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The incorporation of alpha-tocopherol and functional doses of phytosterol esters during cheesemaking does not affect DNA or mRNA dynamics of *Streptococcus thermophilus* and *Lactococcus lactis* throughout and after the end of ripening

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Abstract

Tocopherols and phytosterols are lipid-soluble molecules which have been widely used in the food industry. Nevertheless, the influence of these compounds on the performance of starter lactic acid bacteria (SLAB) in fermented foods has received little attention. Here, we assessed the behavior of *Streptococcus thermophilus* and *Lactococcus lactis* during the ripening of a functional Port Salut light cheese elaborated with these SLAB and with alpha-tocopherol and phytosterol esters as bioactive molecules. Functional and control cheeses were manufactured at an industrial plant and sampled at 7, 21, 40, 60 and 90 days after elaboration for real-time quantitative PCR (qPCR) or reverse transcription-qPCR (RT-qPCR) experiments. Target DNA and mRNA from both SLAB were detected after 90 days of elaboration in both functional and control cheeses, supporting their potential role in generating flavor metabolites. Furthermore, here we showed for the first time that the addition of alpha-tocopherol and functional doses of phytosterols did not affect DNA or mRNA dynamics of these SLAB during cheesemaking, throughout and after the end of ripening. Therefore, our results support the use of cheese manufactured with both *S. thermophilus* and *L. lactis* as an optimal delivery system for these beneficial bioactive compounds.

Keywords

Streptococcus thermophilus; *Lactococcus lactis*; functional food; qPCR; RT-qPCR

Chemical compounds studied in this article

Alpha-tocopherol (PubChem CID: 14985); Campesterol (PubChem CID: 22216479); Beta-sitosterol (PubChem CID: 91746541); Stigmasterol (PubChem CID: 91746470)

1. Introduction

The starter lactic acid bacteria (SLAB) *Streptococcus thermophilus* and *Lactococcus lactis* are the most important microorganisms in the dairy industry (Fernández, Alegría, Delgado, Martín & Mayo, 2011; Mora & Arioli, 2014). Among molecular methods, quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR) have been successfully used to evaluate these SLAB in ripened cheeses or throughout the manufacturing and ripening processes (Pega et al., 2016; Postollec, Falentin, Pavan, Combrisson & Sohier, 2011; Ruggirello, Cocolin & Dolci, 2016).

"Functional" foods are dietary sources of defined biologically active compounds which when in defined quantitative and qualitative amounts, provide a clinically proven health benefit and can thus prevent or treat chronic diseases (Martirosyan, 2011). Phytosterols (sterols and stanols) are a family of plant lipophilic triterpenes, which have been widely used in the food industry (Moreau, Whitaker &, Hicks, 2002), mainly due to their ability to lower cholesterol levels (Rondanelli, Monteferrario, Faliva, Perna & Antoniello, 2013). Tocopherols are also a group of lipid-soluble antioxidants with numerous health benefits and which are commonly used for the fortification of food products (Shahidi & de Camargo, 2016).

In a recent study, we showed for the first time that the addition of alpha-tocopherol (14.4 µg/L of milk) and phytosterols (7.6 g/L of milk) correlates with a significant

increase in *S. thermophilus* DNA and mRNA levels, during the industrial elaboration and ripening of a Port Salut light (reduced fat, semi-soft) cheese manufactured only with this starter (Pega et al., 2016). Lately, there has been great interest in the SLAB *L. lactis*, which is involved in the proteolysis and use of amino acids for the generation of aromatic molecules during the ripening of dairy products (Ruggirello, Cocolin & Dolci, 2016). Moreover, mixed cultures of thermophilic and mesophilic SLAB have been explored to improve cheese production (Champagne, Gagnon, St-Gelais & Vuillemard, 2009). Therefore, the aim of the present study was to assess the behavior of the SLAB *S. thermophilus* and *L. lactis* when added simultaneously in the same cheesemaking process upon the incorporation of alpha-tocopherol and phytosterols, in conditions which have shown to be optimal for the production of functional, reduced-fat, semi-soft cheeses.

2. Materials and methods

2.1. Elaboration and sampling of industrial cheeses

Port Salut light functional and control cheeses were manufactured at an industrial plant as previously described (Pega et al., 2016), with some modifications: *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Danisco Choozit MA14 LYO 50DCU, Copenhagen, Denmark) was used as starter culture together with *S. thermophilus* (STI-14 50U, CHR Hansen, Horsholm, Denmark). Vacuum-packed functional and control cheeses were ripened at 4 °C and sampled at 7, 21, 40, 60 and 90 days of ripening. Triplicate samples from the entire production batch were randomly collected and stored at -80 °C until processing.

