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Effect of the addition of phytosterols and tocopherols on *Streptococcus thermophilus* robustness during industrial manufacture and ripening of a functional cheese as evaluated by qPCR and RT-qPCR

J. Pega [a,b,*], S. Rizzo [a], C.D. Pérez [a,b], L. Rossetti [a], G. Díaz [a], S.M. Ruzal [b,c], M. Nanni [a], A.M. Descalzo [a,d]

[a] Instituto Tecnología de Alimentos, Centro de Investigaciones en Agroindustria, Instituto Nacional de Tecnología Agropecuaria (INTA), Aristizábal y De La Tradición s/n, Hurlingham 1686, Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Av. Rivadavia 1917, CABA, Argentina
[c] Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IQUIBICEN-CONICET, Ciudad Universitaria, CABA, Argentina
[d] INTA-LABINTEX-Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Département PERSYST, UMR Qualisud. TA B-95/16, 34398 Montpellier, France

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ABSTRACT

The quality of functional food products designed for the prevention of degenerative diseases can be affected by the incorporation of bioactive compounds. In many types of cheese, the performance of starter microorganisms is critical for optimal elaboration and for providing potential probiotic benefits. Phytosterols are plant lipophilic triterpenes that have been used for the design of functional dairy products because of their ability to lower serum cholesterol levels in humans. However, their effect on the starter culture behavior during cheesemaking has not yet been studied. Here, we followed DNA and RNA kinetics of the bacterium *Streptococcus thermophilus*, an extensively used dairy starter with probiotic potential, during industrial production of a functional, semi-soft, reduced-fat cheese containing phytosterol esters and alpha-tocopherol as bioactive compounds. For this purpose, real-time quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR) assays were optimized and applied to samples obtained during the manufacture and ripening of functional and control cheeses. An experimental set-up was used to evaluate the detection threshold of free nucleic acids for extraction protocols based on pelleted microorganisms. To our knowledge, this straight-forward approach provides the first experimental evidence indicating that DNA is not a reliable marker of cell integrity, whereas RNA may constitute a more accurate molecular signature to estimate both bacterial viability and metabolic activity. Compositional analysis revealed that the bioactive molecules were effectively incorporated into the cheese matrix, at levels considered optimal to exert their biological action. The starter *S. thermophilus* was detected by qPCR and RT-qPCR during cheese production at the industrial level, from at least 30 min after its inoculation until 81 days of ripening, supporting the possible role of this species in shaping organoleptic profiles. We also showed for the first time that the addition of phytosterols at functional concentrations, not only did not affect starter performance but also correlated with a significant increase in target DNA and cDNA levels in most of the time points evaluated throughout cheesemaking. Therefore, these findings suggest that the growth and metabolism of *S. thermophilus* may be enhanced by the incorporation of these biologically active molecules during cheese production, providing important information for the industrial design of novel fermented foods.

1. Introduction

The combination of different raw materials and processing steps during cheesemaking creates a complex interaction of microorganisms, which are main determinants of the organoleptic characteristics and quality in the final product (Irlinger and Mounier, 2009). Many manufacturing practices of both industrial and artisanal cheeses require the addition of a defined culture of starter lactic acid bacteria (SLAB) to

Abbreviations: AHA, American Heart Association; C, control; Ct, threshold cycle; Cu, curd; C7d, C cheese ripened for 7 days at 4 °C; F, functional; FAMEs, fatty acid methyl esters; F7d, F cheese ripened for 7 days at 4 °C; HPLC, high-pressure liquid chromatography; ISO, International Organization for Standardization; LAB, lactic acid bacteria; Llactis, lyophilized starter culture *Lactococcus lactis* subsp. *lactis* and L. *lactis* subsp. *cremoris*; LOD, limit of detection; MUFA, monounsaturated fatty acids; MTBE, methyl *tert*-butyl ether; ND, not detected; PM, pasteurized milk; PMS, milk pasteurized 30 min after starter addition; PM + St, 45 mL of PM sample spiked with 22 mg of *S. thermophilus* starter; PUFA, polyunsaturated fatty acids; qPCR, quantitative PCR; RM, raw milk; RT, reverse transcription; RT-qPCR, reverse transcription-qPCR; SD, standard deviation; SFA, saturated fatty acids; SLAB, starter lactic acid bacteria; SSD, statistically significant differences; St, lyophilized starter culture *S. thermophilus*; Stherm, primers designed in this study; Tuf, primers reported by (Falentin et al., 2012); VNC, viable but nonculturable; 7d, 7 days of ripening; 20d, 20 days of ripening; 40d, 40 days of ripening; 57d, 57 days of ripening; 81d, 81 days of ripening.

* Corresponding author at: Instituto Tecnología de Alimentos, CIA, INTA. Aristizábal y De La Tradición s/n, Hurlingham 1686, Buenos Aires, Argentina.
E-mail address: juanfpega@gmail.com (J. Pega).

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achieve fast milk acidification rates or specific flavor profiles. Unfortunately, the several processes throughout cheesemaking induce heat-related, osmotic, acidic, oxidative and other types of stresses that can sometimes affect the growth and survival of SLAB (Gatti et al., 2014; Jany and Barbier, 2008). Some of these events may be even more critical when introducing new protocols and/or ingredients for the elaboration of novel products, such as fortified or functional dairy foods.

Streptococcus thermophilus is a Gram-positive, facultative anaerobic, homofermentative, thermophilic lactic acid bacterium (LAB) with restricted natural habitats in the bovine mammary mucosa and raw milk. It is traditionally used as dairy starter, either alone or in combination with other species (Fox et al., 2004), being the second most important microorganism in the dairy industry after *Lactococcus lactis* (Fox et al., 2000). *S. thermophilus* possesses valued metabolic traits, such as efficient production of lactic acid and exopolysaccharides, synthesis of flavoring compounds, galactose fermentation, proteolytic action and urease activity (Mora et al., 2005, Mora and Arioli, 2014). In addition to these well-known technological advantages, human randomized clinical trials have proven the efficacy of this probiotic for a wide variety of health conditions (Agerholm-Larsen et al., 2000; Di Marzio et al., 2003; Gluck and Gebbers, 2003; Hickson et al., 2007; Saavedra et al., 1994).

In the last years, molecular (culture-independent) methods have been widely used to study technologically important LAB in milk and cheese products (reviewed by Cocolin et al., 2013). However, no gold standard has yet been established for molecular detection of microorganisms in dairy products (Sohier et al., 2014). Many of the published reports have used different nucleic acid extraction and quantification methods, amplification protocols, assay controls, expression of results and data interpretation. Nevertheless, culture-independent tools have revealed experimental findings relevant for food research. One example is the evidence regarding SLAB persistence throughout cheese ripening, which has been replicated by different researchers, independently of the methodological procedures and analyses used. For instance, quantitative molecular methods, including real-time quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR), have been used to detect *S. thermophilus* and other SLAB, both in experimental cheeses sampled at different elaboration steps and in ripened cheeses (Achilleos and Berthier, 2013; Carraro et al., 2011; Desfossés-Foucault et al., 2013; Falentin et al., 2012; Ruggirello et al., 2014).

A "functional food" can be defined as a natural or processed food that contains known biologically active compounds which when in defined quantitative and qualitative amounts, provides a clinically proven health benefit, and can thus be used in the prevention, management and treatment of modern chronic diseases (Martirosyan, 2011). Phytosterols (plant sterols and stanols) are a family of lipophilic triterpenes used as cholesterol-lowering agents (reviewed by Rondanelli et al., 2013). Obtaining an adequate amount of certain bacteria in fermented foods is important, not only for industrial but also for probiotic purposes (Sohier et al., 2014). Therefore, when designing a functional fermented food, it is necessary to guarantee that the added molecules do not negatively affect the growth of certain microorganisms and hence their presence in the final product. Although several dairy products with phytosterols and other functional compounds have been designed, experimental data regarding how the incorporation of these bioactive molecules affects their microbiota (including starter cultures), are still lacking.

"Port Salut light" cheese is a reduced-fat version of the full-fat Port Salut cheese, developed in Argentina. It is a semi-cooked product produced from pasteurized cow's milk, of medium moisture (between 46 and 55%) and semi-soft consistency (Argentine Food Code, 2012). Here, a functional Port Salut light cheese containing a mix of phytosterols and tocopherols was successfully produced at the industrial level. The objective of this study was to analyze whether the addition of these functional compounds affected the robustness of the starter *S. thermophilus* throughout cheese manufacture and ripening. To this end, real-time qPCR and RT-qPCR protocols were optimized to study starter dynamics in functional and control cheeses. Samples included raw milk (RM), pasteurized milk (PM), milk pasteurized 30 min after starter addition (PMS), curd (Cu) and cheeses ripened for 7, 20, 40, 57 and 81 days. In addition, we developed an experimental set-up to evaluate for the first time whether DNA and RNA extracted from pelleted material could be used as accurate markers of cell viability.

