Gastro-pancreatic release of phenolic compounds incorporated in a polyphenols-enriched cheese-curd

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Abstract

As functional food, enriched cheese has recently been developed. The main objectives of this study were to investigate the role of casein in the retention of polyphenol during curd formation and the release of polyphenols during *in vitro* gastro-pancreatic digestion of polyphenols-enriched cheese and their contribution to the antioxidant activity of digested curd. Polyphenols showed high retention coefficient in curd. The retention coefficient of polyphenol was related to the binding affinity to casein and to their hydrophilicity. The polyphenols should be added before milk coagulation since the binding decreases as casein molecules aggregate. During *in vitro* gastro-pancreatic digestion steps, polyphenols were released from curd due to the dilution in gastric fluid and to casein proteolysis. The addition of polyphenols to curd determined a relevant increase of antioxidant activity respect to the curd control even a part of polyphenols is degraded by alkaline pH of pancreatic fluid. Our results suggested the possibility of producing highly nutritive value cheese with high release of the polyphenols during digestion. In addition, the whey, which contains polyphenols, can be involved in different products to maximize its utilization.

Keywords: cheese, polyphenols, antioxidant activity, *in vitro* digestion

1. Introduction

Over the last years, much more attention has been paid to polyphenolic compounds. They are the major source of antioxidants in human diet, and show a wide range of activities such as anti-carcinogenic, anti-inflammatory, anti-atherosclerotic, angiogenesis and cell proliferation inhibitory activities (Crozier, Jaganath, & Clifford, 2009; Verzelloni, Tagliazucchi, Del Rio, Calani, & Conte, 2011; Conte, Pellegrini, & Tagliazucchi, 2003). Epidemiological studies and human intervention trials have associated a high intake of fruit and vegetables rich in phenolic compounds with a lower incidence of chronic diseases including diabetes, cardiovascular diseases and cancer (Del Rio et al., 2013).

The incorporation of bioactive compounds during the manufacturing of innovative functional foods became of important interest to improve the nutritional and healthy properties of certain types of food. Recent examples on this topic involved the incorporation of bioactive phenolic compounds in yogurt (Chouchouli et al., 2013), ice cream (Çam, Içyer, & Erdoğan, 2014) and cheese (Han et al., 2011a).

Cheese possesses a unique composition and structure, which actuate the researchers to try to apply different bioactive compounds to cheese with expectation to improve its nutritional and healthy qualities (Joseph, & Akinyosoye, 1997; Prudêncio, Prudêncio, Gris, Tomazi, & Bordignon-Luiz, 2008; Bandyopadhyay, Chakraborty, & Raychaudhuri, 2008; Rinaldoni, Palatnik, Zaritzky, & Campderros, 2014). Recently, Han et al. (2011b) developed a functional cheese product containing polyphenolic compounds.

To exert their biological activity, phenolic compounds must be released from the curd during digestion. While polyphenols contained in the liquid matrices are promptly available for the absorption, this is not true for polyphenols contained in solid matrices

such as polyphenols incorporated in cheese. In these foods, polyphenols must first be released to be bioaccessible, potentially bioavailable and able to exert their beneficial effects (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010; Tagliazucchi, Verzelloni, & Conte, 2012a; Chiang, Kadouh, & Zhou, 2012).

The main objectives of this study were (i) to evaluate the retention coefficients of different types of polyphenol compounds revealing the mechanism by which these compounds are retained in and released from the curd; (ii) to measure the release of incorporated polyphenols during *in vitro* digestion; (iii) to evaluate the antioxidant activity released during gastro-pancreatic digestion steps.

2. Materials and methods

2.1. Materials

Pasteurized whole bovine milk (3.1 g/100 mL protein and 3.6 g/100 mL fat) was purchased in a local market (Reggio Emilia, Italy). Liquid calf rennet was obtained from Educational Dairy Plant (Damanhour University, Damanhour, Egypt). Phenolic compounds, catechin, chlorogenic acid, ferulic acid, vanillic acid, gallic acid, *p*-coumaric acid, 3,4-dihydroxyphenylacetic acid and tannic acid were purchased from Sigma (Milan, Italy). Casein, calcium chloride, bile salts (mixture of sodium cholate and sodium deoxycholate), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP specifications) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were supplied by Sigma (Milan, Italy). Ethanol was supplied by Carlo Erba (Milan, Italy).

2.2. Cheese curd preparation

65 Polyphenols-enriched cheese was manufactured as described by Han et al. (2011b) with
66 some modifications. Calcium chloride was added to the milk obtaining a final
67 concentration of 6 mmol/L to compensate the effect on milk ingredient properties of
68 pasteurization which decreases the concentration of free calcium and homogenization
69 which decreases the dimension of fat micelles and increases the adsorption of κ-casein
70 micelles on the fat globules. Different phenolic compounds were added to the milk as a
71 solid compound to have a final concentration of 0.5 mg/mL, followed by stirring to
72 obtain a homogenized solution. Rennet was firstly submitted to a clotting activity test as
73 described by Berridge (1952), and then 1 mL was added to 20 mL of milk to be
74 completely coagulated within 2h at 35°C. To separate the whey from the curd, the
75 coagulated samples were centrifuged at 1300*g* at 21°C for 15 min. The curd and whey
76 were weighed and measured volumetrically.
77 Casein curd was also prepared using casein solution at the same concentration as in milk
78 (2.48 g/100 mL), in the presence and absence of catechin.
79 The samples containing polyphenols were treated as described above.
80 Curd moisture content (CMC) was calculated according to Pandey, Ramaswamy, & St-
81 Gelais (2000).

