1 **Exploitation of grape marc as functional substrate for lactic acid bacteria and**
2 **bifidobacterial growth and enhanced antioxidant activity**

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19 **Abstract**

20 This study aimed at using a grape marc as substrate for the growth of probiotic lactic acid bacteria
21 and bifidobacteria with the perspective of producing a functional ingredient having live probiotic
22 cells and antioxidant activity. Among the nine tested probiotic strains, only *Lactobacillus plantarum*
23 12A PU1, *Lactobacillus paracasei* 14A and *Bifidobacterium breve* 15A showed the ability to grow
24 on grape marc (GM) based media. The highest cell density of probiotic bacteria (> 9.0 CFU/g) was
25 found in GM added of 1% of glucose (GMG). Compared to un-inoculated and incubated GMC,
26 fermented GMG showed a decrease of carbohydrates and citric acid together with an increase of total
27 titratable and volatile acidities and lactic acid. The content of several free amino acids (FAA) and
28 phenol compounds also differed between GMG fermented by *L. plantarum* 12A PU1, *L. paracasei*
29 14A and *B. breve* 15A. Based on the survival under simulated gastro-intestinal conditions, GMG was
30 a suitable carrier of probiotic strains. Compared to the control, cell-free supernatant (CFS) of GMG
31 fermented by probiotic strains exhibited a marked antioxidant activity in vitro. The increased
32 antioxidant activity was confirmed using Caco-2 (colon adenocarcinoma) cell line after inducing
33 oxidative stress, and determining cell viability and radical scavenging activity through MTT and
34 DCFH-DA assays, respectively. It was confirmed the markedly higher antioxidant activity on Caco-
35 2 cells compared to the un-inoculated control. Supporting these founding, the *SOD-2* gene expression
36 of Caco-2 cells also showed the lowest pro-oxidant effect explained by the four CFS of GMG
37 fermented by probiotic strains.
38 Grape marc is a suitable tool for the growth of probiotic strains showing also antioxidant activity
39 open the way for novel applications as functional food dietary supplements or pharmaceutical
40 preparations.

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42 **Keywords:**

43 Grape marc; Valorization by-product; Fermentation; Lactic acid bacteria and bifidobacteria;
44 Antioxidant activity

45 1. Introduction

46 Grape marc showed a significant impact on the environment due to the high phenols content
47 considerably increasing both the chemical (COD) and biochemical (BOD) oxygen demands (Spigno
48 and De Faveri, 2007), thus making its disposal a serious environmental problem with increasing cost
49 for its treatment. In Europe, grape marc produced in the wine-making process – referred to as "fresh
50 grape marc" – was mandatory utilized by the distilling industry. The Council Regulation (EC) No.
51 479/2008 and Commission Regulation (EC) No. 555/2008 allowed the producers to look for new
52 opportunities for the exploitation of the fresh and also exhausted grape marc (Fiori and Florio, 2010).
53 Italy has always had a great agricultural vocation either in quantitative or qualitative terms, and the
54 Apulian region is leader at national and international levels in wine productive branches with
55 significant economic and occupational outcomes (ISTAT, 2015; OIV, 2015). Several studies
56 suggested different grape marc processing to balance out waste treatment costs. The most of analyses
57 were focused on the extraction of natural antioxidants (polyphenols compounds) with application in
58 pharmacological, cosmetic, and food industries (Palenzuela et al., 2004; Pasqualone et al., 2014;
59 Rockenbach et al., 2011; Sessa et al., 2013; Spigno and De Faveri 2007), cellulose and hemicelluloses
60 (Spigno et al., 2008), composting processes (Bustamante et al., 2009) and energetic exploitation (Fiori
61 and Florio, 2010). Grape marc containing large amounts of hemicellulosic sugars and fatty acids was
62 by *Lactobacillus pentosus* for the production of lactic acid or biosurfactants (Portilla et al., 2007,
63 2008a, 2008b). In other food-chains, e.g. dairy industry, several applications were proposed as
64 efficient and cost-effective methods to eliminate cheese whey by-product without negative
65 environmental issues. Previously, whey was used to produce lactic acid (Arasaratnam et al., 1996;

66 Mostafa, 1996) and ethanol by lactose converting lactic acid bacteria and genetically-engineered yeast
67 (Domingues et al., 2001, Guimaraes et al., 2010), respectively. Production of microbial biomass (e.g.,
68 yeasts) using by-products (e.g., whey) were also proposed (Vamvakaki et al., 2010). In the last
69 decades, the increasing interest for novel processes and products, providing ingredients with new
70 functionalities and cost-effective manufacturing, emphasizes the potential of food-grade
71 fermentations and microbial bioconversion for the production of functional foods/metabolites (De
72 Vos, 2005; Gobbetti et al., 2010). Fermented functional foods have healthy effects on humans by
73 interactions of ingested live microorganisms with the host (probiotic effect) or indirectly as the result
74 of the ingestion of microbial metabolites synthesized during fermentation (biogenic effect) (Stanton
75 et al., 2005; Arena et al., 2014). The use of food by-products to produce functional foods represent a
76 new interesting opportunity. This is already done for whey which is currently used to produce
77 functional beverages (Almeida et al., 2008; Madureira et al., 2005; Magalhaes et al., 2010). The use
78 of grape marc as substrate for production of biomass of probiotic strains has very limited economic
79 costs and due to its chemical composition may deserve interesting nutritional perspective for
80 industrial applications (Iriti and Faoro, 2006). Previously, the consumption of grape must or
81 juice in the human diet increased the serum antioxidant capacity (Zern et al., 2005), decreased
82 peroxide formation and platelet aggregation, and enhanced flow-mediated vasodilation
83 (Castilla et al. 2006; Castilla et al. 2008). Due to the antioxidant activity of grape marc, new
84 opportunities of its use for the production of functional foods and/or compounds can be
85 hypothesized.
86 This study aimed at using grape marc as substrate for the growth of probiotic lactic acid bacteria and
87 bifidobacteria with the perspective of producing a functional ingredient, dietary supplement or
88 pharmaceutical preparation.