2. Materials and methods

2.1. Industrial cheese manufacture and sample collection

Port Salut light functional (F) and control (C) cheeses were produced at the industrial plant Capilla del Señor, located in the city of Villa Maria, Cordoba, Argentina, under International Organization for Standardization (ISO) procedures. Briefly, 2500 L of cow's raw milk obtained from local dairy farms was used for each F and C cheese elaboration batch. Milk was skimmed to achieve a final fat concentration of 1.9% and then it was pasteurized at 72 °C ± 2 for 15 s. Then, 19 kg of phytosterol esters (Advasterol Ester, AOM, Buenos Aires, Argentina) and 36 mg of alpha-tocopherol (SF 900 IU, AOM) were incorporated to the production batch destined for F cheeses only, while the other milk batch (with no added compounds) was previously used for C cheeses. Milk was cooled down to 37–38 °C by homogenization and a defined lyophilized starter culture of *S. thermophilus* (STI-14 CHR Hansen 50 U, Horsholm, Denmark) was added. The pH was monitored until a value of 6.7–6.9 was reached. Then, $CaCl_2$ (Alpha Química, Buenos Aires, Argentina) and rennet (Fromase DSM, Cordoba, Argentina) were incorporated. Milk was heated at 39–40 °C with a pH range between 6.4 and 6.5 until proper consistency was achieved. After coagulation, the Cu was drained and cut to obtain 4-kg cheese blocks, which were brined for 1 h in a saturated NaCl solution. Then, these pieces were divided in ~400 g portions, vacuum-packed in plastic bags and stored at 4 °C. RM, PM, PMS and Cu samples were collected aseptically at each elaboration stage. After collection, samples were immediately stored at 4 °C until their definitive storage at −80 °C. Packed cheeses were ripened at 4 °C and sampled at 7, 20, 40, 57 and 81 days post-elaboration. Packed cheeses from the entire production batch were randomly sampled for each treatment and stored at −80 °C until processing.

2.2. Moisture, sodium chloride and total fat content measurements

Total solids were determined in C and F cheese samples by water evaporation in a gravimetric atmospheric oven at 102 °C, according to the standards of the ISO/International Dairy Federation (ISO5534/IDF4:, 2004). Total fat content was measured by reading directly on a butyrometer scale, after separation of the cheese fat in the butyrometer by centrifugation, according to the van Gulik method (ISO3433/IDF222:, 2008). The concentration of sodium chloride was assessed by potentiometric titration, as specified by the ISO5943/IDF88:, 2006 guidelines.

2.3. Determination of fatty acid composition

The fatty acid profile was determined by gas chromatography based on previous reports (Folch et al., 1957; García et al., 2008), with some modifications. Briefly, total fatty acids were extracted from 2.5 mL of triplicate samples and the chloroform extracts were used to prepare fatty acid methyl esters (FAMEs). These were measured using a Varian CP-3800 equipment fitted with a flame ionization detector. FAMEs were separated using a capillary column (CP-Sil 88,100 m * 0.25 mm * 0.20 μm of Varian CP7489). Nitrogen was used as carrier gas with a 1:10 split ratio. The column temperature was programmed at 70 °C for 4 min, then increased from 70 to 170 °C at 13 °C/min and finally from 170 to 200 °C at 1 °C/min. The injection and detection were carried out at 250 °C. Fatty acids were identified by comparing relative retention times with individual fatty acids standard (PUFA-2 Animal Source

and Grain Fatty acid Methyl Ester Mix, Sigma-Aldrich, Missouri, USA). Analytical results are expressed as percentages of total fatty acids.

2.4. Determination of phytosterols

Phytosterols were extracted from F cheeses as reported by Slavin and Yu (2012), with modifications. Briefly, 0.5 g of each preparation, in triplicate samples, were mixed either with an equal volume of 0.5 M potassium phosphate buffer pH 7.7 or with methyl *tert*-butyl ether (MTBE) (Sigma-Aldrich) and homogenized for 1 min at a speed of 6 m/s (FastPrep-24, MP Biomedicals, California, USA). Thereafter, samples were placed in glass tubes with 2 mL of ethanol 1% butylated hydroxytoluene and 12 N KOH, followed by a saponification step of 2 h at 70 °C. After cooling the samples, phytosterols were extracted twice with 10 mL of *n*-hexane and washed with Na_2CO_3 to eliminate any residual fatty acid. The extracts were suspended in 1 mL of MTBE prior to injection. The analysis was performed by reverse-phase high-pressure liquid chromatography (HPLC) using a Thermo Separation Products instrument (San Jose, USA) connected to a Luna C8 column (Phenomenex, California, USA) of 50 mm × 4.6 mm id, 5 μm. The mobile phase consisted of water (containing 2% methanol to avoid contamination) at 1 mL/min and UV detection was carried out at 210 nm. Quantification was performed using calibration curves with standard campesterol, beta-sitosterol and stigmasterol (Sigma-Aldrich) at five concentration levels. Correlation coefficients ranged from 0.995 to 0.998.

2.5. Determination of tocopherols

Tocopherol in F cheese samples was determined by adapting previously described protocols from our laboratory (Rossetti et al., 2010). Briefly, 0.5 g of each triplicate sample was placed in a glass tube containing 2 mL of pyrogallol 1% ethanol and mixed by vortexing. Thereafter, 1.5 mL of 12 N KOH (Sigma-Aldrich) was added to each tube and samples were saponified for 30 min at 70 °C. After cooling, 2 mL of distilled water was added, and tocopherols were extracted twice with 5 mL of *n*-hexane (Sigma-Aldrich). The organic phases were conserved, evaporated under a nitrogen atmosphere, suspended in methanol and used for HPLC injection. Analysis was conducted with an Ultimate 3000 Dionex (Tokyo, Japan) using a fluorimetric detector. For phase separation, C18 columns were used (250 × 4.6 mm i.d., 5 μm HALO-5 column, AMT, Delaware, USA). The mobile phase consisted of ethanol/methanol (60:40) at isocratic conditions. The flow rate was adjusted to 0.8 mL/min, fluorescence detection was set at 296 nm for excitation and 330 nm for emission and the injection was performed with a volume of 20 μL. Tocopherols were quantified using calibration curves with alpha-tocopherol (Sigma-Aldrich) at five concentration levels. This method separated alpha and gamma tocopherols with correlation coefficients ranging from 0.996 to 0.999.

2.6. Concentration of bacterial cells from milk and cheese samples

Cell pellets were prepared based on previous protocols (Achilleos and Berthier, 2013; Stevens and Jaykus, 2004) with some modifications. Two 50-mL samples of milk were centrifuged at 9700 ×*g* for 15 min at 4 °C. The supernatants were discarded and cell pellets suspended in 40 mL of sterile solution containing 2% trisodium citrate-4% polyethylene glycol (PEG) 8000 (Sigma-Aldrich). The resulting mixtures from the two samples were placed in one tube, mixed by vortexing and divided in 20-mL fractions. Samples were centrifuged at 9700 ×*g* for 15 min at 4 °C and supernatants were decanted. Pellets were stored at −80 °C until nucleic acid extraction. For cheese samples, 4 g of each cheese treatment was mixed with 80 mL of 2% trisodium citrate-4% PEG 8000 solution and stomached for 4 min in a filter bag (Whirl Pack-Nasco, Wisconsin, USA). The homogenized mixture was divided in 20 mL samples. These fractions were then centrifuged at 9700 ×*g* for 15 min at 4 °C. The resulting supernatant was decanted and the remaining pellet was stored at −80 °C until nucleic acid extraction.