82 **2.3. Polyphenols determination and polyphenols retention coefficient (PRC)**

83 To estimate the amount of polyphenols incorporated in curd, retention coefficient was
84 determined for all the samples. Phenolic content was determined using high performance
85 liquid chromatography (Jasco HPLC, Tokyo, Japan) equipped with a C18 column (HxSil
86 C18 Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton Company, Reno, Nevada,
87 USA). A volumetric injector Rheodyne (Cotati, CA, USA), and a temperature-controlled

88 oven were utilized. An amount of 20 μL of each sample was used for injection with a
89 gradient system of solvent A (1 mL/100 mL formic acid in water v/v) and solvent B
90 (acetonitrile) as the mobile phase at a flow rate of 1 mL/min and the temperature was
91 adjusted to 32°C. The gradient system was linear, solvent B started from 4 mL/100 mL at
92 0 min and reached 25 mL/100 mL at 60 min; while in the case of measuring tannic acid,
93 solvent B reached linearly 60 mL/100 mL after 60 min. Peaks for samples and standards
94 were monitored at 280 nm. The calibration curves of standards polyphenols were used to
95 quantify the polyphenols. PRC is the percentage of the amount of the polyphenols added
96 to milk which remains in curd.

97 **2.4. *In vitro* gastro-pancreatic digestion**

98 The two-stage *in vitro* digestive model was adapted from Tagliazucchi et al. (2010) with
99 some modifications. Curds were diluted 10 times with simulated gastric fluid containing 2
100 g/L of NaCl and 60 mmol/L HCl, pH 2.0, and homogenized for 2 min in a laboratory
101 blender. The homogenates were adjusted to pH 2.0 with concentrated HCl and pepsin
102 (315 U/mL) was added. The samples were incubated at 37°C in a shaking bath for 2 h to
103 simulate the gastric phase of digestion. At the end of the gastric digestion, the pH was
104 brought to 7.5 with $NaHCO_3$ before adding 0.8 mg/mL pancreatin and 5 mg/mL of bile
105 salts. The solution was then incubated again at 37°C in a shaking bath to simulate the
106 intestinal phase of digestion. After 2 h incubation, an aliquot of each sample was
107 withdrawn and the pH was lowered to 2.0 to inactivate the enzymes and stabilize the
108 polyphenols.

109 Aliquots of the samples were also withdrawn after the homogenization and after the
110 gastric phase of digestion. A centrifugation was carried out on all aliquots at 1300*g* at

111 21°C for 15 min, the pellet and the supernatant were weighed, measured volumetrically
112 and used for further analysis. Polyphenols were determined using high performance liquid
113 chromatography (HPLC), following the same protocol as described in section 2.3

114 **2.5. Fluorescence spectroscopy**

115 The interaction between casein and the different polyphenols used in cheese was
116 investigated by using fluorescence spectroscopy as reported by Tagliazucchi, Helal,
117 Verzelloni, & Conte (2012b) with some modification.

118 Fluorescence spectra were recorded at 35°C in the range of 290-500 nm at an excitation
119 wavelength of 280 nm using Jasco, FP-6200 spectrofluorometer (Tokyo, Japan). The
120 intensity at 340 nm (tryptophan emission wavelength) was used to calculate the binding
121 constant according to Dufour, & Dangles (2005).

122 Solutions of the following ligands, catechin, tannic acid, chlorogenic acid, coumaric acid,
123 ferulic acid, dihydroxyphenylacetic acid, vanillic acid and gallic acid were prepared in
124 methanol. For each data point, 2 mL of 5 μmol/L casein (a mixture of α-and β-caseins
125 dissolved in 10 mmol/L sodium phosphate buffer, pH 6.5) were transferred into a cuvette.
126 After 5 min of equilibration at 35°C, 0.01 mL of each of the above reported polyphenol
127 methanol solution was added to cuvette. The added solutions of ligands were properly
128 diluted in methanol to have a final ligand concentration between 1 and 100 μmol/L. The
129 change in fluorescence emission intensity was measured after 10 min of the mixing with
130 casein. The effect observed on the casein fluorescence emission spectrum with addition of
131 methanol alone, was calculated and subtracted of the value of casein alone. Catechin,
132 vanillic acid and 3,4-dihydroxyphenylacetic acid possess intrinsic fluorescence at the used

133 excitation wavelength. Therefore, the emission spectrum of these phenolics was
134 determined and subtracted from the emission spectra obtained for casein quenching.
135 The type of binding was assessed using the Stern-Volmer equation (Lakowitz, 2006).
136 For the kinetic analysis of ligand binding, non-linear regression analysis was performed
137 using Graph Pad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). K_D
138 (dissociation constant) and *n* (number of binding site) were calculated according to Rawel,
139 Frey, Meidtner, Kroll, & Schweigert (2006) using the following equation:
140 $F_0-F= F_0*L_0^n/(K_D+L_0^n)$
141 and by plotting the graph of F_0-F versus L_0. F and F_0 are the measured fluorescence
142 emission intensity of the casein solution in the presence and absence of the ligand,
143 respectively, and L_0 the total concentration of the ligand.

