria (LAB) in ripened cheese. The results of molecular quantification and classic bacterial enumeration showed a high level of similarity proving that DNA extraction was carried out in a proper way and that genomic DNA solutions were free of PCR inhibitors. These methods revealed the presence of *L. delbrueckii* and *S. thermophilus*. The real-time PCR enabled quantification with a detection of 10^1–10^3 CFU/g of product. qPCR-standard curves were linear over seven log units down to 10^1 copies per reaction; efficiencies ranged from 77.9% to 93.6%. Cheese samples were analysed with plate count method and qPCR in parallel. Compared with the classic plate count method, the newly developed qPCR method provided faster and species specific identification of two dairy LAB and yielded comparable quantitative results.

Key words: *Lactobacillus delbrueckii*, *Streptococcus thermophilus*, artisanal cheese microflora, LAB identification

Introduction

There is a variety of numerous microorganisms including bacteria, yeasts and moulds,

which constitute cheese microflora and form a complex microbial ecosystem. Lactic acid bacteria (LAB) contribute to forming the desirable aroma and flavour of ripened cheese. They come directly from cow milk and from an environment having contact with milk and cheese curd during manufacture and ripening. Among LAB, different genera including *Lactobacillus, Streptococcus, Enterococcus* and *Leuconostoc* participate in the cheese production (Andrighetto *et al.*, 2002; Aquilanti *et al.*, 2007a; Aquilanti *et al.*, 2007b; Blaiotta *et al.*, 2008; Callon *et al.*, 2004; García *et al.*, 2002; Mannu *et al.*, 1999). Cheeses which are produced from unpasteurized milk using traditional manufacturing procedures may contain a very differentiated microflora (Cronin *et al.*, 2007). Such biodiversity may possess a significant impact on the maintenance of the typical features of traditional cheese products (Bizzarro *et al.*, 2000; Suzzi *et al.,* 2000). In fact, recent investigations have proved that the native microorganisms present in raw milk and the ones coming from the environment are known to contribute to most of the physico-chemical and aromatic transformations taking place during cheese production (Baruzzi *et al.*, 2000; Baruzzi *et al.*, 2002; De Angelis *et al.*, 2001; Dolci *et al.*, 2008a; Dolci *et al.*, 2008b). Artisanal cheese is produced by traditional techniques using unpasteurised milk and without adding any commercial starters which are deliberately added in case of cheese produced from pasteurised milk (Albenzio *et al.*, 2001; Corroler *et al.*, 1998; Terzic-Vidojevic *et al.*, 2007). This cheese ripens at 4°C and is produced in the Podlaskie region of north-eastern Poland. The microflora originally present in raw milk is responsible for ripening of this cheese as no starters are added (Delgado and Mayo, 2004; Delbès *et al.*, 2007, Duthoit *et al.*, 2003). All the phases of manufacturing are carried out in a manual way. This distinctive cheese is closely connected with the territory of production and its tradition which are unique due to its historical and cultural environment.

Microflora of cheese usually comprise lactic acid bacteria which highly influence the human health and nutrition. They are responsible for both spontaneous fermentations and large-scale fermentation processes finding their application in the preservation as well as

* Corresponding author: M.A. Stachelska, Institute of Food Technology and Gastronomy, Lomza State University of Applied Sciences, Lomza, Poland; **e-mail: mstachelska@pwsip.edu.pl**

the transformation of many raw food materials such as milk, meat, fish, cereals, tubers and vegetables. LAB are also known to be commensal inhabitants of the gastrointestinal tract in humans and animals ,in which they are responsible for the complex interactions between the intestinal microbiota and the host. Among LAB, the probiotic species can be identified as they possess a significantly beneficial influence on preventing and treating diarrhoea, improving the digestion of lactose by lactase-deficient individuals (McSweeney *et al.,* 2004). There are also some promising data relating to the prevention and treatment of allergies and inflammatory bowel diseases. The proper enumeration of LAB species is crucial in studying of their role and their dynamics in different niches (Duthoit *et al.*, 2005a; Duthoit *et al.*, 2005b). In spite of the development of various selective culture media, LAB identification and enumeration in dairy products may cause some drawbacks (Dasen *et al.*, 2003). The fast and reliable identification and quantification of LAB species in complex food matrix such as cheese is possible thanks to the application of molecular biology techniques especially when the target population is very low and accounts for at least 1% of the total bacterial population (Amann *et al.*, 1995; Ben Amor *et al.*, 2007; Bouton *et al.*, 2002; Coppola *et al.*, 2001; Coppola *et al.*, 2006; Dahllof 2002). When target population is lower than 1%, PCR techniques are recommended for detection (De Candia *et al.*, 2007).

In the present paper, real-time quantitative PCR protocol was developed to specifically detect and quantify the LAB species comprising *Lactobacillus delbrueckii* and *Streptococcus thermophilus* in artisanal cheese produced from unpasteurised cow milk. The results of the study of molecular quantification were similar to those achieved by application of the classic plate count method.

Experimental

Materials and Methods

Extraction of DNA from pure cultures. *L. delbrueckii* CNRZ69, CNRZ207T, CNRZ334 and CNRZ1105 and *S. thermophilus* CNRZ440 and CNRZ1580 were propagated in 10 ml MRS and M17 broths, respectively, at 42°C. The bacteria coming from a 2-ml late exponential growth phase culture ($A_{650\ nm} = 0.7 – 0.8$) were collected by centrifugation at 3 000 × g for 5 min at 4–6°C and stored at –20°C until DNA extraction. One millilitre of enrichment culture was pipetted into a 2 ml microcentrifuge screw-cap tube and was centrifuged at 13 000 × g for 5 min. Then the supernatant was discarded using a pipet. The care was taken not to disrupt the pellet. Then 200 µl of Fast Lysis Buffer (Syngen Biotech, Germany) was added to the bacterial pellet, the tube was tightly capped and the pellet was resuspended by vigorous vortexing. The microcentrifuge tube was placed into a thermal shaker (800 × g) set to 100°C. The sample was heated for 10 min. The sample was removed and cooled to room temperature (15–25°C) for 2 min. The tube was centrifuged at 13 000 × g for 5 min. After centrifugation, the supernatant was carefully transferred to a new tube and 2 µl of this supernatant was used as the template.

