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1 **In vitro metabolism of elderberry juice polyphenols by lactic acid bacteria.**

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20 **Abstract**

21 In this study, ten strains of *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus*  
22 *rhamnosus* were used to assess the in vitro metabolism of elderberry juice polyphenols. Total  
23 polyphenolic compounds in juice were increased after starters addition, especially with three *L.*  
24 *rhamnosus* and one *L. plantarum* strains, of dairy origin. In these samples, quercetin-3-*O*-rutinoside  
25 was the most abundant compound. Hydroxycinnamic acids, flavonols and anthocyanins were higher  
26 in juices added with these strains, while the highest increase in phenyllactic acids was observed  
27 when *L. plantarum* were used as starters. Interestingly, hydroxycinnamic acids were subjected to  
28 lactic acid bacteria (LAB) metabolism: caffeic acid and protocatechuic acid were consumed during  
29 fermentation while dihydrocaffeic acid and catechol were produced. Anthocyanins, (cyanidin-3-*O*-  
30 glucoside and cyanidin-3-*O*-sambubioside), increased in a strain-specific way. By this study we  
31 highlighted that dairy strains can produced (phenyllactic acids), modified (hydroxycinnamic acids)  
32 or increased (flavonols glycosides and anthocyanins) phenolic compounds.

33

34 **Keywords:**

35 Lactic acid bacteria, elderberry juice fermentation, (poly)phenolic compounds, liquid  
36 chromatography, mass spectrometry

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41 **Highlights:**

- 42 • Lactic acid fermentation was performed with *L. plantarum*, *L. rhamnosus* and *L. casei*.
- 43 • Polyphenolic profile of started and unstarted elderberry juice was characterized
- 44 • Polyphenols content increased after fermentation

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## 59 **1. Introduction**

60 Elderberry (*Sambucus nigra L.*) is the most widespread shrub in Europe, Asia, North Africa and  
61 USA. Berries ripen in late summer and are used for concentrate or juice preparations. The juice  
62 contains sugar and organic acids, conferring a sweet and astringent taste, besides polyphenols,  
63 responsible of the typical black-purple colour of the berries (Sidor et al., 2015). Anthocyanins give  
64 this dark colour to the fruits and are considered an indicator of fruit quality (Veberic et al., 2009).  
65 Cyanidin-3-*O*-glucoside and cyanidin-3-*O*-sambubioside are the most abundant cyanidin  
66 glycosides. Elderberries are also rich in flavonols and phenolic acids. The main flavonols are  
67 quercetin, kaempferol, and isorhamnetin-glycosides, with quercetin-3-*O*-rutinoside being among the  
68 principal components (Lee et al., 2007). The main acyl-quinic acid is 5-*O*-caffeoylquinic acid , with  
69 smaller amounts of 4-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid and other derivatives of  
70 caffeic and *p*-coumaric acid (Lee et al., 2007; Sidor et al., 2015). In the recent years fermentation of  
71 fruit juice with lactic acid bacteria (LAB) has been used to produce functional beverages (Kwaw et  
72 al., 2018; Marsha et al., 2014). Although the evidence is limited, it has been suggested that LABs  
73 improve the bioavailability and bioactivity of phytochemicals present in the juice and lead an  
74 increase of potentially functional microbial metabolites with putative benefits for human health  
75 (Filannino et al., 2018).

76 Polyphenols may negatively affect bacterial viability, only a few LABs are indeed able to grow in  
77 contact with substrates rich in these compounds. High concentrations of polyphenols negatively  
78 affect LAB wall and membrane integrity, dissipate the pH gradient, and delay the metabolism of  
79 carbohydrates (Filannino et al., 2014). Nevertheless, some species such as *Lactobacillus plantarum*  
80 are able to convert polyphenolics to phenolic acids and aromatic compounds which exhibit reduced  
81 antimicrobial activity and, as a consequence partially counteract these negative effects.  
82 Furthermore, it is of interest that some of these low molecular weight metabolites are potentially

83 more bioactive, and have a more substantial impact on human health, that their parent compounds  
84 (Filannino et al.,2018).

85 The present study investigated the ability of ten LAB strains, belonging to three different species (*L.*  
86 *plantarum*, *L. rhamnosus* and *L. casei*) and isolated from different niches, to metabolize  
87 polyphenolic compounds in elderberry juice.