144 **2.6. Radical scavenging activity determination**

145 The radical scavenging activity of samples taken during the *in vitro* simulated digestion
146 procedures was evaluated using the ABTS method as described in Re et al. (1999).
147 Briefly, samples withdrawn after the homogenization and after the gastric and intestinal
148 phase of digestion were centrifuged as described in section 2.5 and 40 µL of supernatant
149 were mixed with 1960 µL of ethanolic $ABTS^{\cdot+}$ solution. The mixture, protected from the
150 light, was incubated in the spectrophotometer at 37°C for 10 min; the decrease in
151 absorbance at 734 nm was measured at the endpoint of 10 min. ABTS units of the
152 samples were measured and calculated as Trolox equivalent antioxidant capacity (TEAC)
153 using a standard curve of Trolox. The results were expressed as µmol/L of TEAC.

154 **2.7 Statistics**

155 Data are presented as mean ± SD for three replicates for each prepared sample. Linear

156 regression analysis was performed using Graph Pad Instat (GraphPad Software, San
157 Diego, CA, USA). Univariate analysis of variance (ANOVA) with Tukey post-test was
158 applied using PASWStatistics 18.0 (SPSS Inc. Chicago, IL, USA) when multiple
159 comparisons were performed. The differences were considered significant when $P < 0.05$.
160

161 **3. Results and Discussion**

162 **3.1. Curd yield, curd moisture content (CMC) and polyphenols retention coefficient**
163 **(PRC)**

164 Table 1 reports the percent of curd formed from 20.68 g of milk (20 mL with a specific
165 weight of 1.034). All the curds formed in the presence of polyphenols showed a non-
166 significant increase in the yields respect to the control.

167 In the same table, curd moisture content, pH, and polyphenol retention coefficient are
168 reported. One of the most important characteristics of cheese curd is the moisture content,
169 which affects many factors like yield, texture properties and calculation of the nutritional
170 values based on dry weight. The addition of different polyphenols to milk had no
171 significant effect on the moisture content in the majority of samples. However, a slight
172 significantly ($P < 0.05$) decrease in CMC in case of the addition of tannic acid was
173 noticed. According to Han et al. (2011a), this decrease can be attributed to hydrophobic
174 interaction between milk proteins and polyphenols, which would reduce the quantity of
175 entrapped water in protein polymeric networks during the formation of cheese curd.

176 The retention coefficient values of phenols investigated ranged between 63.0 ± 1.1 (gallic
177 acid) and 86.8 ± 0.2 g/100 g (tannic acid).

178 Retention coefficient is an important parameter to evaluate the residual amount of

179 additives such as polyphenols. A higher retention coefficient obtained in curd, a lower
180 loss of functional ingredients in whey occurred. A high retention coefficient of the curd
181 predicts a high retention during the cheese processing.
182 To explain the differences in the retention coefficient between the various polyphenols
183 utilized, it is important to consider the media in which they are distributed (curd and
184 whey). The coefficient of retention of polyphenols depends on various factors such as the
185 interaction between specific or non-specific binding sites on the protein molecules, the
186 solubility in water and in lipid micelles, the distribution between solid matrix and liquid
187 phase of the curd. The decrease of pH or temperature decreases the solubility of phenols
188 and a part of the phenols may come out of the liquid phase and be trapped in the pellet
189 curd. An important factor, which can affect the retention coefficient, is the hydrophilicity
190 of the polyphenols. The different types of polyphenols used in our study showed different
191 degree of hydrophilicity. We separated and determined the phenolic compounds by
192 chromatography on C-18 column eluted by gradients of formic acid in water as
193 hydrophilic and acetonitrile as hydrophobic component of mobile phase. The elution of
194 phenolic compounds from C-18 column gives us an evaluation of their hydrophilicity.
195 From elution data, it resulted that the rank order of hydrophilicity at low pH, was gallic
196 acid > 3,4-dihydroxyphenylacetic acid > vanillic acid ≈ catechin ≈ chlorogenic acid > *p*-
197 coumaric acid > ferulic acid > tannic acid.
198 Correlation analysis showed a positive correlation between the retention time value on C-
199 18 column and the polyphenol retention coefficient in curd (Pearson r = 0.644; P =
200 0.0085), which confirms the role of polyphenols hydrophilicity on the retention
201 coefficient in cheese curd.