101

102 *2.2. Determination of quality parameters, phytosterols, tocopherols and fatty acids*

103

104 Total solids, moisture, sodium chloride, total fat content, phytosterols, tocopherols and
105 the fatty acid profile were determined in functional and control cheeses as previously
106 described (Pega et al., 2016).

107

108 *2.3. Real-time qPCR and RT-qPCR experiments*

109

110 Concentration of bacterial cells, nucleic acid extraction and qPCR assays were
111 performed as previously described (Pega et al., 2016). Experiments were carried out in a
112 StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The
113 amplification conditions used and reverse transcription experiments were performed as
114 described previously (Pega et al., 2016).

115

116 *2.4. Primer design and specificity determinations*

117

118 *S. thermophilus* specific primers for DNA amplification in milk and cheese samples
119 have been previously described (Pega et al., 2016). This same methodology was used
120 for the design of *L. lactis* subsp. *lactis* and subsp. *cremoris* specific primers, for
121 assessing *in silico* specificity and cross reaction against *S. thermophilus*. The nucleotide
122 sequence of the primer pair was as follows: 5´- CAT CGT TGA TGA ATA CAT CCC
123 AAC T - 3´ (f), and 5´- CGA CTG GAA GAA GGA GTG GTT T- 3´ (r).

124

125 *2.5. Construction of standard curves for qPCR and RT-qPCR*

The standard curves used for *S. thermophilus* DNA and cDNA quantification in milk and cheese samples have been previously described (Pega et al., 2017). The same protocol was followed to generate *L. lactis* standard curves and to calculate the copy number, efficiency (E%), slope, correlation coefficient R^2 and limit of detection (LOD).

3. Results

3.1. Cheese compositional analysis

The compositional analysis is shown in Table 1. The content of alpha-tocopherol in functional cheeses (5.47 mg/60 g) was equivalent to ~50% of the recommended dietary allowance (RDA) (Institute of Medicine, 2000). Besides, the amounts of phytosterols in functional cheeses (2.42 g/60 g) were above the dosage recommended to exert benefits on human health (~2 g of phytosterols per day), according to the American Heart Association (Lichtenstein et al., 2006).

3.2. Assessment of primer specificity

To determine the specificity of *L. lactis* primers in cheese samples, DNA extracted from *S. thermophilus* was tested and found to be below the detection threshold of the assay (Ct >40), even at the lowest dilution of template evaluated. Moreover, the melting curves generated for *L. lactis* displayed only one peak in every sample, thus confirming the specificity of the primer pair (data not shown).

151 *3.3. Standard curves used for qPCR and RT-qPCR quantifications*

152

153 An optimum linear correlation between Ct values and copy numbers was recorded for
154 both DNA and cDNA standard curves (R^2 values of 0.999) constructed for *L. lactis*. The
155 detection spectrum was linear across a range of 7 log units. Fifty-three copies of
156 DNA/well and 50 copies of cDNA/well were accurately determined (LOD) in cheese
157 samples. Standard curves generated with negative cheese samples and E% values are
158 shown in Fig. 1.

159

160 *3.4. SLAB quantification during ripening of functional and control cheeses by qPCR*
161 *and RT-qPCR*

162

163 Target DNA and cDNA from both SLAB were detected at all time points in both
164 functional and control cheeses and persisted after the end of ripening (90 days after
165 elaboration) (Fig. 2). Slightly higher values were obtained on average for *S.*
166 *thermophilus* (~2.10 x 10^5 genome copies/g) than for *L. lactis* (~1.86 x 10^5 genome
167 copies/g) DNA throughout cheese ripening (Fig. 2, panel A). This pattern was also
168 observed for cDNA during ripening of functional and control cheeses, with average
169 values of ~1.77 x 10^5 and ~1.62 x 10^5 cDNA copies/g for *S. thermophilus* and for *L.*
170 *lactis*, respectively (Fig. 2, panel B).

171

172 Interestingly, the average amount of *S. thermophilus* DNA detected in functional
173 cheeses throughout ripening was the same (~2.10 x 10^5 genome copies/g) to the one
174 detected in control cheeses (~2.10 x 10^5 genome copies/g) (Fig. 2, panel A).
175 Accordingly, *S. thermophilus* average cDNA levels throughout ripening were similar

between functional (~1.79 x 10^5 cDNA copies/g) and control (~1.77 x 10^5 cDNA copies/g) cheeses (Fig. 2, panel B).

The same phenomena were observed for *L. lactis* DNA levels throughout cheese ripening, with average values of ~1.87 x 10^5 and ~1.86 x 10^5 genome copies/g for functional and control cheeses, respectively (Fig. 2, panel A). Furthermore, *L. lactis* average cDNA levels throughout ripening were similar between functional (~1.60 x 10^5 cDNA copies/g) and control (~1.62 x 10^5 cDNA copies/g) cheeses (Fig. 2, panel B).

