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

Ahmed Helal<sup>b</sup>, Davide Tagliazucchi<sup>a</sup>, Elena Verzelloni<sup>a</sup>, Angela Conte<sup>a\*</sup>

<sup>a</sup>Department of Life Sciences, University of Modena and Reggio Emilia, Via Amendola 2,  
42100 Reggio Emilia, Italy

<sup>b</sup>Department of Food and Dairy Sciences and Technology, Damanhour University, 22516  
Damanhour, Egypt

\* Corresponding author. Tel.: +39-0522-522022; fax: +39-0522-522053

1 **Abstract**

2

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

17

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

## 19 **1. Introduction**

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

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

33 Cheese possesses a unique composition and structure, which actuate the researchers to try  
34 to apply different bioactive compounds to cheese with expectation to improve its  
35 nutritional and healthy qualities (Joseph, & Akinyosoye, 1997; Prudêncio, Prudêncio,  
36 Gris, Tomazi, & Bordignon-Luiz, 2008; Bandyopadhyay, Chakraborty, & Raychaudhuri,  
37 2008; Rinaldoni, Palatnik, Zaritzky, & Campderros, 2014). Recently, Han et al. (2011b)  
38 developed a functional cheese product containing polyphenolic compounds.

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

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

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

51

## 52 **2. Materials and methods**

### 53 **2.1. Materials**

54 Pasteurized whole bovine milk (3.1 g/100 mL protein and 3.6 g/100 mL fat) was  
55 purchased in a local market (Reggio Emilia, Italy). Liquid calf rennet was obtained from  
56 Educational Dairy Plant (Damanhour University, Damanhour, Egypt). Phenolic  
57 compounds, catechin, chlorogenic acid, ferulic acid, vanillic acid, gallic acid, *p*-coumaric  
58 acid, 3,4-dihydroxyphenylacetic acid and tannic acid were purchased from Sigma (Milan,  
59 Italy). Casein, calcium chloride, bile salts (mixture of sodium cholate and sodium  
60 deoxycholate), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas  
61 (4xUSP specifications) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)  
62 (ABTS) were supplied by Sigma (Milan, Italy). Ethanol was supplied by Carlo Erba  
63 (Milan, Italy).

### 64 **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  $\kappa$ -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 1300g 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  $\mu$ m 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  $\mu$ L 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<sub>3</sub> 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 1300g 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  $\alpha$ - and  $\beta$ -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:

$$140 \quad F_0 - F = F_0 * L_0^n / (K_D + L_0^n)$$

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  $\mu\text{L}$  of supernatant  
149 were mixed with 1960  $\mu\text{L}$  of ethanolic ABTS<sup>•+</sup> 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  $\mu\text{mol/L}$  of TEAC.

