1 **Effect of cholesterol reduced and zinc fortification treatments on physicochemical,**

2 **functional, textural, microstructural and sensory properties of soft cheese**

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17   Running headline: Evaluation of functional soft cheese

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Abstract

The aim of this study was to produce and evaluate a soft cheese fortified with zinc and with cholesterol-reduced content. To meet this objective a cream base was prepared, from which Cholesterol was removed using β-cyclodextrin as extracting agent. Then, cholesterol-reduced content cheese with and without the addition of zinc salts ($ZnSO_4$ or $ZnCl_2$) was produced. Additionally, a cheese without any treatment was prepared. Furthermore, physicochemical, textural, functional, microstructural and sensory determinations were performed. As a result, 87-94% zinc fortified and 93% cholesterol-reduced cheese samples were obtained which had similar sensorial characteristics to the cheese without treatment.

Keywords: Soft cheese, Zinc Fortification, Cholesterol removal, β-cyclodextrin

INTRODUCTION

In the last years, the "functional foods" concept has achieved a great importance for consumers and between them dairy products are the most required. It has been reported that high cholesterol blood levels represent a major risk factor for developing coronary heart disease. In spite of the relationship between dietary cholesterol and plasma cholesterol levels it is being still investigated, the World Health Organization and the American Heart Association have recommended the reduction of dietary cholesterol consumption (Dias *et al.* 2010). Reduce cholesterol from fat dairy products is a way to avoid the risk of these diseases with keeping the rest of the milk fat, since is an excellent source of energy, vitamins (A, D and E), antioxidants and essential fatty acids (Rehm *et al.* 2015). In addition, it is important to highlight that fat removal from dairy products affects in a negative way their sensorial characteristics (Kim *et al.* 2009).

48   The methods to reduce cholesterol from dairy products include physical,

49   chemical or biological strategies, between them could be mentioned the extraction with

50   high-methoxyl pectins, plant sterols, adsorption with saponin and solvent. However,

51   most of these methods are not suitable, since other molecules are removed at the same

52   time than the cholesterol (Dias *et al.* 2010). The use of β-cyclodextrin (β-CD) is a

53   strategy that effectively removes cholesterol from dairy products improving their

54   nutritional characteristics, leaving the rest of milk fat. This method is selective and

55   avoids the removal of flavours and nutritional components during the cholesterol

56   extraction. β-CD has a central cavity which allows complex formation with the

57   cholesterol molecule (Kwak *et al.* 2001, Kwak *et al.* 2002). In addition, β-CD is

58   nontoxic, edible, non-hygroscopic, chemically stable and easy to separate. Furthermore,

59   in 1998 it was introduced into the Generally Recognized As Safe list of compounds

60   (GRAS), because it is not absorbed in the upper gastrointestinal tract, and it is

61   completely metabolized by the colon microflora (Dias *et al.* 2010) . Moreover, recent

62   works have shown that the use of β-CD as a strategy to remove cholesterol from dairy

63   products does not show significant effects on their chemical, rheological, and sensory

64   properties (Dias *et al.* 2010). All these positives attributes make β-CD use a suitable

65   strategy for cholesterol removal from food.

66   Zinc is one of the most important trace elements present in the body with a great

67   nutritional importance and that has a recognized action on more than 300 enzymes,

68   participating in either their structure or in their catalytic and regulatory action. Due to

69   the great number of zinc-dependent biological processes, the deficiency states of this

70   essential nutrient entails multiclinical consequences, which range from mild to serious

71   dysfunctions (Salgueiro *et al.* 2000, Pomastowski *et al.* 2014).

72   Zn^{2+}deficiency states emerge as a consequence of two groups of causes. The first

73   group includes several syndromes related to metabolic or genetic malfunctions and the

74   second one comprises nutritional causes like decreased Zn^{2+} intake or consumption of

75   poor Zn^{2+} content foods, which represent the most important and the most common

76   (Salgueiro *et al.* 2000)**.** The Recommended Dietary Allowance (RDA), according to the

77   Food and Agriculture Organization of the United Nations and the World Health

78   Organization (FAO/WHO), for Zn^{2+} is 3.9 mg day^{-1} for females and 4.2 mg day^{-1} for

79   males, assuming a bioavailability of dietary zinc of 50 % (diet high in meat), and 9.8 mg

80   day^{-1} for females and 14 mg day^{-1} for males, assuming a bioavailability of dietary zinc

81   of 15 % (vegetarian diets) (WHO/FAO 2005, Kahraman and Ustunol 2012).

82   The available studies clearly show that food fortification with Zn^{2+} can increase

83   the dietary intake and the total daily absorption of this trace element (Hess and Brown

84   2009). In addition, is extremely important to control the quantity of mineral

85   supplementation in food to ensure the desired level in the final products does not exceed

86   the critical limits for an adult man, which is 45 mg/day according to FAO/WHO

87   (WHO/FAO 2005). The selection of an adequate vehicle is crucial for successful

88   fortification. Milk and dairy products are a good option for Zn^{2+} fortification, not only

89   due to their worldwide consumption, but also because of their high nutritional value, the

90   buffer effect in the digestion and absorption processes and the low pH value (in

91   fermented products) that enhances the Zn^{2+}solubilization increasing its bioavailability

92   (Aquilanti *et al.* 2012). Zinc sulfate and zinc chloride salts belong to the list of

93   compounds listed as GRAS (Kahraman and Ustunol 2012).