4. Discussion

To successfully develop functional fermented foods it is necessary to ensure that the added bioactive compounds do not affect the performance of SLAB, which are critical for the fermentation processes (Fernández, Alegría, Delgado, Martín & Mayo, 2011; Mora & Arioli, 2014). Although phytosterols and tocopherols have been extensively used for the development of functional food products (Moreau, Whitaker &, Hicks, 2002; Shahidi & de Camargo, 2016), the evidence regarding the influence of these biologically active compounds on the behavior of SLAB during food fermentations is scarce (Pega et al., 2016).

Here, both target DNA and mRNA from *S. thermophilus* and *L. lactis* were detected in Port Salut light cheeses after 7 days of elaboration and persisted after the end of ripening (90 days after elaboration), supporting the possible role of these SLAB in shaping organoleptic profiles proposed by some authors (Falentin et al., 2012;

Ruggirello, Cocolin & Dolci, 2016; van de Bunt, Bron, Sijtsma, de Vos & Hugenholtz, 2014).

In a recent study, we showed for the first time that the addition of alpha-tocopherol (14.4 µg/L of milk) and phytosterol esters (7.6 g/L of milk) correlates with a significant increase in *S. thermophilus* DNA and mRNA levels, during the industrial elaboration and ripening of a Port Salut light cheese manufactured only with this starter (Pega et al., 2016). Here, the addition of the same amounts of these molecules did not produce such increase in *S. thermophilus* DNA and mRNA levels when *L. lactis* was also used as SLAB in the same cheesemaking process.

Although these differences could be explained by the presence of *L. lactis*, comparisons between different elaboration processes which involve many variables should be approached with caution. Nevertheless, it is evident that the addition of phytosterols and alpha-tocopherol do not negatively affect *S. thermophilus* in any of the two scenarios (presence or absence of *L. lactis*). Moreover, our results are consistent with previous culture-dependent studies showing that soy beverages (Farnworth, Mainville, Desjardins, Gardner, Fliss & Champagne, 2007; Karleskind, Laye, Halpin & Morr, 1991; Shori, 2013) or isolated phytosterols (Monu, Blank, Holley & Zawistowski, 2008) do not affect the growth of *S. thermophilus*.

With regard to *L. lactis*, no reports are available to show whether the addition of phytosterols or tocopherols during food fermentations modifies the behavior of this SLAB. Therefore, the present study provides the first evidence indicating that the

addition of these bioactive compounds during cheesemaking does not affect the dynamics of *L. lactis* DNA or mRNA during cheese ripening.

Preliminary results from our laboratory indicated that several oxidation parameters and organoleptic attributes in ripened cheeses were not modified by the addition of phytosterol esters during cheesemaking, even in the absence of the simultaneous fortification with alpha-tocopherol (unpublished results). However, whether the neutral effect on SLAB could have been obtained without the addition of alpha-tocopherol cannot be known for sure based on the available evidence.

In summary, this study showed that both DNA and mRNA from *S. thermophilus* and *L. lactis* were quantified in cheese samples after 90 days of elaboration, suggesting the persistence of these SLAB even after the end of ripening and thus supporting their possible role in shaping organoleptic profiles. Furthermore, our results highlighted for the first time that the addition of phytosterols and alpha-tocopherol did not affect DNA or mRNA dynamics of *S. thermophilus* and *L. lactis* throughout and after the end of ripening, when used simultaneously during cheesemaking. In addition, the fact that these results were obtained with functional doses of phytosterols (2.42 g/60 g) and ~50% of the RDA (5.47 mg/60g) of alpha-tocopherol, may provide important information for industrial purposes.

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Figure legends

Fig. 1. Standard curves generated for *Lactococcus lactis* by qPCR (panel A) and RT-qPCR (panel B). Each point represents the mean value of triplicate DNA or RNA extractions ± standard deviations (SD). Standard curves were constructed by plotting threshold cycle (Ct) values against the logarithm (log) number of genome or cDNA copies/g of negative cheese samples.