202 It should be pointed out that, besides hydrophobicity, other characteristics of the
203 molecules such as the molecular weight and the shape may affect the elution time on C-
204 18 chromatographic column. Four investigated molecules have very similar molecular
205 weights which are 164.16, 168.15, 170.12, 168.15 and 194.18 Da for coumaric, 3,4-
206 dihydroxyphenylacetic, gallic, vanillic, ferulic acids, respectively. The highest molecular
207 weight is that of tannic acid (1701.20 Da). The molecular weight of catechin and
208 chlorogenic acid are 290.27 and 354.31 Da, respectively. Considering the elution of
209 investigated compounds from C-18 columns and the high solubility in water of tannic
210 acid it is possible that the elution from columns is influenced not only by the
211 hydrophobicity but also from other factors such as the molecular mass and the shape of
212 the molecules. We cannot assume that these factors are operative in the curd, decreasing
213 the release of tannic acid. This is an interesting point to be investigate in the future.
214 The different hydrophilicity affected the distribution of polyphenols between curd and
215 whey. Our results clearly showed that the gallic acid, which had the highest
216 hydrophilicity, exhibited the lowest retention coefficient. Inversely, tannic acid, which
217 had the highest elution time from C-18 column, exhibited the highest retention coefficient
218 in curd.

219 **3.2. The specific binding between polyphenols and casein**

220 Since caseins bind polyphenols to specific binding sites (Tagliazucchi et al., 2012b), we
221 investigated the binding affinity of polyphenols and the number of molecules of
222 polyphenols that bind to the casein, by fluorescence spectroscopy.
223 Table 2 shows the *Ksv* for the binding between different polyphenols and casein. The
224 Stern-Volmer constant can be used to gain information about the type of fluorescence

225 quenching. In fact, the fluorophore can be quenched by collision (dynamic quenching) or
226 by complex formation (static quenching) with the quencher. To understand the type of
227 binding, the bimolecular quenching (K_q) constant is calculated and compared to the
228 maximum value possible for diffusion-limited quenching in water (~10^{10} $mol^{-1}s^{-1}L$). K_q
229 was calculated by dividing the experimentally measured K_{SV} for 6 x 10^{-9} sec that is the
230 maximum lifetime (τ_0) of the fluorophore (tryptophan) in the absence of quencher as
231 reported by Lakowitz (2006). It has been shown that in the case of static quenching, the
232 bimolecular binding constant is several magnitude orders higher than the maximum value
233 of diffusion-limited quenching in water.
234 For all the analyzed compounds the type of quenching was static (K_q 3-4 order of
235 magnitude more than the diffusion-limited quenching in water ~10^{10} $mol^{-1}s^{-1}L$) suggesting
236 that the quenching involved the formation of a complex between the quencher (phenols)
237 and fluorophore (tryptophan).
238 The plotting of corrected fluorescence was analyzed by means of non-linear least-square
239 regression fit for the casein–polyphenols models as reported in Rawel et al. (2006).
240 Figure 1(A-H) shows the emission spectra of casein before and after the addition of
241 different concentrations of polyphenol. As can be seen, the polyphenols caused a decrease
242 in the tryptophan emission with increasing concentration.
243 The K_D value is indicative of the affinity between the protein and the polyphenol. The
244 smaller K_D is, higher the affinity is (Table 2). The rank order of polyphenol affinity to
245 caseins is about the same as the order of their retention in the curds.
246 Linear regression analysis showed an inverse correlation between K_D value and the
247 retention coefficient (Pearson r = 0.759; P = 0.0006).

248 The binding affinity of the different polyphenols to casein can largely explain the
249 differences in the retention coefficient in curd. High binding with casein also led to a
250 decrease in the release of the polyphenol during the whey separation, which implies high
251 retention and high recovery in curd.
252 According to our results, we can conclude that, the retention coefficient of polyphenols in
253 cheese curd is positively affected by their binding affinity versus casein network of curd
254 and negatively affected by their hydrophilicity.
255 **3.3. The binding of polyphenols to curd during milk coagulation**
256 It should be pointed out that the retention coefficients are calculated as percentage of
257 milligrams of phenol added while K_D for casein is reported as μmol/L. There is a large
258 difference about 10 times, between the molecular weight of the *p*-coumaric acid, the
259 compound with the lowest molecular weight we have investigated and the tannic acid.
260 The difference in molecular weight results in relevant differences in the concentration of
261 various phenols used. To compare the data of affinity of polyphenols to casein with the
262 concentration of polyphenols during the various experimental stages we report in Table 3**,**
263 millimolar concentration of phenols in milk, in whey, as well as their apparent
264 concentration in curd. We measured the volume of curd and used this volume to calculate
265 the apparent polyphenol concentrations. It appears that the apparent concentration of
266 polyphenols in curd was higher than in whey for all polyphenols. The difference was
267 greater for those compounds that had greater affinity for casein (tannic acid and catechin).
268 From the concentrations of phenols and casein (1.24 mmol/L calculated with a molecular
269 weight of 20 kDa) in the milk, and from the values of K_D it appeared that, in our
270 experimental conditions, no more than 35% of the binding sites of casein for polyphenols