88

## 89 **2. Materials and methods**

### 90 2.1 Chemicals

91 UHPLC phenolic standard cyanidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside were purchased  
92 from Extrasynthese Genay Cedex; 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 4-*O*-  
93 caffeoylquinic acid, protocatechuic acid, quercetin-3-rutinoside hydrate, quercetin dehydrate,  
94 kaempferol and phenyllactic acid from Sigma-Aldrich, USA; dihydrocaffeic acid, p-  
95 hydroxyphenyllactic acid and catechol from Santa Cruz Biotechnology, USA while luteolin from  
96 Extrasynthese, France.

### 97 2.2 Strains and cultures

98 Ten bacterial strains (Table 1) of three species (*L. plantarum*, *L. casei* and *L. rhamnosus*) were  
99 evaluated in this study, seven strains, of dairy origin, from the microbial collection of Food and  
100 Drug Department, University of Parma, Italy and three strains, of plant origin, from the microbial  
101 collection of the Department of Soil, Plant and Food Science, University of Bari, Italy. Bacterial  
102 cultures were stored at -80 °C in Man Rogosa Sharpe (MRS) medium (Oxoid, Milan, Italy)  
103 containing 15% of glycerol (v/v). Cultures of *L. plantarum*, *L. rhamnosus* and *L. casei* were  
104 routinely grown in MRS broth (AnaeroGen, Oxoid). *L. plantarum* was incubated anaerobically at

105 30 °C while *L. rhamnosus* and *L. casei* at 37 °C, for 15 h. When use for fermentation, the strains  
106 were cultivated until the late exponential phase (ca. 15 h) and the cell were harvested by  
107 centrifugation (10,000 rpm for 10 min at 4°C), washed twice with Ringer's solution (Oxoid, Milan,  
108 Italy), and re-suspended in sterile distilled water to a final concentration of 9.0 Log CFU/mL.

109

### 110 2.3 Elderberry juice fermentation

111 A commercial pasteurized non-filtered elderberry juice was used as substrate for the fermentation  
112 process. Each culture prepared as previously described was inoculated in order to reach 7 Log  
113 CFU/mL. The juice was fermented for 48 h, at 30°C with *L. plantarum*, and 37°C with *L.*  
114 *rhamnosus* and *L. casei* (optimal growth temperatures respectively of the different species), and  
115 then stored for 12 days at 4°C. Each fermentation was performed in triplicate. Samples were  
116 analysed at the end of fermentation process (48 h) and after the storage (14 days). Viable cell counts  
117 were carried out in triplicate by plate count on MRS agar (Oxoid, Milan, Italy), incubating  
118 anaerobically at the optimal temperature of the species (30° C or 37°C) for 48-72 h. Two types of  
119 controls were used: i) elderberry juice subjected to the same temperature treatments (30°C and  
120 37°C), without the use of starters and ii) elderberry juice not subjected to temperature treatment and  
121 without starters. Microbial counts were carried out also in the controls, using Plate Count Agar  
122 medium (Oxoid, Milan, Italy) incubating at 30 °C for 72 h.

123

### 124 2.4 Extraction of polyphenolic compounds

125 The extraction of polyphenolic compounds was performed using 0.6 mL of a methanol-water  
126 solution (70:30 v/v) acidified with 0.2% of formic acid added to 0.3 mL of sample. All samples  
127 were stirred for 15 min at 200 rpm, then treated for 15 min in an ultrasonic bath and finally  
128 centrifuged 10 min at 10,000 rpm. The pellet obtained was subjected to a second extraction

129 suspending it in 0.3 mL of methanol-water solution and the second extraction was performed as  
130 described before.

131

## 132 2.5 Characterization of polyphenolic profile of started and unstarted elderberry juices

133 All samples, started and unstarted (i.e. not incubated and incubated at 30 °C and 37°C), were  
134 analysed with an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS)  
135 (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray  
136 ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Separation was  
137 performed on an Acquity UPLC HSS T3 (2.1 x 100 mm) column coupled with a pre-column  
138 Acquity UPLC HSS T3 VanGuard 3/Pk (2.1x5 mm) (Waters, Milford, MA, USA). Volume injected  
139 was 5 µL and oven temperature was set to 40 °C. For the determination of phenolic profile was  
140 followed the protocol reported by Zaupa et al. (2015) with some modification. Briefly the mobile  
141 phase was 0.1% (v/v) acetonitrile (phase A) and 0.1% (v/v) aqueous formic acid (phase B). Elution  
142 was performed at a flow rate of 0.3 mL/min. The gradient started with 95% B and 5% A, held until  
143 0.5 min, in 9 min B decreased at 49% and A increased at 51%, then after 0.5 min, the column was  
144 flashed setting the eluent percentages at 20% B and 80% A until 11,50 min, and after that the initial  
145 conditions were restored (total run time = 17 min). The anthocyanins analysis was carried out in  
146 positive ionization mode, the MS worked with a capillary temperature set to 275 °C, while the  
147 source heater temperature was 300 °C. The sheath gas flow was 40 unit, while auxiliary and sweep  
148 gases were equal to 5 units, respectively. The analyses were carried out in Full MS<sup>2</sup> mode, using  
149 the selective reaction monitoring (SRM) mode. The other phenolic compounds were detected in  
150 negative ionisation mode, maintaining the same conditions described before, only the sheath gas  
151 flow was changed to 60 unit. The analyses were carried out in Full MS<sup>2</sup> using the selected ion