89 2. Materials and Methods

90 *2.1. Microbial strains and culture conditions*

91 Nine probiotic strains belonging to *Lactobacillus* (*L. rossiae* DSM15814, *L. reuteri* DSM20016, *L.*
92 *plantarum* 12A, PU1, *L. rhamnosus* SP1, *L. casei* FC1-13, *L. paracasei* 14A) and *Bifidobacterium*
93 (*B. breve* 15A and *B. animalis* 13A) were used. Strains were propagated into modified de Man,
94 Rogosa and Sharpe (MRS, Oxoid, Basingstoke, Hampshire, England). The only exception was for *L.*
95 *rossiae* DSM 15814 that was propagated modified MRS broth with the addition of fresh yeast extract
96 (5%, v/v) and 28 mM maltose to a final pH of approximately 5.6 (De Angelis et al., 2002). Strains
97 were cultured at 30°C (lactobacilli) or 37°C (bifidobacteria) for 24 h in anaerobic conditions.

98 *2.2. Grape marc based media and growth of probiotic bacteria*

99 Industrial red grape marc, from the "Negroamaro" variety growth in South Apulia in 2013 was
100 collected. The red grape marc was homogenized with peptone water (0.4 % yeast extract (w/v) and
101 0.1 % peptone (w/v)) in Omni-mixer Homogenizer (Cole-Parmer, Genercontrol S.p.A., Milan, Italy)
102 (rate 1:10) (GM). The homogenate GM was brought to pH ca. 6.0 with 5 N NaOH and sterilized
103 (121°C for 15 min). All strains were inoculated in GM and in GM added of 1% of glucose (GMG).
104 Probiotic cells used for the inoculum were cultured in conditions described above. After 24 h at 30°C
105 or 37°C cells were centrifuged 9000 x *g* for 10 min and washed in sterile 20 mM of potassium
106 phosphate buffer at pH 7.0. Each strain was inoculated in the GM or GMG at the final cell density of
107 ca. 7 log CFU/mL. Fermentation was carried out for 24 h at 30°C (*Lactobacillus*) or 37°C
108 (*Bifidobacterium*) in anaerobic condition. GM and GMG without inoculum were also incubated for
109 24 h at 30°C and 37°C and used as control. Each trial was performed in triplicate.
110 The acidification was monitored by a pH meter (Model 507, Crison, Milan, Italy) prior (T0) and after
111 fermentation (T24). The growth of lactobacilli and bifidobacteria on GM and GMG after 24 h of
112 incubation was determined by plating onto MRS agar. After 24 h at 30°C and 37°C only *L. plantarum*
113 12A, *L. plantarum* PU1, *L. paracasei* 14A and *B. breve* 15A showed acidification and growth on both
114 media. In addition, GMG showing the highest growth of probiotic cells was selected for further
115 studies.

116 *2.3. Chemical characteristics of grape marc fermented by probiotic bacteria*

117 Samples of GMG fermented by selected probiotic bacteria and un-inoculated and incubated GMG
118 (control) were centrifuged at 9000 x *g* for 10 min, and the supernatant was filtered through a Millex-
119 HA 0.22-µm-pore-size filter (Millipore Co., Bedford, MA) (cell-free supernatant, CFS). Analyses of
120 carbohydrates, organic acid and glycerol were carried out through HPLC (High Performance Liquid
121 Chromatography), using an ÄKTA Purifier™ system (GE Healthcare Bio-Sciences, Uppsala,
122 Sweden) equipped with a UV detector (Zeppa et al., 2001). Minerals, vitamins and fiber of GMG
123 samples were determined by using MP/C/473 2006 official method for phosphorus determination;
124 ITISAN 1996/34 for calcium, magnesium, sodium and iron; AOAC 975.03.1988 for potassium;
125 AOAC 991.42 1994 for insoluble fiber; AOAC 993.19 1996 for soluble fiber; MP/C/640 2006 for
126 vitamins B1 and PP. The total and individual Free Amino Acids (FAA) contained in the CFS samples
127 were analyzed using a Biochrom 30 series amino acid analyzer (Biochrom Ltd., Cambridge Science
128 Park, England) with a sodium cation-exchange column (20 by 0.46 cm [inner diameter]) (Siragusa et
129 al., 2007).