94   Previous works present strategies for Zn^{2+} fortification of different kinds of

95   cheese and dairy products, thus supporting the use of fortification with Zn^{2+} as a method

96   to counteract intake deficiency (El-Din *et al.* 2012, Kahraman and Ustunol 2012).

97   Cuartirolo cheese is a soft cheese defined by the Código Alimetario Argentino

98 as a high (46-55%) or very high (>55%) moisture, fatty (45-60%), manufactured with

99 raw milk, acidified by lactic cultures and coagulated by rennet or specific enzymes

100 (ANMAT 2002). The treatments (homogenisation, heating) and the addition of different

101 cosolutes (β-CD, zinc salts) in order to cholesterol-removal and Zn^{2+} fortification may

102 alter dairy products characteristics. The aim of this work is to evaluate physicochemical,

103 sensory, functional, rheological, textural and microstructural characteristics of a

104 cholesterol-reduced and Zn^{2+} fortified Cuartirolo type soft cheese.

105

106 **MATERIALS AND METHODS**

107 <u>**Materials**</u>

108   The Cream (48% fat content) and the skim milk (< 0.05 % w/w fat content) used to

109 obtained the cream base (CB) were purchased from Manfrey (Santa Fe, Argentine).

110 Commercial β-CD (Kleptose®, Roquette, France) was the extracting agent used to

111 remove cholesterol from the CB. The fortification agents, $ZnSO_4.7H_2O$ and $ZnCl_2$

112 (anhydrous), were purchased from Novalquim (Santa Fe, Argentine) and Cicarelli

113 (Santa Fe, Argentine), respectively. Salts belong to the list of compounds listed as

114 GRAS.

115 <u>**Methods**</u>

116 **Cholesterol-reduced and Zn^{2+} fortified Cuartirolo soft cheese manufacture**

117 *Preparation of cholesterol-reduced content cream base*

118 The quantities of cream (48 % w/w fat content) and skim milk (< 0.05 % w/w fat

119 content) required to obtain a cream base (CB: 10 % w/w fat content) were mixed and

120 homogenised using a two stages homogeniser ST2 (Simes S.A., Santa Fe, Argentine)

121 with a total pressure of 100 atm at 70 °C in order to promote cholesterol extraction.

122   The homogenised CB was brought to 30 °C, where β-CD was added at a ratio of

123   2.85 % w/w after which the final mixture was left in mechanic agitation for 30 min. The

124   system was centrifuged at 700 g (Mistral 4L, MSE, Crawley, UK) and 20 °C for 15 min

125   in order to precipitate the β-CD/cholesterol complex, obtaining a CB with cholesterol-

126   reduced content in the supernatant. The optimal conditions for cholesterol extraction

127   were determined using a response surface methodology, which was performed in an

128   early stage (data not shown).

129   *Cheese-making process*

130   The cheese samples were made according to traditional Cuartirolo technology. A bench-

131   top (< 10 L) cheese vat was used. The cheese-making process was made in duplicated

132   for each type of cheese in study.

133   Each type of cheese was made from CB treated with β-CD and diluted with skim

134   milk (Milkaut S.A., Santa Fe, Argentine) to lower down the fat content to 3 % w/w

135   (treated milk, TM), which was pasteurized at 65 °C for 20 min. This TM was then

136   cooled to 40±1 °C, followed by the addition of $CaCl_2$ (200 mg Kg^{-1}, Cicarelli, Santa Fe,

137   Argentine) and the Zn^{2+} salts (16 mg Zn^{2+} kg^{-1}) in the case of fortified cheese samples

138   (Aquilanti *et al.* 2012, Kahraman and Ustunol 2012). The starter lactic culture for

139   Cuartirolo soft cheese (STD, Diagramma S.A., Santa Fe, Argentine) was added at a

140   ratio of 0.01 g kg^{-1} of TM. After that, bacterial rennet (0.94 UR mL^{-1}, Chy-Max®,

141   Chr.Hansen, Denmark) was added at a ratio of 0.5 mL kg^{-1} of TM. Clot was formed

142   after 30 min, cubes of approximately 2 cm^3 were cut and allowed to heal during 15 min.

143   The mixture whey-curd particles were gently stirred for 5 min. This operation

144   repeated thrice. Whey was drained and the curd was moulded. Moulded cheese (0.5 kg

145   cheese) were stored at 40±1 °C, inverted each every 30 min until the pH of the cheese

146   was near 5.3±0.1. The molded cheese was maintained in a cold brine solution (20 %

147   w/w NaCl) for a period of 1 h kg^{-1} of cheese. After that, cheese samples were vacuum

148   packed and ripened at 5 °C for 20 days (Figure 1).

149   Four types of Cuartirolo soft cheese were made: 1) a cholesterol-reduced cheese

150   (ChC-R: made of TM obtained from cholesterol-reduced CB and without Zn^{2+}

151   fortification), 2) a ZnCl$_2$ fortified cheese (ChZnCl$_2$: made of TM obtained from

152   cholesterol-reduced CB and fortified with ZnCl$_2$ salt), 3) ZnSO$_4$ fortified cheese

153   (ChZnSO$_4$: made of TM obtained from cholesterol-reduced CB and fortified with

154   ZnSO$_4$ salt), and 4) a cheese without any treatment (ChWT: made without cholesterol

155   extraction treatment and without fortification). Each cheese sample was made in

156   duplicated.

