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DEVELOPMENT OF A QUANTITATIVE REAL-TIME PCR ASSAY FOR IDENTIFICATION OF *STREPTOCOCCUS THERMOPHILUS* PRESENT IN ARTISANAL RAW COW'S MILK CHEESE

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Abstract: The aim of this paper was to develop a real-time PCR method targeting a gene sequence encoding a bacteriocin ABC transporter ATP-binding protein for the detection of *Streptococcus thermophilus* in cheese produced from raw cow milk. A real-time quantitative PCR assay was designated to identify and count *S. thermophilus* cells in ripened cheese. The developed real-time PCR primers and probe were highly specific for *S. thermophilus* CNRZ1066, CNRZ8232, LMD-9, LMG 18311, CNRZ 002 and CNRZ 03 but not for *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc* spp., *Enterococcus* spp. that are phylogenetically very similar to *S. thermophilus*, and this is the reason why they are difficult to differentiate using culture-based methods. The real-time PCR allowed for 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; PCR efficiencies ranged from 1.00 to 1.03. The newly developed qPCR method could be applied to specific detection and precise enumeration of *S. thermophilus* in raw milk and cheese.

Key words: artisanal cheese, LAB, Real-time PCR, *Streptococcus thermophilus*, a bacteriocin ABC transporter ATP-binding protein gene.

Introduction

Streptococcus thermophilus belongs to thermophilic lactic acid bacteria and finds its wide application in the production of dairy products. *S. thermophilus* plays a particular role in milk fermentation which involves the transformation of lactose into lactic acid, leading to a significant decrease in pH value. It contributes to forming the desirable aroma and flavour of ripened cheese. It is responsible for the production of specific metabolites including exopolysaccharides which give the proper viscous texture and rheological properties for cheese and other fermented milk products [1]. The precise monitoring of the number of *S. thermophilus* cells starting from the fermentation stage and ending with consumption is very crucial. However, it is very hard to precisely identify and count *S. thermophilus* especially in a food matrix as the food samples usually contain phylogenetically closely related bacterial species which due to their very similar physiological and biochemical features are very difficult to differentiate [2, 3].

Cheese microflora include lactic acid bacteria possessing a high influence on human health and nutrition [4]. They carry out both spontaneous fermentations and large-scale fermentation processes which play a significant role in the preservation as well as the transformation of many raw food materials such as milk, meat, fish, cereals, tubers and vegetables. Lactic acid bacteria are also 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 [5, 6]. They possess a significantly beneficial influence of preventing and treating diarrhoea. They improve the digestion of lactose by lactase-deficient individuals. There are also known to actively participate in preventing and treating certain allergies and inflammatory bowel diseases. There is a strong need to properly enumerate lactic acid species in order to study their role and their dynamics in different niches [7, 8].

The identification of *S. thermophilus* using the traditional culture-based methods is usually difficult as it requires the long incubation time and does not give the clear results as the colonies of *S. thermophilus* are very similar to the colonies of *Streptococcus salivarius* and Enteroccoccus spp. There is a need to use specific media with addition of bromocresole purple, bromocresole green and nalidixic acid to discriminate *S. thermophilus* from other streptococci or enterococci [9]. Furthermore, the presumed colonies of *S. thermophilus* require confirmation by PCR-based techniques. The application of a molecular culture-independent method which is quantitative real-time PCR brings many benefits. Such method is specific, sensitive, accurate and fast [10]. The identification of *S. thermophilus* in dairy products by the application of real-time PCR method is mainly based on primers derived from the 16S rDNA gene sequence of *S. thermophilus* [11, 12]. The biggest obstacle is the fact that approximately 80% of the genes in *S. thermophilus* are

similar to other streptococcal genes which means that these genes may also appear in pathogenic species of *S. thermophilus*. It makes the design of species specific real-time PCR primers difficult to develop [13]. It is known that the 16S rDNA sequence of *S. thermophilus* is very similar to those of *S. salivarius, Streptococcus* vestibularis and *Enteroccocus faecium* [14, 15]. As there is the low discriminatory power of primers designed based on *S. thermophilus* 16S rDNA region, there is a need to examine other gene sequences possessing higher discriminatory power to identify *S. thermophilus* with the application of Real-time PCR primers and probes [16, 17].

