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Accepted Version

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Kilic Bayraktar, M., Harbourne, N. and Fagan, C. ORCID: <https://orcid.org/0000-0002-2101-8694> (2019) Impact of heat treatment and acid gelation on polyphenol enriched milk samples. *LWT - Food Science and Technology*, 113. ISSN 0023-6438 doi: <https://doi.org/10.1016/j.lwt.2019.108282> Available at <https://centaur.reading.ac.uk/84834/>

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To link to this article DOI: <http://dx.doi.org/10.1016/j.lwt.2019.108282>

Publisher: Elsevier

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Impact of heat treatment and acid gelation on polyphenol enriched milk samples

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ABSTRACT

The effects of heat treatment and the stage of polyphenol addition to milk before or after heat treatment on the total phenolic content (TPC), ferric-ion reducing antioxidant power (FRAP), pH, casein micelle size (CMS) and whey protein denaturation content of milk-polyphenol mixtures were investigated. Four sources of phenolic compounds (green tea, white grape, tannic acid, gallic acid) were incorporated into pasteurized-skim milk. A heat treatment (85 °C for 30 min) was applied to pasteurized-skim milk either before (M_hP) or after polyphenols addition (M_hP_h). Acid milk gels were produced using M_hP_h samples, and their TPC and FRAP were determined. Heat treatment decreased the TPC and FRAP values of the samples, except for tannic acid, probably due to increased polyphenol-protein interactions. However, M_hP_h tannic acid sample resulted in significantly higher FRAP value than M_hP . The addition of gallic acid before heat treatment (M_hP_h) significantly increased CMS due to the lower pH of this sample during heating. Acid gelation decreased the extractable polyphenols, however there was no significant difference on FRAP between acid gel and M_hP_h milk samples. This study showed that the properties of phenolic source, particularly pH, and the stage of polyphenol addition to milk had an impact on selected properties.

Key words: polyphenol, milk protein, heat treatment, acid milk gel, antioxidant activity

1 **1. Introduction**

2

3 Polyphenols are secondary plant metabolites and there are over 8000 known phenolic structures
4 which consist of a hydroxyl group linked to an aromatic ring (Tresserra-Rimbau, Lamuela-
5 Raventos, & Moreno, 2018). Polyphenols are classified according to their carbon skeleton:
6 phenolic acids, flavonoids, stilbenes and lignans (Tresserra-Rimbau, Lamuela-Raventos, &
7 Moreno, 2018). In recent years, there has been increased interest in the study of polyphenols
8 due to their possible health benefits. Previous studies have suggested that they have strong
9 antioxidant capacities, decrease the risk of cancers and cardiovascular disease (Costa et al.,
10 2017).

11 Milk proteins are natural delivery vehicles of bioactives due to their physicochemical and
12 functional properties (Tavares, Croguennec, Carvalho, & Bouhallab, 2014). Therefore,
13 polyphenols could be delivered by dairy products and the phenolic content and antioxidant
14 activity of the final product might be improved. However, polyphenols may lead to changes in
15 the structural and functional properties of milk proteins as polyphenols interact with milk
16 proteins *via* hydrogen bonding, hydrophobic interactions and covalent bonds, leading to the
17 formation of soluble and insoluble complexes (Jakobek, 2015). These interactions depend on
18 pH, temperature, type and structure of both proteins and polyphenols (Bandyopadhyay, Ghosh,
19 & Ghosh, 2012).

20 The enrichment of various dairy products with polyphenols has been widely studied in recent
21 years (Aliakbarian et al., 2014; Szwajgier & Gustaw, 2015). Past studies reported contradictory
22 results related to the effect of milk on the antioxidant activity of polyphenols (Keogh,
23 McInerney, & Clifton, 2007; Korir, Wachira, Wanyoko, Ngure, & Khalid, 2014) which is
24 possibly a consequence of either various methods used for the measurement of antioxidant
25 activity, or the phenolic types utilised. Therefore, in this study a phenolic acid (gallic acid),