157   **Quantification of cholesterol**

158   Cholesterol content of the CB, before and after β-CD treatment, was determined in

159   quadruplicate. Samples of CB were saponified, followed by cholesterol extraction using

160   n-hexane (Cicarelli, Santa Fe, Argentine) according to the technique published by

161   Pavón *et al*. (2014). Cholesterol quantification was made by an enzymatic method using

162   a commercial kit (Wiener Lab., Argentine). Cholesterol content of all samples was

163   determined by absorption at 510 nm using a UV-VIS spectrophotometer (Jasco V550,

164   Japan) and was compared with the cholesterol standard solution (belong to the

165   commercial kit). Cholesterol extraction percentage (%ExtChol) was calculated as

166   follows:

167   %ExtChol= 100-[(amount of cholesterol in β-CD treated CB*100)/(amount of

168   cholesterol in untreated CB)]   (1)

169   **Chemical cheese analysis and cheese yield determination**

170   *Zinc content determination*

171 Cheese Zn^{2+} content was determined according to the flame atomic absorption

172 spectrophotometric method of the Association of Official Analytical Chemists (1990).

173 The values corresponding to the percentage of Zn^{2+} fortification (%Zn^{2+} Fortification)

174 are calculated as follows:

175

176 %Zn^{2+} Fortification=$[([Zn^{2+}]_{ChF}-[Zn^{2+}]_{ChC-R})/[Zn^{2+}]_{max}]*100$ (2)

177

178 where $[Zn^{2+}]_{ChF}$ and $[Zn^{2+}]_{ChC-R}$ are the Zn^{2+} concentration in the fortified cheese and

179 the ChC-R respectively. $[Zn^{2+}]_{max}$ is the Zn^{2+} maximum concentration present in the

180 cheese if all the Zn^{2+} salt added remains in the curd.

181 *Cheese yield*

182 Cheese yield was determined as the weight of curd obtained per 100 g of TM used for

183 cheese-making, and the yield percentage (%Yield) according to:

184 %Yield= $[(wt.cheese*100)/(wt.milk)]$ (3)

185 where wt.cheese and wt.milk are the weight of cheese and milk respectively (Kwak *et*

186 *al.* 2001, Kwak *et al.* 2002).

187 *Moisture content*

188 For cheese moisture content determinations, 2 g of shredded cheese samples were

189 weighted onto porcelain dishes and dried at 105±1 °C until constant weight was

190 reached, according to the Association of Official Analytical Chemists official method

191 (1990).

192 **Measurement of cheese firmness**

193 Cylindrical samples of cheese (height: 20 mm, diameter: 40 mm) were prepared and

194 tempered at room temperature for 1 h before the determinations. The firmness of the

195 cheese was measured using a motorized test frame Mecmesin Multitest 2.5-d

196 (Mecmesin, Spain) equipped with a dynamometer of a 100 N load cell. Penetration tests

197 were carried out in the middle of the cylindrical samples, always at the same place, to

198 avoid side effects.

199 The force in Newton required to push a cylinder probe (diameter 20 mm), at a

200 speed of 10 mm min^{-1}, into the cheese mesh was measured. Cheese firmness was

201 considered as the force required to insert the probe to a depth of 10 mm into the cheese.

202 **Colorimetric measurement**

203 A high-resolution digital camera (Nikon, Coolpix P520) was used to measure colour by

204 capturing the colour image of cheese samples under proper lighting. The digital images

205 were processed, using Photoshop software (Adobe Systems Inc., USA) according to the

206 method proposed by Soazo *et al*. (2014) in order to obtain the L*, a* and b* parameters.

207 Where L* is the luminance or lightness component that goes from 0 (black) to 100

208 (white), and parameters a* (green to red) and b*(blue to yellow) are both chromatic

209 components, varying from -120 to +120.

210 **Microstructure analysis**

211 *Confocal scanning laser microscopy (CSLM)*

212 CSLM is widely used for the study of food microstructure. Sections of samples,

213 approximately of 5 mm X 5 mm X 2 mm, were cut with a scalpel placed in a

214 microscope slide and one drop (50 μL) of 0.01 mg mL^{-1} Rhodamine B (Sigma-Aldrich,

215 St. Louis, USA) was added to the cut surface. Rhodamine B binds to proteins fraction in

216 the cheese samples, labelling them in red.

217 Images of representative areas of each sample were captured using a confocal

218 microscopy (Nikon Eclipse TE-2000-E, Japan), with an objective magnification of 60X

219 (oil immersion lens) and a numerical aperture of 1.4.

220   The digital images were acquired with a pixel resolution of 1024 X 1024 and stored

221   in a TIFF format in order to be analyzed.

222   *Images textural analysis*

223   Texture parameters: Shannon entropy (S), smoothness (K), uniformity (U) and grey

224   level variance ($\sigma^2(N)$), were determined from the CLSM images according to the

225   method proposed by Ingrassia *et al*. (2013).

226   *Pore diameter determination*

227   In order to determine cheese pore diameter from the CSLM images a plug-in of the

228   image J (version 1.485) program was employed (Doube *et al.* 2010). From the CSLM

229   images, the thickness parameter was obtained using the Bone J plug-in (version 1.3.12).