2.7. Purification of total DNA from starter, milk and cheese samples

Total DNA was extracted using DNAzol BD Reagent (Invitrogen, Massachusetts, USA) by following the manufacturer's instructions with a few modifications. Pellets containing bacterial cells from cheese and milk samples or lyophilized starter were suspended in DNAzol (9 mL of reagent/g of pellet or starter) and lysed by vortexing. Next, 0.4 mL of isopropanol was added, vortexed and incubated for 5 min at room temperature. Samples were centrifuged at 6000 ×*g* for 6 min. The supernatant was discarded and 0.5 mL of DNAzol was added to the DNA pellets which were vortexed until completely dispersed. These mixtures were centrifuged at 6000 ×*g* for 5 min and supernatants were removed. DNA pellets were washed with 1 mL of 75% ethanol and centrifuged at 6000 ×*g* for 5 min. Ethanol was decanted and samples were dried in a speed vacuum for 5 min at 45 °C. DNA pellets were then solubilized in 0.2 mL of GIBCO ultra-pure water (Invitrogen) and incubated at 56 °C for 5 min. For additional purification of the DNA, samples were centrifuged in a QIAshredder column (Qiagen, Hilden, Germany) at 12,000 ×*g* for 2 min. Finally, the filtrate was quantified with the Qubit 2.0 Fluorometer by using the dsDNA Assay HS Kit (Invitrogen). The efficiency of DNA extraction (yield) was maintained across all the matrices used in this work.

2.8. Purification of total RNA from starter, milk and cheese samples

Total RNA was obtained using TRIZol Reagent (Invitrogen) by adapting the manufacturer's instructions. Pellets containing bacterial cells from cheese and milk samples or lyophilized starter were suspended in TRIZol, placing 9 mL of reagent/g of pellet or starter. Samples were homogenized by vortexing and incubated for 5 min at room temperature. Next, 0.2 mL of chloroform per 1 mL of sample was added, mixed vigorously by hand and incubated for 2 min at room temperature. Samples were centrifuged at 12,000 ×*g* for 15 min at 4 °C. The aqueous phase was transferred to tubes containing isopropyl alcohol (0.5 mL for each mL of sample) and mixed by inversion. Samples were incubated for 10 min at room temperature and centrifuged at 12,000 ×*g* for 10 min at 4 °C. The supernatant was removed and the RNA washed once with 1 mL of 75% ethanol for each mL of sample, mixed by vortexing and centrifuged at 7500 ×*g* for 5 min at 4 °C. RNA pellets were dried in a speed vacuum for 5 min at 45 °C and then dissolved by pipetting in ultra-pure water treated with 1.12 g/L of diethyl pyrocarbonate (Sigma-Aldrich). Next, RNA was incubated at 56 °C for 5 min and quantified with the Qubit 2.0 Fluorometer by using the RNA Assay BR Kit (Invitrogen). To digest the DNA in the RNA samples, 1 μg of purified RNA was incubated for 15 min with 1 μL (1 U/μL) of Deoxyribonuclease I (Invitrogen). The efficiency of RNA extraction (yield) was maintained across all the matrices used in this work. Residual DNA was checked by qPCR in controls prepared with non-retrotranscribed RNA from all the samples evaluated, which always showed threshold cycle (Ct) values above the linear range of amplification.

2.9. Primer design and specificity assessment

Primer Express 3.0 (Applied Biosystems, California, USA) was used for the design of a *S. thermophilus* specific primer, based on its *tuf* gene sequence and on the *tuf* gene sequence of other LAB species, which were all retrieved from GenBank. These sequences were aligned with Clustal W2 and BLAST software was used to check *in silico* specificity. The nucleotide sequence of the primer pair was as follows: CGC GAC ACT GAC AAA CCA T (Stherm f), and CCA CGA CCA GTG ATT GAG AAT ACA (Stherm r). Our primer pair was compared against previously designed primers (Tuf) targeting this same gene of *S. thermophilus*

(Falentin et al., 2012). Since these primers are used with the same annealing temperature as our primers, they allowed direct comparison under the same qPCR amplification conditions. For these experiments, both primer sets (Stherm and Tuf) were tested against DNA extracted from selected samples containing varying amounts of target DNA, placing 0.2 ng of total DNA/μL of PCR reaction. For cross amplification experiments with lyophilized *Lactococcus lactis* subsp. *lactis* and L. *lactis* subsp. *cremoris* starter cultures (Danisco Choozit MA 14 LYO, Copenhagen, Denmark), 0.2–30 ng/μL was used. Real-time qPCR amplifications were carried out as described below (Section 2.10).

2.10. Real-time qPCR and RT-qPCR amplification conditions

Assays were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) and the data were analyzed with StepOne software v2.2. Different Stherm primer concentrations, from 40 to 600 nM were evaluated. Based on the results obtained, a concentration of 400 nM was selected. Amplifications were done in a final volume of 25 μL, containing 12.5 μL of QuantiTect SYBR Green PCR Kit (Qiagen), 5 μL of DNA or cDNA (0.2 ng/uL), 1 μL of each 200 nM primer (final concentration of 400 nM) and 5.5 μL of GIBCO ultra-pure water (Invitrogen). No-template controls as well as positive controls with known Ct values were included in every PCR run. Different thermal cycling conditions were evaluated and a working protocol was established as follows: one uracil-*N*-glycosylase preincubation step at 50 °C for 2 min, one cycle at 95 °C for 15 min followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min. A final denaturation step (melting curve) was carried out to assess amplicon melting temperature and to check for non-specific amplification products. The same thresholds defined with the DNA and cDNA standard curves were used to analyze Ct values from all samples. All nucleic acids used for the qPCR runs originated from independent extractions performed in triplicate samples. To obtain cDNA, 1 μg of purified RNA from each sample was incubated with DNase I (1 U/μL) (Invitrogen) for 15 min at room temperature, according to the manufacturer's instructions. Reverse transcription (RT) was carried out with the *M*-MLV Reverse Transcriptase Kit (Promega, Wisconsin, USA), using 5 μL of RNA and following the manufacturer's instructions. All samples were evaluated for the presence of residual DNA by performing RT reactions (without adding reverse transcriptase) in non-retrotranscribed controls. The Ct values obtained for the non-retrotranscribed samples were in all cases below the detection threshold of the assay.

2.11. Construction of standard curves

Autoclaved cheese (2 g; 121 °C for 30 min) samples were spiked with ten-fold amounts of the lyophilized *S. thermophilus* starter culture (0.01 μg – 10 mg) and subjected to DNA and RNA extraction. Samples were amplified by real-time qPCR or RT-qPCR and standard curves were constructed by plotting Ct values (mean of triplicate samples) against the logarithm (log) number of DNA/cDNA copies for *S. thermophilus*/μL of qPCR reaction. Autoclaved cheese samples used for the spiking procedures were previously checked for the absence of target nucleic acids. The same standard curves were constructed by inoculating autoclaved milk samples. The copy number was calculated according to the formula: number of copies = DNA or cDNA mass (ng) × 6.022×10^{23}/genome size (bp) × 650×10^{9}. The efficiency (E%), slope and correlation coefficient R^2 values of each standard curve were calculated with StepOnePlus software version 2.2 (Applied Biosystems). The limit of detection (LOD) was defined as the highest template dilution at which linearity was maintained and corresponded to the lowest value for which copy number calculation was accurate. Results for all samples analyzed are expressed as DNA/cDNA copy number/ng of total DNA/cDNA extracted.

2.12. Evaluation of free DNA and RNA detection threshold

PM samples (45 mL each) were spiked with four ten-fold dilutions (6 ng - 6 μg) of DNA or RNA obtained from the lyophilized *S. thermophilus* starter culture or with 22 mg of the starter itself. Samples were centrifuged at 9700 ×*g* for 15 min at 4 °C and supernatants were discarded. The resulting pellets, as well as cells from PM, were subjected to DNA and/or RNA extraction followed by qPCR or RT-qPCR, respectively. All the conditions were tested in triplicate samples.

2.13. Statistical analysis

Two-factor factorial designs were applied to the Ct values obtained from qPCR and RT-qPCR assays. One factor was assigned to the type of cheese produced (two levels: C and F) and the other one to the ripening day sampled (five levels: 7, 20, 40, 57 and 81 days). Differences in mean Ct values were analyzed by two-way analysis of variance (ANOVA) and all-pairwise comparisons were carried out using a Tukey's HSD test. Analyses were performed using InfoStat software (Di Rienzo et al., 2015).

3. Results

3.1. Cheese compositional analysis

The compositional analysis performed on F and C cheeses is shown in Table 1. As expected, total fat content was higher in F cheeses containing phytosterols and tocopherols than in C cheeses. Despite of this higher fat content, F cheeses could still be classified under the "light (reduced fat) cheese" category, according to the Argentine Food Code (2012). The content of alpha-tocopherol present in F cheeses (5.57 mg/60 g) reflected their efficient incorporation. Importantly, this amount was equivalent to ~50% of the recommended dietary allowance (RDA) (Institute of Medicine, 2000) for this vitamin. Also, total content of phytosterols in F cheeses revealed that the amounts present in the final product were above (2.51 mg/60 g of cheese) the dosage necessary to exert health benefits on human health (~2 mg of phytosterols per day), as recommended by the American Heart Association (AHA) Nutrition Committee (Lichtenstein and Wylie-Rosett, 2006). Accordingly, the fatty acid profile observed in F cheeses, characterized by a higher relative amount of polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) at the expense of saturated fatty acids (SFA), reflected the presence of the added phytosterols in their esterified form (Table 1). In addition, the higher content of plant-derived gamma-tocopherol recorded in F cheeses was also consistent with the presence of phytosterols, since this isomer constitutes a by-product according to the commercial supplier.