Extraction of bacterial genomic DNA from cheese. DNA was isolated from cheese by using Syngen Food DNA Mini Kit (Syngen Biotech, Germany) according to manufacturer protocol. Two hundred mg of the homogenised commercial cheese was placed in 2 ml tube, then 1 ml of buffer DLF was added. The tube was closed and mixed by vortexing. The total volume of supernatant cannot be less than 700 µl. Thirty microlitres of proteinase K was added, the tube was closed and mixed by vortexing, then incubated at 60°C for 30 minutes. During the incubation, the sample was vortexed twice. The sample was incubated for 5 minutes on ice. Then it was centrifuged for 5 minutes at 2 500 × g. Seven hundred microlitres of the supernatant was 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 was transferred to a new 2 ml tube. Then 500 µl of chloroform was added, the tube was closed and vortexed for 15 seconds. Then the tube was centrifuged for 15 minutes at 14 000 × g. Then 350 µl of the upper phase was transferred to a new 2 ml tube, then 350 µl of buffer DWF was added. The lid was closed, the tube was vortexed for 10 seconds then centrifuged. The column DF was placed in a 2 ml tube. All the material was transferred into the column DF. The lid was closed. The tube was centrifuged for 30 seconds at 11 000 × g. The supernatant was discarded, and the column was transferred back to the tube. Seven hundred microlitres of buffer DPF was added to the column, then the lid of the column was closed. The column was centrifuged for 30 seconds at 18 000 × g. The supernatant was discarded, and the column was transferred back to the tube. The column was centrifuged for 3 minutes at 18 000 × g. The column was transferred to a new 1.5 ml tube. From one hundred to two hundred microlitres of pre-warmed DE elution buffer was added at the center of the membrane and incubated at room temperature for 1 minute. The lid was closed and the tube was centrifuged for 1 minute at maximum speed (18 000 × g).

Enumeration of bacteria and identification of LAB isolates. Serial dilutions of pure cultures and cheese sample were prepared in sterile 1% (wt/vol) peptone solution and plated on MRS agar medium with a spiral plating device (Eddy Jet, IUL, Barcelona, Spain). The acidified MRS agar medium (pH 5.5) was also applied to count a specific number of lactobacilli. Counting was

based on the morphology of colonies associated with the morphology of bacterial cells observed by microscopy. The species present in the cheese samples could easily be distinguished based on their respective morphologies. Isolates of representative lactobacilli were the subject of phenotypic characterization to confirm their classification. The facultatively heterofermentative species *L. rhamnosus*, *L. casei* and *L. paracasei*, produced gas when grown in MRS broth containing 4% sodium gluconate as the carbon source (facultative heterofermentation), whereas no gas was produced from glucose. *L. rhamnosus* strains were able to grow in MRS broth containing 0.5% glucose, galactose, mannitol or rhamnose as carbon sources, and could also grow at 45 and 48°C. *L. paracasei* and *L. casei* strains did not use rhamnose as a carbon source and could not grow at 48°C. *Lactobaccilus acidophilus* strains did not use gluconate, mannitol or rhamnose as carbon sources, while *L. delbrueckii* subsp. *bulgaricus* strains did not use galactose or the three carbon sources cited above.

Designing of PCR primers. Specific primers for the detection of *L. delbrueckii* and *S. thermophilus* were designed using an alignment of LAB 16S rRNA gene sequences obtained with the GeneBase software (Applied-Maths, St-Martens-Latem, Belgium). The sequences of the 16S rRNA genes were provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number X52654 for *L. delbrueckii* and Accession Number X68418 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'-ACATGAATCGCATGATTCAAG-3'; reverse primer 5'-AACTCGGCTACGCATCATTG-3'. The sequences for *S. thermophilus* were as follows: forward primer 5'-TTATTTGAAAGGGGCAATTGCT-3'; reverse primer 5'-GTGAACTTTCCACTCTCACAC-3'. The oligonucleotides were synthesised and purchased from Eurofins Genomics (Germany).Fluorescence was detected using an optical detection system installed in the thermocycler of Stratagene Mx3005P (Real-Time PCR Detection System, Agilent Technologies, USA). Fluorescence data were collected during the annealing/elongation step of each PCR cycle. The software automatically plots the relative fluorescence unit (RFU) versus the PCR cycle number. The threshold cycle (Ct), which expresses the amount of a particular nucleic acid sample, is the number of amplification cycles needed for the accumulated fluorescence to achieve a value essentially higher than the background. A Ct value which exceeds 40 meant a negative result.

PCR fragments of 16s rRNA gene, standard curves for molecular quantification. The 16S rRNA gene fragments of *L. delbrueckii* and *S. thermophilus* were synthesised and purchased from Eurofins Genomics (Germany). They were delivered in the lyophilized form. For *L. delbrueckii* there were 1.43×10^{12} DNA copies. They were dissolved in 1430 µl of DE buffer (Syngen Biotech, Germany) achieving the concentration of 1×10^9 DNA copies/µl of eluate. This concentration was used for preparation of standards for standard curve. The dilutions were prepared to achieve 1×10^1 DNA copies/µl of eluate in the highest dilution.