130 *2.4. Concentration of total phenols and low-molecular weight phenol composition*

131 The total phenols content (TPC) of CFS samples was determined using the Folin-Ciocalteu (FC)
132 reagent according to the procedure reported by Di Stefano and Cravero (1991). Results were
133 expressed as milligrams of gallic acid equivalents per liter (mg GAE/L). Profile of low-molecular
134 weight phenols was performed by high performance liquid chromatography (HPLC) using a Waters
135 600 E apparatus (Waters, PA, USA) consisting of a quaternary pump, a photodiode array detector
136 (DAD) and a Reodyne injector with a 5 µL loop. CFS samples were filtered on 0.2 µm regenerated
137 cellulose (RC) membrane filters and injected into a XDB-C18 (50 x 4.6 mm i.d., particle size 1.8 µm,
138 Agilent) column maintained at 30°C and eluted at a flow rate of 0.5 mL/min with 5% acetonitrile in
139 water (solvent A) and water-acetonitrile (50:50) (solvent B) both adjusted to pH 1.8 with perchloric
140 acid. The gradient program of solvent B was as follow: 0–4.8 min 10% isocratic, 4.8–16.8 min 20%,
141 16.8–21.6 min 30%, 21.6–31.2 min 40%, 31.2–40.8 min 45%, 40.8–48 min 100%, 48–58 min 100%
142 isocratic, 58–60 min 10%. Detection was performed at 280, 313 and 350 nm, and quantification was

143 made according to external standard method on the basis on standard calibration curves obtained by
144 the injection of solutions at different concentration of (+)-catechin (R^2 = 0.9913), caffeic acid (R^2 =
145 0.9640) and quercetin (R^2 = 0.9712) for compounds with maximum absorption at 280, 313 and 350
146 nm, respectively. Tentative identification of compounds was achieved by combining elution pattern,
147 UV-VIS spectra and data reported by Revilla and Ryan (2000). Results were expressed as mg/L.

148 *2.5. Antioxidant activity*

149 Antioxidant activity of CFS samples was assessed by ABTS assay according to Ferrara et al. (2014).
150 Trolox standard solutions were prepared at a concentration ranging from 20 to 1000 μM. Results were
151 expressed as μmoles of Trolox Equivalent per liter (μmol TE/L).

152 Antioxidant activity was also measured according to the method of the inhibition of linoleic acid
153 autoxidation (Osawa and Namiki, 1985), with some modifications. After freeze-drying, 1 mg of each
154 CFS samples was suspended in 1 ml of 0.1 M phosphate buffer (pH 7.0), and added to 1 ml of linoleic
155 acid (50 mM), previously dissolved on ethanol (99.5%). Incubation in a glass test tube, tightly sealed
156 with silicon rubber cap, was allowed at 60°C in the dark for 8 days. The degree of oxidation was
157 determined by measuring the values of ferric thiocyanate according to the method described by
158 Mitsuta et al. (1996). One hundred microliters of the above sample were mixed with 4.7 ml of 75%
159 (v/v) ethanol, 0.1 ml of 30% (w/v) ammonium thiocyanate, and 0.1 ml of 0.02 M ferrous chloride,
160 dissolved in 1 M HCl. After 3 min, the degree of color development that represents the oxidation of
161 linoleic acid was measured spectrophotometrically at 500 nm. Butylatedhydroxytoluene (BHT) and
162 α-tocopherol (1 mg/ml) were also assayed as antioxidant references. A negative control (without
163 antioxidants) was also considered (Curiel et al., 2015).

164 *2.6. Resistance to simulated gastric and intestinal fluid*

165 The survival of gastro-intestinal (GI) tract of selected *L. plantarum* 12A, *L. plantarum* PU1, *L.*
166 *paracasei* 14A and *B. breve* 15A grown in GMG or in MRS was assayed. Simulated gastric and
167 intestinal fluids were used (Fernandez et al., 2003). After 24 h of growth on GMG and on MRS, cells
168 of probiotic bacteria were harvested at 9000 x*g* for 15 min at 4°C, washed with physiologic solution

169 (0.85% NaCl), and resuspended in 50 ml of simulated gastric juice (ca. 10 log CFU/mL) which
170 contained NaCl (125 mM/L), KCl (7 mM/L), $NaHCO_3$ (45 mM/ L), and pepsin (3 g/L) (Sigma)
171 (Zarate et al., 2000). The final pH was adjusted to 2.0, 3.0, and 8.0. The value of pH 8.0 was used to
172 investigate the influence of components of the simulated gastric juice apart from the effect of low pH
173 (Fernandez et al., 2003). The suspension was incubated at 37°C with gentle agitation at 100 rpm.
174 Aliquots of this suspension were taken at 0, 90, and 180 min, and viable counts were determined.
175 This condition was assayed to simulate the effect of the matrix during gastric transit (Zarate et al.,
176 2000). After 180 min of gastric digestion, cells were harvested and re-suspended in simulated
177 intestinal fluid which contained 0.1% (w/v) pancreatin and 0.15% (w/v) oxgall bile salt (Sigma) at
178 pH 8.0. The suspension was incubated at 37°C under gentle agitation at 100 rpm, and aliquots were
179 taken at 0, 90, and 180 min (Fernandez et al., 2003).