26 tannin (tannic acid) and polyphenol rich extracts from green tea and white grape were used to
27 understand the effect of phenolic types on the antioxidant activity and acid milk gel product.
28 There is still a lack of information regarding the effect of dairy processing conditions on
29 polyphenols when incorporated in milk. Acid milk gel products (e.g. yogurt) are widely
30 consumed. Thermalization of milk (85 °C for 30 min) is one of the critical steps in the
31 manufacture of this products as it is required to deliver a product of suitable textural quality
32 and water holding capacity (Harbourne, Jacquier & O'Riordan, 2011). Therefore, it is necessary
33 to understand the impact of this thermal process on the polyphenol content, antioxidant activity
34 and stability of proteins in polyphenol enriched dairy products. Furthermore, these properties
35 may also be affected by polyphenol addition before or after the thermal treatment. The
36 objective of the present study was to determine the impact of polyphenol addition, of various
37 types, before or after heat treatment on the total phenolic content, antioxidant activity, casein
38 micelle size & whey protein denaturation of milk and acid gel samples enriched with
39 polyphenols.

40

41 **2. Materials and Methods**

42

43 2.1. Experimental Design

44

45 Polyphenol solutions were prepared by dissolving polyphenol powders in distilled water (1
46 mg/mL) without (P) and with heat treatment (P_h) at 85 °C for 30 min to understand the impact
47 of heat treatment on the reconstituted polyphenol powders. To investigate the effect of heating
48 on milk-polyphenol samples a fully randomised experiment was carried out in triplicate based
49 on a 4 x 3 factorial design. 4 sources of polyphenols: green tea, white grape, tannic and gallic
50 acids and 3 sample preparations: pasteurized skim milk-polyphenol mixtures (MP), pasteurized

51 skim milk heated before polyphenols addition (M_hP) and pasteurized skim milk heated after
52 polyphenols addition (M_hP_h) (Table 1). For each treatment, a control sample which has no
53 polyphenols was prepared by adding distilled water in place of the polyphenols.

54 Acid milk gels were prepared with M_hP_h to determine the effect of acid gelation on the milk-
55 polyphenol mixtures.

56

57 2.2. Materials

58

59 Dried extracts: green tea (Nutraceutica, Monterezenzio, BO, Italy) and white grape (Nutripsy
60 CHR-Hansen, Hørsholm, Denmark) and single phenolic compounds: tannic and gallic acids
61 (Sigma Aldrich, Gillingham, UK) were employed in this study. Their phenolic composition is
62 described in Table 2. Pasteurized skim milk was purchased from a local retailer and
63 composition of milk (0.08 ± 0.01 g fat/100 mL, 3.36 ± 0.02 g protein /100 mL, 8.07 ± 0.04 g
64 total solids /100 mL, 4.75 ± 0.06 g lactose/100 mL) was determined by LactoScope FilterAuto
65 (QuadraChem Laboratories Ltd, Forest Row, UK). Sodium carbonate (Na_2CO_3) was supplied
66 by Thermo Fisher Scientific Ltd (Loughborough, UK). Gluconodelta lactone (GDL),
67 hydrochloric acid (HCl, 12 mol/L), methanol, Folin-Ciocalteu reagent, sodium acetate
68 trihydrate, acetic acid, 2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate,
69 ascorbic acid, sodium chloride (NaCl) and all the polyphenol standards that used to quantify
70 green tea and white grape were from Sigma Aldrich (Gillingham, UK).

71

72 2.2.1 Individual Phenolic Detection of Green Tea and White Grape

73

74 The green tea solution (5 mg/mL) was analysed with Dionex HPLC (Germering, Germany)
75 equipment that contains P680 HPLC pump, ASI-100 automated sample injector, thermostatted

76 column compartment TCC100, PDA-100 photodiode array detector with a Zorbax eclipse
77 XDB-C18 column (4.6 m × 150 mm, 5 μm, 25 °C, Agilent). Separation was carried out by a
78 gradient elution using formic acid/water (0.1: 99.9, v/v) (mobile phase A) and formic
79 acid/acetonitrile (0.1: 99.9, v/v) (mobile phase B) with elution scheme as follows: 0-5 min 4%
80 B; 5-40 min from 4% to 25% B; 40-55 min from 25% to 50% B, 55-60 min 50% B. The
81 protocol used a 1 mL/min flow rate and a 50 μL injection volume. Chromatograms were
82 recorded at 280 nm. Identification was based on retention times by comparison with HPLC
83 grade standards and quantification of green tea solution was performed using calibration curves
84 of epigallocatechin, catechin, epicatechin, epigallocatechin-gallate, epicatechin-gallate, gallic
85 acid.