The aim of the present study was to design qPCR assay for the quantification of *S. thermophilus*, thermophilic lactic acid bacteria, which actively participate in milk fermentation and cheese ripening. It constitutes the typical microflora of examined in this paper the artisanal cheese produced from raw cow milk. In this paper, we developed an assay based on a set of target-specific PCR primers targeting the gene that encodes the bacteriocin ABC transporter ATP-binding protein production. Cheese samples were examined to assess the applicability of qPCR for direct quantification of *S. thermophilus* in ripened cheese. This method occurred to be highly specific for *S. thermophilus* and was applied to quantify these species in cheese.

Bacteria and media

Table 1 presents all the examined strains which were used in the study. They were kept at temperature -80°C in the previously sterilized reconstituted skim milk powder (10%, w/v) and cultivated in M17 broth (Oxoid, Basingstoke, Hampshire, England) at 37°C.

Table 1: A list of strains used to check the specificity of the qPCR protocol.

Type of strain	Origin	Type of source
Streptococcus thermophilus CNRZ1066	Cheese	CNRZ
Streptococcus thermophilus CNRZ8232	Emmental cheese	CNRZ
Streptococcus thermophilus LMD-9	Yoghurt	LMD
Streptococcus thermophilus LMG 18311	Cheese	LMG
Streptococcus thermophilus CNRZ 002	Gruyere cheese	CNRZ
Streptococcus thermophilus CNRZ 03	Yoghurt	CNRZ
Streptococcus agalactiae ATCC 13813	Bovine milk	ATCC
Streptococcus agalactiae CCUG 19094	Bovine milk	CCUG
Streptococcus agalactiae CCUG 24810	Bovine milk	CCUG
Streptococcus agalactiae CCUG 25532	Bovine milk	CCUG
Streptococcus agalactiae LMG 15085	Bovine milk	LMG
Streptococcus agalactiae LMG 15091	Bovine milk	LMG
Streptococcus anginosus CCUG 39159	Human saliva	CCUG
Streptococcus australis ATCC 700641	Human saliva	ATCC
Streptococcus bovis ATCC 700338	Alimentary tract of cattle	ATCC
Streptococcus equinus ATCC 9812	Alimentary tract of a horse	ATCC
Streptococcus infantis ATCC 700779	Upper respiratory tracts of adults	ATCC
Streptococcus infantis CNRZ 1076	Upper respiratory tracts of adults	CNRZ
Streptococcus macacae NCTC 11558	Alimentary tract of cattle	NCTC
Streptococcus mitis NCTC 12261	Human mouth	NCTC
Streptococcus oralis ATCC 35037	Human oral cavity	ATCC
Streptococcus parasanguinis ATCC 15912	Human oral cavity	ATCC
Streptococcus parasanguinis ATCC 903	Human oral cavity	ATCC
Streptococcus parauberis KCTC 11537	Bovine milk	KCTC
Streptococcus parauberis CNRZ 2020	Bovine milk	CNRZ
Streptococcus pasteurianus ATCC 43144	Alimentary tract of cattle	ATCC
Streptococcus peroris ATCC 700780	Alimentary tract of cattle	ATCC
Streptococcus pneumoniae ATCC 700669	Human nasal cavity	ATCC
Streptococcus pneumoniae CCRI 1974	Human nasal cavity	CCRI
Streptococcus pyogenes ATCC 10782	Human skin	ATCC
Streptococcus pyogenes CNRZ 0394	Human skin	CNRZ
Streptococcus salivarius CNRZ 0234	Human oral cavity	CNRZ
Streptococcus sanguinis ATCC 29667	Human mouth	ATCC
Lactococcus lactis subsp. cremoris CNRZ 1117	Cheese	CNRZ
Lactococcus lactis subsp. cremoris CNRZ 1119	Cheese	CNRZ
Lactococcus lactis subsp. cremoris CNRZ 1107	Cheese	CNRZ
Lactococcus lactis subsp. lactis CNRZ 1009	Cheese	CNRZ
Lactococcus lactis subsp. lactis CNRZ 1007	Whey	CNRZ
Leuconostoc lactis KCTC 3528	Butter	KCTC
Leuconostoc mesenteroides subsp. *cremoris* ATCC 19254	Cheese	ATCC
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC 8293	Cheese	ATCC
Enterococcus faecalis ATCC 29200	Human gastrointestinal track	ATCC
Enterococcus faecalis ATCC 4200	Human gastrointestinal track	ATCC
Enterococcus faecium CNRZ 1333	Human gastrointestinal track	CNRZ
Lactobacillus plantarum subsp. *plantarum* ATCC 14917	Yoghurt	ATCC
Lactobacillus plantarum DSM 20053	Yoghurt	DSM
Lactobacillus reuteri DSM 20016	Human gastrointestinal track	DSM
Lactobacillus reuteri ATCC 21052	Human urinary track	ATCC