152 monitoring (SIM) mode for quercetin, luteolin and kaempferol. All the others compounds were  
153 analysed using SRM.

154 Catechol was analysed through a DIONEX Ultimate 3000 UHPLC coupled to a TSQ Vantage triple  
155 quadrupole (Thermo Fisher Scientific Inc., San José, CA, USA) fitted with a heated-electrospray  
156 ionisation (H-ESI) probe (Thermo Fisher Scientific Inc., San José, CA, USA). For UHPLC, mobile  
157 phase A was 0.2% aqueous formic acid and mobile phase B was acetonitrile containing 0.2%  
158 formic acid. Separations were performed using the same analytical column used for UHPLC-ion  
159 trap analyses. The gradient started with 5% B maintaining isocratic conditions for 1 min, and  
160 reached 50% B after 8 min. Then B turned up to 80% keeping these conditions for 2 min before to  
161 re-establish the starting conditions maintaining it 8 min to re-equilibrate the column. The flow rate  
162 was 0.3 mL/min, the injection volume was 5  $\mu$ L, the autosampler temperature was set at 10°C and  
163 the column oven was set at 40 °C. The ESI source was operated in negative ionization mode. The  
164 vaporizer temperature was 300°C, while the capillary temperature was set to 270°C. The spray  
165 voltage was 3 kV and the sheath and auxiliary gases were set to 40 and 10 units respectively. The S-  
166 Lens value was 75 V. Catechol was monitored by SRM with the following characteristic transitions:  
167 109.1  $\rightarrow$  52.9 (CE 22 eV) and 109.1  $\rightarrow$  80.9 (CE 18 eV). However, because of the poor sensitivity  
168 monitoring these product ions, the molecular ion at  $m/z$  109.1 was monitored as base peak product  
169 ion (CE 15 eV). Data processing was performed using Xcalibur 2.1 software (Thermo Scientific  
170 Inc. San José, CA, USA). The quantification of phenolic compounds was performed using  
171 calibration curves with the standards reported in Table 2. All the analyses were performed in  
172 triplicate.

173

174 2.6 Statistical analyses

175 For each fermented sample and its control (elderberry juice incubated at 30°C for *L. plantarum* and  
176 37°C for *L. casei* and *L. rhamnosus* strains) t-test, for independent samples, was applied in order to  
177 identify the differences between the juices. To evaluate the normally distribution for every group of  
178 independent samples Shapiro-Wilk test was used. Differences were considered statistically  
179 significant for p values < 0.05 and highly significant for p values < 0.001. In addition Pearson  
180 correlation analysis was performed to measures the strengths of association and the direction of the  
181 relationship between caffeic acid and dihydrocaffeic acid and between protocatechuic acid and  
182 catechol. Finally a Principal Component Analysis (PCA) was performed using correlation matrix.  
183 All the analyses were carried out using SPSS Statistics 23.0 software (SPSS Inc., Chicago, IL).

184

### 185 **3. Results and discussion**

#### 186 3.1 Elderberry juice fermentation

187 A total of ten strains belonging to three different species of LAB (*L. plantarum*, *L. rhamnosus* and  
188 *L. casei*), isolated from dairy and plant matrices, were used individually for elderberry juice  
189 fermentation.

190 Despite *L. plantarum* isolated from plant niches is considered the most well-adapted species and  
191 more exploitable as starter in fruit juices (Rodríguez et al., 2009), non-autochthonous strains  
192 belonging to *L. rhamnosus* and *L. casei* were also able to grow and steer the fermentation process,  
193 confirming our recent results (Ricci et al., 2018). Indeed, after 48 h of fermentation all strains were  
194 able to grow about two logarithmic cycles, from  $7.20 \pm 0.18$  to  $9.26 \pm 0.20$  Log CFU/mL (mean  
195 values), and cell viability did not decrease at the end of the storage period. The fermentation process  
196 was associated with a moderate shift of pH (3.9 before fermentation), that reached  $3.61 \pm 0.09$  at the  
197 end of fermentation, and remained unchanged after the storage ( $3.55 \pm 0.08$ ).