230   This can be interpreted as the width of cavities or as the distance between structures. It

231   can thus be used to estimate parameters such as pore diameter.

232   **Cheese meltability determinations**

233   A modified Schreiber`s test was used to determine the fusion capability of cylindrical

234   samples of cheese (diameter: 37 mm, height: 12 mm) (Muthukumarappan *et al.* 1999).

235   All cheese meltability determinations were made after 20 days of ripening. Samples

236   were maintained at 4 °C for 30 min before the determinations and then were placed in a

237   stove at 130 °C for 15 min. Subsequently, both areas (before and after heating) were

238   determined in order to quantify the fusion capability of each cheese sample.

239   **Sensory analysis**

240   Cheese samples were evaluated by a trained sensory panel composed of ten members (6

241   female and 4 male from 25 to 60 years old), all of whom had used quantitative

242   descriptive analysis (ISO, 1993) on regular basis over the past 2 years. The panel was

243   calibrated in the use of the chosen attributes during five training sessions. During these

244   sessions, panellists discussed and agreed upon the definitions and how to qualify the

245 attributes on the scale using commercial soft cheese samples and following the

246 recommendations of the International Dairy Federation (IDF Standard, 1997). Different

247 texture, taste and flavour descriptors, on a 10 cm unstructured line scale anchored with

248 appropriate terms at the left and right ends. For texture attributes, the anchor points

249 were: 1= "almost nothing", 9= "a lot" and for taste and flavour properties: 1="barely",

250 9="extremely" perceptible. Test samples of 30 g were presented in plates to the

251 panellist in a randomized order, at 10 °C after 20 days of storage. During tasting, each

252 panel member marked in such scale the perceived intensity of every attribute.

253 Afterwards, the intensities of each descriptor were measured in each scale, in order to

254 assign a numeric value for statistical analysis (Pavón *et al.* 2014).

255 **Statistical analysis**

256 All determinations were performed at least in duplicate. ANOVA test was performed in

257 all experimental determinations and the significance of the results was analyzed by the

258 least significant difference (LSD) test. Differences of $p<0.05$ were considered to be

259 significant.

260

261 **RESULTS AND DISCUSSION**

262 **Quantification of cholesterol**

263 Cholesterol content of the CB was 10 ± 2 mg mL^{-1} and 0.7 ± 0.1 mg mL^{-1} before and after

264 β-CD treatment, respectively. Therefore, the average %ExtChol was 93 ± 1 %. This value

265 agrees with the one previously reported by Kwak *et al.* (2001) for milk cholesterol

266 extraction. These authors report the importance of the homogenisation step in the

267 cholesterol extraction protocol since about 80 % of cholesterol exists in the fat globule.

268 Thus the surface area of the fat globule should be increased for an effective adsorption

269 of β-CD to interact with the cholesterol molecule (Kwak *et al.* 2001).

270

271

Chemical cheese analysis and cheese yield determination

Zinc content determination

Zn^{2+} concentrations, %Yield, % Zn^{2+} Fortification and moisture found in cheese samples are shown in Table 1.

As expected, notably higher Zn^{2+} levels ranging from 129 ± 8 to 131 ± 8 mg kg^{-1}, were found in fortified cheese samples (ChZnCl$_2$ and ChZnSO$_4$). In addition, Zn^{2+} content values found in fortified cheese and in non-fortified ChWT and ChC-R are comparable with those reported by Aquilanti *et al*. (2012) for Zn^{2+} fortified Squacquerone cheese and Caciotta cheese (Italian cheeses that are similar in manufacture and texture to Cuartirolo soft cheese).

The high % Zn^{2+} fortification found, for both salts used, compared to the low yield of the cheese-making process, is in agreement with the existence of an interaction between Zn^{2+} and casein micelles reported by other authors, who studied the Zn^{2+} binding to caseins (Pomastowski *et al.* 2014). Therefore, most of the added Zn^{2+} remains retained in casein fractions and little is lost in the whey during cheese manufacture.

Assuming a low bioavailability (15 %) of Zn^{2+}, a 30 g portion of cheese with the % Zn^{2+} fortification levels (ChZnCl$_2$ and ChZnSO$_4$) represents 40% and 28% of the RDA for women and men, respectively. On the other hand, assuming a high bioavailability (50%) of Zn^{2+} it represents about 100% and 90% of the RDA for women and men, respectively. No significant differences were detected between the fortifying agent used and the % fortification value obtained.

294   The slightly higher yield observed for the cheese obtained from cholesterol-reduced

295 TM compared to ChWT arise as a result of the homogenisation process that involved

296 the cholesterol extraction protocol. Yield increase was previously reported and could be

297 explained by the fact that protein and fat recovery were higher in cheese made from

298 homogenised milk (Kwak *et al.* 2001). Homogenisation creates smaller fat globules

299 with a greater total fat-water interfacial surface area that enabled fat globules to interact

300 with the casein matrix (Everett and Auty 2008).

301   Moreover, Table 1 shows moisture percentages values for each type of cheese.

302 These values slightly varied among samples where the moisture content of the ChC-R

303 was found to be significantly lower than the rest. Additionally, the presence of the Zn^{2+}

304 salts might promote water retention. Previous reports showed that moisture values of

305 other soft cheese increase with the homogenisation process (Kwak *et al.* 2001, Kwak *et*

306 *al.* 2002).

