

# **Bioactivity and cell metabolism of *in vitro* digested sweet cherry (*Prunus avium*) phenolic compounds**

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## 1    **Abstract**

2    In this study, the bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion of  
3    two cherry cultivars was assessed. The phenolic profile was modified during *in vitro* digestion, with  
4    a considerable decrease of total and individual phenolic compounds. Hydroxycinnamic acids and  
5    especially coumaroylquinic acids showed the highest bioaccessibility. Isomerisation of  
6    caffeoylquinic and coumaroylquinic acids was observed after *in vitro* digestion. Modification of the  
7    phenolic profile after digestion resulted in an increased or decreased scavenging activity depending  
8    on the assay. *In vitro* digested phenolic-rich fractions also showed anti-proliferative activity against  
9    SW480 but no effect against Caco-2 cell lines. Both Caco-2 and SW480 cell lines were able to  
10    metabolize cherry phenolic compounds with remarkable differences. An accumulation of  
11    glycosylated flavonols was observed in SW480 medium. In conclusion, phenolic compounds from  
12    cherries and especially hydroxycinnamic acids were efficiently released and remained bioaccessible  
13    after *in vitro* digestion, resulting in antioxidant and anti-proliferative activities.

14  
15    **Keywords:** mass spectrometry, bioaccessibility, anti-proliferative activity, hydroxycinnamic acids,  
16    Caco-2, SW480

## 17    **1. Introduction**

18    Sweet cherry (*Prunus avium* L., Rosacea) is a commercially important table fruit highly appreciated  
19    by consumers due to its taste, colour and sweetness, but also for its nutritional properties and  
20    beneficial effects (Kelley et al. 2018). Sweet cherry fruits contain different phenolic compounds,  
21    including anthocyanins, flavan-3-ols and hydroxycinnamic acids that have been related to the  
22    healthy properties of these fruits (Ferretti et al. 2010). Indeed, cherries were considered as the main  
23    dietary source of anthocyanins within the context of the Mediterranean Diet (Godos et al. 2017).  
24    Recently, our research group thoroughly investigated the phenolic profile of different sweet cherry  
25    varieties, from pale yellow flesh and slightly reddish skin to dark red cultivars, using mass  
26    spectrometry (Martini et al. 2017). Among the 86 identified compounds, 3-coumaroylquinic acid,  
27    epicatechin and cyanidin-3-rutinoside were present at the highest concentrations (Martini et al.  
28    2017). The amount of individual phenolic compounds in sweet cherries is variable and strongly  
29    dependent on the cultivars (Martini et al. 2017; Picariello et al. 2016). The dark red cultivars are  
30    rich in anthocyanins, whereas the slightly reddish cultivars are rich in hydroxycinnamic acids and/or  
31    flavan-3-ols (Martini et al. 2017; Picariello et al. 2016).

32    There are several human intervention studies suggesting that cherry consumption may have  
33    beneficial effects on markers of cardiovascular disease risk (Kelley et al. 2018). Short-term  
34    supplementation of cherries reduced both systolic and diastolic blood pressure in diabetic women or  
35    hypertensive subjects (Keane et al. 2016; Kent et al. 2016). The effect on blood pressure correlated  
36    in time with changes in plasma cyanidin-3-glucoside metabolites such as vanillic and  
37    protocatechuic acids (Keane et al. 2016). Furthermore, cherry intake ameliorated the lipid profile of  
38    overweight and obese subjects by reducing the level of very-low density lipoproteins and increasing  
39    the amount of high-density lipoproteins (Martin et al., 2011). Some mechanisms have been  
40    proposed to explain the protective effect of cherries such as antioxidant activity, increased

41 expression of nitric oxide synthase and decreased expression of endothelin-1 (Kelley et al. 2013;  
42 Edwards et al. 2015).

43 Cherry intake also resulted in an anti-cancer activity at gastro-intestinal tract level (Ferretti et al.  
44 2010). Apc<sup>Min</sup> mice consuming cherries, anthocyanins or cyanidin had significantly fewer and  
45 smaller intestinal adenomas than Apc<sup>Min</sup> mice consuming the control diet (Kang et al. 2003). The  
46 protective effect of cherries against intestinal cancer has been attributed to the biological activities  
47 of anthocyanins. Extracts of sweet cherries as well as anthocyanins and cyanidin aglycone showed  
48 *in vitro* anti-proliferative activity against human cancer cells from the colon (HT-29 and HCT-15)  
49 and stomach (MKN45) (Bastos et al. 2015; Serra et al. 2011; Kang et al. 2003). These *in vitro*  
50 studies with cell culture models, carried out with pure phenolic compounds or cherry extracts, did  
51 not take into account the bioaccessibility and gastro-intestinal tract stability of the phenolic  
52 compounds. Some phenolic compounds, and especially anthocyanins, are unstable under the gastro-  
53 intestinal tract conditions (Yang et al. 2018; Bouayed et al. 2012; Juárez et al. 2017). Indeed, cherry  
54 phenolic compounds are entrapped in a solid food matrix and only the compounds released from the  
55 food matrix may exert their beneficial effects in the gastro-intestinal tract or at systemic level  
56 (Tagliazucchi et al. 2010). Furthermore, only few recent *in vitro* studies investigated the metabolism  
57 and/or the stability of phenolic compounds in cell cultures (Aragonès et al. 2017; Mele et al., 2016;  
58 Sala et al., 2015).

59 Therefore, this work aimed to investigate the effect of *in vitro* gastro-intestinal digestion on the  
60 bioaccessibility and antioxidant properties of cherry phenolic compounds from two different  
61 cultivars (Celeste and Durone Nero I). In addition, the anti-proliferative activity and the cell  
62 metabolism of *in vitro* digested cherry phenolic compounds against two models of human colon  
63 adenocarcinoma cell lines were assessed.

## 64    **2. Materials and methods**

### 65    **2.1. Materials**

66    Phenolic compound standards, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox),  
67    2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-S-triazine  
68    (TPTZ), Folin-Ciocalteu phenol reagent were purchased from Sigma (Milan, Italy). Methanol and  
69    formic acid were obtained from Carlo Erba (Milan, Italy). All MS/MS reagents were from Bio-Rad  
70    (Hercules, CA, U.S.A.). Chemicals and enzymes for the digestion procedure were purchased from  
71    Sigma-Aldrich (Milan, Italy). All the materials and chemicals for cell culture were from Euroclone  
72    (Milan, Italy). MTS cell proliferation assay kit was purchased from Promega (Milan, Italy). Solid  
73    phase extraction (SPE) column (C18, 50 µm, 60 Å, 500 mg) were supplied by Waters (Milan, Italy).  
74    The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer.

### 76    **2.2. Cherry cultivars**

77    Two sweet cherry (*Prunus avium*) cultivars (Celeste and Durone Nero I) were harvested at full  
78    maturity in Vignola (Modena province, Italy). For each variety, about 2 kg of cherries were  
79    randomly sampled from several trees and processed immediately or frozen within 1 h after  
80    harvesting and stored at -80°C until used. The two cherry cultivars were selected according to the  
81    different skin and flesh colours and phenolic composition (Martini et al. 2017). Celeste cultivar  
82    presented a pale red flesh and a bright red skin and predominantly contained hydroxycinnamic acids  
83    and flavan-3-ols. Durone Nero I instead was a dark red cultivar rich in anthocyanins.

### 85    **2.3. *In vitro* gastro-intestinal digestion of cherry cultivars and preparation of the chemical** 86    **extracts**

87    For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST  
88    was followed (Minekus et al. 2014). The fluids (simulated salivary, gastric and intestinal fluids)

89 were prepared according to Minekus et al. (2014). Salivary phase was simulated by homogenizing  
90 with a laboratory blender five grams of each cherry cultivar in presence of 5 mL of the stock  
91 simulated salivary fluid solution. After homogenization, 150 U/mL of porcine  $\alpha$ -amylase were  
92 added and the samples were incubated for 5 min at 37°C in a rotating wheel. The gastric phase of  
93 the digestion was carried out by adding to the bolus 10 mL of simulated gastric fluid. After pH  
94 adjustment to 2.0 with 6 mol/L HCl, 2000 U/mL of pepsin were added. After 2 h of incubation at  
95 37°C, the final intestinal step was carried out by adding 15 mL of simulated intestinal fluid. Then,  
96 the pH was adjusted to 7.0, supplemented with pancreatin and the samples were incubated at 37°C  
97 for 2 h. All samples were immediately cooled on ice and frozen at -80°C for further analysis. The  
98 digestions were performed in triplicate.

99 In addition, phenolic compounds were extracted from each cherry cultivar (chemical extract) as  
100 reported in Martini et al. (2017). The extractions were performed in triplicate.