198

### 199 3.2 Characterization of polyphenols

200 There is a lack of information on the effect of LAB fermentation on polyphenolic profiles in  
201 literature. The few studies investigating this topic, report that LAB were able to increase the  
202 concentration of (poly)phenolic compounds, metabolize phenolic acids, and convert flavonoid  
203 glycosides and tannins (Kwaw et al., 2018; Curiel et al., 2015; Filannino et al., 2015; Filannino et  
204 al., 2013). This is the first report on the impact of fermentation on the (poly)phenolic profile of  
205 elderberry juice. Using LC-MS<sup>n</sup>, 14 compounds were identified and quantified by reference to  
206 authentic standards. The remained 11 phenolic compounds, for which reference compounds were  
207 not available, were tentatively identified based on the interpretation of their fragmentation patterns  
208 obtained from MS<sup>2</sup> and MS<sup>3</sup> spectra and by comparison with data in the literature. The retention  
209 times and mass spectral data along with peak assignments for the compounds identified are  
210 presented in Table 3. Flavonoids were the most predominant to be detected along with small  
211 amounts of phenolic acids. For the evaluation of the phenolic profile, not inoculated elderberry juice  
212 maintained at 30 °C and 37 °C for 48 h and stored at 4 °C for 12 days, was used as reference  
213 respectively for *L. plantarum* and *L. casei*, *L. rhamnosus* fermented counterparts. The  
214 (poly)phenolic profile of fermented elderberry juice was mainly characterized by flavonols,  
215 flavonol glycosides, anthocyanins, hydroxycinnamic acids, phenyllactic acids and hydroxybenzoic  
216 acid, (Figure 1). All fermented elderberry juices showed concentrations of total (poly)phenolic  
217 compounds that were higher than the respective controls. The same trends observed after  
218 fermentation was maintained also at the end of storage. The different contribute of individual strains  
219 to the (poly)phenolic content can be observed in Figure 1. The strains that induced the highest  
220 increase in total (poly)phenolic compounds were *L. plantarum* 285, *L. rhamnosus* 1473, 1019 and  
221 2360, after fermentation and storage, reaching respective concentrations of 338.2 and 302.5 µg/mL  
222 (mean values), resulting in an increase ranging between 65% and 80% compared to controls.

223 Interestingly, all the strains that showed this capacity were isolated from dairy products. Regarding  
224 hydroxycinnamic acids, the highest and the lowest concentration, after fermentation and storage,  
225 were observed with two different strains of the species *L. rhamnosus*, revealing strain-specific  
226 ability to metabolise them. (Figure 1). The increase in total hydroxycinnamic acids was principally  
227 due to the increase of 5-*O*-caffeoylquinic acid, mainly when dairy strains, i.e. *L. plantarum* 285, *L.*  
228 *rhamnosus* 1019, 1473, 2360 and *L. casei* 2107, were inoculated in the juice (Table S1 and S2).  
229 Interestingly, we also observed that *L. plantarum* strains were able to convert caffeic acid in its  
230 reduced metabolite dihydrocaffeic acid, and this is true especially for *L. plantarum* 1LE1 (Figure 2  
231 C and D). The capacity of lactic acid bacteria (*L. plantarum* in particular) to catabolize caffeic acid  
232 has been previously reported (Filannino et al., 2015; Sánchez-Maldonado et al., 2011) and is in  
233 good agreement with results from the present study. It has been hypothesized that lactic acid  
234 bacteria may use hydroxycinnamic acids as external acceptors of electrons, using a phenolic acid  
235 reductase to convert caffeic acid in dihydrocaffeic acid (Filannino et al., 2014).

236 Among hydroxybenzoic acids, protocatechuic acid was the only one identified. Most of the strains  
237 used in this study were able to metabolize it, by a decarboxylation step, into catechol (Figure 2 A  
238 and B), a reaction that was previously reported for *L. plantarum* (Filannino et al., 2018; Filannino et  
239 al., 2015; Tabasco et al., 2011). Instead, to the best of our knowledge, this is the first time *L.*  
240 *rhamnosus* strains were observed able to carry out this specific reaction. However, not all  
241 protocatechuic acid was effectively converted into catechol, suggesting the existence of a different  
242 metabolic pathway (Ludwig et al., 2013).