307 **Firmness determinations**

308 Cheese samples firmness is shown in Figure 2, in which ChWT firmness was

309 statistically higher compared to the other types of cheese analyzed. These results agree

310 with the observation of a soft and brittle curd during cheese manufacture from β-CD

311 treated CB, also reported by Kwak et al. (2002) for cholesterol-reduced Cheddar cheese

312 manufacture. Therefore, these lower firmness values found in cholesterol-reduced

313 cheese samples could be explained by weak coagulum formation as a consequence of

314 CB homogenisation process that caused greater fat globule dispersion in the curd and a

315 reduction in the amount of free casein available to form casein network, resulting in

316 improper curd matting.

317   On the other hand, no statistical differences in firmness value were found

318 between cheese obtained from cholesterol-reduced TM (ChC-R; $ChZnSO_4$; $ChZnCl_2$),

319   indicating that Zn^{2+} salt fortification does not have an effect on firmness value.

320   Therefore, the Zn^{2+} concentrations added seems not to affect the establishment of

321   rearrangements that lead to increase the coagula firmness.

322

323   **Digital colorimetric measurement**

324   Figure 3A and 3B exhibit the images of the different cheese samples under study and

325   the colour parameters for each, respectively. In Figure 3B, a decrease in the parameter

326   values a* and b* could be observed in cheese samples obtained from the cholesterol-

327   reduced TM in comparison with the values found for ChWT, which reflects a decrease

328   in the intensity of the shades of green (a*) and yellow colours (b*). The visual colour

329   differences in ChWT (Figure 3A) are mainly explained by the variation of b* parameter.

330   Moreover, no differences were found in the values for all analyzed cheese samples for

331   the L* parameter. These results agree with previous findings by Rudan *et al*. (1998) and

332   Kwak *et al*. (2001). They had reported a change in the cheese structure as a

333   consequence of the reduction of fat particle size by the homogenisation process, which

334   makes the cheese appearance whiter and less yellow than cheese made from

335   unhomogenised milk (Rudan *et al.* 1998, Kwak *et al.* 2001).

336   **Microstructure analysis**

337   The concept of texture from a computational point of view refers to the spatial

338   arrangement of the brightness of the pixels in a region of the image. Therefore, texture

339   parameter study through images reflects changes of intensity values of pixels which

340   might contain information of geometric structure of objects since a great change of

341   intensity values may indicate a change in geometric structure (Zheng *et al.* 2006).

342   CLSM images of cheese samples sections obtained from the TM with cholesterol-

343   reduced content exhibit a diminution in the values of S, K and $\sigma^2(N)$ and an increment

344   in the U value, in comparison with the parameter values found for ChWT (Table 2). An

345   increment in the value of U implies a tendency to a uniform distribution of the grey

346   scale of the image, while a diminution of S, K and $\sigma^2(N)$ corresponds to structures in

347   which all the particles are dispersed uniformly over the entire extension of the image

348   (Ingrassia *et al.* 2013). On the other hand, as an observation of images of cheese

349   samples obtained from the TM with cholesterol-reduced content exhibit smaller pores

350   size and pores homogeneously distributed around the entire image (Figure 4), as a result

351   of the process of homogenisation. Nevertheless, CLSM micrographs for ChWT images

352   showed large, irregular and sectored pores. However, there are no differences in texture

353   parameter values and pore size distributions between the ChC-R, ChZnSO$_4$ and

354   ChZnCl$_2$, reflecting the fact that the addition of Zn^{2+} salts has no effect on cheese

355   microstructure, thus concluding that microstructural differences are due exclusively to

356   the homogenisation process.

357   **Meltability**

358   Meltability could be defined as the capability of the cheese to flow or spread upon

359   heating. Figure 5 shows the areas before and after cheese melting process. The results

360   obtained from the difference of areas measured agree with Kwak *et al.* (2001), who

361   found that meltability decreased with homogenisation. In addition, other authors

362   reported that milk homogenisation breaks down fat globule membranes, making the

363   spread of melted fat more difficult (Lelievre *et al.* 1990). Therefore, the mechanical

364   homogenisation step over the CB for cholesterol extraction affects in a negative way the

365   meltability of the resulting cheese. On the other hand, Zn^{2+} salt presence would seem

366   not to affect the melting properties of fortified cheese samples.

367

368   **Sensory analysis**

369   The sensory attribute of cheese was studied in appearance, texture and flavour; the

370   average data for all sensory descriptors are summarized in Table 3.

371   *Appearance*

372   Cheese appearance was homogeneous with no significant differences among all

373   analyzed cheese samples. Colour was more intense in ChWT than in the other types,

374   which is in consonance with the results found in the digital colour determinations

375   presented. Appearance is one of the most important attributes for cheese and is closely

376   related to consumer's acceptance. From this point of view, whiteness of cholesterol-

377   reduced cheese would grant a desirable feature for the consumer, avoiding the frequent

378   use of chemical strategies, such the addition of titanium dioxide to reach such desirable

379   whiteness (Rudan *et al.* 1998).

380   *Texture*

381   Elasticity for ChZnSO$_4$ showed a significantly lower value than the rest. Adherence to

382   the palate was higher in the case of fortified cheese than for ChC-R and ChWT,

383   indicating than Zn^{2+} salts increase the resistance to remove the product from the palate.