271 could be occupied by the tannic acid, because its concentration (0.30 mmol/L) was lower
272 than that of casein. For the other phenols the 90-98% of the binding site of casein could be
273 occupied. However, the phenol retention coefficient obtained and the concentration of
274 phenols that remained in whey showed that these values were far from being reached.
275 Between 13% (tannic acid) and 37% (gallic acid) of the added compounds remained in the
276 supernatant whey. The presence in milk of compounds bound to phenol binding site of
277 casein, and the partial loss of the capacity of casein to bind phenol during milk
278 coagulation, are two possible reasons for the observed inconsistency. To evaluate the
279 possible role of compounds which in milk compete with the binding of polyphenols to
280 casein we determined the polyphenol retention coefficient of curd prepared from a
281 solution of commercial casein at the same concentration and experimental condition of
282 milk. The catechin retention coefficient significantly increased from 84.0 ± 0.6 to 96.9 ±
283 0.7 g/100 g with commercial casein, which suggested that some compounds present in
284 milk compete with catechin for polyphenols binding site of casein.
285 We also determined the coefficient of retention of catechin when it was added to milk
286 after coagulation as well as to curd after centrifugation and separation from the whey. In
287 both the experiments, the samples were maintained for 2h at 35°C after catechin addition
288 before final centrifugation. When catechin was added to milk after coagulation and to the
289 curd after centrifugation the retention coefficients decreased to 78.7 ± 0.5 and to 50.5 ±
290 0.7 g/100 g, respectively. We added the catechin to curd of casein solution and also in this
291 case we observed that the catechin retention coefficient decreased to 61.5 ± 0.7 from 96.9
292 ± 0.7 g/100g. These data demonstrated that during coagulation the interactions between
293 the molecules of casein decreased the number of binding sites available for the

294 polyphenols. These data also suggested that the coagulation process did not remove
295 polyphenols from casein binding site when they were bound before coagulation.

296 **3.4. Release of polyphenols from curd matrix during simulated gastro-pancreatic**
297 **digestion**

298 Table 4 reports the phenol released during the digestion steps. For comparison the phenol
299 content in curd before digestion is also reported in the table. The dilution and
300 homogenization of curd in simulated gastric fluid determined a relevant polyphenol
301 release. In the supernatant obtained after centrifugation the amount of polyphenol ranged
302 from 69% (tannic acid) to 90% (gallic acid) of the compound present in the curd. It should
303 be pointed out that the phenols present in curd are in part bound to the solid fraction and
304 in part are present in the liquid fraction of the curd, which has a composition similar to
305 whey.

306 Table 3 reports the millimolar concentration of polyphenols in the supernatant obtained
307 after curd dilution and centrifugation. The apparent concentration in pellet obtained after
308 curd dilution and centrifugation is also reported in the same table.

309 With dilution in gastric fluid the concentration of polyphenols and casein decreased 11
310 times and for some polyphenols such as gallic acid the concentration was near to K_D
311 value.

312 The rank order of phenol content, in the curd before dilution and homogenization was
313 tannic acid > catechin > chlorogenic acid > *p*-coumaric acid > ferulic acid > vanillic acid
314 > 3,4-dihydroxyphenylacetic acid > gallic acid. The rank order of phenols that remained
315 in pellet after dilution of curd was tannic acid > *p*-coumaric acid > ferulic acid > catechin
316 > 3,4-dihydroxyphenylacetic acid > chlorogenic acid > vanillic acid > gallic acid.

317 The different rank order may be due to the different pH before (pH 6.70-6.79) and after
318 (pH 2.0) dilution. The decrease of pH results in the protonation of the carboxylic acids of
319 some polyphenols with modification of their solubility in the liquid phase.
320 The affinity of polyphenols to coagulated casein and to protein-lipid micelles may also
321 change with pH variation.
322 At the end of gastric digestion some variation in phenols concentration measured in liquid
323 phase of digest respect to the beginning of the digestion were observed. The significant
324 increase in catechin and chlorogenic acid concentrations that was observed, suggested a
325 further release from curd of these phenols. A small, non-significant increase was also
326 observed for tannic acid, *p*-coumaric and vanillic acids.
327 At the end of the pancreatic digestion the significant decrease in catechin, *p*-coumaric
328 acid, and gallic acid suggested that these compounds were partially degraded during this
329 phase of digestion. Tannic, ferulic, 3,4-dihyddroxyphenylacetic and vanillic acids were
330 quite stable during digestion.
331 It should be pointed out that the concentration of phenols in the supernatant was the result
332 of the negative effect of their degradation and positive effect of their release from casein.
333 From our digestion data it appears that chlorogenic acid, vanillic acid, catechin and tannic
334 acid are the best candidate as phenol additives to curd since they are recovered in higher
335 amounts in the supernatant after pancreatic digestion.

336 **3.5. Curd antioxidant activity**

337 The antioxidant activities of cheese curds are shown in Table 5. The control curd showed
338 antioxidant activity (102.4 ± 4.7 TEAC/L). This antioxidant activity occurred as a result
339 of the content of several compounds in milk, especially high molecular weight caseins

340 (Clausen, Skibsted, & Stagsted, 2009).

341 All the curd samples with polyphenols showed more antioxidant activity than the control
342 sample after homogenization. These results evidenced the higher nutritive value of those
343 polyphenols-enriched cheese.

344 The curd with gallic acid exhibited the highest value followed by ferulic acid, 3,4-
345 dihydroxyphenylacetic acids, *p*-coumaric acid and catechin. Vanillic, tannic and
346 chlorogenic acids containing curd were those with the lowest antioxidant activity.
347 However, it should be taken into account that there are differences in the millimolar
348 concentration of phenols as it results from Table 4**,** column "supernatant of diluted curd".
349 For example, the concentration of gallic acid was 1.56 and 8.3 times higher than that of
350 catechin and tannic acid, respectively.