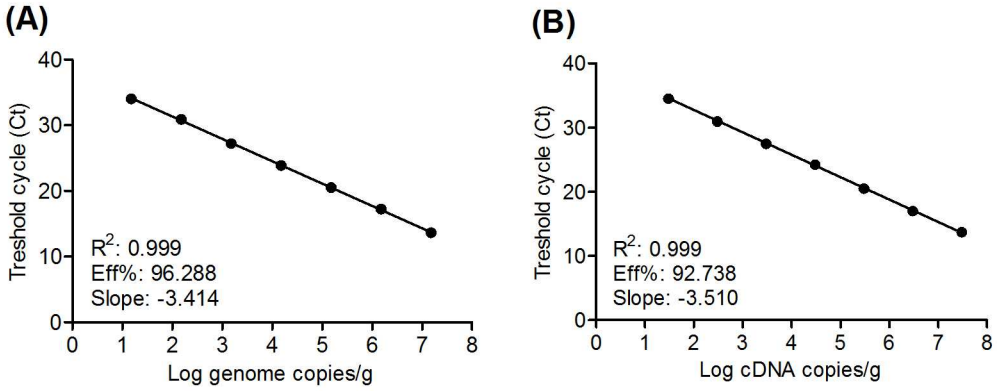
Fig. 2. Quantification of *Streptococcus thermophilus* and *Lactococcus lactis* by qPCR (panel A) and RT-qPCR (panel B) during ripening of functional and control cheeses. Results are expressed as the number of genome or cDNA copies/g of sample. Each bar represents the mean value of triplicate samples ± SD.

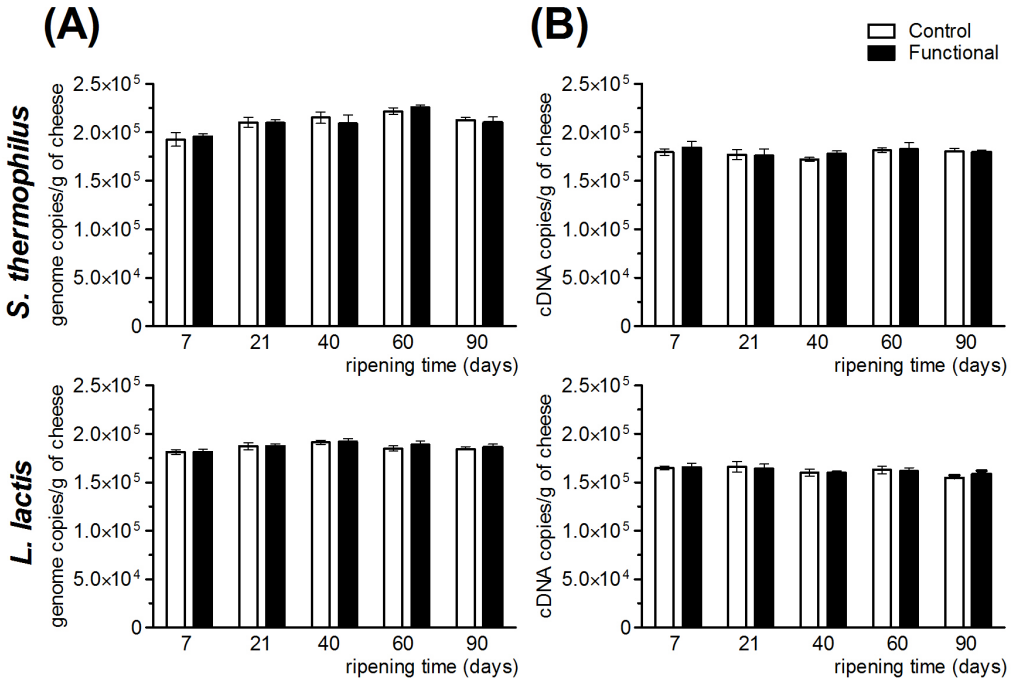
Table 1

Compositional analysis for Port Salut light cheeses manufactured at industrial level.

Parameter	Functional	Control
g/100g		
Moisture	51.76	55.85
Sodium chloride	1.32	1.14
Protein	19.58	22.12
Fat	23.75	17.00
(%)		
PUFA	5.02	7.42
MUFA	31.22	33.91
SFA	63.76	58.67
g/60g		
Stigmasterol	0.81	ND
Campesterol	0.64	ND
Beta-sitosterol	0.96	ND
Total phytosterols	2.42	ND
mg/60g		
Alpha-tocopherol	5.47	0.26
Gamma-tocopherol	4.96	0.09
Total tocopherols	10.43	0.35

PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids; ND: not detected. Results are mean values obtained for samples after 90 days of elaboration, except for moisture, sodium chloride, protein and fat contents which were determined after 15 days of elaboration. PUFA, MUFA and SFA are expressed as percentage of total fatty acids.





Highlights

- We produced industrial cheeses with alpha-tocopherol and functional phytosterols.
- *Streptococcus thermophilus* and *Lactococcus lactis* were used as starter bacteria.
- DNA and mRNA from both bacteria persisted after the end of ripening (90 days).
- The bioactive molecules did not affect the behavior of the bacteria during ripening.