101

#### 102 ***2.4. Preparation of the phenolic-rich fractions***

103 Chemical extracts and samples collected at the end of the *in vitro* digestion were then passed  
104 through a SPE column preconditioned with 4 mL of acidified methanol (containing 0.1% of formic  
105 acid), followed by 5 mL of acidified water (containing 0.1% of formic acid). Elution was carried  
106 out with acidified water (6 mL) to eliminate the unbound material and phenolic compounds were  
107 then desorbed with 3 mL of acidified methanol. The obtained phenolic-rich extracts were then used  
108 for the subsequent analysis. Each sample was extracted in triplicate.

109

#### 110 ***2.5. Identification and quantification of phenolic compounds by liquid chromatography*** 111 ***electrospray ionization ion trap mass spectrometry (LC-ESI-IT-MS)***

112 Phenolic-rich fractions from chemical extracts and *in vitro* digested samples were analysed on  
113 HPLC Agilent 1200 Series system equipped with a C18 column (HxSil C18 Reversed phase,

114 250×4.6 mm, 5 µm particle size, Hamilton Company, Reno, Nevada, USA) as reported in Martini et  
115 al. (2017). The mobile phase composition was (A) H<sub>2</sub>O/formic acid (99:1, v/v) and (B)  
116 acetonitrile/formic acid (99:1, v/v). After 0.5 min at 4% B, the gradient linearly ramped up to 30%  
117 B in 60 min. The mobile phase composition was raised up to 100% B in 1 min and maintained for 5  
118 min in order to wash the column before returning to the initial condition. The flow rate was set at 1  
119 mL/min. The samples were injected in the amount of 20 µL. After passing through the column, the  
120 eluate was split and 0.3 mL/min were directed to an Agilent 6300 ion trap mass spectrometer. Two  
121 MS experiments were performed, one in negative ion mode and one using positive ionization (for  
122 anthocyanins), under the same chromatographic conditions. Identification of phenolic compounds in  
123 all samples was carried out using full scan, data-dependent MS<sup>2</sup> scanning from *m/z* 100 to 800 and  
124 selected reaction monitoring. MS operating conditions, limits of detection (LOD) and limits of  
125 quantification (LOQ) for the different standards are reported in Martini et al. (2017).  
126 Quantitative results were expressed as µmol of compounds per 100 g of cherry.

127

## 128 **2.6. Bioaccessibility calculation**

129 The percentage bioaccessibility of phenolic compounds after *in vitro* gastro-intestinal digestion was  
130 calculated as follow:

$$131 \text{ Bioaccessibility} = \frac{CCd}{CCe} * 100$$

132 Where CCd is the concentration of total or individual phenolic compounds in the phenolic-rich  
133 fraction from *in vitro* digested sample and CCe is the concentration in the phenolic-rich fraction  
134 from the chemical extract.

135

## 136 **2.7. Antioxidant activity assays**

137 The antioxidant properties of the phenolic-rich fractions obtained from chemical extracts and *in*  
138 *vitro* digested samples were detailed by using four different assays.

139 The ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and ferric reducing power  
140 (FRAP) assays were performed according to the protocols described by Re et al. (1999) and Benzie  
141 and Strain (1996), respectively. The capacity to scavenge hydroxyl and superoxide anion radicals  
142 were evaluated according to the methods reported by Martini et al. (2017).

143 The results were expressed as  $\mu\text{mol}$  of trolox equivalent per  $\text{mmol}$  of total phenolic compounds.

144

## 145 ***2.8. Cell cultures and anti-proliferative activity of cherry phenolic-rich fractions***

146 Human adenocarcinoma Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium  
147 (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotic mix (streptomycin and  
148 penicillin) and 2  $\text{mmol/L}$  L-glutamine. Human adenocarcinoma SW480 cells were cultured in  
149 Leibowitz medium supplemented with 10% FBS, 1% antibiotic mix (streptomycin and penicillin)  
150 and 2  $\text{mmol/L}$  L-glutamine. Cells were maintained at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ .  
151 Cells were seeded at  $5 \times 10^3/100 \mu\text{L}$  and  $10 \times 10^3/100 \mu\text{L}$  for Caco-2 and SW480, respectively, in 96-  
152 well plates for 24 h to allow cell adhesion to the bottom of the wells.

153 For the anti-proliferative assays, a colorimetric method for the sensitive quantification of viable  
154 cells was performed, using MTS assay kit. 100  $\mu\text{L}$  of digested and un-digested cherry phenolic-rich  
155 fractions diluted in cell culture media at different concentrations were added to the cell plates and  
156 incubated for 24 h. At the end of the treatments, media were refreshed with 180  $\mu\text{L}$  of culture media  
157 and 20  $\mu\text{L}$  of MTS reagent were added to each well. After 4 h of incubation at  $37^{\circ}\text{C}$ , the absorbance  
158 was measured at the wavelength of 490 nm using a microplate reader and results were expressed as  
159  $\text{IC}_{50}$ .  $\text{IC}_{50}$  was defined as the concentration of phenolic compounds required to inhibit 50% cell  
160 proliferation and expressed as  $\mu\text{mol}$  of total phenolic compounds/ $\text{L}$ . The  $\text{IC}_{50}$  values were  
161 determined using nonlinear regression analysis and fitting the data with the log (inhibitor) vs.  
162 response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

163



164 **2.9. Liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis of cell media**

165 Cell culture supernatants were collected at the end of the experiments and analysed by LC-MS/MS  
166 to determine the cell metabolism of the cherry phenolic compounds. Cell media were extracted  
167 according to Sala et al. (2015) and analysed using a HPLC Agilent 1200 Series system equipped  
168 with an Agilent 6300 ion trap mass spectrometer as reported above. Separations were performed  
169 using a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 µm particle size, Hamilton  
170 Company, Reno, Nevada, USA), with an injection volume of 40 µL and elution flow rate of 1  
171 mL/min. The mobile phase composition, the gradient and MS operating conditions are the same as  
172 reported above.

173

174 **2.10. Statistic**

175 All data are presented as mean ± SD for three replicates for each prepared sample. One-way  
176 analysis of variance (one-way ANOVA) with Tukey's post-hoc test was applied using Graph Pad  
177 prism 6.0 (GraphPad software, San Diego, CA, U.S.A.). The differences were considered  
178 significant with  $P < 0.05$ .

### 179 3. Results and discussion

#### 180 3.1. *In vitro* bioaccessibility of phenolic compounds in cherry cultivars

181 **Table 1** shows data about the bioaccessibility of individual phenolic compounds in the two analysed  
182 cherry cultivars. The MS data of the individual phenolic compounds are reported in Martini et al.  
183 (2017).

184 A total of 57 and 56 phenolic compounds were identified and quantified in the chemical extracts of  
185 cherry cultivars Celeste and Durone Nero I, respectively (**Table 1**). In both the cultivars, 3-  
186 coumaroylquinic acid (found as *cis* and *trans* isomers) was the main phenolic compound accounting  
187 for 40.6% and 31.3% of the total compounds in Celeste and Durone Nero I, respectively. In general,  
188 hydroxycinnamic acids were the most representative class of phenolic compounds in both the  
189 cultivars (54.2% and 42.2% of the total phenolic compounds in Celeste and Durone Nero I,  
190 respectively). From a quantitative point of view, flavan-3-ols were the second class of phenolic  
191 compounds in both the cultivars (40.7% and 33.4% of the total phenolic compounds in Celeste and  
192 Durone Nero I, respectively). Anthocyanins accounted for the 15.4% of total compounds in the  
193 cultivar Durone Nero I whereas their incidence was about 3% in the cultivar Celeste. Finally, small  
194 amounts of others flavonoids were also detected in both the cultivars.

195 The phenolic compounds profile was significantly modified from a qualitative and quantitative  
196 point of view after *in vitro* gastro-intestinal digestion (**Figure 1** and **Table 1**). Only 25 and 39 of the  
197 original phenolic compounds were identified after simulated digestion of the cultivars Celeste and  
198 Durone Nero I, respectively. This meant that 56% and 30% of individual phenolic compounds were  
199 not bioaccessible.