243 In order to measure the strengths of association and the direction of the relationship between caffeic  
244 acid/dihydrocaffeic acid and protocatechuic acid/catechol Pearson's correlation analysis was  
245 performed. A negative significant correlation was observed between caffeic acid/ dihydrocaffeic  
246 acid ( $r = -0.700$ ;  $p$  value = 0.024) and protocatechuic acid/catechol ( $r = -0.856$ ;  $p$  value <0.001)

247 proving that caffeic acid and protocatechuic acid were consumed by lactic acid bacteria during  
248 fermentation while dihydrocaffeic acid and catechol were produced. These catabolic steps exerted  
249 by specific microorganisms may end up forming metabolites that are able to exert biological  
250 activities that are more relevant to human health with respect to their parent phenolics. It is reported  
251 that dihydrocaffeic acid is a more potent antioxidant than caffeic acid (Silva et al., 2000), and that it  
252 can bioaccumulate inside the endothelial cells employing an intracellular antioxidant activities  
253 (Huang et al., 2004).

254 All fermented elderberry juices showed an increase in phenyllactic acids when compared to  
255 controls, confirming existing results (Valerio et al., 2004; Lavermicocca et al., 2003). After 48 h of  
256 incubation and further 12 days of cold storage, all *L. plantarum* strains generated the greatest  
257 increase. It is well known that phenyllactic acids may derive from amino acid metabolism, in  
258 particular as phenylalanine converted into phenylpyruvic acid by a reaction of transamination, and  
259 finally metabolized into phenyllactic acid by hydroxyl acid dehydrogenase, while p-  
260 hydroxyphenyllactic acid may originate from tyrosine metabolism (Lavermicocca et al., 2003).  
261 Since this is the first study fully focused on the polyphenolic fraction of fermented elderberry juice,  
262 we could only speculate that phenylalanine and tyrosine present in elderberry (Künsch & Temperli,  
263 1978) can be used as precursors. The relevance of phenyllactic acids consist in their well-known  
264 antimicrobial ability against pathogenic strains such as *Listeria spp.*, *Staphylococcus*  
265 *aureus* and *Enterococcus faecalis*, their antifungal and antimicotoxin (aflatoxins) properties (for  
266 example against *Aspergillus spp.* and *Penicillium spp.* especially those isolated from bakery  
267 products) (Valerio et al., 2004; Guimarães et al., 2018).

268 Regarding flavonoids, the highest levels were principally observed in *L. plantarum* 285, *L.*  
269 *rhamnosus* 1019, 1473 and 2360 fermented juices. The capacity of *L. plantarum* to increase total  
270 polyphenols, flavonoids and anthocyanins after fermentation had already been documented (Curiel

271 et al., 2015; Kwaw et al., 2018), but, to our knowledge, this is the first evidence for *L. rhamnosus*.  
272 After fermentation and storage, total flavonols reached the lowest concentration when *L. rhamnosus*  
273 2178 was used as starter, whereas the highest level was obtained when *L. rhamnosus* 1473 was  
274 applied (Figure 1). Among flavonol glycosides, the most concentrated was quercetin-3-*O*-  
275 rutinoid, followed by quercetin-3-*O*-glucoside (Table S1 and S2), and the variability among  
276 samples was extreme. Anthocyanins were also observed in fermented elderberry juices, being  
277 relevant for both their recognised antioxidant properties and for their colours, ranging from orange  
278 to blue, that makes them natural alternatives to replace synthetic colorants in food industry (Falcone  
279 Ferreyra et al. 2012). Notably, for four strains (*L. plantarum* 285, *L. rhamnosus* 1019,1473 and  
280 2360), the same which have shown high content of flavonols, flavonol glycosides and total  
281 polyphenols, the increase in anthocyanins was observed after fermentation. Even if glycosylated  
282 flavonoids have been often described as systematically deconjugated by lactic acid bacteria thanks  
283 to  $\beta$ -glucosidase activity (Di Cagno et al., 2013; Svensson et al., 2010), giving rise to a stable  
284 decrease in their level during fermentation (Svensson et al., 2010; Flannino et al., 2015), in this  
285 work a net increase (in particular of quercetin-3-*O*-rutinoside, cyanidin-3-*O*-sambubioside,  
286 cyanidin-3-*O*-glucoside) was observed, especially when dairy strains were used. As a possible  
287 explanation, recently, bacteria have been reported able to express glycosyltransferases, known to be  
288 involved in the glycosylation of different compounds, such as glucans. Their ability to glycosylate  
289 phenolic structures has been hypothesised (Thai Huynh et al., 2014) as a way to enhance  
290 detoxification and solubilisation of phenolic substrates. Other reports describe the expression of  
291 glucansucrases in LABs, which have been described to be involved in luteolin, quercetin, myrcetin  
292 and catechol glycosylation (Bertrand et al., 2006; Te Poele et al., 2016; Devlamynck et al., 2016).  
293 In addition, Hur et al. (2014) reported that, during fermentation, enzymes such as glucosidase,  
294 amylase, cellulase, chitinase, inulinase, phytase, xylanase, tannase, and esterase could be produced,  
295 and they could be able to break down plant cell wall making specific compounds, such as