384   Cohesiveness was lower for ChZnSO$_4$ and higher for ChC-R. Chewiness and mouthfeel

385   were similar in all samples.

386   *Flavour*

387   Cream odour was detected with more intensity in ChWT than in the other types of

388   cheese. Sweet flavour was detected in low intensity in all the cheese samples studied,

389   ruling out the possibility that a remainder of β-CD could impart sweetness to the cheese

390   mass. Bitter, salty and acid flavours were barely detected in all samples, presenting no

391   significant differences among them. Cream flavour was detected with the same intensity

392   in all samples, indicating that the process of cholesterol extraction does not alter the

393   organoleptic properties of the traditional cheese. Metallic flavour was not detected and

394   astringency was low for all samples.

395   Although negative changes in sensory properties of foods fortified with $ZnSO_4$

396   have been reported (Salgueiro *et al.* 2002, Boccio and Monteiro 2004), metallic flavour,

397   acid taste and astringency were detected without statistically significant differences

398   among all samples, indicating that fortification with Zn^{2+} salts does not have a

399   significant negative effect on organoleptic characteristics of cheese. In addition, similar

400   sensorial scores were obtained for the fortified cheese samples with the two salts under

401   study, except for elasticity and cohesiveness sensory attributes, in which $ChZnCl_2$

402   showed to be more similar to ChWT.

403

404   **CONCLUSIONS**

405   In the present study, a functional dairy product with a satisfactory level of cholesterol

406   extraction (93%) and a high content of Zn^{2+} level, with adequate physical and sensory

407   characteristics, comparable to those found in traditional Cuartirolo soft cheese, was

408   achieved. However, the differences found between the ChWT and the cholesterol-

409   reduced content cheese samples were due to the mechanical homogenising process used

410   to maximize the cholesterol extraction percentage and were not related with the use of

411   β-CD or with the Zn^{2+} fortification strategy. On the other hand, both Zn^{2+} salts tested

412   showed to be effective and appropriate for Cuartirolo soft cheese fortification.

413   Nevertheless, the use of $ZnCl_2$ showed to be more adequate from a sensorial point of

414   view.

415   Based on the overall results we can demonstrate the suitability of the manufacture

416   of a cholesterol-reduced and Zn^{2+} fortified Cuartirolo soft cheese that may meet

417   nutritional needs of risk groups.

418

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REFERENCES

ANMAT (2002). *Código Alimentario Argentino. Capítulo VIII: Alimentos lácteos.* Buenos Aires: De la Canal y Asociados.

Association of Official Analytical Chemists (1990) *Official methods of analysis*, 15th edn. Helrich K, ed. Arlington, VA: Association of Official Analytical Chemists.

Aquilanti L, Kahraman O, Zannini E, Osimani A, Silvestri G, Ciarrocchi F, Garofalo C, Tekin E and Clementi F (2012) Response of lactic acid bacteria to milk fortification with dietary zinc salts. *International Dairy Journal* **25** 52-59.

Boccio J and Monteiro J B (2004) Food fortification with iron and zinc: pros and cons from a dietary and nutritional viewpoint. *Revista de Nutrição* **17** 71-78.

Dias H M A M, Berbicz F, Pedrochi F, Baesso M L and Matioli G (2010) Butter cholesterol removal using different complexation methods with beta-

443   cyclodextrin, and the contribution of photoacoustic spectroscopy to the

444   evaluation of the complex. *Food Research International* **43** 1104-1110.

445 Doube M, Kłosowski M M, Arganda-Carreras I, Cordelières F P, Dougherty R P,

446   Jackson J S, Schmid B, Hutchinson J R and Shefelbine S J (2010) BoneJ: Free

447   and extensible bone image analysis in ImageJ. *Bone* **47** 1076-1079.

448 El-Din A G, Hassan A, El-Behairy S and Mohamed E (2012) Impact of zinc and iron

449   salts fortification of buffalo's milk on the dairy product. *World Journal of Dairy*

450   *& Food Sciences* **7** 21-27.

451 Everett D W and Auty M A (2008) Cheese structure and current methods of analysis.

452   *International Dairy Journal* **18** 759-773.

453 Hess S Y and Brown K H (2009) Impact of zinc fortification on zinc nutrition. *Food &*

454   *Nutrition Bulletin* **30** 79S-107S.

455 Ingrassia R, Costa J P, Hidalgo M E, Mancilla Canales M, Castellini H, Riquelme B and

456   Risso P (2013) Application of a digital image procedure to evaluate

457   microstructure of caseinate and soy protein acid gels. *LWT - Food Science and*

458   *Technology* **53** 120-127.

459 Kahraman O and Ustunol Z (2012) Effect of zinc fortification on Cheddar cheese

460   quality. *Journal of Dairy Science* **95** 2840-2847.

461 Kim S Y, Hong E K, Ahn J and Kwak H S (2009) Chemical and sensory properties of

462   cholesterol-reduced processed cheese spread. *International journal of dairy*

463   *technology* **62** 348-353.

464 Kwak H, Jung C, Shim S and Ahn J (2002) Removal of cholesterol from Cheddar

465   cheese by β-cyclodextrin. *Journal of Agricultural and Food Chemistry* **50**

466   7293-7298.