Strain collection: ATCC: American Type Culture Collection, Corporate, 10801 University Boulevard, Manassas, VA 20110-2209, USA Products & Services Orders, Manassas, VA 20108-1549, USA; CNRZ: Centre National de Recherches Zootechniques, Jouy-en-Josas, France; KCTC: Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology, Yusong, Taejon 305-600, Republic of Korea; CCRI: Collection du Centre de Recherche en Infectiologie, Universite Laval, 2705 Boulevard Laurier, RC709, Quebec, Quebec, G1V 4G2, Canada; CCUG: Culture Collection, University of Göteborg, Department of Clinical Bacteriology, Institute of Clinical Bacteriology, Immunology, and Virology, Guldhedsgatn 10A s-413, 46 Göteborg, Sweden; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, D-38124 Braunschweig, Germany; LMD: Laboratorium voor Microbiologie der Technische Hogeschool, Delft University of Technology, Julianalaan 67A, 2628 BC Delft, The Netherlands; LMG: Collection of the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Ledeganckstraat 35, B-9000, Gent, Belgium; NCTC: National Collection of Type Cultures, Public Health, England.

Isolation of *S. thermophilus* from cheese

Cheese samples were aseptically grated, and 10 g of cheese was put into sterile stomacher bag. The samples were homogenized with 90 ml of 0,1% peptone water at temperature 40°C (10 g/L peptone from casein, 5 g/L sodium chloride, 20 g/L tri-sodium citrate dihydrate, pH 7.0) (Oxoid, UK) for 2 min in a stomacher (Colworth Stomacher 400, Seward Ltd, London, UK). Consequently, the serial dilutions of the previously prepared homogenate were plated on *Streptococcus thermophilus* agar (Oxoid, UK), which was the selective media for the counting of *S. thermophilus* under aerobic incubation at 37°C for 24 h. A spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain) was used for plating.

Extraction of DNA from the pure culture

Streptococcus thermophilus CNRZ1066, CNRZ8232, LMD-9, LMG 18311, CNRZ 002, CNRZ 03 were grown in 10 ml M17 broth at 42°C. The cells coming from a 2-ml late exponential growth phase culture (A_{650nm} = 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. 1 ml 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. 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. Then 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

The DNA was isolated from cheese by using Syngen Food DNA Mini Kit (Syngen Biotech, Germany). Commercial cheese in the amount of 200 mg of the homogenised sample was placed in 2 ml tubel; then 1 ml of buffer DLF was added. The tube was closed and mixed by vortexing. The total volume of supernatant could not be less than 700 μl. 30 μl 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 2500×g. 700 μl of the supernatant was transferred to a new 2 ml tube. In some food samples the three phases were able to 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. 700 μl 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. 100-200 μl of pre-warmed DE elution buffer was added in the centre 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).