3.2. Primer specificity

To determine primer specificity of the qPCR assays, we compared our primer pair (Stherm) against primers (Tuf) also targeting the *tuf* gene from *S. thermophilus* previously reported by Falentin et al. (2012), across selected samples containing varying amounts of target DNA (Table 2). The microbial diversity of RM used for these experiments was confirmed by denaturing gradient gel electrophoresis (DGGE) analysis (data not shown). In addition, both primer sets were evaluated against DNA obtained from lyophilized L. *lactis* subsp. *lactis* and L. *lactis* subsp. *cremoris*, which was found in our RM samples by DGGE experiments (data not shown) but was not studied by Falentin et al. (2012).

In all cases, qPCR results showed that the samples analyzed with both primer pairs had similar Ct values. Although Stherm primers produced slightly higher Ct values, this pattern was maintained across all samples, including those like RM or PM, where little or no target DNA should be expected. This indicated that the sensitivity of both primer

Table 1
Compositional analysis for semi-soft, reduced-fat cheeses produced at the industrial level.

Parameter	C cheeses[a]	F cheeses[a]
g/100 g		
Moisture	54.29	52.9
NaCl	1.93	1.64
Fat	15.2	20.61
(%)		
PUFA	4.8	7.34
MUFA	30.25	33.79
SFA	64.95	58.87
mg/60 g		
Stigmasterol	ND	0.85
Campesterol	ND	0.67
Beta-sitosterol	ND	0.68
Total phytosterols	ND	2.51
Alpha-tocopherol	0.25	5.57
Gamma-tocopherol	0.05	5.18
Total tocopherols	0.3	10.75

[a] Results are mean values obtained from cheeses sampled at 90 days of ripening, except for moisture, NaCl, and fat determinations which were performed at 20 days after elaboration. C: control, F: functional, PUFA: polyunsaturated fatty acids, MUFA: monounsaturated fatty acids, SFA: saturated fatty acids, ND: not detected. PUFA, MUFA and SFA are expressed as percentage of total fatty acids detected.

sets was similar (Table 2). Most importantly, the ratio between RM and the other samples containing *S. thermophilus* DNA (PM, PM + St, PMS, St) was very similar for both Stherm and Tuf primers, showing that they are also similar in terms of specificity. As expected, DNA extracted from PM and L. *lactis* starter culture presented the highest Ct numbers (below the detection threshold of the assay) with both primer sets. Melting curves for Stherm and Tuf primer pairs always presented one peak only (data not shown), confirming the specificity of the amplification.

3.3. Standard curves for Streptococcus thermophilus

To calculate *S. thermophilus* concentration by real-time qPCR and RT-qPCR, standard curves were generated from autoclaved cheese (without detectable target DNA) inoculated with ten-fold concentrations of lyophilized starter culture (Fig. 1). Correlation coefficients between Ct values and copy numbers presented R^2 values of 0.999 for both DNA and cDNA standard curves, indicating a good linear correlation. These data showed that the detection spectrum was linear across a range of 7 log units. Efficiency values were 94.91% for DNA and 93.47% for cDNA curves, respectively. The LOD in the spiked-cheese matrix corresponded to 74 copies of DNA/well and 103 copies of cDNA/well. Similar results were obtained for standard curves performed by spiking autoclaved milk samples (data not shown).

Table 2
Comparison between primer pairs targeting dairy starter *Streptococcus thermophilus*.

Sample	Stherm[a]	Tuf[a]
PM	36.58 ± 0.23	36.12 ± 0.05
RM	31.53 ± 0.24	31.01 ± 0.15
St	12.71 ± 0.28	12.23 ± 0.23
PM + St	18.56 ± 0.10	18.08 ± 0.19
PMS	18.58 ± 0.31	17.93 ± 0.66
C7d	23.18 ± 0.46	22.34 ± 0.17
F7d	21.35 ± 0.18	21.11 ± 0.17
Llactis	39.73 ± 0.05	38.26 ± 0.21
PM/RM	1.16	1.16
RM/PMS	1.69	1.72
RM/St	2.48	2.53
RM/PM + St	1.69	1.71

[a] Results are expressed as mean Ct values of triplicate samples. PM/RM, RM/PMS, RM/St and RM/PM + St represent Ct ratios. Stherm: primers designed in this study, Tuf: primers reported by Falentin et al. (2012). Total DNA extracted from selected samples: PM (pasteurized milk), RM (raw milk), St (lyophilized starter culture *S. thermophilus*), PM + St (45 mL of PM sample spiked with 22 mg of *S. thermophilus* starter), PMS (PM sampled at the industrial plant 30 min after starter addition), C7d (control cheese ripened for 7 days at 4 °C), F7d (functional cheese containing phytosterol esters and alpha-tocopherol ripened for 7 days at 4 °C), Llactis (lyophilized starter culture *Lactococcus lactis* subsp. *lactis* subsp. *cremoris*).

3.4. Effect of free DNA and RNA concentration on qPCR and RT-qPCR results

An experimental set-up was developed to evaluate whether our nucleic acid extraction protocols could be affected by genetic material from sources other than pelleted cells. For this purpose, PM samples (negative for *S. thermophilus*) were spiked with ten-fold amounts (6 ng – 6 μg) of either DNA or RNA obtained from lyophilized starter. Alternatively, 22 mg of the same starter were placed in another group of PM samples. The amount chosen was 22 mg, because it yielded 6000 ± 100 ng of DNA when inoculated on PM, which is the same mass of free DNA present in the most concentrated dilution evaluated. Pellets from DNA-spiked milk treatments, as well as cells harvested from PM only (negative control) and lyophilized starter (positive control) were subjected to DNA and RNA extraction followed by qPCR or RT-qPCR, respectively (Fig. 2).

As expected, pelleted cells obtained from milk samples containing lyophilized starter showed the presence of both target DNA (2.53×10^5 mean copies/ng of total DNA) and cDNA (1.94×10^5 mean copies/ng of total cDNA), while PM samples were negative for both *S. thermophilus* DNA and RNA. In the same way, PM samples contaminated with *S. thermophilus* RNA were negative, even at the highest amount (6 μg) evaluated. The same negative results were also observed for PM samples containing up to 600 ng of DNA. However, 45-mL milk samples which had been spiked with the highest amounts (6 μg) of free DNA (added in levels that tested positive for PM inoculated with lyophilized starter), showed the presence of target DNA (8.91×10^4 mean copies/ng of total DNA). These results demonstrate that the bacterial DNA detected by qPCR cannot be used as an accurate target to assess cell integrity, even if using nucleic acid extraction protocols which involve pellet concentration. At the same time, the failure to detect free prokaryotic RNA using this experimental set-up suggests that these molecules may provide a better molecular signature to estimate bacterial viability by real-time qPCR (Fig. 2).

3.5. Streptococcus thermophilus quantification during industrial elaboration of F and C cheeses

To study the dynamics of the *S. thermophilus* starter culture at the industrial level, milk and cheese sampled at different steps throughout F and C cheesemaking, were subjected to qPCR and RT-qPCR according to the protocols optimized in this study (Fig. 3). Additionally, cheeses were sampled at different time points after vacuum-packing and ripening at 4 °C, including 7, 20, 40, 57 and 81 days of ripening (Fig. 4).