296 flavonoids, more accessible. Another hypothesis is that fermentation with lactic acid bacteria could  
297 contribute to depolymerisation of high molecular weight phenolic compounds (Othman et al.,  
298 2009), increasing therefore the concentration of monomeric and simpler structures.

299

### 300 3.3 Chemometrics analysis

301 To obtain more information on the parameters that could influence differences and similarities  
302 among the fermented elderberry juices, the concentrations of the (poly)phenolic compounds  
303 identified after fermentation and storage were used as variable vectors for Principal Component  
304 Analysis (PCA). Figure 3 reports score plot and loading plot obtained from PCA analysis. Applying  
305 PCA on correlation matrix, two components were extracted representing together the 75% of total  
306 variance. In particular, component 1 explained the 55% of variance while the last 20% was  
307 explained by component 2. Observing the score plot, it is possible to highlight that the  
308 characteristics found in the samples after fermentation were maintained after storage.

309 The main differences observed could be related to the strains applied. Samples analysed can be  
310 gathered in four groups according to the score plot. The first group is formed by elderberry juices  
311 fermented with *L. plantarum* 1LE1, POM1 and C1, and is characterized by those variables with  
312 negative values on component 1 and positive values on component 2, such as catechol,  
313 dihydrocaffeic acid, phenyllactic acid and *p*-hydroxyphenyllactic acid. The variables with negative  
314 values on component 1 and around zero, or weakly negative, on component 2, such as luteolin,  
315 isorhamnetin-*O*-hexoside and coumaroylquinic acid (1) characterized instead the second group of  
316 samples, represented by juices fermented with *L. rhamnosus* 2178, 2140, and the unfermented  
317 samples. The third group was characterized by those variables positive on component 1 and around  
318 zero or weakly negative on component 2, like 5-*O*-caffeoylquinic acid, quercetin, quercetin-3-*O*-  
319 glucoside, quercetin-3-*O*-rutinoside, quercetin-*O*-rutinoside-*O*-hexoside, isorhamnetin-*O*-hexoside,

320 cyanidin-3-*O*-glucoside, cyanidin-3-*O*-sambubioside. The last group is formed solely by the juice  
321 inoculated with *L. plantarum* 285. This sample was separated from the other groups and  
322 characterized by negative variables on component 1, such as quercetin-3-*O*-rutinoside,  
323 caffeoylquinic acids, cyanidin-3-*O*-sambubioside, cyanidin-3-*O*-glucoside, and positive on  
324 component 2, like phenyllactic acid and *p*-hydroxyphenyllactic acid.

325 Interestingly, the score plot shows a clear separation among the considered species, as all elderberry  
326 juices fermented with *L. plantarum* strains were characterized by positive variables on component  
327 2, whereas the juices inoculated with *L. casei* and *L. rhamnosus* strains were characterized by  
328 around zero or weak negative variables. On the base of component 1, microorganisms (with positive  
329 variables) able to increase more the total (poly)phenolic compound quantities, were separated from  
330 the others, characterized by negative variables. In particular these microorganisms are *L. plantarum*  
331 285, *L. rhamnosus* 1019, 1473, 2360 and *L. casei* 2107.

332

#### 333 **4. Conclusions**

334 Only few studies available in literature have reported the modification of (poly)phenolic profile of  
335 fruit juices after lactic acid fermentation. More specifically, this is the first work that considers the  
336 effects of *L. rhamnosus* and the different behaviours of plant and dairy isolates. Moreover, this is  
337 the only one study describing polyphenolic conversion of elderberry juice, a matrix which is  
338 recently used in LAB fermentation.