Oligonucleotide primer and probe design

The sequence of the gene was delivered from GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number CP012588.1 for *Streptococcus thermophilus*. The nucleotide sequences of the locus_tag AMD33_06980 gene encoding the bacteriocin ABC transporter ATP-binding protein that are specific for *S. thermophilus* were the subject of comparison with the sequences belonging to closely related strains. A set of primer/probe was developed using Primer Express Software v3.0 (Applied Biosystems, Foster City, CA, USA). Consequently, the set was validated using NCBI BLAST (Basic Local Alignment Search Tool: www.ncbi.nlm.nih.gov/blast/). The region from nucleotide position 1348636 to 1348685 within the locus_tag AMD33_06980 gene occurred to be a potential target site because it was identical in all the examined *S. thermophilus* strains. Simultaneously, it was found to be different in the rest of examined species. The sequences were as follows: forward primer 5' – CATGATTGGTAATAGCTTGAGAG – 3'; reverse primer 5' – CGGTTCTGCTATTGATAACCAG – 3'; probe 5' – FAM – CAGACTCAGGTCTCAGAGTATGAGGCG – 3' – MGB-NFQ. The oligonucleotides were synthesised and purchased from Eurofins Genomics (Ebersberg, Germany).

Construction of standard curves for qPCR

The locus_tag AMD33_06980 gene belonging to *S. thermophilus* and encoding the bacteriocin ABC transporter ATP-binding protein was synthesised and purchased from Eurofins Genomics (Ebersberg, Germany). The sequence of the gene was provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number CP012588.1, region from 1347901 to 1349221). They were bought in the lyophilized form. Standard curves were prepared with serial dilutions of genomic DNA isolated from *S. thermophilus*. There were 1.56×10^{12} DNA copies in a delivered tube. They were dissolved in 1270 μl of DE buffer (Syngen Biotech, Wrocław, Poland) achieving the concentration of 1×10^{9} DNA copies/μl of eluate. This concentration was used for the preparation of standards for the standard curve. The dilutions were prepared to achieve 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, were used to assess the sensitivity of the method.

The qPCR conditions

The reaction's total volume amounted to 20 μl. Real-time PCR analysis was made using Stratagene Mx3005P thermocycler (Agilent Technologies, Santa Clara, CA, USA). The PCR mixture contained 5 μl DNA template, 4 μl of Quantum Probe Mix (Syngen Biotech, Cambridge, United Kingdom), 0.8 μl of primers F and R respectively, 0.5 μl of hydrolysis probe and 8.9 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

Table 2: A set of primers, a probe and plasmid applied in our research.

Primer/probe/gene	Sequence from 5' do 3'	Application
Forward primer	CATGATTGGTAATAGCTTGAGAG	Specific detection of *S. thermophilus*
Reverse primer	CGGTTCTGCTATTGATAACCAG	Specific detection of *S. thermophilus*
Hydrolysis probe	CAGACTCAGGTCTCAGAGTATGAGGCG	Specific detection of *S. thermophilus*
A fragment of locus_tag AMD33_06980 gene	Plasmid pGEM which contains a fragment of the locus_tag AMD33_06980 gene encoding the bacteriocin ABC transporter ATP-binding protein	Preparation of standard curve

5 min) and 35 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 90 s). The fluorescence of the reporter dye (FAM) was measured during amplification at 510 nm. The real-time PCR reaction and amplification step were carried out using 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, Santa Clara, CA, USA). All standard and sample reactions were run three times.