For *S. thermophilus* there were also $1{,}43 \times 10^{12}$ DNA copies. They were dissolved in 1430 µl of DE buffer (Syngen Biotech, Germany) achieving the concentration of 1×10^9 DNA copies/µl. This concentration was used for preparation of standards for standard curve. The dilutions were prepared to achieve 1×10^1 DNA copies/µl of eluate in the highest dilution.

A 10-fold dilution series of the PCR fragment solution for each bacterial species, covering 7 logs ranging from 10^1 to 10^7 DNA copies per reaction, was used to estimate the sensitivity of the method. The correlation coefficients (r^2) of the standard curves for both *L. delbrueckii* and *S. thermophilus* were equal to 0.999 for the initial copy numbers of standard PCR fragments within the range of 10^1–10^7 per reaction, indicating that the exponential amplification patterns were reliable under these conditions. All assays were performed at least in triplicate and the average values were used for analysis.

Construction of standard curves. Standard curves were prepared with serial dilutions of genomic DNA isolated from *L. delbrueckii* and *S. thermophilus* and cheese. Accession Number X52654 for *L. delbrueckii* and Accession Number X68418 for *S. thermophilus*). The number of bacterial DNA copies were calculated on the basis of the size of the *L. delbrueckii* (GenBank accession number X52654) and *S. thermophilus* (GenBank accession number X68418) using Avogadro's constant (6.023×10^{23}) and the molecular weight of DNA (660 Da/bp). Genomic DNA was tenfold serially diluted in ultrapure water to final concentrations ranging from 10^7 to 10^1 genome copies per 1 µl, equivalent to concentrations of 33.2 to 3.32×10^{-6} ng. The Cq versus log CFU was estimated using genomic DNA extracted from the bacteria culture grown until stationary growth phase (OD 0.8) or from cheese with *L. delbrueckii* and *S. thermophilus*. Ten times serial dilutions of DNA extracted from *L. delbrueckii* and *S. thermophilus* was performed and the corresponding CFU was calculated based on plate counting of the same sample, bacterial culture or cheese with *L. delbrueckii* and *S. thermophilus*. Standard curves were generated by the plot cycle threshold (Cq) values versus logarithm of bacterial DNA copy number (pure culture) or CFU (pure culture and cheese). Amplification

efficiencies were determined using the following equation: $E = 10^{(-1/S)} - 1$; where E is the efficiency and s is the slope obtained from the standard curve.

Relationship between colony forming units and number of target copies. The number of *L. delbrueckii* was 7.3×10^6 CFU/ml. In comparison, 1.2×10^7 copies/ml were achieved in the qPCR assay. Based on these results, a ratio of 1.6 copies per CFU was calculated for *L. delbrueckii* (1.2×10^7 copies/ml amounted to 7.3×10^6 CFU/ml). Similar ratios were achieved for *S. thermophilus*. (1.9 copies per CFU); The enumeration of the liquid stock culture of *S. thermophilus* amounted to 6.9×10^6 CFU/ml. In comparison, 1.3×10^7 copies/ml were achieved in the qPCR assay. Based on these results, a ratio of 1.9 copies per CFU was calculated for *S. thermophilus* (1.3×10^7 copies/ml amounted to 6.9×10^6 CFU/ml) (Table I). Since cells of LAB occur sometimes in pairs, it is plausible that the CFU counts are usually lower than the number of target copies obtained in the qPCR assay.

Real-time PCR conditions. The reaction total volume was 20 µl. Real-time PCR analysis was performed using the thermocycler of Stratagene Mx3005P (Agilent Technologies, USA). The PCR mixture contained 5 µl DNA template, 4 µl of Quantum EvaGreen PCR Mix (Syngen Biotech, United Kingdom), 0.5 µl of primers F and R respectively, and 10 µl of PCR water. A non-template control (NTC) contained 5 µl of water instead of DNA and was included in each run. 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). The real-time PCR reaction and amplification step were carried out using a DNA amplification curves which were the subject of analysis. The calculation of the threshold cycle (C_T) value was carried out using Stratagene Mx3005P software version 2.1 (Agilent Technologies, USA). The C_T value was described as the real-time PCR cycle, at which the generated fluorescence increased exponentially and exceeded its background level.

Table I
Counts of *L. delbrueckii* and *S. thermophilus* in stock culture.

Strain	Quantification in stock culture with PCM* (CFU/ml)	Quantification in stock culture with qPCR (copies/ml)	Number of DNA copies per CFU
L. delbrueckii	7.3×10^6	1.2×10^7	1.6
S. thermophilus	6.9×10^6	1.3×10^7	1.9

* PCM, plate count method.

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

Assessment of the real-time PCR assay performance. The qPCR-standards (linearised plasmids containing the target sequences) for *L. delbrueckii* and *S. thermophilus* species were tenfold serial diluted to achieve a concentration in the range from 10^7 to 10^1 DNA copies per reaction. For both primer-systems, the result of the qPCR indicated a linear correlation between the concentrations of the qPCR-standards and the threshold cycles (Ct) over a 7-log range (Fig. 1