180 *2.7. MTT assay and protection from oxidative stress induced on human adenocarcinoma Caco-2*
181 *cells*

182 Caco-2 (colon adenocarcinoma) ICLC HTL97023 cell line was obtained from the National Institute
183 for Cancer Research of Genoa, Italy. The cell line was cultured in RPMI (Roswell Park Memorial
184 Insitute) medium supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 1%
185 penicillin (10000 U/mL)/streptomycin (10000 μg/mL) mixture, 0.1% gentamicin and 0.1% β-
186 mercaptoethanol and maintained in 25 cm^2 culture flasks at 37°C, 5% CO_2. Every two days confluent
187 cultures were splitted 1:3 – 1:6, after washing with PBS 1X (without Ca^{2+} and Mg^{2+}), using
188 trypsin/EDTA and seed at 5 x 10^4 cell/cm^2, 37°C, 5% CO_2. Caco-2 cells were treated with different
189 concentrations of CFS (0.01-0.1-1-10-50 mg/mL) at 37°C, 5% CO_2 for 16h. After incubation, the
190 viability of Caco-2 cells was measured using the MTT [3-(4,5-dimethyl-2-yl)-2,5-
191 diphenyltetrazolium bromide] method (Mosmann et al., 1983). The concentrations that permitted the
192 highest cell viability (0.01-0.1-1 mg/mL of CFS) were selected for further analyses. When the Caco-
193 2 cells were exposed to the above concentrations of each CFS and incubated at 37°C, 5% CO_2 for 16
194 h. A negative control, without addition of CFS samples, was used. α-Tocopherol (0.01-0.1-1 mg/mL)

195 was used as the positive control. After treatment, medium was removed from each well and, after
196 washing, Caco-2 cells were exposed to 1 mM hydrogen peroxide (100 µl/well) for 2 h. Cells exposed
197 to RPMI complete medium with and without H_2O_2 treatment were used as additional controls. After
198 the incubation, MTT assay was performed as described by Mosmann et al. (1983). Data were
199 expressed as the mean percentage of viable Caco-2 cells compared to the control culture (cells
200 exposed to RPMI complete medium) without oxidative stress. Each experiment was carried out in
201 triplicate.

202 *2.8. Intracellular reactive oxygen species (ROS) generation by Caco-2 cells*

203 Production of ROS by Caco-2 cells was monitored spectrofluorometrically using the 2',7'-
204 dichlorofluorescein diacetate (DCFH-DA) method, as described by Tobi et al. (2000). Caco-2 cells
205 (~80% confluent) were treated with 0.1, 0.1 and 1 mg/mL of CFS as described above. α-Tocopherol
206 at the same concentrations was used as positive control. After the test compound treatment, the
207 medium was removed and cells were rinsed twice with PBS and loaded with 100 µM DCFH-DA for
208 30 min at 37°C in the dark. Cells were then exposed to treatment with 100 µL pre-warmed RPMI
209 (2.5% FBS) containing 1mM hydrogen-peroxide at 37°C for 2 h in the dark in 5% CO_2. At the end
210 of the treatment, cells were washed twice, lysed with Cell Lytic M lysis buffer (Sigma Aldrich) and
211 transferred into a black 96-well plate. Fluorescent 2',7' dichlorofluorescein (DCF) was read
212 fluorometrically using a Fluoroskan Ascent FL Microplate Fluorescence Reader (Thermo Scientific)
213 at excitation and emission wavelengths of 485 and 538 nm, respectively. Cells exposed to RPMI
214 complete medium only and H_2O_2-stressed cells were used as controls. Each experiment was carried
215 out in triplicate.

216 *2.9. Expression of superoxide dismutase (SOD-2) in Caco-2 cells*

217 After treatment with freeze dried CFS, the expression of *SOD-2* gene from Caco-2 cells was
218 investigated through real-time PCR (RT-PCR). Caco-2 cells were treated with CFS samples and H_2O_2
219 as described above. Cells exposed to RPMI complete medium only was used as negative controls.
220 After 2 h, supernatants were collected and Caco-2 cells were washed with PBS1x. Total RNA was

221 extracted from the Caco-2 cells using a commercial kit (Ribospin Minikit-GeneAll, Seoul, Korea).
222 The cDNA was synthesized from 2 μg RNA template in a 20 μl reaction volume, using the High-
223 Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy). Ten microliters of
224 total RNA were added to the Master Mix and subjected to reverse transcription in a thermal cycler
225 (Stratagene Mx3000P Real Time PCR System, Agilent Technologies Italia S.p.A., Milan, Italy). The
226 conditions were as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 60 s. The cDNA was
227 amplified and detected through TaqMan® assay (Applied Biosystems, Monza, Italy).
228 Hs00167309_m1 (*SOD-2*) and Hs999999_m1 (human glyceraldehyde-3-phosphate dehydrogenase,
229 *GAPDH*) were used for Taqman gene expression assays. Human *GAPDH* was the housekeeping gene
230 (Marzani et al., 2012). PCR amplifications were carried out using 40 ng of cDNA on a 20 μl of total
231 volume. The mixture reaction contained 10 μl of 2× TaqMan Universal PCR Master Mix, 1 μl of 20×
232 TaqMan gene expression assay, 5 μl of water and 4 μl of cDNA. PCR conditions were as follows:
233 50°C for 2 min (for optimal AmpErase® UNG activity) and 95°C for 10 min, followed by 40
234 amplification cycles (95°C for 15 s; 60°C for 1 min). Analyses were carried out in triplicate. The
235 average value of target gene was normalized using *GAPDH* gene and the relative quantification of
236 the levels of gene expression was determined by comparing the Δ cycle threshold (ΔCt) value (Vigetti
237 et al., 2008). Analyses were carried out in triplicate.