86 The white grape solution (5 mg/mL) was analysed with a Waters UPLC-MS and Quattro
87 Ultima mass spectrometer (Waters, Manchester, UK) with a C-18 guard column (1.7μ / 50 x
88 2.1 mm, Kinetex, C18 column, Phenomenex, Macclesfield, UK) was used for the analyses.
89 Separation was carried out by a gradient elution using formic acid/water (0.1: 99.9, v/v) (mobile
90 phase A) and formic acid/acetonitrile (0.1: 99.9, v/v) (mobile phase B) with elution scheme as
91 follows: B was increased from 7% to 75% (0.2 min to 8.3 min), B was decreased from 75% to
92 7% (9.3 min to 10 min), then the column was equilibrated for 5 min at initial condition (7%
93 B). The total run time was 15 min with flow rate of 0.1 mL/min and injection volume of 10μL.
94 The oven temperature was set at 35°C. Detection was performed using retention time and
95 multiple reaction monitoring transition using positive ion mode (3.35 kV). The quantification
96 of white grape solution was estimated based on the area of 10 μM of each polyphenol standard:
97 resveratrol, quercetin-3-O-glucoside, catechin, epicatechin, epigallocatechin-gallate,
98 procyanidin B2.

99

100 2.3. Preparation of Polyphenols in Pasteurized Skim Milk

101

102 The stock polyphenol solutions (5 mg/mL) were freshly prepared by dissolving polyphenol
103 powders in distilled water before each experiment. They were added to pasteurized skim milk
104 (MP) and stirred for 30 min at room temperature. The final concentration of polyphenol
105 powders in milk samples was 1 mg/mL. A thermalization step of 85 °C for 30 min, typically
106 used in acid milk gel manufacture to denature whey proteins and enhance gel texture, was
107 applied to pasteurized skim milk before (M_hP) and after (M_hP_h) polyphenols addition. Samples
108 were placed (5 mL) in a shaking (90 rev/min) water bath (Grant Instrument Ltd, Cambridge,
109 UK). After heating samples were rapidly cooled by immersion in ice-water. After preparation,
110 all MP, M_hP and M_hP_h samples were stored at 4 °C for 2 h until analysis.

111

112 2.4. Preparation of Acid Milk Gels

113

114 Gluconodelta lactone (GDL) (1.7 g/100 g sample) was added to M_hP_h samples and stirred for
115 2 min. The samples were incubated in Sanyo Gallenkamp incubator (Leicestershire, UK) at
116 30°C for 3 h and 45 min until the pH reached a value of 4.6.

117

118 2.5. Measurement and Adjustment of pH of the Milk-Polyphenol Mixtures

119

120 The pH of each sample was determined using an Orion 3-star benchtop pH meter (Fisher
121 Scientific Ltd, UK) fitted with a glass combination electrode. On addition of gallic acid to
122 pasteurized skim milk, the pH decreased to 6.4. Therefore, to have a representative control for
123 this sample, pasteurized skim milk was adjusted to 6.4 using HCl (1 mol/L) and stored at 4°C
124 overnight to equilibrate.

125

126 2.6. Casein Micelle Size and Undenatured Whey Protein Content of Milk-Polyphenol Mixtures

127

128 The average CMS of samples was measured using a Zetasizer 5000 (Malvern Instruments Ltd,
129 Worcestershire, UK) according to Chen, Grandison, & Lewis (2012). Undenatured whey
130 protein nitrogen (WPN) of samples was determined by applying the GEA Niro method (GEA
131 NIRO, 2009)