339 The results obtained by a deep molecular analysis of the tested juices showed the power of  
340 fermentation to significantly increase total polyphenol content when *L. rhamnosus* and *L.*  
341 *plantarum* of dairy origin were applied. We especially highlighted that the studied strains were  
342 able to produce (e.g. phenyllactic acids), modify (e.g. hydroxycinnamic acids) or increase (e.g.  
343 flavonoid glycosides) phenolic compounds. Thanks to statistical analysis (PCA), a clear

344 clusterization of strains was observed, showing how the metabolism of phenolics is species and  
345 strain dependent and that the features of each strain may be influenced by the isolation source.

346

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349 providing *L. plantarum* strains (POM1, 1LE1, C1).

350

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438 changes during domestic cooking. *Food Chemistry*, 187, 338-347.

439

Strain	Species	Origin	
			440
			441
POM1	<i>L. plantarum</i>	Tomato	442
1LE1	<i>L. plantarum</i>	Pineapple	443
C1	<i>L. plantarum</i>	Carrot	444
285	<i>L. plantarum</i>	Brazilian cheese	445
1473	<i>L. rhamnosus</i>	Parmigiano Reggiano cheese	446
1019	<i>L. rhamnosus</i>	Parmigiano Reggiano cheese	447
2360	<i>L. rhamnosus</i>	Grana Padano cheese	448
2140	<i>L. rhamnosus</i>	Grana Padano cheese	449
2178	<i>L. rhamnosus</i>	Grana Padano cheese	450
2107	<i>L. casei</i>	Grana Padano cheese	451

452 Table 1. Strains used in elderberry juice fermentation.

Compounds identified	Standard used for quantification	Modality of acquisition
Cyanidin-3- <i>O</i> -sambubioside	Cyanidin-3- <i>O</i> -rutinoside	SRM
Cyanidin-3- <i>O</i> -glucoside	Cyanidin-3- <i>O</i> -glucoside	SRM
Cyanidin-3- <i>O</i> -sambubioside-glucoside	Cyanidin-3- <i>O</i> -rutinoside	SRM
Cyanidin-3- <i>O</i> -rutinoside	Cyanidin-3- <i>O</i> -rutinoside	SRM
3- <i>O</i> -caffeoylquinic acid	3- <i>O</i> -caffeoylquinic acid	SRM
5- <i>O</i> -caffeoylquinic acid	5- <i>O</i> -caffeoylquinic acid	SRM
4- <i>O</i> -caffeoylquinic acid	4- <i>O</i> -caffeoylquinic acid	SRM
Coumaroylquinic acid (1)	3- <i>O</i> -caffeoylquinic acid	SRM
Coumaroylquinic acid (2)	5- <i>O</i> -caffeoylquinic acid	SRM
Protocatechuic acid	Protocatechuic acid	SRM
Caffeic acid	Caffeic acid	SRM
Dihydrocaffeic acid	Dihydrocaffeic acid	SRM
Quercetin-3- <i>O</i> -rutinoside	Quercetin-3-rutinoside hydrate	SRM
Quercetin-3- <i>O</i> -glucoside	Quercetin-3-rutinoside hydrate	SRM
Kaempferol- <i>O</i> -rutinoside	Quercetin-3-rutinoside hydrate	SRM
Isorhamnetin- <i>O</i> -hexoside	Quercetin-3-rutinoside hydrate	SRM
Isorhamnetin- <i>O</i> -rutinoside	Quercetin-3-rutinoside hydrate	SRM
Quercetin- <i>O</i> -rutinoside- <i>O</i> -hexoside	Quercetin-3-rutinoside hydrate	SRM
Quercetin	Quercetin dihydrate	SIM
Luteolin	Luteolin	SIM
Kaempferol	Kaempferol	SIM
Isorhamnetin	Quercetin dihydrate	SIM
Phenyllactic acid	Phenyllactic acid	SRM
<i>p</i> -hydroxyphenyllactic acid	<i>p</i> -hydroxyphenyllactic acid	SRM
Catechol	Catechol	

454

455 Table 2. Standard and acquisition mode used for the quantification of phenolic compounds in

456 started and unstarted elderberry juices.