Evaluation of the qPCR specificity, sensitivity, and efficiency

The specificity of the primer/probe design was evaluated by the NCBI Primer-BLAST tool. It was examined against strains of *S. thermophilus* and bacteria acid belonging to other genera (Table 1). The nucleotide sequences of primers and a probe are presented in Table 2. The sensitivity of the qPCR assay was checked by preparing a serial tenfold dilution of a plasmid containing the target sequence as well as *S. thermophilus* which was diluted in milk and cheese homogenate. The plasmid containing the target sequence was treated as a positive control. The dilutions were examined by qPCR, and a number of DNA copies contained in each dilution was used to calculate the limit of detection (LOD) and the linearity of the qPCR assay. The standard curve was created by plotting the Ct values of all dilutions as a function of the concentration of DNA copies and calculating the linear regression in R^2. qPCR efficiency was assessed using standard curves with an equation PCR efficiency of $(10^{\frac{-1}{slope}})$-1.

The efficiency of the sum of DNA extraction plus qPCR was assessed by comparing the calculated copy numbers with the colony forming units (CFU). In order to evaluate it, a culture of *S. thermophilus* MN-BM-A01 containing 5.71×10^7 cfu per ml was serially diluted tenfold in raw milk. Two dilution series were prepared, and the DNA coming from each dilution sample was extracted as was described above before giving it to qPCR. The concentration of CFU in the M17 broth was assessed by plate counting using M17 agar, pH 7.1 (Oxoid, Basingstoke, UK) at 42°C for 48 h.

Inoculating of milk and cheese with *S. thermophilus*

Raw milk and cheese were used to be inoculated with *S. thermophilus*. The milk was examined using the qPCR protocol to check that it did not contain indigenous *S. thermophilus*. *S. thermophilus* MN-BM-A01 culture was used for tenfold serial dilution in milk. Its total number was assessed using plate counting. The qPCR was used for DNA extracted from inoculated milk. The inoculation of milk was carried out twice. Milk samples which contained a low level of *S. thermophilus* with 100 colony forming units per 1 ml, which was checked by plate counting, were used to evaluate the sensitivity and specificity of the qPCR protocol.

In the case of cheese, an artisanal raw cow milk cheese homogenate was used. At first, the qPCR protocol was used to confirm the absence of native *S. thermophilus*. Next, *S. thermophilus* MN-BM-A01 culture was used to prepare a tenfold serial dilution of a cheese sample. The number of S. thermophilus in inoculated cheese was assessed by plate counting. The extraction of DNA was carried out from each dilution step; then the DNA was examined by qPCR. The efficiencies of qPCR were assessed by plotting of Cq against the log10 cfu per ml and cfu per gram of milk and cheese, respectively.

Assessment of *S. thermophilus* in milk and cheese

A number of gene copies in milk and cheese were estimated with the plasmid standard curves which were carried out for each analysis. The results of milk and cheese samples were multiplied with dilution factors.

Statistical analysis

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

Preparing of qPCR assay

In order to prepare a qPCR assay, we analysed the whole genomic DNA coming from 6 *S. thermophilus* isolates. Our analysis of the whole genomes proved that a gene sequence encoding a bacteriocin ABC transporter ATP-binding protein was present as a single copy gene in each isolate. The genome sequence of *S. thermophilus* MN-BM-A01 was deposited in the GenBank database (Accession Number CP012588.1), and the specific gene was accessible with the locus_tag AMD33_06980. We used this gene sequence to develop a primer/probe set for the qPCR assay. We examined 6 *S. thermophilus* strains to assess the sensitivity and specificity of the designed primer/probe set. This set was also examined against other streptococci including *Streptococcus agalactiae, Streptococcus anginosus, Streptococcus australis, Streptococcus bovis, Streptococcus equines, Streptococcus infantis, Streptococcus macacae, Streptococcus mitis, Streptococcus oralis, Streptococcus parasanguinis, Streptococcus parauberis, Streptococcus pasteurianus, Streptococcus peroris, Streptococcus pneumonia, Streptococcus pyogenes, Streptococcus salivarius, Streptococcus sanguinis as well as Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. lactis, Leuconostoc lactis, Leuconostoc mesenteroides subsp. cremoris, Leuconostoc mesenteroides subsp. mesenteroides, Enterococcus faecalis, Enterococcus faecium, Lactobacillus plantarum* and *Lactobacillus reuteri* (Table 2). In PCR reaction only *S. thermophilus* strains were able to be identified using the developed primer/probe set. In the case of the rest of species, it was not possible to detect any signal.