467   Kwak H, Nam C and Ahn J (2001) Low cholesterol Mozzarella cheese obtained from

468   homogenized and β-cyclodextrin-treated milk. *Asian-Australasian Journal of*

469   *Animal Sciences* **14** 268-275.

470   Lelievre J, Shaker R R and Taylor M W (1990) The role of homogenization in the

471   manufacture of halloumi and mozzarella cheese from recombined milk.

472   *International Journal of Dairy Technology* **43** 21-24.

473   Muthukumarappan K, Wang Y C and Gunasekaran S (1999) Modified Schreiber test for

474   evaluation of Mozzarella cheese meltability. *Journal of Dairy Science* **82** 1068-

475   1071.

476   Pavón Y L, Lazzaroni S M, Sabbag N G and Rozycki S D (2014) Simultaneous effects

477   of gelatin and espina corona gum on rheological, physical and sensory properties

478   of cholesterol-reduced probiotic yoghurts. *International Journal of Food Science*

479   *& Technology* **49** 2245-4451.

480   Pomastowski P, Sprynskyy M and Buszewski B (2014) The study of zinc ions binding

481   to casein. *Colloids and Surfaces B: Biointerfaces* **120** 21-27.

482   Rehm C D, Drewnowski A and Monsivais P (2015) Potential Population-Level

483   Nutritional Impact of Replacing Whole and Reduced-Fat Milk With Low-Fat

484   and Skim Milk Among US Children Aged 2–19 Years. *Journal of Nutrition*

485   *Education and Behavior* **47** 61-68.

486   Rudan M A, Barbano D M, Guo M R and Kindstedt P S (1998) Effect of the

487   modification of fat particle size by homogenization on composition, proteolysis,

488   functionality, and appearance of reduced fat Mozzarella cheese. *Journal of*

489   *Dairy Science* **81** 2065-2076.

490 Salgueiro M J, Zubillaga M, Lysionek A, Caro R, Weill R and Boccio J (2002)

491   Fortification strategies to combat zinc and iron deficiency. *Nutrition Reviews* **60**

492   52-58.

493 Salgueiro M J, Zubillaga M, Lysionek A, Sarabia M I, Caro R, De Paoli T, Hager A,

494   Weill R and Boccio J (2000) Zinc as an essential micronutrient: a review.

495   *Nutrition Research* **20** 737-755.

496 Soazo M, Pérez L M, Rubiolo A C and Verdini R A (2014) Prefreezing application of

497   whey protein-based edible coating to maintain quality attributes of strawberries.

498   *International Journal of Food Science & Technology*.

499 WHO/FAO (2005). *Vitamin and mineral requirements in human nutrition, 230-243*.

500   Switzerland: World Health Organizationand and Agriculture Organization of the

501   United Nations.

502 Zheng C, Sun D-W and Zheng L (2006) Recent applications of image texture for

503   evaluation of food qualities—a review. *Trends in Food Science & Technology*

504   **17** 113-128.

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506 **Figure captions**

507 **Figure 1.** Flow diagrams for Cuartirolo cheese manufacture.

508 **Figure 2.** Firmness estimated at 25°C by penetrometry on Cuartirolo cheese samples.

509 The same letters indicate that there were no significant differences among the samples

510 ($p > 0.05$)

511 **Figure 3.** A) Cheese sample images B) Parameters of colour of cheese samples. The

512 same letters indicate that there was no significant difference among the samples

513 ($p > 0.05$).L*=Lightness (0=black; 100=white); a*= red-green component ; b*= yellow-

514 blue component.

515   **Figure 4.** Box plots of the cheese samples pore size distribution. The same letters

516   indicate that there was no significant difference among the samples (p>0.05).

517   **Figure 5.** Areas before and after melting test. The same letters indicate that there was

518   no significant difference among the samples (p>0.05).

519   **Table 1** Data represent the means and standard deviation of cheese chemical

520   composition and cheese yield measurements per sample.[*]

521   **Table 2** Data represent the means and standard deviation of textural parameters

522   obtained from digital images of different cheese samples: Shannon entropy (S),

523   smoothness (K), uniformity (U), and mean normalized grey-level variance (σ^2 (N)).[*] All

524   texture parameters are dimensionless.

525   **Table 3** Data represent the means and standard deviation of sensory characteristics of

526   different cheese samples storage at 4°C for 20 days.[*]

527

528   **Table 3** Data represent the means and standard deviation of sensory characteristics of

529   different cheese samples storage at 4°C for 20 days.[*]

Sample	ODOR	COLOR	CHEESE APPEARANCE	ELASTICITY	ADHERENCE	COHESIVENESS
ChWT	4.15 ± 1.70^c	4.71 ± 1.75^b	8.39 ± 0.55^a	4.83 ± 2.12^b	2.77 ± 1.37^a	5.54 ± 1.77^{ab}
ChC-R	2.40 ± 1.96^{ab}	2.98 ± 1.35^a	7.98 ± 0.98^a	5.70 ± 1.47^b	3.88 ± 1.58^{ab}	6.91 ± 1.19^b
ChZnCl₂	3.54 ± 2.10^{cd}	2.71 ± 1.20^a	7.23 ± 2.03^a	5.17 ± 2.57^b	5.01 ± 2.44^b	5.65 ± 1.83^b
ChZnSO4	1.69 ± 1.88^a	1.87 ± 1.07^a	7.32 ± 2.24^a	2.76 ± 2.28^a	5.58 ± 3.44^b	4.09 ± 1.39^a
p-value	0.0190	0.0012	0.4103	0.0243	0.0413	0.0075