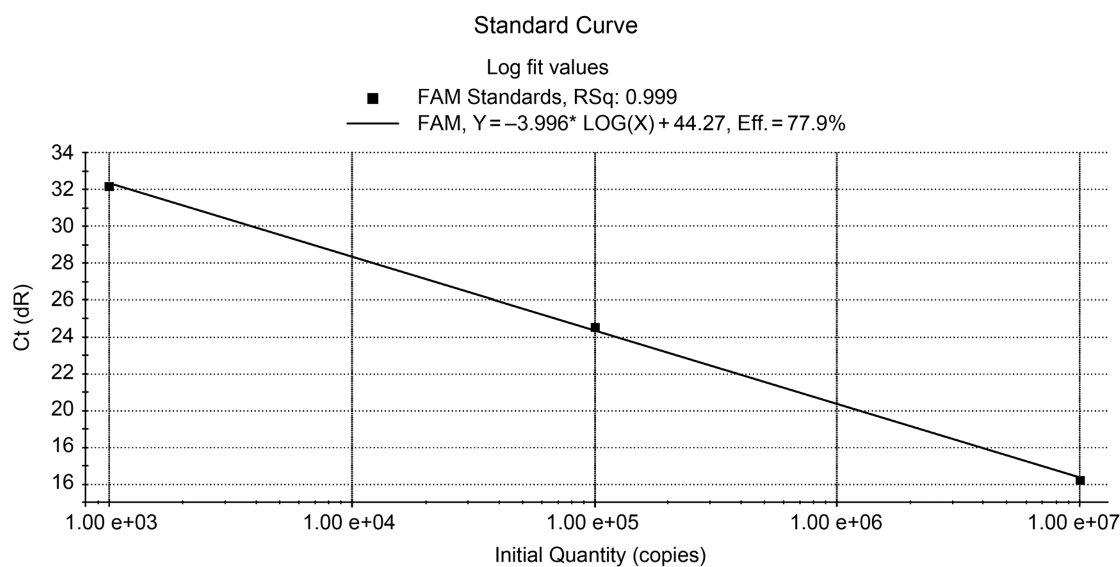

Fig. 1. Performance of the qPCR assays for the species-specific quantification of *L. delbrueckii*. The standard curve was made by plotting the inputs of the three different concentrations of qPCR standard against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

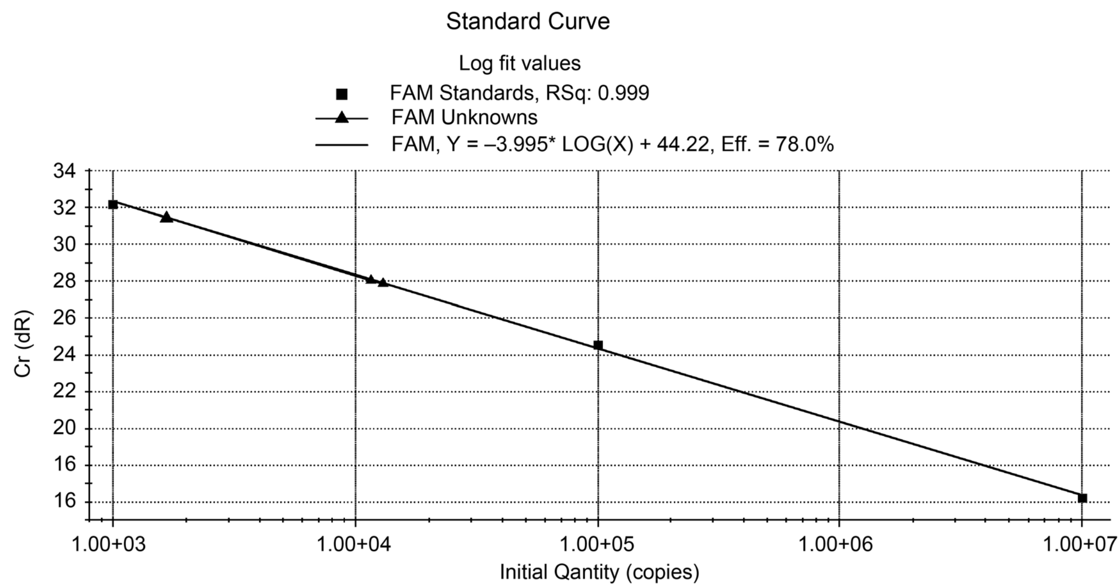

Fig. 2. Performance of the qPCR assays for the species-specific quantification of *L. delbrueckii*. The standard curve was made by plotting the inputs of the four different concentrations of qPCR standard against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

and Fig. 3). The coefficients of correlation (R^2) over the whole range were 0.999 for *L. delbrueckii* and *S. thermophilus*. The amplification efficiency (Eff.) was 77.9% for *L. delbrueckii* and 93.5% for *S. thermophilus*.

Assessment of primer specificity for *L. delbrueckii* and *S. thermophilus*. Specificity test was carried out using DNA extracted from *L. delbrueckii* and *S. thermophilus* strains and other bacterial genera to evaluate if the primer pairs with the sequences described in Materials and Methods were able to detect exclusively *L. delbrueckii* and *S. thermophilus*. Specificity test was carried out using bacterial DNA extracted from pure medium cultures and from cheese produced from unpasteurised milk (10 ng of template DNA). *L. delbrueckii* strains CNRZ69, CNRZ207T, CNRZ334 and CNRZ1105 presented mean Ct values ±standard deviation equal to 16.2 ± 0.7, 17.2 ± 0.4, 18.2 ± 0.2 and 18.7 ± 0.2 respectively, and amplicon presented Tm of 78.3 ± 0.2, 78.0 ± 0.2, 77.9 ± 0.2 and 77.3 ± 0.2 respectively (Table II).