200 Total phenolic compounds determined by LC-ESI-IT-MS/MS in the cherry cultivars chemical  
201 extracts were significantly higher ( $P<0.05$ ) than those found after *in vitro* gastro-intestinal digestion  
202 (**Figure 1A** and **Table 1**). The bioaccessibility of total phenolic compounds was 39.7% and 29.9%  
203 in the cultivars Celeste and Durone Nero I, respectively. Hydroxycinnamic acids were the most

204 abundant phenolic compounds released from cherry matrices with a bioaccessibility of 71.7% and  
205 46.5% in the cultivars Celeste and Durone Nero I, respectively (**Figure 1B** and **Table 1**). At the end  
206 of the digestion, hydroxycinnamic acids represented the 98.0% and 65.5% of total phenolic  
207 compounds in the cultivars Celeste and Durone Nero I, respectively (**Figure 1H**). These compounds  
208 were particularly stable under gastro-intestinal conditions and easily released from the cherry  
209 matrices. The stability of hydroxycinnamic acids under gastro-intestinal conditions has been already  
210 reported. For example, Tagliazucchi et al. (2012) found that only the 30% of hydroxycinnamic  
211 acids were degraded during *in vitro* digestion of a coffee beverage whereas Monente et al. (2015)  
212 assessed a 14% decrease during *in vitro* digestion of a spent coffee water extract. Previous studies  
213 with solid food matrices confirmed the easy release of hydroxycinnamic acids during *in vitro*  
214 digestion. The bioaccessibility of hydroxycinnamic acids was 60-67% in cooked cardoon, 32-57%  
215 in apple and about 77% in frozen sweet cherries subjected to gastro-intestinal digestion (Juániz et  
216 al. 2017; Fazzari et al. 2008; Bouayed et al. 2012). This is also in accordance with the recorded  
217 recovery of caffeoylquinic acids (59-77%) in ileal fluid after coffee or apple ingestion by  
218 ileostomists (Erk et al. 2014; Stalmach et al. 2010). The highest release after incubation with  
219 digestive fluids was found for coumaroylquinic acids, followed by feruloylquinic and  
220 caffeoylquinic acids (**Table 1** and **Figure 2**). These results are in accordance with Monente et al.  
221 (2015) and Tagliazucchi et al. (2012) who found that feruloylquinic and caffeoylquinic acids were  
222 quite stable under gastro-intestinal conditions. No data about the stability or bioaccessibility of  
223 coumaroylquinic acids have been published until now. Otherwise, the minor hydroxycinnamic acids  
224 identified in cherries (such as di-hydroxycinnamic acids and lactone derivatives) were not  
225 bioaccessible probably because of their instability in the gastro-intestinal environment (Monente et  
226 al. 2015; Tagliazucchi et al. 2012). The differences between the recoveries of hydroxycinnamic  
227 acids in the two different cherry cultivars could be indicative of a food matrix impact (**Figure 2** and  
228 **Table 1**).

229 As reported above, overall coumaroylquinic, caffeoylquinic and feruloylquinic acids were easily  
230 released from food matrices, although changes mainly due to variations in isomers level were  
231 recorded after *in vitro* digestion. While the 5- and 3-caffeoylquinic acid isomers were found at  
232 lower concentrations after *in vitro* digestion compared to the chemical extracts, the 4-caffeoylquinic  
233 acid isomers concentration increased significantly (**Figure 2A** and **2B** and **Table 1**). Acyl migration  
234 of caffeoylquinic acids has been already demonstrated both *in vitro* with pure standard compounds  
235 or after digestion of coffee and *in vivo* in ileostomy volunteers (Erk et al. 2014; Monente et al.  
236 2015; Tagliazucchi et al. 2012). Similar results were observed for feruloylquinic acids (**Figure 2C**  
237 and **2D** and **Table 1**). Otherwise, a different behaviour was observed for coumaroylquinic acids. In  
238 this case, we found remarkable higher concentrations of 4- and 5-coumaroylquinic acid isomers  
239 after simulated digestion (**Figure 2E** and **2F** and **Table 1**). On the contrary, the concentration of 3-  
240 coumaroylquinic acid isomers was lower after digestion than that recorded after chemical  
241 extraction. Previously, Kahle et al. (2011) reported isomerization of 4-coumaroylquinic acid to 3-  
242 and 5-coumaroylquinic acids after incubation of standard compounds with intestinal fluids. These  
243 results, instead, suggested that coumaroylquinic acids were subjected to isomerization from the 3-  
244 acyl to 4- and 5-acyl structures.

245 Simple hydroxycinnamic acids (coumaric, caffeic and ferulic acids) were not found after intestinal  
246 digestion, suggesting that relevant hydrolysis of ester bond did not occur during *in vitro* digestion.  
247 On the other hand, incubation of caffeoylquinic and coumaroylquinic acids standards resulted in the  
248 appearance of simple acids after 6 h of incubation in intestinal fluids (Kahle et al. 2011).

249 Flavan-3-ols, which were the second most abundant class of phenolic compounds in cherry  
250 chemical extracts, were characterized by a very low bioaccessibility (**Figure 1C** and **Table 1**). In  
251 the cultivar Celeste, only (epi)catechin-3-gallate was detected after gastro-intestinal digestion. In  
252 the cultivar Durone Nero I we also found monomeric and dimeric (epi)catechins, although  
253 (epi)catechin-3-gallate was still the flavan-3-ol with the highest bioaccessibility. The

254 bioaccessibility of monomeric catechins seems to be highly dependent on the food matrix. During  
255 *in vitro* digestion of green tea, Green et al. (2007) found that only the 32% of epicatechin was  
256 degraded. High stability of monomeric catechins was also described when the standard compounds  
257 were incubated with gastro-intestinal fluids (Tagliazucchi et al. 2010). However, *in vitro* digestion  
258 of apple varieties, grape or wine resulted in an important degradation of monomeric catechins  
259 (Bouayed et al. 2012; Lingua et al. 2018). Whether catechin and epicatechin were degraded or not  
260 released from cherry matrices remained to be established.

261 Cultivar Durone Nero I was also characterized by a high content of anthocyanins. In this cherry  
262 cultivar, anthocyanins represented the phenolic class with highest bioaccessibility (55.0%),  
263 constituting the 28.2% of total phenolic compounds after *in vitro* digestion (**Figure 1G** and **1H** and  
264 **Table 1**). Anthocyanins are generally considered very unstable compounds under slightly alkaline  
265 conditions as found in the intestinal milieu. For example, *in vitro* digestion of red wine, chokeberry,  
266 and pomegranate juice resulted in a substantial degradation of monomeric anthocyanins (Bermúdez-  
267 Soto et al. 2007; Lingua et al. 2018; Perez-Vicente et al. 2002). Anthocyanins stability in the  
268 digestive system appeared to be related to their molecular structure. The presence of a methoxy  
269 group in the B-ring stabilized the anthocyanin structure (Yang et al. 2018). The acetylated  
270 derivatives of anthocyanins showed relatively higher stability during the gastro-intestinal transit  
271 than simple anthocyanins (Yang et al. 2018). As shown in **Table 1**, the rutinoside derivatives of  
272 cherry anthocyanins were more stable than the corresponding glycosidic forms. These results are in  
273 agreement with Fazzari et al. (2008). It can be speculated that fruits containing high levels of  
274 rutinoside derivatives are likely to provide larger amounts of anthocyanins in the colon. Indeed,  
275 peonidin-3-*O*-rutinoside, which is characterized by the presence of a rutinose moiety and a methoxy  
276 group in the B-ring showed 100% of bioaccessibility.

277 Some newly identified compounds were indicative of anthocyanins degradation (**Table 1**).

278 Protocatechuic acid appeared after *in vitro* digestion of cherry cultivars and plausibly came from the

279 degradation of cyanidin-3-*O*-glucoside. Moreover, cyanidin-3-*O*-glucoside chalcone was also found  
280 after *in vitro* digestion of Durone Nero I due to the presence of its  $[M+H]^+$  precursor ion at 467 and  
281 product ions at  $m/z$  305 (-162 amu; loss of a glucosyl moiety),  $m/z$  449 (-18 amu; loss of water) and  
282  $m/z$  287 (-18-162 amu; loss of water and glucosyl moiety) (Lopes et al. 2007). After *in vitro*  
283 digestion of Durone Nero I, a new compound with a  $[M+H]^+$  mass signal at 737 was detected. In  
284 ESI positive experiments, it gave a base peak product ion at  $m/z$  575 (-162 amu) corresponding to  
285 the loss of a glucosyl moiety. Additional product ions were detected at  $m/z$  557 (loss of water from  
286  $m/z$  575) and  $m/z$  287 (loss of a (epi)catechin unit from  $m/z$  575). Finally, a product ion at  $m/z$  423  
287 was detected corresponding to the loss of 152 amu from  $m/z$  575 resulting from the retro Diels-  
288 Alder decomposition of the flavan-3-ol unit. The described fragmentation pattern corresponded to  
289 that previously observed for a (epi)catechin-cyanidin-3-*O*-glucoside dimer (Sentandreu et al. 2010).  
290 The bioaccessibility of the other phenolic classes was very low with the exception of flavonols  
291 (52.2% and 39.3% of bioaccessibility in the cultivars Celeste and Durone Nero I, respectively)  
292 (**Figure 1D** and **Table 1**). However, due to their low concentration in the cherries, they represented  
293 only the 1.5% and 4.4% of total phenolic compounds determined after *in vitro* digestion in the  
294 cultivars Celeste and Durone Nero I, respectively (**Figure 1H** and **Table 1**).

295

### 296 **3.2 Effect of *in vitro* digestion on the antioxidant properties**

297 To fully characterize the antioxidant properties of the phenolic-rich fractions of cherries, the  
298 ability to scavenge some physiologically relevant radicals (superoxide anion and hydroxyl radical)  
299 and the organic nitro-radical ABTS as well as the reducing power were evaluated before and after  
300 digestion (**Figure 3**).