RM analysis revealed low levels of both *S. thermophilus* DNA (3.01×10^4 copies/ng of total DNA) and RNA (1.72×10^3 cDNA copies/ng of total cDNA). After pasteurization, neither target DNA nor RNA could be accurately measured (mean Ct values were outside the linear range of quantification (>36) in PM samples (Fig. 3)). Milk samples obtained 30 min after lyophilized *S. thermophilus* starter culture incorporation showed a substantial increase in both target DNA and RNA levels. This way, the amount of starter DNA in PMS increased to 2.50×10^5 copies/ng of total DNA whereas the amount of cDNA increased to 2.51×10^5 copies/ng of total cDNA. The mean concentration of *S. thermophilus* genome copies was ~8 fold higher than that observed in RM. Interestingly, cDNA copies presented even more differences, with mean values ~145 fold higher in PMS than in RM. No differences were recorded in mean starter DNA and cDNA concentrations between PMS obtained from the production batch corresponding to F cheeses and that corresponding to C cheeses. Overall, PMS showed the highest levels of both starter DNA and RNA from all the samples evaluated along the

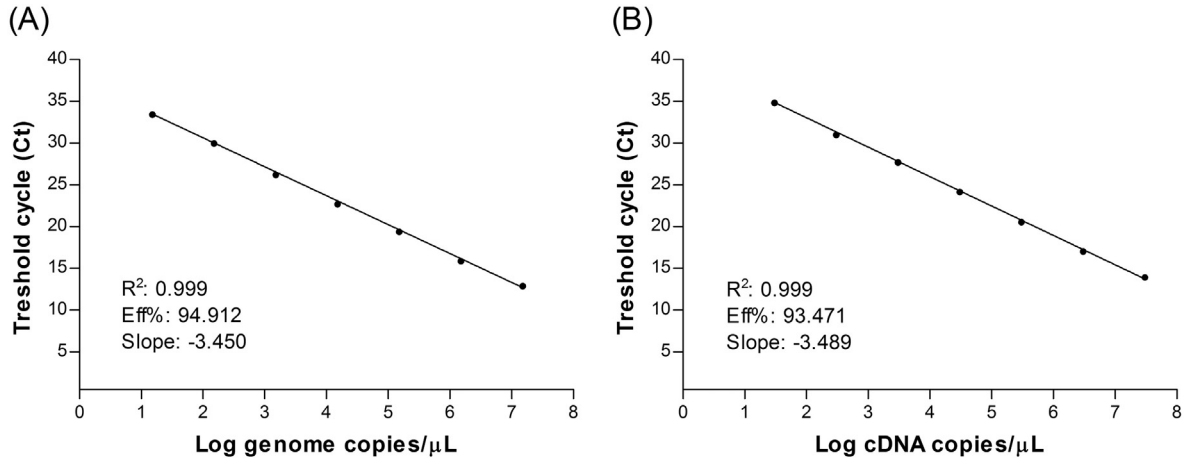

Fig. 1. Standard curves obtained by qPCR and RT-qPCR for *Streptococcus thermophilus*. Cheese samples (2 g each) were spiked with ten-fold amounts of the lyophilized starter (0.01 µg – 10 mg) and subjected to DNA and RNA extraction for subsequent amplification by real-time qPCR (panel A) or RT-qPCR (panel B), respectively. Each point represents the mean value of triplicate samples ± standard deviations (SD). Standard curves were constructed by plotting threshold cycles (Ct) values against the logarithm (log) number of DNA (panel A)/cDNA (panel B) copies for *S. thermophilus*/µL of qPCR reaction.

manufacturing steps. These same profiles were also observed for the next elaboration step (Cu) evaluated (Fig. 3).

However, cheeses sampled after 7 days of ripening revealed a scenario different from that of the earlier production stages studied. Although a marked decrease in *S. thermophilus* DNA and RNA concentration was observed, this decrease was less pronounced for F cheeses. C cheeses presented mean copy numbers of 1.67×10^5 copies/ng of total DNA and 1.57×10^5 copies/ng of total cDNA, respectively, while F cheeses displayed mean copy numbers of 2.02×10^5 copies/ng of total DNA and 1.84×10^5 copies/ng of total cDNA, respectively. Moreover, statistically significant differences in mean copy numbers were recorded between F and C cheeses, both for DNA ($p = 0.0003$) and cDNA (<0.0001) levels (Fig. 3).

At 20 days of ripening, mean levels of *S. thermophilus* DNA were maintained in relation to the previous time point sampled at 7 days, recording a non-significant increase in both F (2.10×10^5 copies/ng of total DNA) and C cheeses (1.70×10^5 copies/ng of total DNA). RNA analysis showed a similar picture, with mean levels of target cDNA of 1.54 copies/ng of total cDNA and 1.89 copies/ng of total cDNA, for C and F cheeses respectively. Differences between starter DNA and RNA levels in F and C cheeses also maintained significant differences at this time point (Fig. 4).

F cheeses evaluated at 40 days of ripening showed a level of starter genome copies (2.09×10^5 copies/ng of total DNA) similar to that recorded at 20 days, while C cheeses revealed a moderate increase (1.89×10^5 copies/ng of total DNA) which did not reach statistical significance. Interestingly, RNA data showed a different situation where a significant decrease in mean concentration of *S. thermophilus* cDNA copies was observed for F (1.57×10^5 copies/ng of total cDNA) and C cheeses (1.25×10^5 copies/ng of total cDNA). Also, the higher (statistically significant) values recorded at 20 days for F than for C cheeses, remained only in terms of cDNA copies but not for DNA (Fig. 4).

At 57 days of ripening, mean *S. thermophilus* genome copies showed almost the same concentration for C and F cheeses, with values of 2.23×10^5 copies and 2.20×10^5 copies/ng of total DNA, respectively. In relation to the previous time point evaluated (40 days), these numbers represented a significant relative increase for C cheeses (Fig. 4). A marked increase was observed for mean *S. thermophilus* RNA levels at 57 days in both F (2.25×10^5 copies/ng of total cDNA) and C cheeses (1.98×10^5 copies/ng of total cDNA). These were the highest values observed in all ripened cheeses, and were significantly higher than those recorded at 7, 20 and 40 days. However, they were lower than the cDNA concentrations found in PMS samples. Importantly, C and F cheeses showed similar mean cDNA kinetics throughout ripening until

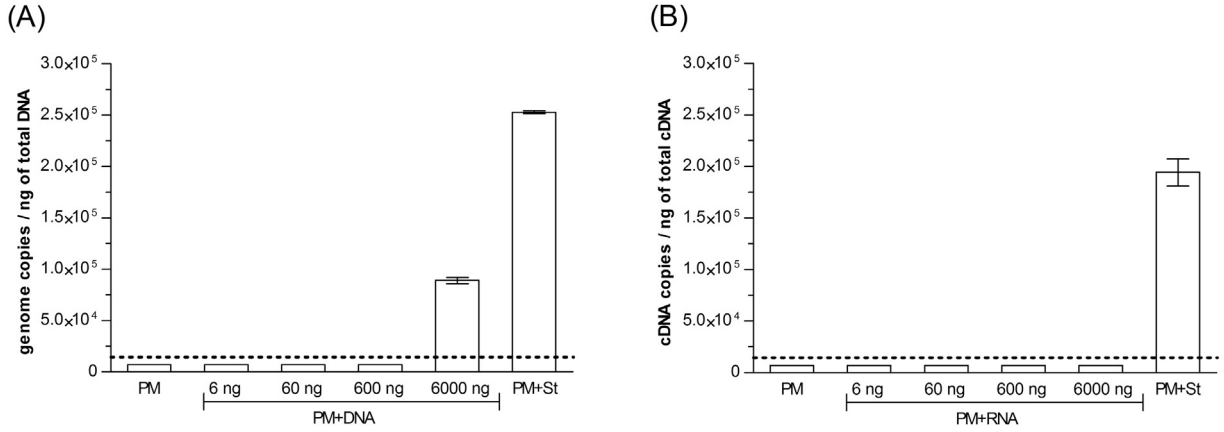

Fig. 2. Free DNA and RNA detection threshold determined by qPCR and RT-qPCR for nucleic acids extraction methods based on pelleted microorganisms. Pasteurized milk (PM) samples (45 mL each) were spiked with ten-fold dilutions (6 ng – 6000 ng) of DNA (PM + DNA) or RNA (PM + RNA) extracted from the lyophilized *Streptococcus thermophilus*. Another group of PM samples were mixed with 22 mg of this same starter culture (PM + St). Concentrated pellets harvested from these samples and from PM only (negative control) were subjected to nucleic acid purification for qPCR (panel A) or RT-qPCR (panel B) assays. Results are expressed as the number of *S. thermophilus* DNA/cDNA copies per ng of total DNA/cDNA obtained from milk samples. Each bar represents the mean value of triplicate samples ± SD. The detection limit of the qPCR protocol is represented by a dotted line.