S. thermophilus strains CNRZ440 and CNRZ1580 presented mean Ct values ± standard deviation equal to 17.2 ± 0.4 and 18.2 ± 0.4 respectively, and amplicon presented Tm of 78.8 ± 0.2 and 78.2 ± 0.2 respectively (Table II). *Bifidobacterium* BB-12 and *Lactobacillus plantarum* did not show amplification as expected for all qPCR assays. Other negative samples indicated some unspecific amplification, with late Ct and different Tm values in comparison to *L. delbrueckii* and *S. thermophilus* Tm values in a range from 77.3 to 78.8. *Bacillus cereus* indicated Ct = 31.7. *Escherichia coli*, *Pseudomonas* spp., and *L. acidophilus* also showed unspecific amplification at late Ct values (Table II). *L. acidophilus* indicated a late Ct (Ct > 39.42). *E. coli* and *Pseudomonas* presented late Ct (Ct > 37.89 and Ct > 38.73, respectively) with different Tm values.

qPCR parameters for enumeration of *L. delbrueckii* and *S. thermophilus*. The reaction parameters such as efficiency and correlation coefficient of the qPCR assay with the application of specific primer pairs were determined based on standard curves achieved from tenfold serial dilution of the qPCR-standards (linearised plasmids containing the target sequences of bacterial DNA) for *L. delbrueckii* (Fig. 1) and *S. thermophilus* species (Fig. 3). The standard curves indicated the

Table II
Ct and Tm achieved by qPCR assay using DNA extracted from *L. delbrueckii* and *S. thermophilus* (positive controls) and other bacterial species (negative controls).

Sample*	Ct	Tm
L. delbrueckii CNRZ69	16.2 ± 0.7	78.3 ± 0.2
L. delbrueckii CNRZ207T	17.2 ± 0.4	78.0 ± 0.2
L. delbrueckii CNRZ334	18.2 ±0.2	77.9 ±0.2
L. delbrueckii CNRZ1105	18.7 ± 0.2	77.3 ± 0.2
S. thermophilus CNRZ440	17.2 ± 0.4	78.8 ± 0.2
S. thermophilus CNRZ1580	18.2 ± 0.4	78.2 ± 0.2
Bifidobacterium BB-12	nd	nd
L. plantarum	nd	nd
B. cereus	34.7	78.2 ± 0.2
E. coli	37.89	65.2 ± 0.2
Pseudomonas spp.	38.73	75.2 ± 0.2
Lactobacillus acidophilus	39.42	72.2 ± 0.2

* 10 ng of template DNA; nd; not detected with Ct > 40

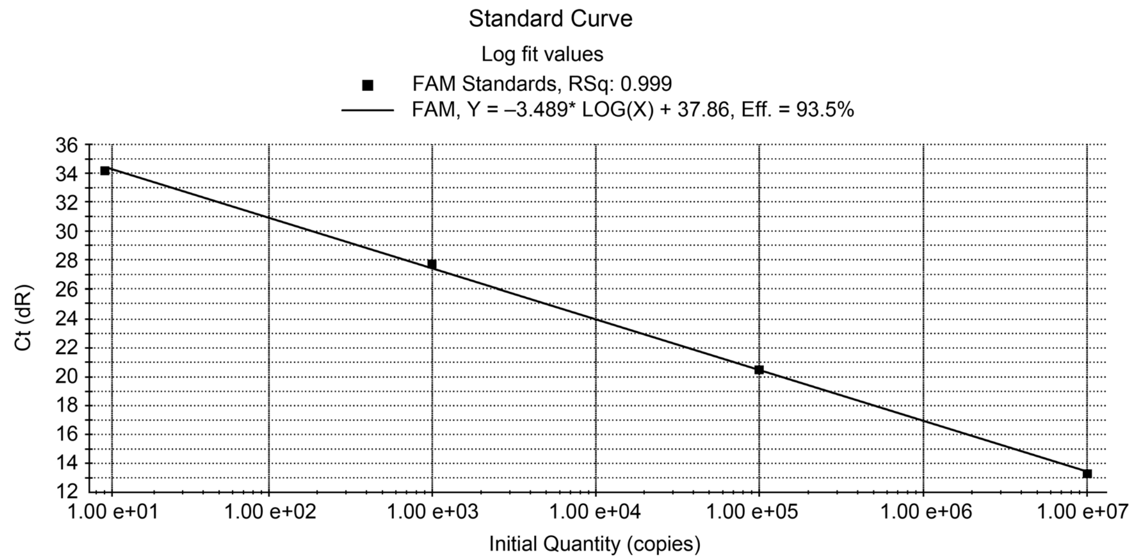

Fig. 3. Performance of qPCR assays for the species-specific quantification of *S. thermophilus* in cheese (▲). The standard curve was made by plotting the inputs of the three different concentrations of qPCR standard (■) against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

suitable linear correlation coefficient $R^2 = 0.999$ and mean efficiency Eff = 77.9% for *L. delbrueckii* and the suitable linear correlation coefficient $R^2 = 0.999$ and mean efficiency Eff = 93.5% for *S. thermophilus*. The limit of detection (LOD) for *L. delbrueckii* was 3 log DNA copy number, corresponding to 3.32 pg of DNA (Fig. 1). For *L. delbrueckii* LOD corresponded to a mean Ct = 32.0. The Ct versus log CFU of *L. delbrueckii* was estimated using genomic DNA extracted from the *L. delbrueckii* bacterial culture plate counted in parallel, so tenfold serial dilutions of bacterial DNA were performed and the corresponding CFU values were calculated based on plate counting. LOD corresponded to 2.78 log CFU of *L. delbrueckii*.

The qPCR enumeration of *L. delbrueckii* in cheese samples was carried out on the base of the construction of standard curve achieved from tenfold serial dilution of the qPCR-standards (linearised plasmids containing the target sequences of bacterial DNA) for *L. delbrueckii* and bacterial DNA coming from *L. delbrueckii* present in cheese. In this case, the efficiency value was 78.0% and R^2 was 0.99 (Fig. 2). The number of DNA copies in the cheese samples taken from the three different places amounted to 1.68×10^3/µl; $1,17 \times 10^4$/µl; 1.30×10^4/µl of eluate per reaction (Fig. 2). The standard curve equation Ct versus log CFU of samples of cheese for *L. delbrueckii* (Fig. 2) was used to calculate CFU per reaction well of all cheese samples from obtained Ct values. It was possible to obtain *L. delbrueckii* count (CFU/g of cheese) of cheese samples by qPCR taking into consideration the fact that 1.6 DNA copies present in *L. delbrueckii* corresponds to 1 CFU (Table I and Table III). On the other hand, 1.9 DNA copies present in *S. thermophilus* corresponds to 1 CFU (Table I and Table III).