132

133 2.7. Extraction of free Polyphenols from Milk and Acid Milk Gel

134

135 The polyphenols were extracted from the milk and acid milk gel according to Karaaslan,
136 Ozden, Vardin, & Turkoglu (2011). Briefly, an aliquot of the milk-polyphenol mixture (5 mL)
137 or acid milk gel (10 g) was centrifuged (Sorvall RC 6) at 25,860 x g for 15 min at 20 °C with
138 10 mL (milk) or 15 mL (gel) of acidified methanol (methanol containing 100 µL and 150 µL
139 conc. HCl for milk and gel, respectively). TPC and FRAP analysis was carried out on the
140 supernatants. For each replicate, the extracts of milk-polyphenol mixtures were freshly
141 prepared and were used the same day for chemical analyses. The same treatment was applied
142 to pasteurized skim milk without polyphenols for comparison.

143

144 2.8. Chemical Analyses

145

146 2.8.1. Total Phenolic Content

147

148 The total phenolic content (TPC) was determined according to Folin Ciocalteu method as
149 described by (Singleton, 1985), using gallic acid as the standard. The results were expressed as
150 milligrams of gallic acid equivalents (GAE) per millilitre of sample (mg GAE/mL).

151

152 2.8.2. FRAP (ferric ion reducing antioxidant power) Assay

153

154 The FRAP assay was performed to determine the antioxidant activity (AA) of samples
155 according to Benzie and Strain (1996). An ascorbic acid calibration curve was prepared ($R^2 =$
156 0.99). The results were expressed as ascorbic acid equivalents ($\mu\text{mol AAE}$) per millilitre of
157 sample ($\mu\text{mol AAE/mL sample}$).

158

159 2.9. Statistical Analysis

160

161 Results in the text are given as mean values \pm standard error (SE). The normality of data
162 distribution was analysed by Kolmogorov-Smirnov test. A one-way analysis of variance
163 (ANOVA) and Tukey's pairwise comparisons were used to identify significant differences
164 between the means of treatment methods and samples. An independent t-test was used for
165 comparison of two means. Results with $p < 0.05$ were considered significantly different.
166 Analyses were performed using SPSS Software for Windows (Version 21.0, Armonk, NY:
167 IBM Corp., USA).

168

169 **3. Results and Discussions**

170

171 3.1. Selected Properties of Polyphenol Powders

172

173 The dominant compounds in green tea were epigallocatechin-gallate (413.99 ± 14.18 mg/g
174 powder) and catechin (373.77 ± 9.24 mg/g powder) (Table 2), this is in agreement with
175 previous studies (Jaziri, Ben Slama, Mhadhbi, Urdaci, & Hamdi, 2009). The phenolic
176 compounds analysed in white grape (Table 2) were also in agreement with previous studies
177 (Wittenauer, Maeckle, Sussmann, Schweiggert-Weisz, & Carle, 2015).

178 Gallic acid had the highest TPC among the samples, followed by tannic acid, green tea and
179 white grape in descending order (Table 3). There was no significant difference between the
180 TPC of tannic acid and green tea. Similarly, the TPC of green tea and white grape are not
181 significantly different. However, when the concentration of individual phenolic compounds
182 were detected via HPLC and LC-MS, the concentration of the sum of all phenolic compounds
183 in green tea was nearly 8 times more than the concentration of the sum of all phenolic
184 compounds in white grape (Table 2). This indicated that there are some other phenolic
185 compounds in white grape that contributed the TPC, whereas those phenolics were not
186 quantified. The polyphenol powders used in this study have a much higher TPC than regular
187 teas, vegetables and fruits (Gharras, 2009; Dubeau, Samson, & Tajmir-Riahi, 2010). This is
188 possibly due to dehydrated form of polyphenol powders used. The levels of TPC in the
189 polyphenol powders that used in the present study are within the expected range as compared
190 to the commercial grape extracts in the study of da Silva, Matumoto-Pintro, Bazinet, Couillard,
191 and Britten (2015). The pH of green tea, white grape, tannic acid and gallic acid solutions were
192 6.71, 6.79, 6.40, 3.62 respectively (Table 4).

193 The FRAP of polyphenol powders correlated ($r: 0.796$, $p: 0.01$) with the total phenolic content
194 results, gallic acid had the highest FRAP and it was followed by tannic acid, green tea and
195 white grape (Table 3). The FRAP value of green tea and tannic acid were not significantly
196 different.