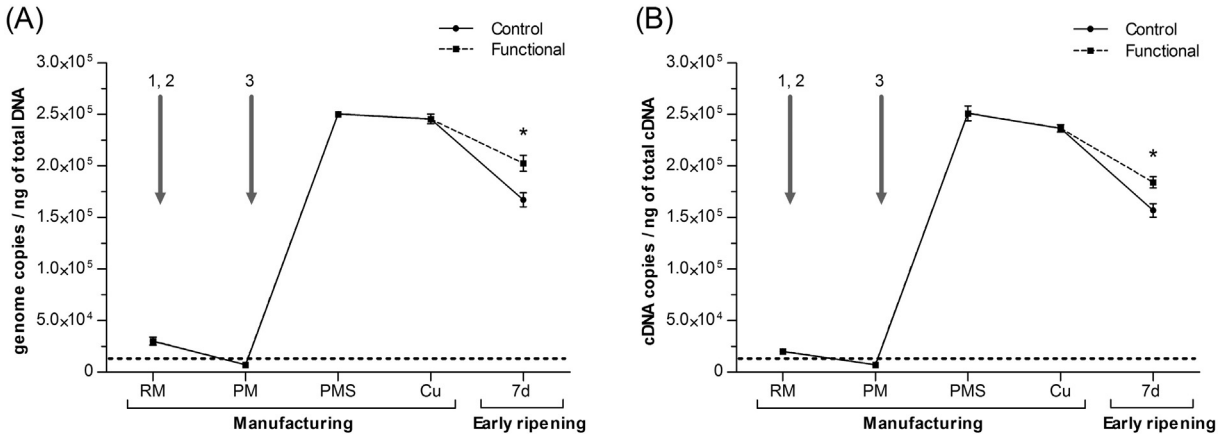

Fig. 3. Nucleic acids kinetics for the starter culture *Streptococcus thermophilus* throughout industrial production of functional (F) and control (C) cheeses. Raw milk (RM), pasteurized milk (PM), milk pasteurized 30 min after starter incorporation (PMS), curd (Cu), and cheeses at 7 days of ripening (7d) were subjected to total DNA and RNA extraction for qPCR (panel A) and RT-qPCR (panel B), respectively. Results are expressed as the number of starter DNA/cDNA copies per ng of total DNA/cDNA obtained from each sample. Each point represents the mean value of triplicate samples ± SD. The detection limit of the qPCR protocol is represented by a dotted line. Relevant manufacture procedures performed at the industrial plant are indicated by arrows: (1) pasteurization (72 °C for 15 s), (2) incorporation of phytosterol esters (7.6 g/L of milk) and α-tocopherol (14.4 μg/L of milk). (3) Addition of lyophilized starter *S. thermophilus* (0.02 U/L of milk). (*) Statistically significant differences (SSD) in mean concentration of starter DNA ($p = 0.0003$) or cDNA ($p < 0.0001$) between C and functional F cheeses.

57 days, while conserving significant differences between C and F cheeses (Fig. 4).

After 81 days of ripening, mean starter genome copies continued to increase in F (2.26×10^5 copies/ng of total DNA) but not in C cheeses. The same as with samples from 7 and 20 days, the higher values in target DNA copies obtained for F than for C cheeses at this time point recovered statistical significance. RNA analysis showed a significant decrease in the mean concentration of *S. thermophilus* cDNA for F cheeses only (1.70×10^5 copies/ng of total cDNA), and only a small decline for C cheeses (1.91×10^5 copies/ng of total cDNA) (Fig. 4).

4. Discussion

The elaboration of functional food products for the prevention of degenerative diseases represents a technological challenge, since the incorporation of biologically active compounds can modify rheological and/or organoleptic attributes. It is also known that the performance of starter microorganisms is critical for optimal cheesemaking and for providing potential probiotic benefits. Phytosterols (plant sterols and stanols) are a family of lipophilic triterpenes which have been used for the design of functional dairy products due to their positive outcomes on cardiovascular disease risk. However, their effect on the starter culture robustness during cheesemaking had not been yet studied.

In this study, we analyzed the dynamics of the extensively used dairy starter *S. thermophilus* during manufacture and ripening of a semi-soft, reduced-fat functional cheese containing phytosterol esters (campesterol, beta-sitosterol and stigmasterol) and alpha-tocopherol. For this goal, real-time qPCR and RT-qPCR assays were optimized and applied to samples obtained during the production of functional and control cheeses. Importantly, phytosterol esters were incorporated into the cheese matrix at optimal amounts to provide health benefits, while alpha-tocopherol was present at levels equivalent to ~50% of the RDA (Institute of Medicine, 2000). Therefore, the success in the elaboration process of functional cheeses allowed studying the impact of phytosterols and alpha-tocopherol on the kinetics of *S. thermophilus* nucleic acids, with the certainty that any change in starter performance could be correlated only to the incorporation of these bioactive compounds. Besides, any possible effect of phytosterol esters would be the result of functional concentrations, providing important information for industrial applications.

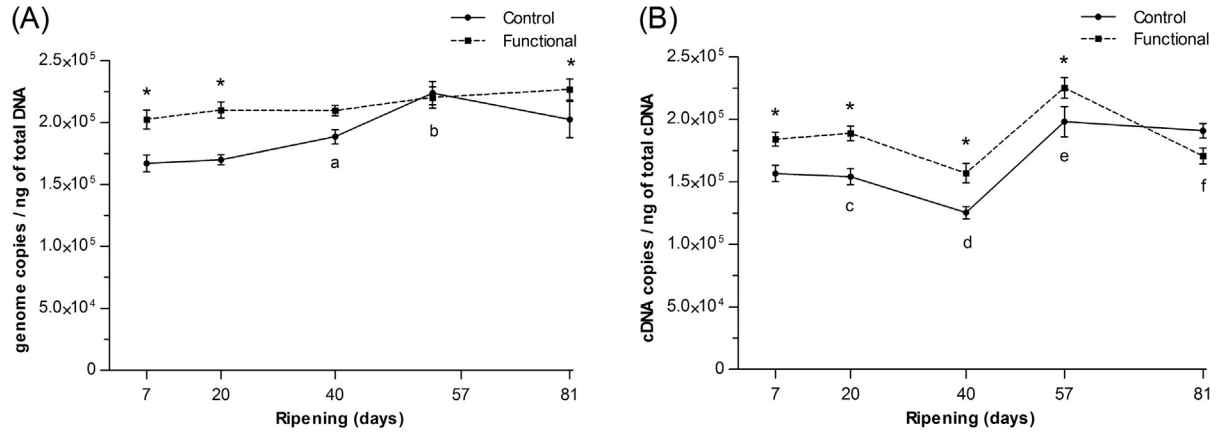

Fig. 4. Nucleic acids kinetics for the starter culture *Streptococcus thermophilus* during ripening of functional (F) and control (C) cheeses. All cheeses manufactured at the industrial plant were vacuum packed and ripened at 4 °C. Samples from F and C cheeses were randomly obtained at different time-points (7, 20, 40, 57, 81 days of ripening) and subjected to total DNA and RNA extraction for qPCR (panel A) and RT-qPCR (panel B), respectively. Results are expressed as the number of *S. thermophilus* DNA/cDNA copies per ng of total DNA/cDNA obtained from each sample. Each point represents the mean value of triplicate cheeses ± SD. (*) Statistically significant differences (SSD) in mean concentration of starter DNA ($p = 0.0003$) or cDNA ($p < 0.0001$) between C and F cheeses. (a, b) SSD in mean concentration of starter DNA between 40 and 57 days of ripening for C cheeses ($p = 0.0003$). (c, d, e) SSD in mean concentration of starter cDNA between 20 and 40 days of ripening, and between 40 and 57 days of ripening for both F and C cheeses ($p < 0.0001$ in both cases). (e, f) SSD in mean concentration of starter cDNA between 57 and 81 days of ripening for F cheeses ($p < 0.0001$).

Most of the studies analyzing microorganisms by molecular methods rely on DNA or RNA extraction protocols based on the concentration of microbial cells prior to nucleic acid extraction (Stevens and Jaykus, 2004). However, it is not known whether these extraction techniques could be affected by genetic material from sources other than pelleted cells. To clarify these issues, we employed an in-house experimental set-up based on spiking negative milk samples with known amounts of nucleic acids obtained from lyophilized starter *S. thermophilus* or with the bacterium itself, followed by DNA and RNA extraction from pelleted cells and qPCR or RT-qPCR, respectively. To our knowledge, this simple and straight-forward approach constitutes the first experimental evidence demonstrating that microbial DNA cannot be used to estimate cell integrity or viability for quantification purposes, even with nucleic acid extraction protocols based on concentration of bacterial cells. In contrast, RNA molecules may be a more reliable target to assess bacterial viability and metabolic activity.