301 The hydroxyl radical scavenging capacity of phenolic-rich extracts decreased after *in vitro* digestion  
302 of both the cherry cultivars (**Figure 3A**). This means that cherry phenolic compounds in the  
303 chemical extracts were more efficient scavengers of hydroxyl radical than the phenolic compounds

304 present in the digested cherries. This decrease may reflect the different composition of the chemical  
 305 and *in vitro* digested phenolic-rich extracts. In both the cherry cultivars, the *in vitro* digested  
 306 extracts were enriched in hydroxycinnamic acids (**Table 1** and **Figure 1H**) that showed the lowest  
 307 hydroxyl scavenging activity among phenolic compounds (Özyürek et al. 2008). On the contrary,  
 308 ferric reducing power of the phenolic-rich extracts increased after *in vitro* digestion (**Figure 3B**).  
 309 This clearly reflected the different composition of the chemical and digested extracts, since the  
 310 ferric reducing ability of hydroxycinnamic acids is considered higher than that of flavan-3-ols  
 311 (Pulido et al. 2000). The superoxide anion scavenging activity instead was similar between the  
 312 phenolic-rich extracts before and after *in vitro* digestion (**Figure 3C**). Finally, the ABTS radical  
 313 scavenging activity assay displayed a different behaviour in the two cherry cultivars (**Figure 3D**).  
 314 In Celeste cultivar, the phenolic-rich extract obtained after *in vitro* digestion showed a lower  
 315 scavenging ability than the extract before digestion. This is in keeping with the observed percent  
 316 increase in hydroxycinnamic acids that showed lower ABTS radical scavenging activity respect to  
 317 flavan-3-ols (Rice-Evans et al. 1996). Instead, in the cultivar Durone Nero I we observed an  
 318 increase in the ABTS radical scavenging activity in the phenolic-rich extract after *in vitro* digestion.  
 319 This can be related to the percentage increase of anthocyanins and flavonols in the digested extracts  
 320 (**Table 1** and **Figure 1H**), which displayed greater ABTS radical scavenging activity than flavan-3-  
 321 ols (Rice-Evans et al. 1996).  
 322 Indeed, it is important to note that the different incidence of the phenolic classes in the digested  
 323 phenolic-rich extracts gave rise to significant different behaviours in the scavenging capacity or  
 324 reducing power in relation to the type of assay and mechanism involved, leading to an increase or  
 325 decrease of the considered activities.  
 326  
 327 **3.3 Anti-proliferative activity of cherry phenolic-rich fractions on human colon adenocarcinoma**  
 328 **cell lines**

329 The effect of cherry phenolic-rich fractions extracted before and after *in vitro* gastro-intestinal  
330 digestion on the proliferation of human colon adenocarcinoma Caco-2 and SW480 cells was  
331 investigated. Caco-2 and SW480 cells were incubated with different concentrations of phenolic-rich  
332 extracts for 24 h. Cherry phenolic-rich extracts did not affect the proliferation of Caco-2 (data not  
333 shown). Instead, the phenolic compounds from the chemical extracts of both the cultivars were able  
334 to inhibit in a dose-dependent manner the proliferation of SW480 cell line (**Figure 4**). The chemical  
335 extract of cultivar Durone Nero I was about 2.5 times more efficient than Celeste with IC<sub>50</sub> values  
336 of  $48.01 \pm 3.02$  and  $121.90 \pm 5.03$   $\mu\text{mol/L}$ , respectively. *In vitro* digestion positively affected the  
337 anti-proliferative activity of Celeste phenolic-rich fraction. As reported in **Figure 4**, the IC<sub>50</sub> value  
338 of phenolic compounds extracted after digestion of cultivar Celeste halved to  $61.22 \pm 4.02$   $\mu\text{mol/L}$ .  
339 No significant differences were found between the IC<sub>50</sub> values calculated for cultivar Durone Nero I  
340 before and after digestion.

341 According to the literature, this is the first report investigating the anti-proliferative activity of  
342 sweet cherry phenolic-rich extracts against human colon adenocarcinoma Caco-2 and SW480 cell  
343 lines and the influence of *in vitro* digestion processes.

344 Previous *in vitro* studies have shown anti-proliferative properties of sweet cherry extracts against  
345 colon cancer cell lines such as HCT-15 and HT29 (Bastos et al. 2015; Serra et al. 2011). This effect  
346 has been attributed to the anthocyanin content of the cherry extracts (Wang and Stoner 2008).  
347 However, Olsson et al. (2008) found no correlation between proliferation of HT29 cells and  
348 the concentration of anthocyanins in ten fruits and berries (including cherries). In accordance, also  
349 in this study we did not find any correlation between the anthocyanin content and the anti-  
350 proliferative activity. In fact, Durone Nero I chemical extract contained five times more  
351 anthocyanins than Celeste chemical extract but exhibited only 2.5 times more anti-proliferative  
352 activity. Indeed, *in vitro* digested phenolic fraction from Celeste was as effective as Durone Nero I  
353 chemical extract although it did not contain anthocyanins.



354 Our results suggested that other compounds rather than anthocyanins can be responsible for the  
355 observed anti-proliferative effect of phenolic-rich fractions extracted before and at the end of the *in*  
356 *vitro* gastro-intestinal digestion.

357

### 358 **3.4. Metabolism of cherry phenolic compounds in cell cultures**

359 In an attempt to unravel the metabolism of cherry phenolic compounds by Caco-2 and SW480, cell  
360 media were collected after 24 h of incubation with *in vitro* digested Durone Nero I phenolic-rich  
361 fraction and subjected to LC-MS ion trap analysis. Some parent compounds and newly formed  
362 metabolites were detected in both cell types and reported in **Table 2**.

363 Total coumaroylquinic acids final concentration accounted for the 13.9 and 14.7% of the initial  
364 concentration in the cultured Caco-2 and SW480, respectively. In addition to the coumaroylquinic  
365 acid isomers, one newly formed metabolite was tentatively identified as coumaroylquinic acid  
366 sulphate in the cell media of both the cell lines. The rate of sulphation of coumaroylquinic acid  
367 isomers in SW480 was 0.5%, while in Caco-2 coumaroylquinic acid sulphate was found only at  
368 trace levels. To the best of our knowledge, this is the first report showing sulphation of  
369 coumaroylquinic acids by Caco-2 and SW480. Coumaric and (iso)ferulic acids were found in the  
370 media after incubation with both cell lines, whereas caffeic acid only after incubation with SW480  
371 (**Table 2**). The appearance of these simple hydroxycinnamic acids, which were not present in the  
372 digested sample, was accompanied by the disappearance of caffeoylquinic and feruloylquinic acids  
373 (with the exception of trace amounts of 3-feruloylquinic acid) and the decrease in concentration of  
374 coumaroylquinic acids. These results can be indicative of hydroxycinnamoyl-quinic acids  
375 degradation in the media or to the hydrolysis catalysed by esterases. Considering the initial  
376 concentration of feruloylquinic acids, their complete hydrolysis during incubation with cells should  
377 result in a (iso)ferulic acid concentration of 10.47  $\mu\text{mol/L}$ . However, the amount of (iso)ferulic acid  
378 found in cell medium after 24 h of incubation with Caco-2 was about 5.5 times higher (**Table 2**).

379 Previous studies have shown that Caco-2 possess catechol-*O*-methyltransferase able to metabolize  
380 caffeic acid to (iso)ferulic acid (Monente et al. 2015; Farrell et al. 2011; Kern et al. 2003).  
381 Therefore, the exceeded amount of (iso)ferulic acid can be a consequence of the methylation of  
382 caffeic acid released from hydrolysis of caffeoylquinic acids. The mass balance recovery of  
383 (iso)ferulic acid also considering the initial amount of caffeoylquinic acids present in the digested  
384 sample was 35.6%, suggesting the partial methylation of caffeic acid released after hydrolysis of  
385 caffeoylquinic acids. This conclusion is also supported by the lack of identification of caffeic acid  
386 in the medium after 24 h of incubation with Caco-2.

387 The same conclusions can not be drawn for SW480. In the medium of this cell line, we found some  
388 residual caffeic acid and the amount of (iso)ferulic acid was 1.2 times higher respect than the  
389 amount found at the end of the digestion (**Table 2**). It can be speculated that SW480 expressed  
390 lower amount of catechol-*O*-methyltransferase or that the isoform in these cells had a lower affinity  
391 for caffeic acid respect to the isoform in Caco-2 cells.

392 Another difference between the two cell lines was related to the ability to metabolize glycosylated  
393 flavonols. Caco-2 cells were found to be able to hydrolyse quercetin-3-*O*-glucoside to the  
394 corresponding aglycone. Furthermore, a newly formed metabolite, quercetin-3-*O*-glucoside  
395 sulphate appeared in the cell medium after 24 h of incubation with Caco-2. The ability of Caco-2  
396 cells to hydrolyse glycosylated flavonols was previously reported (Boyer et al. 2004). Finally,  
397 glycosylated flavonols tended to be accumulated after 24 h of incubation with SW480 cell line.