197

198 3.2. pH of Polyphenol Enriched Skim Milk

199

200 Table 4 demonstrates that, as expected (Fox, 1981), the application of a heat treatment (M_hP
201 and M_hP_h) reduced the pH of all samples as compared to MP samples. The pH of the samples
202 containing gallic acid was significantly lower than all the other samples studied. This is due to
203 the acidic properties of gallic acid (pKa 4.41). The stage of polyphenols addition to milk had
204 no impact on pH (M_hP versus M_hP_h).

205

206 3.3. Effect of Heat Treatment on Polyphenol Enriched Skim Milk

207

208 3.3.1. Total Phenolic Content and FRAP

209

210 Before examining the impact of heat treatment on the polyphenol enriched milks the effect of
211 heat treatment (85°C for 30 min) on TPC and FRAP of the polyphenol powders reconstituted
212 in water (1 mg/mL) was determined. Heating the polyphenol solutions had no significant effect
213 on the FRAP of the solutions. Furthermore, there were no significant differences between the
214 TPC of green tea and white grape solutions after heating. However, the TPC of both tannic and
215 gallic acid solutions significantly decreased by 16% and 7% respectively after heating ($p =$
216 0.038 , $p = 0.033$) (Table 5). This suggests that multiple phenolic compounds present in a
217 solution may combine to have a protective effect in comparison with solutions with individual
218 compounds. This is supported by the results of previous studies (Sari, Wijaya, Sajuthi, &
219 Supratman, 2012; Volf, Ignat, Neamtu, & Popa, 2014).

220 Fig. 1A represents the TPC of samples subjected to MP, M_hP and M_hP_h treatments. Adding
221 polyphenols significantly increased the TPC of all the samples as expected. The TPC of control
222 pasteurized skim milk is not affected by heat treatment. Pasteurized skim milk heated before

223 polyphenols addition (M_hP) resulted in a significant decrease in the TPC of milk containing
224 tea, grape and tannic acid as compared to MP samples. This is probably due to increased
225 interactions between milk proteins and polyphenols (Arts et al., 2002; Wu et al., 2013). It was
226 previously observed that the binding of epigallocatechin gallate (EGCG) was higher with
227 preheated beta lactoglobulin at 75-85 °C for 20 min as compared to native protein at room
228 temperature (Shpigelman, Israeli, & Livney, 2010). The binding interaction between EGCG
229 and beta lactoglobulin was attributed to hydrophobic interactions and hydrogen bonding.
230 Heating results in denaturation of whey proteins revealing hydrophobic and sulphur containing
231 groups (Taterka & Castillo, 2015); which increases the probability that polyphenols will bind
232 to the protein. It has also been shown that heat-induced denatured whey proteins played a role
233 in strengthening casein-polyphenol interactions (Yazdi & Corredig, 2012).

234 With the exception of samples containing tannic acid, a similar decrease in TPC was evident
235 when pasteurized skim milk heated after polyphenols addition (M_hP_h) was compared to MP
236 (Fig. 1A). However, the TPC was only significantly lower for green tea samples. As mentioned
237 above, when the tannic acid solution was heated the TPC decreased by 16%. However, no
238 significant decrease was evident when tannic acid was heated with milk (M_hP_h v MP). This
239 suggests that milk may have a protective effect on the phenolic compound in the sample. This
240 has been previously demonstrated for anthocyanins extracted from corncob (Jing & Giusti,
241 2005). Overall, heat treatment had a significant effect on the TPC of milk-polyphenol mixtures.
242 The stage of polyphenol addition (M_hP v M_hP_h) had no significant effect on the TPC of milk-
243 polyphenols.