To evaluate primer specificity of the qPCR assays, we compared our primer pair against previously reported primers (Falentin et al., 2012) targeting also the *tuf* gene from *S. thermophilus*. These were chosen because, unlike the rest of published primers targeting this bacterium, they have been tested for the absence of cross reactivity against six genera of microorganisms which are commonly found in dairy products. It is important to mention that the use of raw milk in the present study, well-known for its high richness and diversity of microbial species (Aldrete-Tapia et al., 2014; Delcenserie et al., 2014; Delgado et al., 2013; De Pasquale et al., 2014), allowed comparing non-specific amplification of both primer pairs in a real setting where other LAB represent a potential but genuine source of quantification bias. Overall, the information obtained in the present work confirms the usefulness of the *tuf* gene for designing primers aimed to quantify LAB species (Achilleos and Berthier, 2013; Falentin et al., 2012; Ruggirello et al., 2014), indicating that they can be reliable also when tested in a real dairy ecosystem.

In the present study, samples obtained from raw milk showed low levels of both *S. thermophilus* DNA and RNA. These findings are consistent with recent data from several reports showing that this bacterium can be detected in cow's raw milk in varying levels, both by culture-dependent (Delgado et al., 2013; Bennama et al., 2012) and culture-independent methods (Aldrete-Tapia et al., 2014; Delgado et al., 2013; De Pasquale et al., 2014; Dolci et al., 2014; Masoud et al., 2012). Neither starter DNA nor RNA was detected after milk pasteurization at 72 °C for 15 s. To our knowledge, only the work by Delgado et al. (2013) analyzed the presence of *S. thermophilus* in pasteurized milk by molecular methods, which detected DNA from this microorganism by PCR-DGGE and 16S rRNA pyrosequencing. Delcenserie et al. (2014) did not analyze pasteurized milk, but similarly to our results, by pyrosequencing analysis, they found that *S. thermophilus* was present only in Herve cheese samples produced with raw milk but not with pasteurized milk. Therefore, the available evidence strongly suggests that the initial quantity of this species in raw milk, rather than the assay sensitivity, is the ultimate determinant of detection after milk pasteurization.

The results obtained in this study during the industrial manufacture and ripening of a semi-soft, reduced-fat cheese indicates that *S. thermophilus* DNA and RNA could be quantified until the last ripening time point evaluated (81 days), both in functional and control cheeses. This information confirms previous studies obtained also by qPCR and RT-qPCR (Carraro et al., 2011, Falentin et al., 2012) and by other molecular methods, such as DGGE or next-generation sequencing (Aldrete-Tapia et al., 2014; Cocolin et al., 2013; De Pasquale et al., 2014; Dolci et al., 2014; Masoud et al., 2011; Masoud et al., 2012). Based on the differences obtained between culture-independent and culture-dependent experiments performed in many of these works, it is possible that SLAB remain during ripening mainly in a viable but nonculturable (VNC) state, playing an active role not only in the initial acidification events but also in the shaping of rheological and sensory attributes (Cocolin et al., 2013; Ruggirello et al., 2014). The study of this VNC condition may be of great technological interest, especially if we consider experimental evidence indicating that several volatile metabolites synthesized by the SLAB L. *lactis* are only generated under a system involving non-growing cells (van de Bunt et al., 2014).

Here we showed that the addition of phytosterols during cheesemaking, at concentrations that produced functional amounts in the final product, correlated with a significant increase in *S. thermophilus* DNA and cDNA levels in most of the time points evaluated. In the present manufacturing conditions, these results were achieved with the addition of both phytosterol esters (7.6 g/L of milk) and alpha-tocopherol (14.4 μg/L of milk). While the existence of antioxidant mechanisms operating in LAB have been well established both *in vitro* and *in vivo* (Amaretti et al., 2013; Bruno-Bárcena et al., 2010), to our knowledge no studies are available regarding the effect of isolated tocopherols on the redox activity or growth of these microorganisms. Results from previous industrial elaborations (unpublished data from our laboratory) have shown that several oxidation parameters were not different between phytosterol-enriched cheeses (with or without added tocopherols) and control cheeses up to 92 days of ripening. This indicates that despite their PUFA content, phytosterol esters may not promote lipid-mediated oxidation throughout ripening when incorporated during cheesemaking, independently of the presence or absence of tocopherols. Regardless of this information, whether the effect on starter performance could have been obtained without the incorporation of alpha-tocopherol cannot be known based on our results. However, our findings are in accordance with previous studies evaluating the effect of plant compounds, including isolated phytosterols, on *S. thermophilus* behavior during fermented dairy formulations. Indeed, the ability of this bacterium to thrive in soy beverages has been demonstrated by several culture-dependent studies (Angeles and Marth, 1971; Chumchuere and Robinson, 1999; Karleskind et al., 1991). Besides, it has been found that this species grows faster in a soy formulation than in cow's milk (Farnworth et al., 2007). Similarly, Shori (2013) showed that *S. thermophilus* achieves higher plate counts in cow's or camel's milk if they contain soybean water extract. Although the data obtained by these authors cannot reveal whether the phytosterol content in soy contributed in some way to the results achieved, it has been shown that only the addition of phytosterols (beta-sitosterol, campesterol, beta-sitostanol) to yogurt (up to 1.8% w/v) either does not affect or slightly increases the performance of the SLAB *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Monu et al., 2008). Although this finding was recorded for yogurt by culture-dependent methods, our results were obtained by studying cheesemaking. Thus, these previous observations were confirmed here by molecular methods in a system where the fermentation periods and hence the time of exposure of SLAB to biologically active compounds, are much longer. Since the expiry date for Port-Salut light cheese is 60 days after elaboration, this report indicates that *S. thermophilus* was present until the end of ripening presumably in a metabolically active state. Therefore, phytosterol esters and alpha-tocopherol, in our manufacturing conditions, could enhance growth of the starter culture in semi-soft, low-fat cheese.

As a whole, our results may have relevant implications for the study of microbial viability based on nucleic acid extraction and quantification protocols, as well as for the design of fermented dairy products containing plant sterols or other related bioactive molecules. Further experiments are needed to assess the molecular mechanisms underlying the favorable adaptations in the starter *S. thermophilus* which, in the present work, correlated with the addition of phytosterols and tocopherols during industrial cheese production.

Acknowledgments

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References

Achilleos, C., Berthier, F., 2013. Quantitative PCR for the specific quantification of *Lactococcus lactis* and *Lactobacillus paracasei* and its interest for *Lactococcus lactis* in cheese samples. Food Microbiol. 36 (2), 286–295.

Agerholm-Larsen, L., Bell, M.L., Grunwald, G.K., Astrup, A., 2000. The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. Eur. J. Clin. Nutr. 54 (11), 856–860.

Aldrete-Tapia, A., Escobar-Ramírez, M.C., Tamplin, M.L., Hernández-Iturriaga, M., 2014. High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese. Food Microbiol. 44, 136–141.

Amaretti, A., di Nunzio, M., Pompei, A., Raimondi, S., Rossi, M., Bordoni, A., 2013. Antioxidant properties of potentially probiotic bacteria: in vitro and in vivo activities. Appl. Microbiol. Biotechnol. 97 (2), 809–817.

Angeles, A.G., Marth, E.H., 1971. Growth and activity of lactic-acid bacteria in soymilk. I: growth and acid production. J. Milk Food Technol. 34, 30–36.

Argentine Food Code (CAA), 2012. Código Alimentario Argentino (Art. 625). Ed. De La Canal & Asociados SRL, Buenos Aires, Argentina.

Bennama, R., Fernández, M., Ladero, V., Alvarez, M.A., Rechidi-Sidhoum, N., Bensoltane, A., 2012. Isolation of an exopolysaccharide-producing *Streptococcus thermophilus* from Algerian raw cow milk. Eur. Food Res. Technol. 234, 119–125.

Bruno-Bárcena, J.M., Azcárate-Peril, M.A., Hassan, H.M., 2010. Role of antioxidant enzymes in bacterial resistance to organic acids. Appl. Environ. Microbiol. 76 (9), 2747–2753.

Carraro, L., Maifreni, M., Bartolomeoli, I., Martino, M.E., Novelli, E., Frigo, F., Marino, M., Cardazzo, B., 2011. Comparison of culture-dependent and –independent methods for bacterial community monitoring during Montasio cheese manufacturing. Res. Microbiol. 162 (3), 231–239.

Chumchuere, S., Robinson, R.K., 1999. Selection of starter cultures for the fermentation of soya milk. Food Microbiol. 16, 129–137.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., Rantsiou, K., 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. Int. J. Food Microbiol. 167 (1), 29–43.