351 Following simulated gastric digestion phase, we found a significant increase in the control
352 curd antioxidant activity, which was more evident after pancreatic digestion. This increase
353 was due to the release of antioxidant peptides from milk protein during digestion.

354 During digestion (gastric and pancreatic) all the supernatants of cheese enriched with
355 polyphenols maintain a higher antioxidant activity than control.

356 **3.6. Conclusion**

357 The polyphenol retention coefficients of enriched cheese curds were positively related to
358 polyphenol affinity to a single high affinity binding site on casein molecules, while was
359 negatively affected by their hydrophilicity. The polyphenols should be added before milk
360 coagulation since the binding decreases as casein molecules aggregate. The polyphenols
361 released from enriched curds at the end of digestion depend from their stability in gastro
362 and pancreatic fluids and from their affinity to casein.

363 All the tested polyphenols increased the antioxidant activity of enriched curds. This
364 antioxidant activity is released during gastric and pancreatic digestion.
365 This study represents a model for further investigations at molecular level, for the
366 preparation of cheese enriched with bioactive compounds.

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Figure captions

Figure 1. Fluorescence emission spectra of polyphenol–casein interactions. In all samples the casein concentration was 5 μmol/L. (A) Catechin concentrations: 0 (a), 1 (b), 2.5 (c), 5 (d), 7.5 (e), 10 (f), 15 (g), 20 (h), 25 (i), and 30 (l) μmol/L. (B) Tannic acid concentrations: 0 (a), 1 (b), 2 (c), 3 (d), 5 (e), 6 (f), 7 (g), 8 (h), 9 (i), 10 (l), 11 (m) and 12 (n) μmol/L. (C) Chlorogenic acid concentrations: 0 (a), 1 (b), 2 (c), 5 (d), 10 (e), 15 (f), 20 (g), 25 (h), 30 (i), 40 (l), 50 (m) and 100 (n) μmol/L. (D) Coumaric acid concentrations: 0 (a), 2.5 (b), 5 (c), 7.5 (d), 10 (e), 15 (f), 20 (g), 25 (h), 30 (i), 35 (l), and 40 (m) μmol/L. (E) Ferulic acid concentrations: 0 (a), 2.5 (b), 5 (c), 7.5 (d), 10 (e), 15 (f), 20 (g), 25 (h), 30 (i), 35 (l), 40 (m) and 50 (n) μmol/L. (F) Dihydroxyphenylacetic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 30 (g), 40 (h), 50 (i), and 60 (l) μmol/L. (G) Vanillic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 30 (g), 40 (h), and 60 (i) μmol/L. (H) Gallic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 25 (g), 30 (h), 35 (i), 40 (l), 50 (m) and 60 (n) μmol/L.