Variable	Compounds	Rt (min)	[M-H] <sup>-</sup> (m/z)	MS <sup>2</sup> (m/z)	MS <sup>3</sup> (m/z)
Var1	3- <i>O</i> -caffeoylquinic acid	3.80	353	<b>191</b> ,179,135,173	85,127,93,111,173
Var2	5- <i>O</i> -caffeoylquinic acid	4.45	353	<b>191</b> ,179	173,127,85,93,111
Var3	4- <i>O</i> -caffeoylquinic acid	4.57	353	173,179,191	
Var4	Coumaroylquinic acid (1)	4.40	337	163,191,173	
Var5	Coumaroylquinic acid (2)	5.25	337	191,173,163	
Var6	Caffeic acid	4.90	179	135	
Var7	Dihydrocaffeic acid	5.29	181	109,119, <b>137</b>	
Var8	Protocatechuic acid	3.45	253	109	
Var9	Catechol	5.3	109	53,81,109	
Var10	Phenylactic acid	6.03	165	147,119	
Var11	<i>p</i> -hydroxyphenylactic acid	3.99	181	163, 135	
Var12	Luteolin	7.94	285	241,243,199,175,217,151	
Var13	Quercetin	7.92	301	193	
Var14	Kaempferol	8.99	285	151,257,229,213,243,241	
				, 239,185,169	
Var15	Isorhamnetin	9.20	351	300,247	
Var16	Quercetin-3- <i>O</i> -glucoside	5.92	463	301	179,151,273,239,193
Var17	Kaempferol- <i>O</i> -rutinoside	6.17	593	<b>285</b>	257,267,241,239,229,213,199,197,195,223163,151
Var18	Quercetin- <i>O</i> -rutinoside- <i>O</i> -hexoside	5.59	771	<b>301</b>	179,151
Var19	Isorhamnetin- <i>O</i> -hexoside	6.55	477	<b>314</b> ,315,357,151,179	300,271,285,286,299,275,243
Var20	Isorhamnetin- <i>O</i> -rutinoside	6.29	623	300,271,255	300,287,271
Var21	Quercetin-3- <i>O</i> -rutinoside	5.94	609	<b>301</b> ,343	179,151,193,257
Var22	Cyanidin-3- <i>O</i> -sambubioside	3.43	581	<b>287</b> ,449	287,231,241,259,213
Var23	Cyanidin-3- <i>O</i> -glucoside	4.38	449	287	
Var24	Cyanidin-3- <i>O</i> -rutinoside	4.47	595	<b>287</b> ,449	
Var25	Cyanidin-3- <i>O</i> -sambubioside-hexoside	3.74	743	<b>287</b> ,449,581	287,259,231,213,241

458

459 Table 3. Mass spectral characteristics of (poly)phenolic compounds detected in started and  
460 unstarted elderberry juices. For each compound a specific variable, used in PCA analyses, was  
461 assigned.

462

463 **Figure captions**

464 **Figure 1.** Total (poly)phenolic compounds and phenolic profile, express in  $\mu\text{g/mL}$ ,  
465 (hydroxycinnamic acids, phenyllactic acids, flavonols, flavonols glycosides, anthocyanins) of  
466 elderberry juices inoculated with *L. plantarum* (1LE1, 285, C1, POM1), *L. casei* (2107), *L.*  
467 *rhamnosus* (1019, 1473, 2140, 2178, 2360), unstarted juice (incubated at 30 °C and 37 °C) and  
468 unstarted unincubated juice (elderberry, green bar). Blue bars refer to 48 h of fermentation while  
469 red bars to further 12 days of refrigerate storage. Significant differences ( $p < 0.05$ ) between controls  
470 (30°C and 37°C) and samples are indicated by an asterisk, highly significant differences ( $p < 0.001$ )  
471 by two asterisks.

472 **Figure 2.** Quantification of protocatechuic acid (orange bars) and catechol (grey bars), caffeic acid  
473 (blue bars) and dihydrocaffeic acid (red bars) after 48 h (A and C) and after storage (B and D), in  
474 elderberry juices fermented with *L. plantarum* 1LE1, 285, C1, POM1 and *L. rhamnosus* 1019, 2140,  
475 2178, 2360; in elderberry juice incubated at 30°C and 37°C without bacterial inoculum (30°C,  
476 37°C). Data were reported as mean values  $\pm$  standard deviation of three different experiment.

477 **Figure 3.** Principal component analysis (PCA). Score plot and loading plot based on (poly)phenolic  
478 compounds identify in started (1LE1, POM1, C1, 4186, 285, 2178, 2140, 1019, 1473, 2360 and  
479 2107) and unstarted elderberry juices (30°C, 37°C and T0) after fermentation (48 h) and storage (14  
480 d). The compounds used as variables were detailed in Table 2.