398

#### 399 **4. Conclusions**

400 Bioactivity of phenolic compounds is primarily conditioned by their bioaccessibility in the gastro-  
401 intestinal tract, and secondly on their cellular uptake and internal transformation. The present study  
402 determined the amounts of bioaccessible phenolic compounds from two cherry cultivars after *in*  
403 *vitro* gastro-intestinal digestion. Results showed that the digestion process modified from a

404 qualitative and quantitative point of view the profile of phenolic compounds in the samples.  
405 Hydroxycinnamic acids and flavonols showed the highest bioaccessibility. Isomerisation of  
406 caffeoylquinic and coumaroylquinic acids was observed after *in vitro* gastro-intestinal digestion.  
407 We have also demonstrated that Caco-2 and SW480 cell lines showed metabolic activity resulting  
408 in a partial modification of cherry phenolic compounds. Methylation, sulphation, de-glycosylation  
409 and hydrolysis of esters were the main pathways of phenolic compounds metabolism in the tested  
410 cell lines. Moreover, we demonstrated that bioaccessible cherry phenolic compounds showed anti-  
411 proliferative activity against two models of human colon adenocarcinoma cell lines. The presence  
412 of (iso)ferulic acid in Caco-2 cell medium at higher concentration than in SW480 cell medium or  
413 the presence of caffeic acid only in SW480 cell medium and quercetin and quercetin-3-*O*-glucoside  
414 sulphate only in Caco-2 cell medium may suggest the intrinsic differences among the two cell lines  
415 and the metabolic mechanisms involved. The different effects on the two tested cell lines can be  
416 related to the accumulation of glycosylated flavonols in the cell medium of SW480 or to a different  
417 sensitivity of the cell lines to the phenolic compounds. Nevertheless, it could be interesting to  
418 underline how the sensitivity to the presence of phenolic-rich extracts increased after digestion,  
419 where the hydroxycinnamic acids were the major phenolic class present.  
420 Further studies are necessary in order to confirm the proposed pathways of metabolism of cherry  
421 phenolic compounds during incubation with cell lines and the potential role of hydroxycinnamic  
422 acids.

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The authors report no conflict of interest.

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## References

- Aragonès G, Danesi F, Del Rio D, Mena P. 2017. The importance of studying cell metabolism when testing the bioactivity of phenolic compounds. *Trends Food Sci Technol.* 69:230-242.
- Bastos C, Barros L, Dueñas M, Calhelha RC, Queiroz MJRP, Santos-Buelga C, Ferreira ICFR. 2015. Chemical characterization and bioactive properties of *Prunus avium* L.: The widely studied fruits and the unexplored stems. *Food Chem.* 173:1045-1053.
- Benzie IFF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal Biochem.* 239:70-76.
- Bermúdez-Soto MJ, Tomás-Barberán FA, García-Conesa MT. 2007. Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to *in vitro* gastric and pancreatic digestion. *Food Chem.* 102:865–874.
- Bouayed J, Deußer H, Hoffmann L, Bohn T. 2012. Bioaccessible and dialyzable polyphenols in selected apple varieties following *in vitro* digestion vs their native pattern. *Food Chem.* 131:1466-1472.
- Boyer J, Brown D, Liu RH. 2004. Uptake of quercetin and quercetin 3-glucoside from whole onion and apple peel extracts by Caco-2 cell monolayers. *J Agric Food Chem.* 52:7172-7179.
- Edwards M, Czank C, Woodward GM, Cassidy A, Kay CD. 2015. Phenolic metabolites of anthocyanins modulate mechanisms of endothelial function. *J Agric Food Chem.* 63:2423–2431.
- Erk T, Renouf M, Williamson G, Melcher R, Steiling H, Richling E. (2014). Absorption and isomerization of caffeoylquinic acids from different foods using ileostomist volunteers. *Eur J Nutr.* 53:159-166.
- Farrel TL, Dew TP, Poquet L, Hanson P, Williamson G. 2011. Absorption and metabolism of chlorogenic acids in cultured gastric epithelial monolayers. *Drug Metab Dispos.* 39:2338-2346.

- Fazzari M, Fukumoto L, Mazza G, Livrea MA, Tesoriere L, Marco LD. (2008). In vitro bioavailability of phenolic compounds from five cultivars of frozen sweet cherries (*Prunus avium* L.). *J Agric Food Chem.* 56:3561-3568.
- Ferretti G, Bacchetti T, Belleggia A, Neri D. 2010. Cherry antioxidants: from farm to table. *Molecules.* 15:6993-7005.
- Godos J, Marventano S, Mistretta A, Galvano F, Grosso G. 2017. Dietary sources of polyphenols in the Mediterranean healthy Eating, Aging and Lifestyle (MEAL) study cohort. *Int J Food Sci Nutr.* 68:750-756.
- Green RJ, Murphy AS, Schulz B, Watkins BA, Ferruzzi MG. 2007. Common tea formulations modulate in vitro digestive recovery of green tea catechins. *Mol Nutr Food Res.* 51:1152-1162.
- Juániz I, Ludwig IA, Bresciani L, Dall'Asta M, Mena P, Del Rio D, Cid C, de Peña MP. 2017. Bioaccessibility of (poly)phenolic compounds of raw and cooked cardoon (*Cynara cardunculus* L.) after simulated gastrointestinal digestion and fermentation by colonic microbiota. *J Funct Foods.* 32:195-207.
- Kahle K, Kempf M, Schreier P, Scheppach W, Schrenk D, Kautenburger T, Hecker D, Huemmer W, Ackermann M, Richling E. 2011. Intestinal transit and systemic metabolism of apple polyphenols. *Eur J Nutr.* 50:507-522.
- Kang SY, Seeram NP, Nair MG, Bourquin LD. 2003. Tart cherry anthocyanins inhibit tumor development in Apc<sup>Min</sup> mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* 194:13-19.
- Keane KM, George TW, Constantinou CL, Brown MA, Clifford T, Howatson G. 2016. Effects of Montmorency tart cherry (*Prunus cerasus* L.) consumption on vascular function in men with early hypertension. *Am J Clin Nutr.* 103:1531–1539.

- Kelley DS, Adkins Y, Reddy A, Woodhouse LR, Mackey BE, Erickson KL. 2013. Sweet bing cherries lower circulating concentrations of markers for chronic inflammatory diseases in healthy humans. *J Nutr.* 143:340–344.
- Kelley DS, Adkins Y, Laugero KD. 2018. A review of the health benefits of cherries. *Nutrients.* E368.
- Kent K, Charlton KE, Jenner A, Roodenrys S. 2016. Acute reduction in blood pressure following consumption of anthocyanin-rich cherry juice may be dose-interval dependant: A pilot cross-over study. *Int J Food Sci Nutr.* 67:47–52.
- Kern SM, Bennett RN, Needs PW, Mellon FA, Kroon PA, García-Conesa MT. 2003. Characterization of metabolites of hydroxycinnamates in the in vitro model of human small intestinal epithelium Caco-2 cells. *J Agric Food Chem.* 51:7884-7891.
- Lingua MS, Wunderlin DA, Baroni MV. 2018. Effect of simulated digestion on the phenolic components of red grapes and their corresponding wines. *J Funct Foods.* 44:86-94.
- Lopes P, Richard T, Saucier C, Teissedre PL, Monti JP, Glories Y. 2007. Anthocyanone A: A quinone methide derivative resulting from malvidin 3-*O*-glucoside degradation. *J Agric Food Chem.* 55:2698-2704.
- Martin KR, Bopp J, Burrell L, Hook G. 2011. The effect of 100% tart cherry juice on serum uric acid levels, biomarkers of inflammation and cardiovascular disease risk factors. *FASEB J.* 25
- Martini S, Conte A, Tagliazucchi D. 2017. Phenolic compounds profile and antioxidant properties of six sweet cherry (*Prunus avium*) cultivars. *Food Res Int.* 97:15-26.
- Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. 2014. A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct.* 5:1113–1124.



- Mele L, Mena P, Piemontese A, Marino V, López-Gutiérrez N, Bernini F, Brighenti F, Zanotti I, Del Rio D. 2016. Antiatherogenic effects of ellagic acid and urolithins *in vitro*. Arch Biochem Biophys. 599:42-50.
- Monente C, Ludwig IA, Stalmach A, de Peña MP, Cid C, Crozier A. 2015. In vitro studies on the stability in the proximal gastrointestinal tract and bioaccessibility in Caco-2 cells of chlorogenic acids from spent coffee grounds. Int J Food Sci Nutr. 66:657-664.
- Olsson ME, Gustavsson KE, Andersson S, Nilsson A, Duan RD. 2004. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. J Agric Food Chem. 52:7264-7271.
- Oziürek M, Bektaşoğlu B, Güçlü K, Apak R. 2008. Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. Anal Chim Acta. 616:196-206.
- Pérez-Vicente A, Gil-Izquierdo A, García-Viguera C. 2002. In vitro gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. J Agric Food Chem. 50:2308-2312.
- Picariello G, De Vito V, Ferranti P, Paolucci M, Volpe MG. 2016. Species- and cultivar-dependent traits of Prunus avium and Prunus cerasus polyphenols. J Food Comp Anal. 40:50-57.
- Pulido R, Bravo L, Saura-Calixto F. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem. 49:3396-3402.
- Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med. 26:1231-1237.
- Rice-Evans CA, Miller NJ, Paganga G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 20:933-956.