238 *2.10. Statistical analysis*

239 Each sample was produced and analyzed in triplicate. Data were subjected to one-way ANOVA; pair-
240 comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical
241 software, Statistica for Windows (Statistica7.0 per Windows).

242 3. Results

243 *3.1. Growth of probiotic bacteria on grape marc based media*

244 Among the nine tested probiotic strains, only *L. plantarum* 12A, *L. plantarum* PU1, *L. paracasei* 14A
245 and *B. breve* 15A showed the ability to grow on grape marc based media (Table 1). The cell density

246 of these strains significantly increased ($P < 0.05$) after 24 h (initial cell density ca. 7 log CFU/mL)
247 reaching values from 8.80 to 9.16 log CFU/mL on GM and from 9.07 to 9.55 log CFU/mL on GMG
248 medium. According to cell density, only *L. plantarum* 12A, *L. plantarum* PU1, *L. paracasei* 14A and
249 *B. breve* 15A showed acidification of GM and GMG media. Compared to GM medium, the addition
250 of 1% of glucose (GMG medium) increased both cell density and acidification for all the assayed
251 strains. Thus, all further experiments were referred to GMG fermented for 24 h with *L. plantarum*
252 12A, *L. plantarum* PU1, *L. paracasei* 14A or *B. breve* 15A.

253 *3.2. Chemical characteristics of grape marc fermented by probiotic bacteria*

254 *L. plantarum* PU1 and *B. breve* 15A cultivated on GMG showed the highest total titratable and
255 volatile acidities (Table 2). Compared to control, carbohydrates, citric acid and glycerol were almost
256 or completely depleted during GMG fermentation. Compared to control, lactic acid increased in all
257 GMG fermented samples, especially for *L. plantarum* PU1 and *B. breve* 15A. Minerals and vitamins
258 content did not show significant increase ($P < 0.05$) in fermented samples compared to control. The
259 only exceptions were for calcium and iron that showed the highest concentration in samples of GMG
260 fermented by *L. paracasei* 14A and *B. breve* 15A, respectively. The concentration of total free amino
261 acids (FAA) in the control was of ca. 3051 mg/kg. Compared to control, the FAA concentration
262 decreased in all fermented samples (Table 3). The only exceptions were tryptophan that was found
263 slightly increased in fermented samples compared to control. Indeed, the highest consumption of
264 phenylalanine, tyrosine and serine in fermented samples supported these data via Shikimate and
265 serine pathway.

266 *3.3. Concentration of total phenols and low-molecular weight phenol composition*

267 Compared to control (un-inoculated and incubated GMG), the fermentation of GMG with *L.*
268 *plantarum* 12A and *L. paracasei* 14A showed no significant ($P > 0.05$) modification of the level of
269 total phenols content (TPC) (Table 4). A decrease of the TPC was found for the other strains. Linear
270 correlation between total titratable acidity (TTA) and TPC content was found (Pearson correlation =
271 0.849; P level 0.032). Phenol compounds were also identified. In particular, gallic acid,

272 protocatechuic acid, procyanidin B1, caftaric acid, (+)-catechin, caffeic acid, syringic acid, (-)-
273 epicatechin, and rutin were quantified (Figs. S1 and S2). Other peaks appear on the chromatograms,
274 but they were not identified. Compared to acidified control, similar ($P > 0.05$) level of TPC was found
275 in fermented samples. Among identified phenols of the acidified control, gallic acid and (-)-
276 epicatechin were the mainly representative compounds, followed by (+)-catechin, syringic acid,
277 procyanidin B1, protocatechuic acid, caffeic acid, and rutin. Dramatic loss in gallic acid was observed
278 in all fermented GMG samples, while less noticeable losses were found for (-)-epicatechin and
279 syringic acid.

280 *3.4. Antioxidant activity in vitro*

281 The total antioxidant capacity of CFS samples was determined based on the scavenging activity
282 towards radical cation 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS). Compared to
283 control (GMG un-inoculated and incubated), samples of GMG fermented by probiotic strains showed
284 no significant ($P > 0.05$) variation of the antioxidant activity estimated by ABTS (data not shown).
285 The quantification of the inhibition of linoleic acid peroxidation was also used to measure the
286 antioxidant activity (Fig. 1). All samples showed antioxidant activity compared to the negative control
287 obtained without addition of GMG samples or antioxidants. Compared to control, samples of GMG
288 fermented by probiotic strains showed a higher ($P > 0.05$) inhibition of the oxidation of linoleic acid.
289 In particular, *L. plantarum* PU1 showed the highest antioxidant activity which was similar ($P > 0.05$)
290 to those found for the well-known antioxidant compounds (α-tocopherol and BHT).