Figure 1

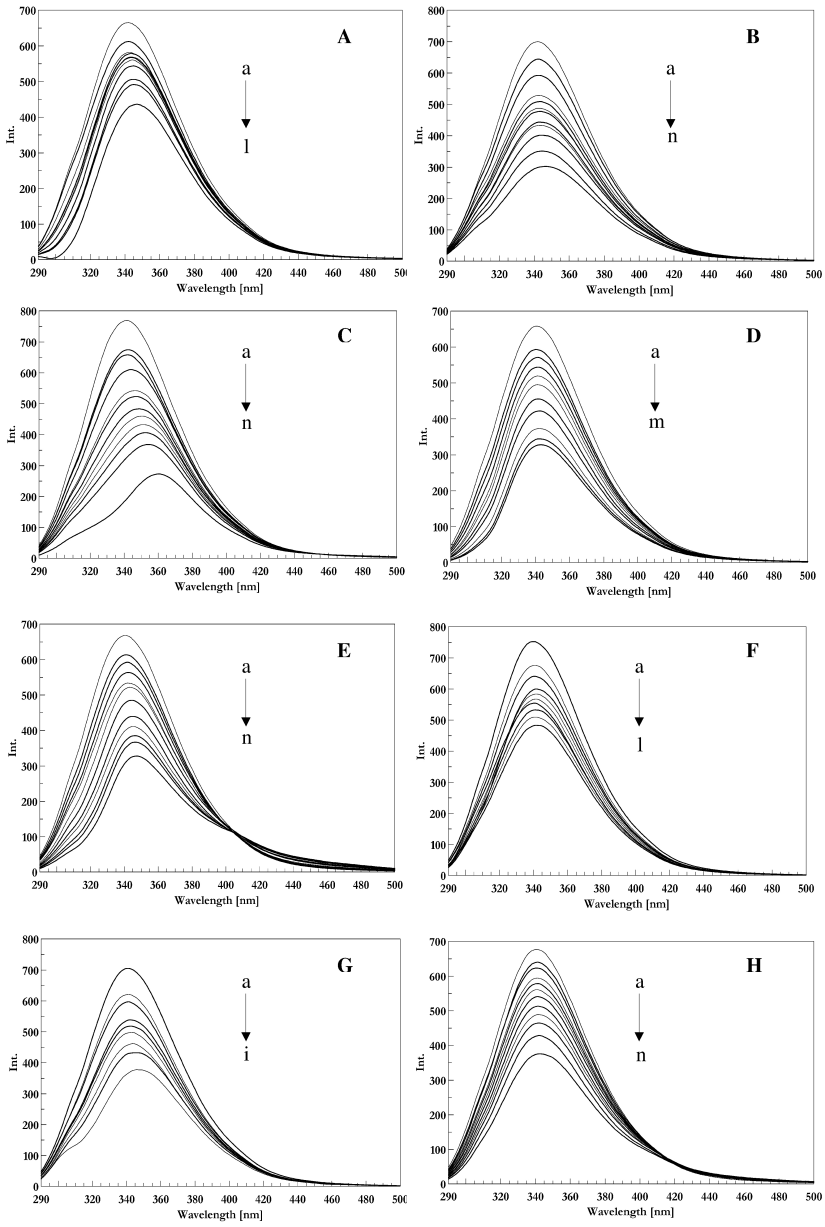

Table 1. Curd yield, curd moisture content (CMC) and pH values of cheese curds and polyphenol retention coefficient (PRC) in cheese curd.

Treatment	*Curd yield (g/100 g)*	*CMC (g/100 g)*	*pH*	*PRC (g/100 g)*
Control	61.9 ± 1.5	68.9 ± 1.0[a]	6.79	/
Catechin	63.8 ± 1.8	67.5 ± 1.4[a,b]	6.78	84.8 ± 0.6[a]
Tannic acid	65.7 ± 2.1	66.0 ± 1.8[b]	6.70	86.8 ± 0.2[a]
Chlorogenic acid	65.1 ± 1.2	66.5 ± 0.9[a,b]	6.72	73.0 ± 1.4[b]
p-Coumaric acid	62.0 ± 1.6	68.8 ± 1.2[a]	6.75	70.3 ± 2.6[b,c]
Ferulic acid	63.4 ± 1.5	67.8 ± 1.1[a,b]	6.75	69.3 ± 0.3[c,d]
3,4-Dihydroxyphenylacetic acid	63.7 ± 1.7	67.6 ± 1.3[a,b]	6.77	63.7 ± 2.3[e,f]
Vanillic acid	62.1 ± 1.4	68.8 ± 0.9[a]	6.74	66.5 ± 1.1[d,e]
Gallic acid	63.6 ± 0.5	67.7 ± 0.4[a,b]	6.72	63.0 ± 1.1[f]

Values in the same columns with different lowercase letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$).

Table 2. Binding constants, quenching type and number of binding sites (*n*) for polyphenols-casein complexes as determined by the quenching of the tryptophan fluorescence.

Polyphenol	*Ksv* ($\times 10^3$ mol^{-1})	*Kq* ($\times 10^{10}$ $mol^{-1}s^{-1}$)	*Quenching type*	K_D (*μmol/L*)	*n*
Catechin	50.3 ± 3.8	838.3 ± 63.3	static	21.9 ± 1.6	0.95 ± 0.09
Tannic acid	509.9 ± 39.3	8498.3 ± 655.0	static	1.8 ± 0.1	0.91 ± 0.08
Chlorogenic acid	27.9 ± 0.6	465.0 ± 10.0	static	37.9 ± 2.3	0.88 ± 0.06
p-Coumaric acid	18.6 ± 0.8	310.5 ± 13.8	static	75.2 ± 4.4	1.14 ± 0.09
Ferulic acid	16.9 ± 0.7	281.7 ± 11.7	static	73.2 ± 5.1	1.20 ± 0.11
3,4-Dihydroxyphenylacetic acid	11.4 ± 0.7	189.0 ± 11.7	static	92.2 ± 5.5	1.27 ± 0.11
Vanillic acid	17.4 ± 0.8	290.0 ± 13.3	static	90.1 ± 8.3	1.16 ± 0.15
Gallic acid	4.1 ± 0.1	68.3 ± 1.7	static	240.4 ± 11.2	0.99 ± 0.06

Kq was calculated by dividing the experimentally measured *Ksv* for 6 x 10^{-9} sec that is the maximum lifetime (τ_0) of the fluorophore (tryptophan) in the absence of quencher as reported by Dufour, & Dangles (2005). Data are means ± SD (*n* = 3).

Table 3. Polyphenol concentration in milk and whey. Concentration in supernatant of diluted curd. Apparent concentration in curd and in pellet of diluted curd.