Table III
Comparison of *L. delbrueckii* and *S. thermophilus* count (log CFU/g) in cheese obtained by qPCR and plate count method.

Samples	CFU/g with plate count method	CFU/g with qPCR method	DNA copies with qPCR method
L. delbrueckii	1.02×10^3 7.23×10^3 8.05×10^3	1.05×10^3 7.31×10^3 8.12×10^3	1.68×10^3 1.17×10^4 1.30×10^4
S. thermophilus	5.42×10^4 8.65×10^4 2.02×10^5	5.52×10^4 8.74×10^4 2.09×10^5	1.05×10^5 1.66×10^5 3.98×10^5

The numbers of DNA copies coming from *S. thermophilus* present in the cheese samples are present in Figure 4.

Cheese samples were analysed with plate count method and qPCR in parallel. Compared with the classic plate count method, the newly developed qPCR method gave faster and species specific determination of two dairy LAB and yielded comparable quantitative results.

Discussion

The bacterial quantification in food with the application of a DNA-based method requires the proper DNA extraction method. It is very difficult to extract bacterial DNA, especially from a dairy product, as it may potentially contain PCR inhibitors like calcium and fat. In the present study, Syngen Food DNA Mini Kit protocol was used to extract DNA from commercial cheese produced from unpasteurised milk. Such DNA

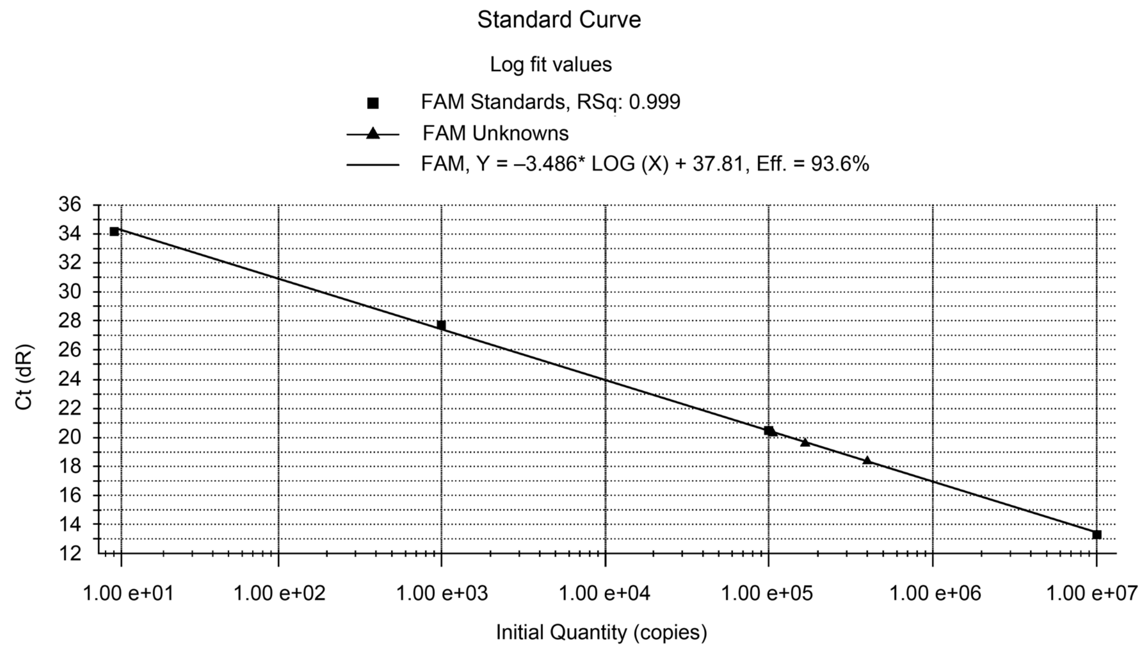

Fig. 4. Performance of the qPCR assays for the species-specific quantification of *S. thermophilus* in cheese (▲). The standard curve was made by plotting the inputs of the four different concentrations of qPCR standard (■) against Ct outcomes. Each data point constitutes a mean value and standard deviation of three qPCR replicates.

was used for qPCR enumeration assay of *L. delbrueckii* and *S. thermophilus* in cheese. A qPCR assay based on 16S rRNA gene sequences was successfully applied for enumeration of *L. delbrueckii* and *S. thermophilus* in cheese (Jany and Barbier, 2008; Randazzo *et al.*, 2002). Several other lactic acid bacteria were used as negative controls by other authors, and the pairs of primers used in this qPCR assay were specific enough for the identification of *L. delbrueckii* and *S. thermophilus* (Justé *et al.,* 2008). The specificity of these primers was also examined with other bacterial species (Table II).

It was found that some bacterial species showed unspecific amplification; however, Ct values were always above the Ct value corresponding to LOD for *L. delbrueckii* (Ct = 32) and LOD for *S. thermophilus* (Ct = 34), and they showed amplicons with different Tm values in comparison to compared to *L. delbrueckii* and *S. thermophilus* samples (Table II). The ΔCt between DNA samples (10 ng) of *L. delbrueckii* and *S. thermophilus* strains (Cq < 18) and the other bacteria (Cq < 34) is sufficiently high to reinforce the use of the specific primer pairs used in this study because the amplifications of other bacterial DNA of *Bifidobacterium* BB-12, *L. plantarum*, *B. cereus*, *E. coli*, *Pseudomonas* spp. and *L. acidophilus* are unspecific and they are easily distinguishable by their Tm. The DNA extraction method possesses a huge impact on the quantification by real-time PCR and it is essential to achieve an optimal yield of DNA and to avoid the appearance of substances that might influence PCR efficiency (Mannu *et al.,* 2002; Sánchez *et al.*, 2006). The standard curve for *L. delbrueckii* showed a relatively high efficiency value of 77.9% and a very high efficiency value of 93.5% for *S. thermophilus*.