291 *3.5. Resistance to simulated gastric and intestinal fluids of selected probiotic bacteria grown on*
292 *grape marcs*

293 Selected probiotic bacteria grown on GMG medium and in optimal condition (MRS medium) were
294 incubated at 37°C in simulated gastric fluid at pH 2.0, 3.0, and 8.0. Strains showed different survival
295 in the conditions assayed. After 180 min of incubation in simulated gastric juice at pH 3.0, all the
296 strains showed decreases lower than 1 logaritmic cycle compared to their initial cell density (10 ±
297 0.44 to 9.19 ± 0.39 log CFU/mL), excepted *L. paracasei* 14A grown on GMG which survival was

298 found decreased from 10 ± 0.42 to 8.6 ± 0.38 log CFU/mL (Fig. 2). At pH 8.0 no decrease in survival
299 was observed for any strains grown on MRS. After 180 min at pH 2.0, all strains decreased in any
300 conditions. Nevertheless, all probiotic bacteria grown on GMG showed more resistance in simulated
301 gastric juice at pH 2.0 compared to those grown in MRS with the exception of *L. paracasei* 14A.
302 After 180 min of gastric digestion, cells were exposed to simulated intestinal fluid for a subsequent
303 180 min at pH 8.0 (Fig. 2). Cell survival depended on the pH of gastric digestion. The same trend of
304 resistance to gastric acidity was found at the intestinal level. The decrease for cells previously treated
305 at pH 8.0 was always lower than 1 log cycle. *L. plantarum* 12A incubated at pH 2.0 during gastric
306 digestion showed the highest cell density on grape marc (7.58 ± 0.25 log CFU/mL) revealing a better
307 adaptation if compared to that grown in MRS.

308 *3.6. Protection of grape marc preparations toward oxidative-induced stress in human*
309 *adenocarcinoma Caco-2 and intracellular ROS generation*

310 To further investigate the capacity of fermented grape marc to act as radical scavenger, Caco-2 cells
311 were grown in the presence of lyophilized CFS grape marc. Afterwards, cells were treated with
312 hydrogen peroxide. As revealed by MTT assay, concentrations of CFS higher than 1 mg/ml were not
313 assayed to avoid cytotoxic effects (data not shown). Cell viability was assayed through the capacity
314 of functional mitochondria to catalyze the reduction of MTT to formazan salt via mitochondrial
315 dehydrogenases. Compared to control (10.6 ± 0.20 % of cell viability after oxidative stress), α-
316 tocopherol and all grape marc CFS significantly ($P < 0.05$) increased human cell survival (Fig. 3).
317 Positive control, α-tocopherol, at concentration of 0.01-0.1 mg/mL (1 mg/ml showed citotoxicity),
318 produced a percentage of cells viability higher than control treated with hydrogen peroxide (22.74 ±
319 0.5 e 28.13 ± 0.6 %, respectively). As regards treatments of Caco-2 cells by un-inoculated control
320 CFS, 1mg/mL showed cytotoxicity. Among grape marc CFS fermented by *L. paracasei* 14A and *B.*
321 *breve* 15A were the most effective in protect cells against oxidative stress at 0.01 and 0.1 mg/mL. *L.*
322 *paracasei* 14A was effective also at 1mg/mL. *L. plantarum* 12A was effective in protect cells against
323 oxidative stress only at 0.1 mg/mL. Also *L. plantarum* PU1 cultured on grape marc was found to

324 protect cells (0.01 and 0.1 mg/mL) with a lower effectiveness than *L. paracasei* 14A and *B. breve*
325 15A.
326 These results were confirmed by determining the concentration of intracellular reactive oxygen
327 species (ROS; Fig. 4). Data are reported as percentage of Radical scavenging activity (RSA).
328 Compared with H_2O_2-stressed control cells, cells treated with 0.01 and 0.1 mg/mL α-tocopherol
329 showed a significative (P<0.05) percentage of RSA (68.29 ± 0.22 and 68.93 ± 0.24%, respectively).
330 The highest percentage of RSA activity was found for GMG fermented by *L. plantarum* 12A and *L.*
331 *paracasei* 14A at 0.01mg/ml (72.90 ± 2.21 and 70.30 ± 3.33%, respectively) and especially for *L.*
332 *plantarum* PU1 at 0.01, 0.1 and 1mg/mL (68.31 ± 3.22, 77.91 ± 4.11 and 74.41 ± 1.55%,
333 respectively).
334 *3.7. Effect of grape marc preparations on the expression of SOD-2 gene*
335 After 16 h of incubation (Fig. 5) *SOD-2* gene expression was enhanced (pro-oxidant effect) by
336 treatment with only H_2O_2 1mM (+185% respect negative control). Compared to negative control (cell
337 subjected to oxidative stress), α-tocopherol act as antioxidant by reducing *SOD-2* gene expression to
338 82 and 48% at the concentration of 0.1 and 1 mg/mL, respectively. Among tested compounds, with
339 the exception of lyophilized CFS of grape marc fermented by *L. paracasei* 14A 0.1 and 1mg/mL, all
340 grape marc preparation under study acted as antioxidant, with values of *SOD-2* gene expression lower
341 than positive control. The most active compounds were grape marc preparation fermented by *L.*
342 *plantarum* 12A 0.01 mg/mL and *L. plantarum* PU1 1 mg/mL that showed 8-40 and -70 %,
343 respectively.