For the evaluation of how the qPCR assay worked, at the beginning the tenfold serial dilutions of the plasmid pGEM-T/ locus_tag AMD33_06980 were assayed. The linearity of plasmid dilution series amounted to 7 logs and the correlation coefficient (R2) amounted to 0.998 (Fig. 1). The PCR efficiency amounted to 1.03. The limit of detection (LOD) amounted to 10 gene copies per reaction. The limit of quantification (LOQ) amounted to 100 gene copies per reaction.

The cultures of *S. thermophilus* MN-BM-A01 with concentration from 4×10^7 to 7×10^7 cfu per ml was serially diluted tenfold in raw milk. The milk was examined to eliminate the presence of *S. thermophilus*. DNA samples extracted from spiked milk samples indicated a linearity at a level of over 7 logs and a correlation coefficient (R2) of 0.997 (Fig. 2). The PCR efficiency amounted to 1.03. The precision of reaction with a low number of *S. thermophilus* in milk was evaluated on the basis of five DNA extractions per dilution. It was observed that it was possible to detect between 10 and 100 gene copies per reaction as they gave a positive signal with Cq value below 35.

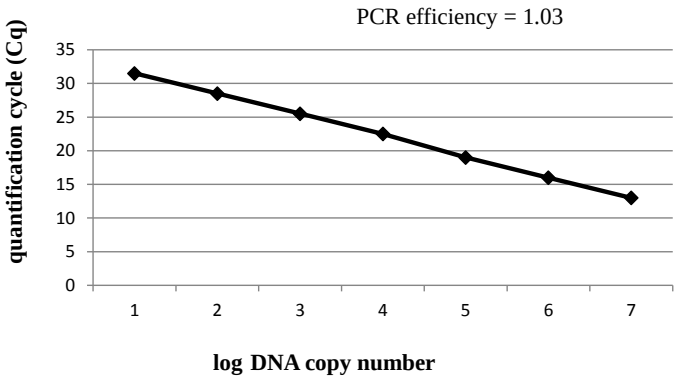

Fig. 1: Standard curve achieved by qPCR for pGEM-T/ locus_tag AMD33_06980. The plasmid standard curve was obtained by plotting the threshold cycle (Ct) values against the calculated gene copy numbers per reaction from serial tenfold dilutions of plasmid in which the bacteriocin ABC transporter ATP-binding protein was inserted.

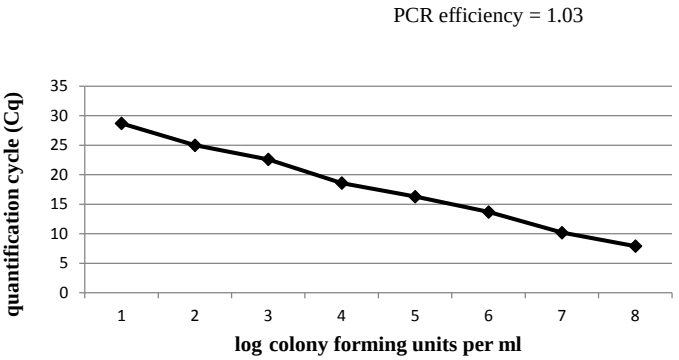

Fig. 2: The standard curve achieved by qPCR for *S. thermophilus* MN-BM-A01 inoculated in raw milk. The standard curve was obtained by plotting the threshold cycle (Ct) values against log cfu/ml for milk.