- Sala R, Mena P, Savi M, Brighenti F, Crozier A, Miragoli M, Stilli D, Del Rio D. 2015. Urolithins at physiological concentrations affect the levels of pro-inflammatory cytokines and growth factor in cultured cardiac cells in hyperglucidic conditions. *J Funct Foods*. 15:97-105.
- Sentandreu E, Navarro JL, Sendra JM. 2010. LC-DAD-ESI/MS<sup>n</sup> determination of direct condensation flavanol-anthocyanin adducts in pressure extracted pomegranate (*Punica granatum* L.) juice. *J Agric Food Chem*. 58:10560-10567.
- Serra AT, Duarte RO, Bronze MR, Duarte CMM. 2011. Identification of bioactive response in traditional cherries from Portugal. *Food Chem*. 125:318-325.
- Stalmach A, Steiling H, Williamson G, Crozier A. (2010). Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch Biochem Biophys*. 501:98-105.
- Tagliazucchi D, Verzelloni E, Bertolini D, Conte A. 2010. In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chem*. 120:599-606.
- Tagliazucchi D, Helal A, Verzelloni E, Conte A. 2012. The type and concentration of milk increase the in vitro bioaccessibility of coffee chlorogenic acids. *J Agric Food Chem*. 60:11056-11064.
- Tagliazucchi D, Helal A, Verzelloni E, Conte A. 2016. Bovine milk antioxidant properties: effect of in vitro digestion and identification of antioxidant compounds. *Dairy Sci Technol*. 96:657-676.
- Wang LS, Stoner GD. 2008. Anthocyanins and their role in cancer prevention. *Cancer Lett*. 269:281-290.
- Yang P, Yuan C, Wang H, Han F, Liu Y, Wang L, Liu Y. 2018. Stability of anthocyanins and their degradation products from cabernet sauvignon red wine under gastrointestinal pH and temperature conditions. *Molecules*. 23:E354.

## Figure captions

**Figure 1. Bioaccessibility of individual phenolic compounds identified and quantified by LC-ESI-IT MS/MS grouped by classes.** (A) Sum of the different classes; (B) hydroxycinnamic acids; (C) flavan-3-ols; (D) flavonols; (E) other flavonoids; (F) hydroxybenzoic acids; (G) anthocyanins. Dark grey columns represent the amount of the individual classes found in the chemical extract whereas light grey columns the amount at the end of the digestion. (H) Percent incidence of the different classes. UC: un-digested Celeste; DC: digested Celeste; UDN: un-digested Durone Nero I; DDN: digested Durone Nero I. ( ) Anthocyanins; ( ) other flavonoids; ( ) flavan-3-ols; ( ) flavonols; ( ) hydroxycinnamic acids; ( ) hydroxybenzoic acids. Results are expressed as mean  $\pm$  standard deviation. Values in the same graph with different lowercase letters are significantly different ( $P < 0.05$ ).

**Figure 2. Bioaccessibility of individual hydroxycinnamic acids isomers.** (A, C and E) Celeste cultivar; (B, D and F) Durone Nero I cultivar. Black columns represent the amount of the individual isomers found in the chemical extracts whereas grey columns the amount at the end of the digestion. CQA: caffeoylquinic acids; FQA: feruloylquinic acids; CoQA: coumaroylquinic acids. Results are expressed as mean  $\pm$  standard deviation. Values in the same graph with different lowercase letters are significantly different ( $P < 0.05$ ).

**Figure 3. Antioxidant properties of un-digested and *in vitro* digested cherry phenolic-rich fractions.** Antioxidant capacity measured as hydroxyl radical scavenging capacity (A), ferric reducing power (FRAP) (B), superoxide anion scavenging capacity (C) and ABTS radical scavenging activity (D). Black columns show data from the chemical extracts whereas grey columns from the extracts at the end of the digestion. Results are expressed as mean  $\pm$  standard

deviation. Values in the same graph with different lowercase letters are significantly different ( $P < 0.05$ ).

**Figure 4. Anti-proliferative activity of un-digested and *in vitro* digested cherry phenolic-rich fractions.** IC<sub>50</sub> is defined as the concentration of phenolic compounds required to inhibit 50% of cell proliferation. The amount of phenolic compounds was determined by LC-ESI-IT MS/MS analysis. Dark grey columns represent the activity of the chemical extracts whereas light grey columns the activity of the extracts at the end of the digestion. Results are expressed as mean  $\pm$  standard deviation. Values in the same graph with different lowercase letters are significantly different ( $P < 0.05$ ).

**Table 1.** Quantitative results ( $\mu\text{mol}/100\text{ g}$  fresh weight fruit) for phenolic compounds identified in the cherry phenolic-rich fractions obtained from both chemical extraction and after gastro-intestinal digestion. Values represent means  $\pm$  standard deviation of triplicate determination.\*