244 The addition of polyphenols to milk significantly increased the antioxidant activity as measured
245 by FRAP for all samples (Fig. 1B) Additionally, heat treatment had no significant effect on
246 FRAP of control sample. Heat treatment, regardless of stage of polyphenol addition, either
247 decreased or had no impact on the FRAP of the samples. These results are inline with the TPC

248 results in Fig. 1A, indicating that in general increased interaction between milk proteins and
249 the polyphenols occurred. This is most likely due to the interaction between polyphenols and
250 milk proteins which have been denatured, as previously mentioned. The FRAP of tannic acid
251 added to pasteurized skim milk before heat treatment (M_hP_h) was significantly higher than
252 tannic acid added after heat treatment (M_hP). Heat treatment may increase the hydrolysis of
253 tannic acid, producing gallic acid and galloyl groups when tannic acid added to milk before
254 heat treatment (Kim, Silva, & Yung, 2011). Hydrolysed gallic acid and the hydroxyl groups
255 newly formed on the galloyl group as a result of thermal hydrolyses could be responsible for
256 the increased antioxidant activity of tannic acid (Kim, Silva, Kim, & Yung, 2010). The stage
257 of polyphenol addition had no effect on FRAP of any other sample.

258 Overall, with the exception of tannic acid, while heat treatment reduced the TPC and FRAP of
259 polyphenol enriched samples, polyphenol addition before or after heat treatment did not
260 significantly impact on TPC and FRAP values.

261

262 3.3.2. Casein Micelle Size and Undenatured Whey Protein Amount of Polyphenol Enriched 263 Skim Milk

264

265 Fig. 1C presents the CMS of MP, M_hP and M_hP_h samples. Regardless of the sample, the CMS
266 increased after heat treatment for samples where polyphenols were added after the milk was
267 heat treated (M_hP), with the exception of gallic acid, as compared to MP samples and these
268 increases were significant. This is in agreement with previous studies that show whey proteins
269 become denatured following heat treatment. The denatured whey proteins, mainly β -LG and
270 α -LA, interact with κ -casein by forming disulfide bonds on the surface of micelle and this
271 attachment leads to ultimately increasing the micelles average diameter (Dalglish & Corredig,
272 2012; Martin, Williams, & Dunstan, 2007). However, when polyphenols were added prior to

273 heat treatment (M_hP_h) in all cases, except gallic acid, the CMS tended to be smaller than for
274 the M_hP sample, albeit this decrease was insignificant for green tea. This is probably because
275 the M_hP_h samples were more dilute during the heat treatment which leads to lower levels of
276 whey protein denaturation and hence smaller CMS. This is supported by the levels of
277 undenatured whey protein that were detected in the samples (Fig. 1D). In general, M_hP_h
278 samples did show higher levels of undenatured whey protein in comparison to M_hP sample,
279 albeit the differences were not statistically significant for control, green tea or white grape. The
280 trend for the gallic samples was different. Specifically, when gallic acid was heated together
281 with the milk (M_hP_h) the CMS was larger than all other samples. This is probably related to the
282 heat treatment of the sample at a lower pH than other samples (Table 4). Heating milk proteins
283 at lower pH values results in a higher attachment of denatured whey proteins to casein micelles
284 (Taterka & Castillo, 2015). To understand the effect of the pH of pasteurized skim milk on the
285 CMS and undenatured whey protein amount, the pH of pasteurized skim milk was adjusted to
286 6.41 and heated at 85°C for 30 min. The CMS and undenatured whey level of the pH adjusted
287 sample was not significantly different to the M_hP_h gallic acid sample (Table 6).

288

289 3.4. Effect of Acid Gel Formation on TPC and FRAP

290

291 The effect of polyphenol addition on the TPC of M_hP_h samples was compared to acid milk gels
292 prepared with M_hP_h (Fig. 2A). As expected the acid milk gels containing polyphenols had a
293 significantly higher TPC than the control gel (Chouchouli et al., 2013; Karaaslan, Ozden, Vardin,
294 & Turkoglu, 2011). There was a significant decrease in the TPC of acid gel samples in
295 comparison to M_hP_h milk samples for all types of polyphenols studied. The decrease in the
296 level of extractable polyphenols in the acid milk gels is probably because they are very tightly
297 bound to the casein.