Polyphenol	*Milk mmol/L*	*Curd mmol/L*	*Whey mmol/L*	*Pellet of diluted curd mmol/L*	*Supernatant of diluted curd mmol/L*
Catechin	1.72 ± 0.02^{a}	2.61 ± 0.12^{a}	0.59 ± 0.05^{a}	0.56 ± 0.06^{a}	$0.17 \pm 0.01^{a,d}$
Tannic acid	0.30 ± 0.01^{b}	0.44 ± 0.06^{b}	0.09 ± 0.02^{b}	0.14 ± 0.03^{b}	0.03 ± 0.01^{b}
Chlorogenic acid	1.41 ± 0.01^{c}	1.77 ± 0.09^{c}	0.92 ± 0.06^{c}	$0.31 \pm 0.03^{b,c}$	0.13 ± 0.02^{a}
p-Coumaric acid	3.05 ± 0.02^{d}	3.79 ± 0.21^{d}	2.09 ± 0.09^{d}	1.14 ± 0.10^{c}	$0.24 \pm 0.01^{c,e,f}$
Ferulic acid	2.57 ± 0.01^{e}	3.17 ± 0.19^{e}	1.82 ± 0.10^{e}	0.92 ± 0.07^{d}	$0.21 \pm 0.02^{d,c}$
3,4-Dihydroxyphenylacetic acid	2.97 ± 0.01^{f}	$3.40 \pm 0.20^{d,e}$	2.61 ± 0.11^{f}	0.76 ± 0.05^{d}	$0.23 \pm 0.01^{c,f}$
Vanillic acid	2.97 ± 0.01^{f}	$3.54 \pm 0.14^{d,e}$	2.24 ± 0.07^{d}	$0.42 \pm 0.05^{a,e}$	$0.28 \pm 0.03^{e,f}$
Gallic acid	2.94 ± 0.02^{f}	3.22 ± 0.10^{e}	2.55 ± 0.11^{f}	$0.31 \pm 0.05^{b,e}$	0.27 ± 0.02^{f}

Values in the same column with different lowercase letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$).

Table 4. Amount of the phenols retained in curd and in curd pellet after dilution and amount of phenols released from curd after dilution and during *in vitro* digestion. Data referred to curd samples prepared with 20 mL of milk with a specific weight of 1.034 added of 10 mg of phenolic compounds.

Phenol added	*Phenols retained in curd (mg)*		*Phenols released in supernatant (mg)*		
	Before dilution	*After dilution (pellet)*	*After dilution*	*After gastric digestion*	*After pancreatic digestion*
Catechin	8.5 ± 0.1^{a}	1.8 ± 0.02^{b}	6.6 ± 0.3^{c}	7.3 ± 0.1^{d}	6.0 ± 0.3^{e}
Tannic acid	8.7 ± 0.1^{a}	2.7 ± 0.05^{b}	6.0 ± 0.1^{c}	6.3 ± 0.5^{c}	5.8 ± 0.2^{c}
Chlorogenic acid	7.3 ± 0.1^{a}	1.4 ± 0.02^{b}	6.0 ± 0.3^{c}	$7.0 \pm 0.1^{a,d}$	$6.6 \pm 0.5^{a,c,d}$
p-Coumaric acid	7.0 ± 0.2^{a}	2.2 ± 0.04^{b}	4.9 ± 0.1^{c}	5.3 ± 0.1^{c}	3.9 ± 0.1^{d}
Ferulic acid	6.9 ± 0.1^{a}	2.0 ± 0.03^{b}	4.9 ± 0.2^{c}	4.6 ± 0.1^{c}	4.4 ± 0.1^{d}
3,4-Dihydroxyphenylacetic acid	6.4 ± 0.2^{a}	1.4 ± 0.04^{b}	4.9 ± 0.1^{c}	$4.6 \pm 0.2^{c,d}$	4.5 ± 0.1^{d}
Vanillic acid	6.7 ± 0.1^{a}	0.7 ± 0.01^{b}	5.9 ± 0.2^{c}	$6.1 \pm 0.1^{c,d}$	6.3 ± 0.1^{d}
Gallic acid	6.3 ± 0.1^{a}	0.5 ± 0.01^{b}	5.7 ± 0.1^{c}	5.5 ± 0.1^{c}	4.9 ± 0.2^{d}

Values in one row not sharing the same superscript letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$)

Table 5. Antioxidant activity of supernatant of *in vitro* digested curds determined by ABTS assay at pH 2.0. Results are expressed as μmol of TEAC/L.

Treatment	*Before digestion*	*Post-gastric*	*Post-pancreatic*
Control	102.4 ± 4.7[a]	260.4 ± 51.8[b]	808.2 ± 126.0[c]
Catechin	560.3 ± 3.6[a]	885.1 ± 68.7[b]	1317.3 ± 129.3[c]
Tannic acid	323.7 ± 25.5[a]	506.9 ± 56.2[b]	1116.8 ± 75.8[c]
Chlorogenic acid	268.9 ± 24.5[a]	560.9 ± 52.8[b]	1101.3 ± 111.1[c]
p-Coumaric acid	576.1 ± 51.5[a]	739.8 ± 30.3[b]	1137.8 ± 119.7[c]
Ferulic acid	592.4 ± 32.5[a]	729.4 ± 136.2[a]	1202.3 ± 51.5[c]
3,4-Dihydroxyphenylacetic acid	579.9 ± 56.4[a]	808.0 ± 48.9[a]	1332.3 ± 80.3[c]
Vanillic acid	396.4 ± 25.9[a]	496.3 ± 43.8[a]	1047.2 ± 105.5[c]
Gallic acid	637.4 ± 27.9[a]	964.3 ± 78.8[b]	1474.9 ± 97.0[c]

Values in one row not sharing the same superscript letter are significantly different ($P < 0.05$). Data are means ± SD ($n = 3$)