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

4

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24

25

26 Abstract

27

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

44

45 Keywords

46

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

48

49 Chemical compounds studied in this article

50

51 Alpha-tocopherol (PubChem CID: 14985); Campesterol (PubChem CID: 22216479);

52 Beta-sitosterol (PubChem CID: 91746541); Stigmasterol (PubChem CID: 91746470)

53

54 **1. Introduction**

55

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

63

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

73

74 In a recent study, we showed for the first time that the addition of alpha-tocopherol
75 (14.4 µg/L of milk) and phytosterols (7.6 g/L of milk) correlates with a significant

76 increase in *S. thermophilus* DNA and mRNA levels, during the industrial elaboration
77 and ripening of a Port Salut light (reduced fat, semi-soft) cheese manufactured only
78 with this starter (Pega et al., 2016). Lately, there has been great interest in the SLAB *L.*
79 *lactis*, which is involved in the proteolysis and use of amino acids for the generation of
80 aromatic molecules during the ripening of dairy products (Ruggirello, Cocolin & Dolci,
81 2016). Moreover, mixed cultures of thermophilic and mesophilic SLAB have been
82 explored to improve cheese production (Champagne, Gagnon, St-Gelais & Vuilleumard,
83 2009). Therefore, the aim of the present study was to assess the behavior of the SLAB
84 *S. thermophilus* and *L. lactis* when added simultaneously in the same cheesemaking
85 process upon the incorporation of alpha-tocopherol and phytosterols, in conditions
86 which have shown to be optimal for the production of functional, reduced-fat, semi-soft
87 cheeses.

88

89 **2. Materials and methods**

90

91 *2.1. Elaboration and sampling of industrial cheeses*

92

93 Port Salut light functional and control cheeses were manufactured at an industrial plant
94 as previously described (Pega et al., 2016), with some modifications: *L. lactis* subsp.
95 *lactis* and *L. lactis* subsp. *cremoris* (Danisco Choozit MA14 LYO 50DCU,
96 Copenhagen, Denmark) was used as starter culture together with *S. thermophilus* (STI-
97 14 50U, CHR Hansen, Horsholm, Denmark). Vacuum-packed functional and control
98 cheeses were ripened at 4 °C and sampled at 7, 21, 40, 60 and 90 days of ripening.
99 Triplicate samples from the entire production batch were randomly collected and stored
100 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*

126

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

131

132 **3. Results**

133

134 *3.1. Cheese compositional analysis*

135

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

142

143 *3.2. Assessment of primer specificity*

144

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

150

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* ($\sim 2.10 \times 10^5$ genome copies/g) than for *L. lactis* ($\sim 1.86 \times 10^5$ 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 $\sim 1.77 \times 10^5$ and $\sim 1.62 \times 10^5$ 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 ($\sim 2.10 \times 10^5$ genome copies/g) to the one
174 detected in control cheeses ($\sim 2.10 \times 10^5$ genome copies/g) (Fig. 2, panel A).

175 Accordingly, *S. thermophilus* average cDNA levels throughout ripening were similar

176 between functional ($\sim 1.79 \times 10^5$ cDNA copies/g) and control ($\sim 1.77 \times 10^5$ cDNA
177 copies/g) cheeses (Fig. 2, panel B).

178

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

184

185 **4. Discussion**

186

187 To successfully develop functional fermented foods it is necessary to ensure that the
188 added bioactive compounds do not affect the performance of SLAB, which are critical
189 for the fermentation processes (Fernández, Alegría, Delgado, Martín & Mayo, 2011;
190 Mora & Arioli, 2014). Although phytosterols and tocopherols have been extensively
191 used for the development of functional food products (Moreau, Whitaker & Hicks,
192 2002; Shahidi & de Camargo, 2016), the evidence regarding the influence of these
193 biologically active compounds on the behavior of SLAB during food fermentations is
194 scarce (Pega et al., 2016).

195

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

200 Ruggirello, Cocolin & Dolci, 2016; van de Bunt, Bron, Sijtsma, de Vos & Hugenholtz,
201 2014).

202

203 In a recent study, we showed for the first time that the addition of alpha-tocopherol
204 (14.4 µg/L of milk) and phytosterol esters (7.6 g/L of milk) correlates with a significant
205 increase in *S. thermophilus* DNA and mRNA levels, during the industrial elaboration
206 and ripening of a Port Salut light cheese manufactured only with this starter (Pega et al.,
207 2016). Here, the addition of the same amounts of these molecules did not produce such
208 increase in *S. thermophilus* DNA and mRNA levels when *L. lactis* was also used as
209 SLAB in the same cheesemaking process.

210

211 Although these differences could be explained by the presence of *L. lactis*, comparisons
212 between different elaboration processes which involve many variables should be
213 approached with caution. Nevertheless, it is evident that the addition of phytosterols and
214 alpha-tocopherol do not negatively affect *S. thermophilus* in any of the two scenarios
215 (presence or absence of *L. lactis*). Moreover, our results are consistent with previous
216 culture-dependent studies showing that soy beverages (Farnworth, Mainville,
217 Desjardins, Gardner, Fliss & Champagne, 2007; Karleskind, Laye, Halpin & Morr,
218 1991; Shori, 2013) or isolated phytosterols (Monu, Blank, Holley & Zawistowski, 2008)
219 do not affect the growth of *S. thermophilus*.