Compound	<i>Celeste</i>		<i>Durone Nero I</i>	
	Chemical extraction	After digestion	Chemical extraction	After digestion
<i>Hydroxycinnamic acids</i>				
3-Caffeoylquinic acid <i>cis</i>	31.90 $\pm$ 1.76 <sup>b</sup>	8.44 $\pm$ 0.45 <sup>c</sup>	37.87 $\pm$ 0.28 <sup>a</sup>	3.54 $\pm$ 0.51 <sup>d</sup>
3-Caffeoylquinic acid <i>trans</i>	123.91 $\pm$ 5.43 <sup>b</sup>	33.06 $\pm$ 0.72 <sup>c</sup>	197.17 $\pm$ 0.75 <sup>a</sup>	33.36 $\pm$ 0.40 <sup>c</sup>
4-Caffeoylquinic acid <i>cis</i>	1.44 $\pm$ 0.03 <sup>c</sup>	6.23 $\pm$ 0.45 <sup>a</sup>	2.51 $\pm$ 0.22 <sup>b</sup>	2.19 $\pm$ 0.01 <sup>b</sup>
5-Caffeoylquinic acid <i>trans</i>	85.03 $\pm$ 3.50 <sup>b</sup>	17.23 $\pm$ 0.31 <sup>c</sup>	170.27 $\pm$ 6.56 <sup>a</sup>	14.64 $\pm$ 0.17 <sup>c</sup>
4-Caffeoylquinic acid <i>trans</i>	2.75 $\pm$ 0.07 <sup>b</sup>	37.85 $\pm$ 0.42 <sup>a</sup>	n.d.	36.57 $\pm$ 0.17 <sup>a</sup>
5-Caffeoylquinic acid <i>cis</i>	11.91 $\pm$ 0.08 <sup>b</sup>	3.70 $\pm$ 0.06 <sup>c</sup>	26.37 $\pm$ 0.11 <sup>a</sup>	2.05 $\pm$ 0.01 <sup>d</sup>
3-Coumaroylquinic acid <i>cis</i>	464.38 $\pm$ 11.74 <sup>a</sup>	89.38 $\pm$ 4.12 <sup>c</sup>	383.90 $\pm$ 20.47 <sup>b</sup>	54.94 $\pm$ 2.66 <sup>d</sup>
3-Coumaroylquinic acid <i>trans</i>	551.01 $\pm$ 5.45 <sup>b</sup>	212.71 $\pm$ 3.28 <sup>d</sup>	1278.30 $\pm$ 14.14 <sup>a</sup>	351.02 $\pm$ 5.85 <sup>c</sup>
4-Coumaroylquinic acid <i>cis</i>	2.09 $\pm$ 0.04 <sup>d</sup>	136.06 $\pm$ 3.46 <sup>a</sup>	38.18 $\pm$ 0.96 <sup>c</sup>	102.24 $\pm$ 1.10 <sup>b</sup>
4-Coumaroylquinic acid <i>trans</i>	29.45 $\pm$ 1.43 <sup>d</sup>	287.86 $\pm$ 2.17 <sup>b</sup>	50.91 $\pm$ 0.76 <sup>c</sup>	319.44 $\pm$ 2.67 <sup>a</sup>
5-Coumaroylquinic acid <i>trans</i>	1.54 $\pm$ 0.06 <sup>c</sup>	78.95 $\pm$ 1.25 <sup>a</sup>	4.32 $\pm$ 0.16 <sup>b</sup>	81.85 $\pm$ 2.69 <sup>a</sup>
5-Coumaroylquinic acid <i>cis</i>	2.07 $\pm$ 0.11 <sup>c</sup>	49.25 $\pm$ 0.72 <sup>a</sup>	2.72 $\pm$ 0.11 <sup>c</sup>	32.77 $\pm$ 0.36 <sup>b</sup>
3-Feruloylquinic acid <i>cis</i>	5.30 $\pm$ 0.07 <sup>a</sup>	3.05 $\pm$ 0.03 <sup>b</sup>	4.94 $\pm$ 0.09 <sup>a</sup>	1.84 $\pm$ 0.05 <sup>c</sup>
3-Feruloylquinic acid <i>trans</i>	6.98 $\pm$ 0.19 <sup>a</sup>	2.59 $\pm$ 0.07 <sup>b</sup>	2.03 $\pm$ 0.01 <sup>c</sup>	2.13 $\pm$ 0.02 <sup>c</sup>
4-Feruloylquinic acid <i>cis</i>	0.97 $\pm$ 0.07 <sup>c</sup>	2.79 $\pm$ 0.05 <sup>a</sup>	1.10 $\pm$ 0.03 <sup>c</sup>	1.96 $\pm$ 0.03 <sup>b</sup>
5-Feruloylquinic acid <i>trans</i>	0.39 $\pm$ 0.02 <sup>c</sup>	1.02 $\pm$ 0.06 <sup>a</sup>	0.69 $\pm$ 0.03 <sup>b</sup>	n.d.
5-Feruloylquinic acid <i>cis</i>	5.59 $\pm$ 0.05 <sup>b</sup>	0.69 $\pm$ 0.01 <sup>c</sup>	8.24 $\pm$ 0.24 <sup>a</sup>	0.35 $\pm$ 0.01 <sup>c</sup>
Caffeoylquinic acid-hexoside isomer	1.21 $\pm$ 0.03 <sup>a</sup>	n.d.	1.20 $\pm$ 0.03 <sup>a</sup>	n.d.
Caffeoylquinic acid-hexoside isomer	1.05 $\pm$ 0.02 <sup>b</sup>	1.04 $\pm$ 0.08 <sup>b</sup>	2.07 $\pm$ 0.07 <sup>a</sup>	0.78 $\pm$ 0.02 <sup>c</sup>
3,5-diCaffeoylquinic acid	3.38 $\pm$ 0.03 <sup>b</sup>	n.d.	5.60 $\pm$ 0.12 <sup>a</sup>	n.d.
4,5-diCaffeoylquinic acid	1.64 $\pm$ 0.02 <sup>b</sup>	n.d.	2.06 $\pm$ 0.17 <sup>a</sup>	n.d.
Caffeoylshikimic acid isomer	1.58 $\pm$ 0.04 <sup>a</sup>	n.d.	0.75 $\pm$ 0.09 <sup>b</sup>	n.d.
3- and 4-Caffeoylquinic lactone	1.99 $\pm$ 0.01 <sup>a</sup>	n.d.	2.26 $\pm$ 0.15 <sup>a</sup>	n.d.
Caffeoylshikimic acid isomer	0.67 $\pm$ 0.03	n.d.	n.d.	n.d.
3-Coumaroylquinic lactone	1.09 $\pm$ 0.03	n.d.	n.d.	n.d.
4-Coumaroylquinic lactone	1.90 $\pm$ 0.04 <sup>b</sup>	n.d.	5.46 $\pm$ 0.10 <sup>a</sup>	n.d.
3-Coumaroyl-5-caffeoylquinic acid	0.94 $\pm$ 0.05 <sup>b</sup>	n.d.	1.78 $\pm$ 0.08 <sup>a</sup>	n.d.
3-Caffeoyl-4-coumaroylquinic acid	0.90 $\pm$ 0.07	n.d.	n.d.	n.d.

Caffeoyl-hexose isomer	0.57 ± 0.02 <sup>b</sup>	n.d.	2.21 ± 0.21 <sup>a</sup>	n.d.
Caffeic acid-hexoside isomer	2.94 ± 0.13 <sup>b</sup>	n.d.	8.70 ± 0.26 <sup>a</sup>	n.d.
Caffeoyl alcohol 3/4-O-hexoside	2.21 ± 0.11	n.d.	n.d.	n.d.
Feruloyl-hexose isomer	6.17 ± 0.05	n.d.	n.d.	n.d.
Sinapoyl-hexose isomer	n.d.	n.d.	0.94 ± 0.02 <sup>a</sup>	0.82 ± 0.02 <sup>b</sup>
Sinapic acid-hexoside	0.93 ± 0.02	n.d.	n.d.	n.d.
<b><u>Total hydroxycinnamic acids</u></b>	<b><u>1354.99 ± 4.49<sup>b</sup></u></b>	<b><u>971.91 ± 3.22<sup>d</sup></u></b>	<b><u>2242.57 ± 5.69<sup>a</sup></u></b>	<b><u>1042.51 ± 2.87<sup>c</sup></u></b>
<b><u>Flavan-3-ols</u></b>				
Catechin	127.92 ± 3.20 <sup>b</sup>	n.d.	229.51 ± 6.86 <sup>a</sup>	1.86 ± 0.08 <sup>c</sup>
Epicatechin	772.30 ± 4.82 <sup>b</sup>	n.d.	1369.61 ± 12.58 <sup>a</sup>	11.16 ± 0.17 <sup>c</sup>
(Epi)catechin-3-gallate	10.75 ± 0.23 <sup>a</sup>	4.30 ± 0.22 <sup>c</sup>	8.41 ± 0.09 <sup>b</sup>	4.86 ± 0.03 <sup>c</sup>
(Epi)catechin-hexoside isomer	16.03 ± 0.59 <sup>b</sup>	n.d.	26.78 ± 0.42 <sup>a</sup>	1.35 ± 0.05 <sup>c</sup>
Procyanidin tetramer B type isomer	3.40 ± 0.19 <sup>b</sup>	n.d.	10.17 ± 0.14 <sup>a</sup>	n.d.
Procyanidin dimer B type isomer	13.17 ± 1.80 <sup>b</sup>	n.d.	21.66 ± 2.58 <sup>a</sup>	0.77 ± 0.01 <sup>c</sup>
Procyanidin dimer B type isomer	65.66 ± 1.08 <sup>b</sup>	n.d.	91.27 ± 1.98 <sup>a</sup>	1.05 ± 0.04 <sup>c</sup>
Procyanidin dimer B type isomer	7.11 ± 0.46 <sup>b</sup>	n.d.	12.22 ± 0.15 <sup>a</sup>	0.94 ± 0.02 <sup>c</sup>
Propelargonidin dimer isomer	n.d.	n.d.	2.65 ± 0.06	n.d.
Procyanidin pentamer B type isomer	2.59 ± 0.21 <sup>b</sup>	n.d.	5.79 ± 0.23 <sup>a</sup>	n.d.
<b><u>Total flavan-3-ols</u></b>	<b><u>1018.94 ± 6.21<sup>b</sup></u></b>	<b><u>4.30 ± 0.22<sup>d</sup></u></b>	<b><u>1778.07 ± 14.71<sup>a</sup></u></b>	<b><u>21.99 ± 0.20<sup>c</sup></u></b>
<b><u>Flavonols</u></b>				
Quercetin-3-O-rutinoside	15.69 ± 0.42 <sup>c</sup>	6.09 ± 0.07 <sup>d</sup>	102.25 ± 2.09 <sup>a</sup>	29.72 ± 0.14 <sup>b</sup>
Quercetin-3-O-glucoside	1.40 ± 0.03 <sup>c</sup>	0.64 ± 0.02 <sup>d</sup>	40.90 ± 0.22 <sup>a</sup>	26.41 ± 0.15 <sup>b</sup>
Quercetin-7-O-hexoside-3-O-rutinoside	7.53 ± 0.06 <sup>b</sup>	6.07 ± 0.14 <sup>b</sup>	11.90 ± 0.13 <sup>a</sup>	3.53 ± 0.07 <sup>c</sup>
Kaempferol-3-O-hexoside	n.d.	n.d.	2.93 ± 0.07 <sup>a</sup>	2.39 ± 0.05 <sup>a</sup>
Kaempferol-3-O-rutinoside	2.97 ± 0.06 <sup>c</sup>	1.31 ± 0.02 <sup>d</sup>	17.53 ± 0.14 <sup>a</sup>	7.04 ± 0.06 <sup>b</sup>
Kaempferol-7-O-hexoside-3-O-rutinoside	1.44 ± 0.07 <sup>b</sup>	1.05 ± 0.01 <sup>c</sup>	2.33 ± 0.02 <sup>a</sup>	0.76 ± 0.01 <sup>c</sup>
<b><u>Total flavonols</u></b>	<b><u>29.02 ± 0.44<sup>c</sup></u></b>	<b><u>15.16 ± 0.16<sup>d</sup></u></b>	<b><u>177.83 ± 2.11<sup>a</sup></u></b>	<b><u>69.85 ± 0.23<sup>b</sup></u></b>
<b><u>Other flavonoids</u></b>				
Naringenin-hexoside	0.81 ± 0.01 <sup>b</sup>	n.d.	4.18 ± 0.24 <sup>a</sup>	n.d.
Taxifolin-rutinoside isomer	10.01 ± 0.26 <sup>b</sup>	n.d.	151.92 ± 11.33 <sup>a</sup>	2.09 ± 0.08 <sup>c</sup>
Taxifolin-rutinoside isomer	6.70 ± 0.12 <sup>b</sup>	n.d.	38.12 ± 0.19 <sup>a</sup>	2.02 ± 0.05 <sup>c</sup>
Taxifolin-hexoside isomer	n.d.	n.d.	78.53 ± 1.02 <sup>a</sup>	2.27 ± 0.03 <sup>b</sup>
Taxifolin-hexoside isomer	n.d.	n.d.	24.13 ± 0.09 <sup>a</sup>	2.10 ± 0.04 <sup>b</sup>