It should be noted that the qPCR assay designed for one matrix may not occur to be proper for other matrices. Limit of detection (LOD) which is defined as the lowest amount of sample that can be reliably detected amounts to 3 log DNA copy number for *L. delbrueckii* and 1 log DNA copy number for *S. thermophilus*. This *L. delbrueckii* qPCR assay enabled to detect *L. delbrueckii* DNA ranging between 7 log genome copies to 3 log genome copies in the reaction well in comparison to *S. thermophilus* qPCR assay which enabled to detect *S. thermophilus* DNA ranging between 7 log genome copies to 1 log genome copy in the reaction well (Ventura *et al.*, 2003). It is a proper amount of LAB in cheese samples, once they should be contained in relatively high numbers.

L. delbrueckii species were presented in the range 1.02×10^3–8.05×10^3 log CFU/ml using the plate count method and in the range 1.05×10^3–8.12×10^3 log CFU/ml using qPCR method. *S. thermophilus* species were presented in the range 5.42×10^4–2.02×10^5 log CFU/ml using the plate count method and in the range 5.52×10^4–2.09×10^5 log CFU/ml using qPCR method. It was observed that both plate count method and qPCR method gave very similar results concerning a number of *L. delbrueckii* and *S. thermophilus* in cheese samples. However, in comparison to the classic plate count method, the newly developed qPCR method gave faster and species specific determination of two dairy LAB (Giraffa and Neviani, 2000).

The results of the present work constitute a molecular approach to identify the characteristic microflora of traditional Polish artisanal cheese. Further isolation of the LAB strains together with further examination and enumeration of other specific species could provide us with a wider knowledge of bacterial ecosystem of this traditional cheese.

Literature

Albenzio M., M.R. Corbo, S.U. Rehman, P.F. Fox, M. de Angelis, A. Corsetti, A. Sevi and M. Gobbetti. 2001. Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *Int. J. Food Microbiol.* 67: 35–48.

Amann R.I., W. Ludwing and K.H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59: 143–169.

Andrighetto C., F. Borney, A. Barmaz, B. Stefanon and A. Lombardi. 2002. Genetic diversity of *Streptococcus thermophilus* strains isolated from Italian traditional cheeses. *Int. Dairy J.* 12: 141–144.

Aquilanti L., G. Silvestri, E. Zannini, A. Osimani, S. Santarelli and F. Clementi. 2007a. Phenotypic, genotypic and technological characterization of predominant lactic acid bacteria in Pecorino cheese from central Italy. *J. Appl. Microbiol.* 103: 948–960.

Aquilanti L., E. Zannini, A. Zocchetti, A. Osimani and F. Clementi. 2007b. Polyphasic characterization of indigenous lactobacilli and lactococci from PDO Canestrato Pugliese cheese. *LWT* 40: 1146–1155.

Baruzzi F., M. Morea, A. Matarante and P.S. Cocconcelli. 2000. Changes in the Lactobacillus community during Ricotta Forte cheese natural fermentation. *J. Appl. Microbiol.* 89: 807–814.

Baruzzi F., A. Matarante, M. Morea and P.S. Cocconcelli. 2002. Microbial community dynamics during the Scamorza Altamurana cheese natural fermentation. *J. Dairy Sci.* 85: 1390–1397.

Ben Amor K., E.E. Vaughan and W.M. De Vos. 2007. Advanced molecular tools for the identification of lactic acid bacteria. *J. Nutr.* 741–747.

Bizzarro R., G. Torri Tarelli, G. Giraffa and E. Neviani. 2000. Phenotypic and genotypic characterisation of lactic acid bacteria isolated from Pecorino Toscano cheese. *Int. J. Food Sci.* 12: 303–316.

Blaiotta G., V. Fusco, D. Ercolini, M. Aponte, O. Pepe and F. Villani. 2008. *Lactobacillus* strains diversity based on partial hsp60 gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. *Appl. Environ. Microbiol.* 74: 208–215.

Bouton Y., P. Guyot, E. Beuvir, P. Tailliez and R. Grappin. 2002. Use of PCR based method and PFGE for typing and monitoring homofermentative lactobacilli during Comtè cheese ripening. *Int. J. Food Microbiol.* 76: 27–38.

Callon C., L. Millet and M.C. Montel. 2004. Diversity of lactic acid bacteria isolated from AOC Salers cheese. *J. Dairy Res.* 71: 231–244.

Coppola R., M. Nanni, M. Succi, A. Sorrentino, M. Iorizzo, C. Chiavari and L. Grazia. 2001. Enumeration of thermophilic lactic bacteria in ripened cheese manufactured from raw milk. *Milchwissenscaft* 56: 140–142.

Coppola S., V. Fusco, R. Andolfi, M. Aponte, G. Blaiotta, D. Ercolini and G. Moschetti. 2006. Evaluation of microbial diversity during the manufacture of Fior di Latte di Agerola, a traditional raw milk pasta-filata cheese of the Naples area. *J. Dairy Res.* 73: 264–272.

Corroler D., I. Magin, N. Desmasures and M. Guèguen. 1998. An ecological study of lactococci isolated from raw milk in the Camembert cheese registered designation of origin area. *Appl. Environ. Microbiol.* 64: 4729–4735.