220

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

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

226

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

233

234 In summary, this study showed that both DNA and mRNA from *S. thermophilus* and *L.*
235 *lactis* were quantified in cheese samples after 90 days of elaboration, suggesting the
236 persistence of these SLAB even after the end of ripening and thus supporting their
237 possible role in shaping organoleptic profiles. Furthermore, our results highlighted for
238 the first time that the addition of phytosterols and alpha-tocopherol did not affect DNA
239 or mRNA dynamics of *S. thermophilus* and *L. lactis* throughout and after the end of
240 ripening, when used simultaneously during cheesemaking. In addition, the fact that
241 these results were obtained with functional doses of phytosterols (2.42 g/60 g) and
242 ~50% of the RDA (5.47 mg/60g) of alpha-tocopherol, may provide important
243 information for industrial purposes.

244

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246

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250 “Design of functional dairy products” involving INTA, Ministerio de Ciencia,
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252

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254

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315 metabolites with direct relevance for flavor formation during dairy fermentations.
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- 317

318 **Figure legends**

319

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

325

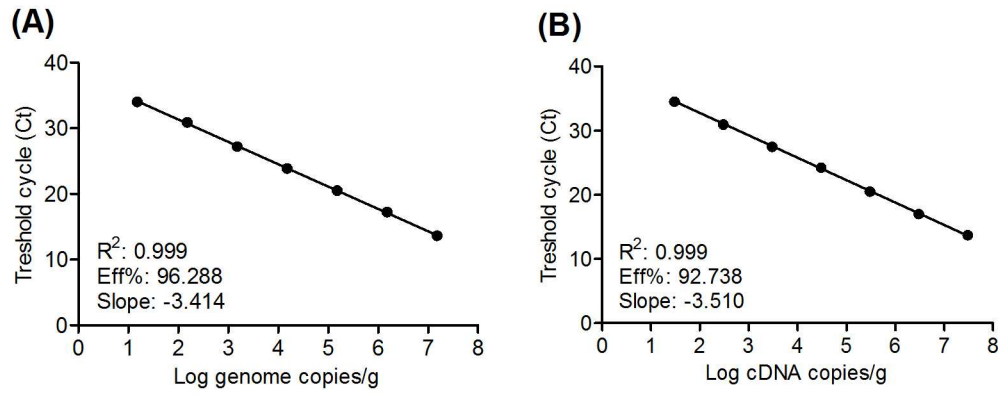
326 **Fig. 2.** Quantification of *Streptococcus thermophilus* and *Lactococcus lactis* by qPCR
327 (panel A) and RT-qPCR (panel B) during ripening of functional and control cheeses.
328 Results are expressed as the number of genome or cDNA copies/g of sample. Each bar
329 represents the mean value of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

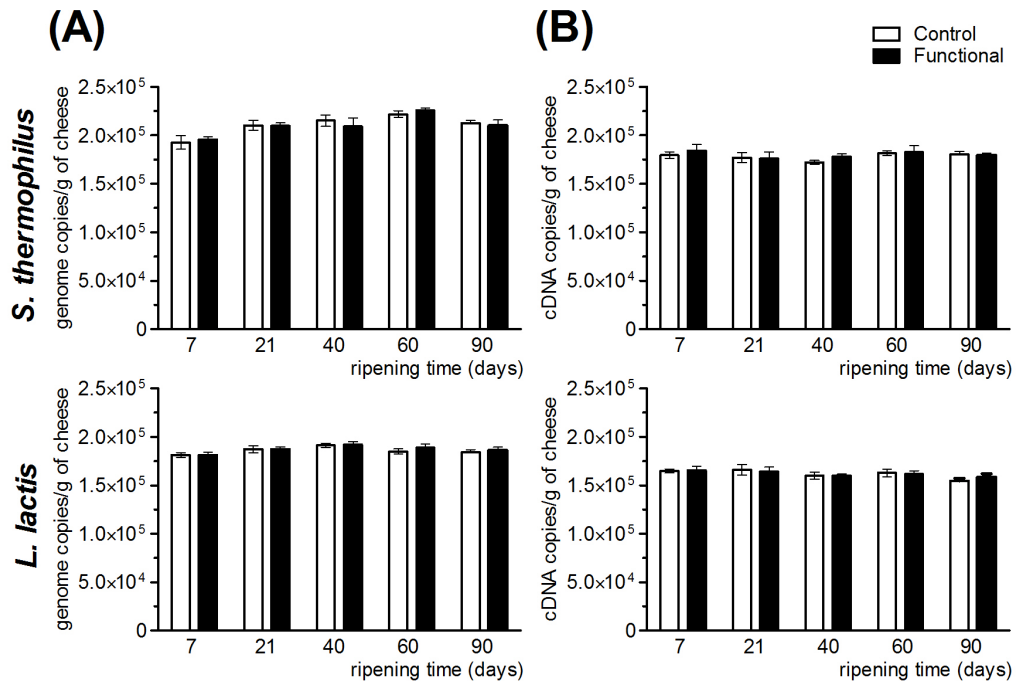
Table 1

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

Parameter	Functional	Control
<i>g/100g</i>		
Moisture	51.76	55.85
Sodium chloride	1.32	1.14
Protein	19.58	22.12
Fat	23.75	17.00
<i>(%)</i>		
PUFA	5.02	7.42
MUFA	31.22	33.91
SFA	63.76	58.67
<i>g/60g</i>		
Stigmasterol	0.81	ND
Campesterol	0.64	ND
Beta-sitosterol	0.96	ND
Total phytosterols	2.42	ND
<i>mg/60g</i>		
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.