In the last stage of our study the cheese samples were inoculated with S. thermophilus MN-BM-A01 culture containing 7×10^7 cfu per 1 ml. DNA after extraction of tenfold dilution series was used for PCR reaction. The linearity over 7 logs was observed and a correlation coefficient (R^2) amounted to 0.998 (Fig. 3). The PCR efficiency amounted to 1.00. Cq value amounted to 32 in the lower limit of linearity.

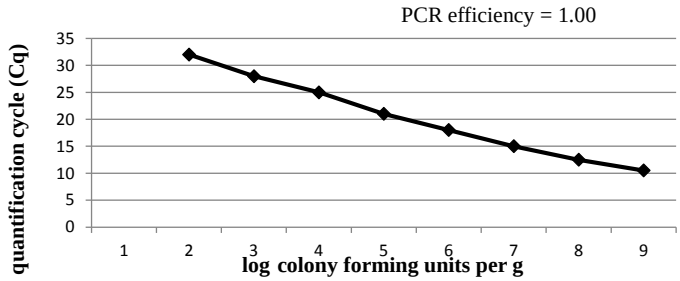

Fig. 3: The standard curve achieved by qPCR for *S. thermophilus* MN-BM-A01 inoculated in cheese. The standard curve was obtained by plotting the threshold cycle (Ct) values against log cfu/g for cheese.

Discussion

S. thermophilus is Gram-positive thermophilic lactic acid bacteria that contributes to milk acidification and aroma formation. As the methods that made it possible to quantify selectively still require improvement; we designed an assay based on a set of target-specific PCR primers targeting the gene that encodes the bacteriocin ABC transporter ATP-binding protein production. We managed to isolate and identify *S. thermophilus* in raw milk and cheese. The set of primers and probe targets the locus_tag AMD33_06980 gene. It was found to successfully generate amplicons from genomic DNA of *S. thermophilus*. This set was designed to be used in quantitative analysis involving LOD and LOQ amounting to 10 and 100 GE per reaction which constituted 125 and 1250 GE per 1 ml of milk as well as 500 and 5000 GE per 1 g of cheese). We found that PCR efficiencies both for milk and cheese extracts indicated similar value to PCR efficiency of the plasmid standard, which means that the plasmid standard might find its application in estimating the number of *S. thermophilus* in cheese samples. Moreover, our qPCR protocol is also appropriate to be used for the rapid detection of milk samples which contain *S. thermophilus*. Thus, such samples can be categorised as possessing low (which means below LOQ) and high (which means above LOQ) numbers of *S. thermophilus* [18, 19].

The newly designed qPCR method has been found as a valuable device in the identification and quantitative assessment of *S. thermophilus* in cheese produced from raw cow milk. Moreover, the qPCR protocol may also find its application in examining the factors which have an influence on the growth of *S. thermophilus* in cheese including food additives, starter culture and adjunct cultures. Our study demonstrates a fast and highly specific method of detection of *S. thermophilus* in milk and cheese.

Molecular methods find their applications in monitoring the microflora of dairy products. Molecular techniques, specifically polymerase chain reaction-based methods, deliver sensitive, rapid, and quantitative analytical tools to investigate the growth of lactic acid bacteria during the fermentation and ripening of cheese. However, they also possess some drawbacks. The molecular techniques available today and those under development need further refinement in order to be standardized and applicable to a diversity of matrices. The pros and cons of molecular techniques for the detection and quantification of lactic acid bacteria should be the subject of further investigations as there is a huge variety of different food matrices and more efficient lysis methods for extracting DNA from cheese samples are needed to overcome this limit [20].

The results of the current work present an essential molecular approach for the detection, identification and quantitative assessment of typical microflora contained in traditional Polish cheese produced from raw cow milk. The subsequent isolation and identification of lactic acid bacteria combined with the enumeration of other specific species could deliver a broader command of bacterial ecosystem present in this traditional cheese.

Acknowledgments

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