298 There was no significant difference in FRAP values between M_hP_h milk and acid milk gel
299 samples, with the exception of acid milk gels containing green tea which had a significantly
300 higher FRAP value than milk containing green tea (Fig. 2B). Overall, there is poor correlation
301 between the total phenolic content and antioxidant activity in polyphenol enriched acid milk
302 gels, which is in agreement with previous studies on yoghurt (Trigueros, Wojdylo, & Sendra,
303 2014). They attributed this poor correlation to the complex nature of yogurt. It is possible that
304 whey protein and polyphenol complexes in the supernatant exhibited antioxidant activity,
305 which may explain the reduction in TPC without an impact on FRAP. Almajano, Delgado, and
306 Gordon (2007) found that mixing whey proteins (BSA, beta-lactoglobulin, alpha-lactalbumin)
307 with EGCG (antioxidant from green tea) resulted in the formation of a complex with
308 antioxidant activity. The antioxidant of the complex formed increased during 7-d storage at 30
309 °C possibly due to increased interactions between the polyphenol and protein. In the present
310 study, it is possible that the acidification conditions may have resulted in increased interactions
311 between the whey proteins in the supernatant and polyphenols resulting in complexes with
312 antioxidant activity (Zhang et al., 2014).

313

314 **4. Conclusions**

315

316 This study showed that heating the polyphenol solutions at 85°C for 30 min had no significant
317 effect on the FRAP of the solutions. However, heat treatment of milk reduced the TPC and
318 FRAP of polyphenols, except for tannic acid, regardless of whether polyphenols were added to
319 milk before (M_hP_h) or after (M_hP) heat treatment. This suggests that thermal denaturation of
320 whey proteins may increase the binding capacity of proteins to polyphenols. The FRAP value
321 of tannic acid was significantly higher when it is added to milk before heat treatment in
322 comparison to addition of after heat treatment. The lower pH of gallic acid resulted in a

323 significant increase in the CMS of milk when the gallic acid was added before heat treatment.
324 This is related to higher attachment of denatured whey proteins to casein micelle due to lower
325 pH of milk with gallic acid addition during heat treatment. Acid gel processing decreased the
326 extractable polyphenols as compared to M_hP_h milk samples, however there was no significant
327 decrease in FRAP of polyphenol enriched milk gels.
328 The findings of this study demonstrated that commercial polyphenol extracts, and single
329 phenolic compounds could successfully be incorporated into milk used for production of
330 acidified dairy products. However, the acid dissociation constant of a polyphenol and the stage
331 at which it is added to the milk needs to be considered when enriching milk samples as this
332 may impact on selected properties of the samples. The current study can be applied to yogurt
333 and dairy based deserts and the effect of starter culture on the polyphenols under processing
334 conditions could be investigated.

335

336 **5. Acknowledgments**

337

338 Mukaddes Kilic Bayraktar are grateful to Turkish Ministry of Education for the financial
339 support and sponsorship of her PhD study.

340

341 **6. References**

342

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444 **Figure Captions**



445

446 **Fig. 1.** (A) Total phenolic content (TPC, mg GAE/mL), (B) ferric ion reducing antioxidant power
447 (FRAP, $\mu\text{mol AAE/mL}$), (C) casein micelle size (d.nm), (D) undenatured whey protein amount (mg
448 N/g) of milk-polyphenol mixtures following three different treatments (■: MP, ■: M_hP, □: M_hP_h).

449 MP: pasteurized skim milk-polyphenol mixtures, M_hP: pasteurized skim milk heated before
450 polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition, GAE: gallic acid
451 equivalents, AAE: ascorbic acid equivalents, d.nm: diameter.nanometer, N: nitrogen. Different letters
452 indicate significant differences at $p < 0.05$ among three treatments for each sample. Error bars represent
453 means of three replicates \pm the standard errors.

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Fig. 2. The effect of heat treatment and acid gelation on (A) total phenolic content (TPC, mg GAE/mL) and (B) ferric ion reducing antioxidant power (FRAP, $\mu\text{mol AAE/mL}$) of milk-polyphenol mixtures. GAE: gallic acid equivalents, AAE: ascorbic acid equivalents. Different letters indicate significant differences at $p < 0.05$ between : M_hP_h milk and : M_hP_h acid milk gel for each sample. M_hP_h : pasteurized skim milk heated after polyphenols addition. Error bars represent means of three replicates \pm the standard errors.