344 ## 4. Discussion

345 By-products of plant food processing represent a major disposal problem for the industry concerned,
346 but they are also promising sources of compounds which may be used because of their favorable
347 technological or nutritional properties. The purpose of this study is to highlight the potential of
348 selected by-products as a source of functional compounds. A large number of plants were screened

349 to be sources of novel phenolic compounds for alimentary, cosmetic and pharmaceutical uses
350 (Tuberoso et al., 2010). Grape may be the paradigm of the great variety of natural products
351 synthesized and contained in plant tissues. These products, namely, phenylpropanoids, isoprenoids,
352 and alkaloids combined together, contribute to the beneficial effects derived from the large fruit and
353 vegetable consumption (Iriti and Faoro, 2006). First, this study reported the capacity of probiotic
354 selected lactic acid bacteria and Bifidobacteria to grow on grape marc rich of polyphenols. In
355 particular, it was dealing with the valorization of grape marc through both production of probiotic
356 biomass and supplying of antioxidant compounds. Previously, biotechnological valorization of whey,
357 the major by-product of the dairy industry, also refers to its conversion into microbial mass and
358 production of microbial specific lipids (Vamvakaki et al., 2010). To support the hypothesis that grape
359 marc fermented by probiotic bacteria might have an interest if used as ingredient in functional food
360 or beverage it was considered the chemical and nutritional composition. As expected, fermented grape
361 marc added of 1% of glucose (GMG) showed a decrease of carbohydrates together with an increase
362 of total titratable and volatile acidities and lactic acid. Chemical data also showed that citric acid and
363 glycerol supported the probiotic growth on GMG. According to Zaunmüller et al. (2006), many lactic
364 acid bacteria use citrate as substrate (electron acceptor) for co-metabolism with sugars like glucose,
365 fructose, lactose, or xylose providing NADH for pyruvate reduction. da Cun and Fsoter (1992) found
366 that the simultaneous fermentation of glycerol and sugar by lactobacilli increased both the growth
367 rate and final cell density. Besides, the marked catabolism of FAA in all fermented samples compared
368 to un-inoculated and incubated control demonstrated their usage as alternative carbon source by
369 selected bacteria. Overall, the ability of lactic acid bacteria to efficiently transport and metabolize
370 carbohydrates and other carbon sources (e.g., citric acids, FAA) is crucial to grow and persist under
371 non-optimal condition or environmental stress conditions, i.e. low pH (Kunji et al., 1993). Data
372 regarding the quantification of phenols in CFS grape-marc samples, showing a decrease in fermented
373 samples compared to un-inoculated control, could suggest that bacteria use some phenols, such as
374 gallic acid, for their metabolism. This finding is in agreement with the study by Landete et al. (2007)

375 that demonstrated that *Lactobacillus* genus was able to degrade phenolic compounds to other high-
376 added-value molecules.
377 Antioxidant activity of fermented grape marc was investigated, and compared to an un-inoculated
378 and chemically acidified control. Oxidative stress and lipid peroxidation are believed to play a
379 significant role in the development of tissue damage and in several pathologies of the human body
380 (Tuberoso et al., 2010). The antioxidant activity of cell-free supernatant (CFS) grape marc was first
381 estimated *in vitro*. A stronger inhibition of the linoleic acid peroxidation was found for all the samples
382 tested compared to un-inoculated control. It is to point out that the antioxidant activity depends not
383 only on phenolic content but also on the chemical structure of each phenol, as demonstrated by Rice-
384 Evans and Miller (1997). Overall, several phenols together with low pH reached by probiotics on
385 GMG after 24 h of fermentation could affect the survival of bacterial cells during the gastro-intestinal
386 transit. Indeed, probiotic strains grown on GMG showed tolerance under simulated gastro-intestinal
387 conditions. Acid and bile salt treatments were combined in this study, since they have both individual
388 and combined effects (Chou and Weimer, 1999). The time chosen for treatments in simulated gastric
389 (180 min) and intestinal (further 180 min) fluids mimicked the in vivo times between entrance to and
390 release from the stomach and intestine during digestive processes (Chou and Weimer, 1999). Before
391 reaching the intestine, probiotic bacteria must first survive the deleterious action of gastric juice
392 during passage through the stomach. In general, the acid tolerance of lactic acid bacteria depends on
393 the pH profile for H^+ transport relative to ATPase activity and on the composition of the cytoplasmic
394 membrane, which is largely influenced by the type of bacterium, the type of growth medium and the
395 incubation conditions (Hood and Zotolla, 1988; De Angelis and Gobbetti, 2004). Compared to MRS,
396 selected probiotic strains cultivated in GMG showed a higher cell survival, especially, in gastric juice
397 at pH 2.0. The only exception was for *L. paracasei*. Also Vinderola et al. (2000), studying the viability
398 of probiotic bacteria on Fresco cheese, concluded that *L. paracasei* was the only probiotic specie that
399 underwent a significant decrease in viable cell numbers during exposure to artificial gastric juice. The
400 enhanced cell survival under gastro-intestinal tract of probiotics cultivated on GMG medium support