De Pasquale, I., Di Cagno, R., Buchin, S., De Angelis, M., Gobbetti, M., 2014. Microbial ecology dynamics reveal a succession in the core microbiota involved in the ripening of pasta filata caciocavallo pugliese cheese. Appl. Environ. Microbiol. 80 (19), 6243–6255.

Delcenserie, V., Taminiau, B., Delhalle, L., Nezer, C., Doyen, P., Crevecoeur, S., Roussey, D., Korsak, N., Daube, G., 2014. Microbiota characterization of a Belgian protected designation of origin cheese, Herve cheese, using metagenomic analysis. J. Dairy Sci. 97 (10), 6046–6056.

Delgado, S., Rachid, C.T., Fernández, E., Rychlik, T., Alegría, A., Peixoto, R.S., Mayo, B., 2013. Diversity of thermophilic bacteria in raw, pasteurized and selectively-cultured milk, as assessed by culturing, DGGE and pyrosequencing. Food Microbiol. 36 (1), 103–111.

Desfossés-Foucault, É., LaPointe, G., Roy, D., 2013. Dynamics and rRNA transcriptional activity of lactococci and lactobacilli during Cheddar cheese ripening. Int. J. Food Microbiol. 166 (1), 117–124.

Di Marzio, L., Centi, C., Cinque, B., Masci, S., Giuliani, M., Arcieri, A., Zicari, L., De Simone, C., Cifone, M.G., 2003. Effect of the lactic acid bacterium *Streptococcus thermophilus* on stratum corneum ceramid levels and signs and symptoms of atopic dermatitis patients. Exp. Dermatol. 12 (5), 615–620.

Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW, 2015. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. (URL) http://www.infostat.com.ar)

Dolci, P., De Filippis, F., La Storia, A., Ercolini, D., Cocolin, L., 2014. rRNA-based monitoring of the microbiota involved in Fontina PDO cheese production in relation to different stages of cow lactation. Int. J. Food Microbiol. 185, 127–135.

Falentin, H., Henaff, N., Le Bivic, P., Deutsch, S.M., 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.

Farnworth, E.R., Mainville, I., Desjardins, M.P., Gardner, N., Fliss, I., Champagne, C., 2007. Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. Int. J. Food Microbiol. 116, 174–181.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Fox, P.F., Guinee, T.P., Cogan, T.M., McSweeney, P.L.H., 2000. Fundamentals of Cheese Science. AN Aspen Publishers Inc., Gaithersburg, Ma., USA.

Fox, P.F., McSweeney, P.L., Cogan, T.M., Guinee, T.P., 2004. Cheese: Chemistry, Physics and Microbiology: General Aspects, 3rd Ed. Vol. 1. Elsevier Academic, Oxford, United Kingdom.

García, P.T., Pensel, N.A., Sancho, A.M., Latimori, N.J., Kloster, A.M., Amigone, M.A., Casal, J.J., 2008. Beef lipids in relation to animal breed and nutrition in Argentina. Meat Sci. 79, 500–508.

Gatti, M., Bottari, B., Lazzi, C., Neviani, E., Mucchetti, G., 2014. Invited review: microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. J. Dairy Sci. 2, 573–591.

Gluck, U., Gebbers, J.O., 2003. Ingested probiotics reduce nasal colonization with pathogenic bacteria (*Staphylococcus aureus*, Streptococcus pneumonia and β haemolytic streptococci). Am. J. Clin. Nutr. 77 (2), 517–520.

Hickson, M., D'Souza, A.L., Muthu, N., Rogers, T.R., Want, S., Rajkumar, C., Bulpitt, C.J., 2007. Use of probiotic Lactobacillus preparation to prevent diarrhoea associated with antibiotics: randomised double blind placebo controlled trial. Br. Med. J. 335 (7610), 80.

Institute of Medicine, 2000. Food and nutrition board. Dietary Reference Intakes: Vitamin C, Vitamin E, Selenium and Carotenoids. National Academy Press, Washington DC.

International Organization for Standardization/International Dairy Federation (ISO/IDF), 2004a. ISO 5534/IDF 4:2004: Reference Method for the Determination of the total Solids Content of Cheese and Processed Cheese.

International Organization for Standardization/International Dairy Federation (ISO/IDF), 2006a. ISO 5943/IDF 88:2006: Potentiometric Titration Method for the Determination of the Chloride Content of Cheese and Processed Cheese Products.

International Organization for Standardization/International Dairy Federation (ISO/IDF), 2008a. ISO 3433/IDF 222:2008: Van Gulik Method for the Determination of the Fat Content, as a Mass Fraction, of Cheese.

Irlinger, F., Mounier, J., 2009. Microbial interactions in cheese: implications for cheese quality and safety. Curr. Opin. Biotechnol. 20, 142–148.

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

Karleskind, D., Laye, I., Halpin, E., Morr, C.V., 1991. Improving acid production in soy-based yogurt by adding cheese whey proteins and mineral salts. J. Food Sci. 56, 999–1001.

Lichtenstein, A.H., Wylie-Rosett, J., 2006. Diet and lifestyle recommendations revision 2006. A scientific statement from the American Heart Association Nutrition Committee. Circulation 114, 82–96.

Martirosyan, D.M., 2011. Introduction to Functional Food Science. second ed. Food Science Publisher.

Masoud, W., Takamiya, M., Vogensen, F.K., Lillevang, S., Abu Al-Soud, W., Sørensen, S.J., Jakobsen, 2011. Characterization of bacterial populations in Danish raw milk cheeses made with different starter cultures by denaturating gradient gel electrophoresis and pyrosequencing. Int. Dairy J. 21, 142–148.

Masoud, W., Vogensen, F.K., Lillevang, S., Abu Al-Soud, W., Sørensen, S.J., Jakobsen, M., 2012. The fate of indigenous microbiota, starter cultures, *Escherichia coli*, Listeria innocua and *Staphylococcus aureus* in Danish raw milk and cheeses determined by pyrosequencing and quantitative real time (qRT)-PCR. Int. J. Food Microbiol. 153 (1–2), 192–202.

Monu, E., Blank, G., Holley, R., Zawistowski, J., 2008. Phytosterols effects on milk and yogurt microflora. J. Food Sci. 73 (3), M121–M126.

Mora, D., Arioli, S., 2014. Microbial urease in health and disease. PloS One Pathogens 10 (12), e1004472.

Mora, D., Monnet, C., Parini, C., Guglielmetti, S., Mariani, A., Pintus, P., Molinari, F., Daffonchio, D., Manachini, P.L., 2005. Urease biogenesis in *Streptococcus thermophilus*. Res. Microbiol. 156 (9), 897–903.

Rondanelli, M., Monteferrario, F., Faliva, M.A., Perna, S., Antoniello, N., 2013. Key points for maximum effectiveness and safety for cholesterol-lowering properties of plant sterols and use in the treatment of metabolic syndrome. J. Sci. Food Agric. 93, 2605–2610.

Rossetti, L., Langman, L., Grigioni, G.M., Biolatto, A., Sancho, A.M., Comerón, E., Descalzo, A.M., 2010. Antioxidant status and odor profile in milk from silage or alfalfa-fed cows. Aust. J. Dairy Technol. 65 (1), 3–9.

Ruggirello, M., Dolci, P., Cocolin, L., 2014. Detection and viability of *Lactococcus lactis* throughout cheese ripening. PLoS One 9 (12), e114280.

Saavedra, J.M., Bauman, N.A., Perman, J.A., Yolken, R.H., Oung, I., 1994. Feeding of Bifidobacterium bifidum and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 344, 1046–1049.

Shori, A.B., 2013. Antioxidant activity and viability of lactic acid bacteria in soybean-yogurt made from cow and camel milk. J. Taibah Univ. Sci. 7, 202–208.

Sohier, D., Pavan, S., Riou, A., Combrisson, J., Postollec, F., 2014. Evolution of microbial analytical methods for dairy industry needs. Front. Microbiol. 5, 16.

Slavin, M., Yu, L.L., 2012. A single extraction and HPLC procedure for simultaneous analysis of phytosterols, tocopherols and lutein in soybeans. Food Chem. 135 (4), 2789–2795.

Stevens, K.A., Jaykus, L.A., 2004. Direct detection of bacterial pathogens in representative dairy products using a combined bacterial concentration-PCR approach. J. Appl. Microbiol. 97, 1115–1122.

van de Bunt, B., Bron, P.A., Sijtsma, L., de Vos, W.M., Hugenholtz, J., 2014. Use of non-growing *Lactococcus lactis* cell suspensions for production of volatile metabolites with direct relevance for flavor formation during dairy fermentations. Microb. Cell Factories 13, 176.