#### 154 **2.7 Statistics**

155 Data are presented as mean  $\pm$  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  $63.0 \pm 1.1$  (gallic  
177 acid) and  $86.8 \pm 0.2$  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  $\approx$  catechin  $\approx$  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  $K_{sv}$  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 ( $\sim 10^{10} \text{ mol}^{-1}\text{s}^{-1}\text{L}$ ).  $K_q$   
229 was calculated by dividing the experimentally measured  $K_{SV}$  for  $6 \times 10^{-9}$  sec that is the  
230 maximum lifetime ( $\tau_0$ ) 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  $\sim 10^{10} \text{ mol}^{-1}\text{s}^{-1}\text{L}$ ) 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  $\mu\text{mol/L}$ . 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  $84.0 \pm 0.6$  to  $96.9 \pm$   
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  $78.7 \pm 0.5$  and to  $50.5 \pm$   
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  $61.5 \pm 0.7$  from  $96.9$   
292  $\pm 0.7$  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-dihydroxyphenylacetic 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 ( $102.4 \pm 4.7$  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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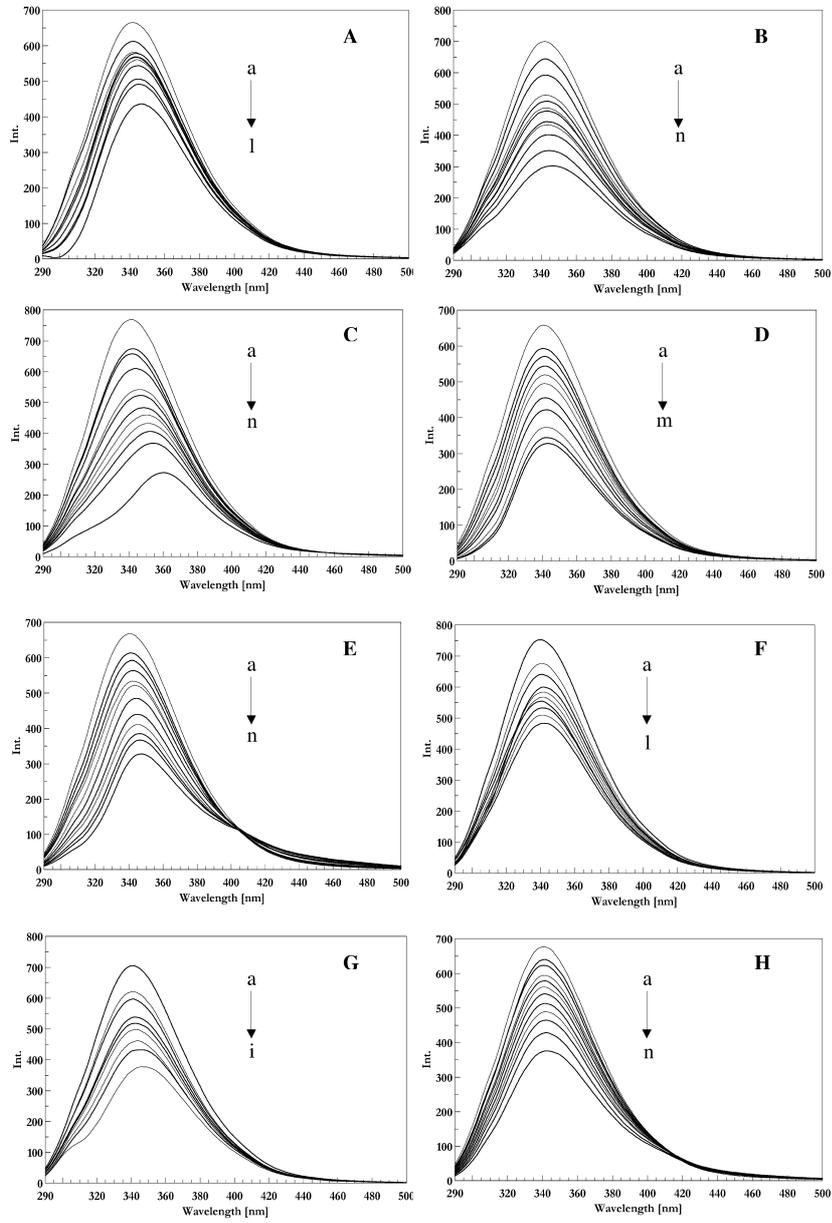
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## Figure captions

**Figure 1.** Fluorescence emission spectra of polyphenol–casein interactions. In all samples the casein concentration was 5  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ . (G) Vanillic acid concentrations: 0 (a), 5 (b), 7.5 (c), 10 (d), 15 (e), 20 (f), 30 (g), 40 (h), and 60 (i)  $\mu\text{mol/L}$ . (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)  $\mu\text{mol/L}$ .

Figure 1



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

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

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.

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

$K_q$  was calculated by dividing the experimentally measured  $K_{sv}$  for  $6 \times 10^{-9}$  sec that is the maximum lifetime ( $\tau_0$ ) of the fluorophore (tryptophan) in the absence of quencher as reported by Dufour, & Dangles (2005). Data are means  $\pm$  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.

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

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.

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

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  $\mu\text{mol}$  of TEAC/L.

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

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