<b><u>Total other flavonoids</u></b>	<b><u>17.53 ± 0.29<sup>b</sup></u></b>	<b><u>n.d.</u></b>	<b><u>296.88 ± 11.38<sup>a</sup></u></b>	<b><u>8.49 ± 0.10<sup>c</sup></u></b>
<b><u>Hydroxybenzoic acids</u></b>				
Protocatechuic acid	n.d.	0.13 ± 0.01 <sup>b</sup>	n.d.	0.34 ± 0.01 <sup>a</sup>
Protocatechuoyl-hexose	0.52 ± 0.01 <sup>b</sup>	n.d.	1.05 ± 0.08 <sup>a</sup>	n.d.
Hydroxybenzoic acid-hexoside	0.41 ± 0.01	n.d.	n.d.	n.d.
Vanillic acid-hexoside	2.96 ± 0.10 <sup>b</sup>	n.d.	5.24 ± 0.24 <sup>a</sup>	n.d.
<b><u>Total hydroxybenzoic acids</u></b>	<b><u>3.89 ± 0.10<sup>b</sup></u></b>	<b><u>0.13 ± 0.01<sup>d</sup></u></b>	<b><u>6.29 ± 0.25<sup>a</sup></u></b>	<b><u>0.34 ± 0.01<sup>c</sup></u></b>
<b><u>Anthocyanins and derivatives</u></b>				
Cyanidin-3- <i>O</i> -glucoside	2.49 ± 0.03 <sup>c</sup>	n.d.	317.02 ± 12.32 <sup>a</sup>	86.91 ± 0.56 <sup>b</sup>
Cyanidin-3- <i>O</i> -rutinoside	68.77 ± 0.74 <sup>c</sup>	0.59 ± 0.02 <sup>d</sup>	480.05 ± 4.82 <sup>a</sup>	346.74 ± 2.14 <sup>b</sup>
Cyanidin-5- <i>O</i> -hexoside-3- <i>O</i> -coumaroyl-hexoside	n.d.	n.d.	0.97 ± 0.01	< LOQ
Cyanidin-3- <i>O</i> -glucoside chalcone	n.d.	n.d.	n.d.	0.07 ± 0.01
(Epi)catechin-cyanidin-3- <i>O</i> -glucoside dimer	n.d.	n.d.	n.d.	0.09 ± 0.01
Peonidin-3- <i>O</i> -rutinoside	5.07 ± 0.09 <sup>b</sup>	n.d.	13.43 ± 0.14 <sup>a</sup>	13.94 ± 0.17 <sup>a</sup>
Pelargonidin-3- <i>O</i> -rutinoside	0.27 ± 0.01 <sup>c</sup>	n.d.	3.85 ± 0.50 <sup>a</sup>	1.47 ± 0.04 <sup>b</sup>
Pelargonidin-3- <i>O</i> -hexoside	n.d.	n.d.	1.59 ± 0.20 <sup>a</sup>	0.32 ± 0.01 <sup>b</sup>
<b><u>Total anthocyanins</u></b>	<b><u>76.60 ± 0.74<sup>c</sup></u></b>	<b><u>0.59 ± 0.02<sup>d</sup></u></b>	<b><u>816.92 ± 13.24<sup>a</sup></u></b>	<b><u>449.53 ± 2.22<sup>b</sup></u></b>
<b><u>Total phenolic compounds</u></b>	<b><u>2500.96 ± 3.99<sup>b</sup></u></b>	<b><u>992.10 ± 3.23<sup>d</sup></u></b>	<b><u>5318.56 ± 23.61<sup>a</sup></u></b>	<b><u>1592.71 ± 3.67<sup>c</sup></u></b>

\*Hydroxycinnamic acids were quantified as 5-caffeoylquinic acid equivalent; flavan-3-ols were quantified as epicatechin equivalent; flavonols and other flavonoids were quantified as quercetin-3-glucoside equivalent; hydroxybenzoic acids were quantified as gallic acid equivalent; anthocyanins and derivatives were quantified as cyanidin-3-glucoside equivalent.

Different superscript letters within the same row indicate that the values are significantly different ( $P < 0.05$ ).

<LOQ means the compound was detected but it was below the limit of quantification.

n.d. means not detected

**Table 2.** Phenolic compounds extracted from *in vitro* digested Durone Nero I identified in the cell media after 24 h of incubation with Caco-2 and SW480. Results are expressed as  $\mu\text{mol/L}^*$ . In bracket the percentage of recovery respect to the time zero (before incubation with cells).

Compounds	[M-H] <sup>-</sup>	Fragment ions	Caco-2 $\mu\text{mol/L}$	SW480 $\mu\text{mol/L}$
3-Coumaroylquinic acid <i>cis</i>	337	163, 119, 191	19.56 $\pm$ 0.20 (21.4) <sup>b</sup>	55.49 $\pm$ 0.16 (60.6) <sup>a</sup>
3-Coumaroylquinic acid <i>trans</i>	337	163, 119, 191	47.18 $\pm$ 2.79 (8.1) <sup>a</sup>	44.35 $\pm$ 0.06 (7.6) <sup>a</sup>
4-Coumaroylquinic acid <i>cis</i>	337	173, 163	22.69 $\pm$ 0.47 (4.3) <sup>b</sup>	48.80 $\pm$ 2.29 (9.2) <sup>a</sup>
4-Coumaroylquinic acid <i>trans</i>	337	173, 163	59.96 $\pm$ 4.77 (32.8) <sup>a</sup>	37.31 $\pm$ 0.32 (21.9) <sup>b</sup>
5-Coumaroylquinic acid <i>trans</i>	337	191, 173, 163	45.44 $\pm$ 1.26 (33.3) <sup>a</sup>	21.49 $\pm$ 0.55 (15.8) <sup>b</sup>
5-Coumaroylquinic acid <i>cis</i>	337	191, 163, 173	27.50 $\pm$ 0.51 (50.4) <sup>a</sup>	23.71 $\pm$ 1.22 (43.4) <sup>a</sup>
Total coumaroylquinic acids	/	/	218.33 $\pm$ 5.71 (13.9) <sup>b</sup>	231.14 $\pm$ 7.30 (14.7) <sup>a</sup>
Coumaroylquinic acid sulphate	417	337, 163	< LOQ	7.83 $\pm$ 0.54
Caffeoylquinic acid-hexoside isomer	515	341, 353, 179	< LOQ	n.d.
3-Feruloylquinic acid	367	193	< LOQ	n.d.
Caffeic acid	179	135	n.d.	< LOQ
(Iso)ferulic acid	193	178	58.47 $\pm$ 4.40 <sup>a</sup>	12.62 $\pm$ 0.52 <sup>b</sup>
Coumaric acid	163	119	4.11 $\pm$ 5.71	< LOQ
Dihydro-caffeic acid	181	137	n.d.	< LOQ
Quercetin-3- <i>O</i> -rutinoside	609	301	n.d.	26.84 $\pm$ 0.15 (54.2)
Quercetin-3- <i>O</i> -glucoside	463	301	n.d.	9.80 $\pm$ 0.19 (22.3)
Quercetin-3- <i>O</i> -glucoside sulphate	543	381, 301	< LOQ	n.d.
Quercetin	301	179, 151	< LOQ	n.d..
Kaempferol-3- <i>O</i> -rutinoside	593	285, 255	5.99 $\pm$ 0.08 (51.0) <sup>b</sup>	9.17 $\pm$ 0.49 (78.1) <sup>a</sup>

\*Hydroxycinnamic acids were quantified as 5-caffeoylquinic acid equivalent, whereas flavonols were quantified as quercetin-3-glucoside equivalent.

Different superscript letters within the same row indicate that the values are significantly different ( $P < 0.05$ ).

<LOQ means the compound was detected but it was below the limit of quantification.

n.d. means not detected