Sample	CHEWINESS	MOUTHFEEL	SWEET TASTE	ACID	SALTY	BITER
ChWT	3.34 ± 1.69^a	5.43 ± 2.10^a	1.39 ± 1.03^a	3.37 ± 1.69^a	4.29 ± 1.61^a	1.98 ± 1.47^a
ChC-R	4.29 ± 1.46^a	6.39 ± 1.20^a	1.51 ± 1.04^a	3.10 ± 1.05^a	3.67 ± 1.69^a	1.98 ± 0.66^a
ChZnCl₂	3.79 ± 0.87^a	5.61 ± 1.55^a	1.59 ± 1.18^a	3.61 ± 2.20^a	3.81 ± 1.11^a	1.70 ± 1.48^a
ChZnSO₄	2.77 ± 1.01^a	6.14 ± 1.72^a	1.58 ± 1.11^a	3.53 ± 1.62^a	3.97 ± 1.94^a	2.64 ± 1.40^a
p-value	0.1128	0.6060	0.9797	0.9244	0.8717	0.2703

Sample	CREAM FLAVOR	METALLIC FLAVOR	ASTRINGENCY	RESIDUAL FLAVOR		
CWT	3.44 ± 1.84^a	2.07 ± 1.67^a	1.84 ± 1.93^a	4.29 ± 2.87^a		
ChC-R	4.38 ± 1.43^a	1.34 ± 1.43^a	2.30 ± 1.15^a	3.49 ± 1.63^a		
ChZnCl₂	4.10 ± 1.92^a	1.63 ± 0.88^a	2.68 ± 2.16^a	4.00 ± 1.70^a		
ChZnSO₄	2.80 ± 1.75^a	1.89 ± 1.07^a	3.53 ± 1.88^a	4.42 ± 2.03^a		
p-value	0.1821	0.6292	0.1042	0.8001		

530   [*]The same letters indicate that there was no significant difference among the samples

531   (p>0.05)

532

533

Table 1 Data represent the means and standard deviation of cheese chemical composition and cheese yield measurements per sample.[*]

Sample	$[Zn^{2+}]$ (mg kg^{-1})	Yield of cheese (% w/w)	Zn^{2+}Fortification (% w/w)	Moisture (% w/w)
ChWT	$(33\pm2)^a$	$(12.2\pm0.5)^a$	-	$(56.4\pm0.5)^a$
ChC-R	$(26\pm2)^b$	$(13.1\pm0.5)^{ab}$	-	$(53.9\pm0.4)^b$
ChZnCl$_2$	$(129\pm8)^c$	$(13.5\pm0.1)^b$	$(87\pm7)^a$	$(55.8\pm0.9)^a$
ChZnS0$_4$	$(131\pm8)^c$	$(14\pm1)^b$	$(94\pm7)^a$	$(55.8\pm0.1)^a$

[*] The same letters in the same column indicate that there was no significant difference among the samples (p>0.05).

Table 2 Data represent the means and standard deviation of textural parameters obtained from digital images of different cheese samples: Shannon entropy (S), smoothness (K), uniformity (U), and mean normalized grey-level variance (σ^2 (N)).[*] All texture parameters are dimensionless.

Texture parameters	ChWT	ChC-R	ChZnSO$_4$	ChZnCl$_2$
S	5.8 ± 0.2^a	5.4 ± 0.1^b	5.24 ± 0.02^b	5.28 ± 0.08^b
K	0.043 ± 0.005^a	0.017 ± 0.002^b	0.014 ± 0.001^b	0.016 ± 0.001^b
σ^2(N)	$2{,}900\pm300^a$	$1{,}100\pm100^b$	930 ± 60^b	$1{,}050\pm80^b$
U	0.023 ± 0.004^a	0.031 ± 0.003^b	0.031 ± 0.001^b	0.030 ± 0.002^b

[*]The same letters indicate that there was no significant difference among the samples ($p > 0.05$)

CB (10% w/w fat content)

(With and without Cholesterol-reduced content)

```
                        ┌──────────────┐
                    ◄───│  Skim milk   │
                        └──────────────┘
```

TM (3 % w/w fat content)

↓

Heating (65 °C, 20min)

↓

Cooling to 40 °C

```
                    ┌──────────────────────────┐
                ◄───│   Addition of Ca²⁺        │
                    └──────────────────────────┘
                    ┌──────────────────────────┐
                ◄───│   Fortification with Zn²⁺ │
                    └──────────────────────────┘
                    ┌──────────────────────────┐
                ◄───│   Starter inoculum        │
                    └──────────────────────────┘
                    ┌──────────────────────────┐
                ◄───│   Rennet addition         │
                    └──────────────────────────┘
```

Coagulation

(40 °C, 30 min)

↓

Cutting of the curd

↓

Maintenance

(40 °C, 15 min)

↓

Molding and whey drainage

(40 °C, final pH=5.3±0.1)

↓

Brining

(20 % w/w NaCl, 4-8 °C, 60min kg^{-1})

↓

Ripening

(5 °C, 20 days)