Cronin T., M. Ziino, C. Condurso, P.L.H. McSweeney, S. Mills, R.P. Ross and C. Stanton. 2007. A survey of the microbial and chemical composition of seven semi-ripened Provola dei Nebrodi Sicilian cheeses. *J. Appl. Microbiol.* 103: 128–1139.

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

Dasen A., F. Berthier, R. Grappin, A.G. Williams and J. Banks. 2003. Genotypic and phenotypic characterization of the dynamics of the lactic acid bacterial population of adjunct-containing Cheddar cheese manufactured from raw and microfiltered pasteurised milk. *J. Appl. Microbiol.* 94: 595–607.

De Angelis M., A. Corsetti, N. Tosti, J. Rossi, M.R. Corbo and M. Gobbetti. 2001. Characterization of non-starter LAB from Italian ewe cheeses based on phenotypic, genotypic and cell wall protein analyses. *Appl. Environ. Microbiol.* 67: 2011–2020.

De Candia S., M. De Angelis, E. Dunlea, F. Minervini, P.L.H. McSweeney, M. Faccia and M. Gobbetti. 2007. Molecular identification and typing of natural whey starter cultures and microbiological properties of related traditional Mozzarella cheeses. *Int. J. Food Microbiol.* 119: 182–191.

Delgado S. and B. Mayo. 2004. Phenotypic and genotypic diversity of Lactococcus lactis and Enterococcus spp. strains isolated from Northern Spain starter-free farmhouse cheeses. *Int. J. Food Microbiol.* 90: 309–319.

Dolci P., V. Alessandria, G. Zeppa, K. Rantsiou and L. Cocolin. 2008a. Microbiological characterization of artisanal Raschera PDO cheese: analysis of its indigenous lactic acid bacteria. *Food Microbiol.* 25: 392–399.

Dolci P., V. Alessandria, K. Rantsiou, L. Rolle, G. Zeppa and L. Cocolin. 2008b. Microbial dynamics of Castelmagno PDO, a traditional Italian cheese, with a focus on lactic acid bacteria ecology. *Int. J. Food Microbiol.* 122: 302–311.

Delbès C., L. Ali-Mandjee and M.C. Montel. 2007. Monitoring bacterial communities in raw milk and cheese by culture-dependent and -independent 16S rRNA gene-based analysis. *Appl. Environ. Microbiol.* 73: 1882–1891.

Duthoit F., J.J. Godon and M.C. Montel. 2003. Bacterial community dynamics during production of Registered Designation of Origin Salers cheese as evaluated by 16S rRNA gene single strand conformation polymorphisms analysis. *Appl. Environ. Microbiol.* 69: 3840–3848.

Duthoit F., C. Callon, L. Tessier and M.C. Montel. 2005a. Relationships between sensorial characteristics and microbial dynamics in "Registered Designation of Origin" Salers cheese. *Int. J. Food Microbiol.* 103: 259–270.

Duthoit F., L. Tessier and M.C. Montel. 2005b. Diversity dynamics and activity of bacterial population in "Registered Designation Origin" Salers cheese by single-strand conformation polymorphism analysis of the 16S rRNA genes. *J. Appl. Microbiol.* 98: 1198–1208.

García M.C., M.J. Rodríguez, A. Bernardo, M.E. Tornadijo and J. Carballo. 2002. Study of enterococci and micrococci isolated throughout manufacture and ripening of San Simón cheese. *Food Microbiol.* 19: 23–33.

Giraffa G. and E. Neviani. 2000. Molecular identification and characterization of food associated lactobacilli. *Int. J. Food Sci.* 4: 403–423.

Jany J.L. and G. Barbier. 2008. Culture-independent methods for identifying microbial communities in cheese. *Food Microbiol.* 25: 839–848.

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

Mannu L., A. Paba, M. Pes, R. Floris, M.F. Scintu and L. Morelli. 1999. Strain typing among enterococci isolated from home made Pecorino Sardo cheese. *FEMS Microbiol. Lett.* 170: 25–30.

Mannu L., G. Riu, R. Comunian, M.C. Fozzi and M.F. Scintu. 2002. A preliminary study of lactic acid bacteria in whey starter culture and industrial Pecorino Sardo ewes' milk cheese: PCR-identification and evolution during ripening. *Int. Dairy J.* 12: 17–26.

McSweeney P.L.H., G. Ottogalli and P.F. Fox. 2004. Diversity of cheese varieties: an overview. In: Fox, P.H., McSweeney, P.L.H., Cogan, T., Guinee, T. (Eds.), Cheese: Chemistry, Physics and Microbiology. Elsevier Academic Press, San Diego, pp. 1–22.

Randazzo C.L., S. Torriani, A.D.L. Akkermans, W.M. de Vos and E.E. Vaughan. 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Appl. Environ. Microbiol.* 68: 1882–1892.

Sánchez I., S. Seseña, J.M. Poveda, L. Cabezas and L. Palop. 2006. Genetic diversity, dynamics and activity of Lactobacillus community involved in traditional processing of artisanal Manchego cheese. *Int. J. Food Microbiol.* 107: 265–273.

Suzzi G., M. Caruso, F. Giardini, A. Lombardi, L. Vannini, M.E. Guerzoni, C. Andrighetto and M.T. Lanorte. 2000. A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto Caprino). *J. Appl. Microbiol.* 89: 267–274.

Terzic-Vidojevic A., M. Vukasinovic, K. Veljovic, M. Ostojic and L. Topisirovic. 2007. Characterization of microflora in homemade semi-hard white Zlatar cheese. *Int. J. Food Microbiol.* 114: 36–42.

Ventura M., C. Canchaya, V. Meylan, T.R. Klaenhammer and R. Zink. 2003. Analysis, characterization, and loci of the tuf genes in Lactobacillus and Bifidobacterium species and their direct application for species identification. *Appl. Environ. Microbiol.* 69: 6908–6922.