401 the hypothesis that grape marc could be used as medium for the growth of probiotics. Based on the
402 chemical composition of grape marc, the probiotic preparations could improve the human antioxidant
403 defenses. First, the MTT assay on Caco-2 (colon adenocarcinoma) cell line was used to determine
404 the cytotoxicity of the CFS grape marc. As expected, a cytotoxic effect was found at high
405 concentrations (>1 mg/ml, data not showed) of CFS grape marc. A proliferative effect occurred at
406 lower concentrations, with treatment lasting 48 h. The MTT assay on Caco-2 cells was also used to
407 show the protective effect of the fermented grape marc samples towards induced oxidative stress. The
408 antioxidant effect on cultured intestinal line cells was higher than that of the un-inoculated sample.
409 The protective effect was investigated through the determination of the intracellular ROS production
410 and detoxification by DCFH-DA assay. Also in this case, it was confirmed the markedly higher
411 antioxidant activity on Caco-2 cells compared to the un-inoculated control. Supporting these
412 founding, the *SOD-2* gene expression also showed the lower pro-oxidant effect explained by all grape
413 marc preparation if compared to positive control, with the exception of *L. paracasei* 14A at the
414 concentration of 0.1 and 1 mg/mL.
415 In conclusion, this study demonstrated the capacity of probiotic lactic acid bacteria and Bifidobacteria
416 to grow on by-product of winery industry such as grape marc. Furthermore, biotechnological
417 valorization and reutilization of grape marc is based on probiotic biomass production and supply of
418 antioxidant compounds. This study also demonstrates how grape marc act as a vector protecting
419 probiotic strains during stomach passage and the antioxidant properties of grape marc on intestinal
420 cells could be enhanced through lactic acid fermentation. Based on this founding, grape marc
421 fermented by probiotic strains may be used in the future as functional food dietary supplements or
422 pharmaceutical preparations in aqueous or freeze-dried forms.
423

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Legend to figures

Figure 1. Lipid peroxidation inhibitory activity (Abs at 500 nm) in cell free supernatants (CFS) of grape marc added of 1% of glucose (GMG) inoculated by selected probiotic bacteria (*Lactobacillus plantarum* 12A, *L. plantarum* PU1, *Lactobacillus paracasei* 14A and *Bifidobacterium breve* 15A) or un-inoculated (control) and incubated for 24 h at 30 °C (*Lactobacillus*) or 37°C (*Bifidobacterium*). The activity was measured under a linoleic acid oxidation system for 8 days. BHT and α-tocopherol (1 mg/ml) were used as the positive controls. A negative control, without antioxidants, also was considered. Data are the means from three independent experiments. Bars represent standard deviations.

Figure 2. Survival of *Lactobacillus plantarum* 12A (GM1), PU1 (GM2), *Lactobacillus paracasei* 14A (GM3) and *Bifidobacterium breve* 15A (GM4) grown on GMG (dashed lines) and in optimal medium (MRS; solid lines) under gastric condition (0-180 min) at pH 8.0(♦), 3.0 (▲) and 2.0 (■) and further intestinal digestion (180-360 min) at pH 8.0. The values were the averages of three replicates and standard deviations are indicated by vertical bars.

Figure 3. Effect of different concentrations (0.01-0.1-1 mg/ml) of grape marc cell-free supernatant (CFS) on the cell viability of adenocarcinoma Caco-2 after oxidative stress. Caco-2 cells were cultured in RPMI and incubated with re-suspended freeze-dried CFS for 16 h. Oxidative stress was artificially induced by incubating cultured cells with 1 mM hydroxide peroxide for 2 h. The percentage of viable cells was measured through MTT assay. CFS of grape marc samples fermented by *Lactobacillus plantarum* 12A, PU1, *Lactobacillus paracasei* 14A, and *Bifidobacterium breve* 15A and GMG un-inoculated and incubated (Control un-inoculated) were assayed at concentration ranging from 0.01 to 1 mg/mL. α-Tocopherol (α-tp) was used as the positive control at same concentrations. Control H_2O_2-stressed cells incubated without antioxidant compounds (control + H_2O_2) was also included. Data are the means of three independent experiments twice analysed.

Figure 4. Effect of different concentrations (0.01-0.1-1 mg/ml) of fermented freeze-dried grape marc cell free supernatant (CFS) on the radical scavenging activity (RSA) of Caco-2 after oxidative stress as estimated by 2'.7'-dichlorofluorescein diacetate (DCFH-DA) assay. Adenocarcinoma Caco-2 cells were cultured on RPMI and incubated with re-suspended freeze-dried homogenates for 16 h. Oxidative stress was artificially induced by incubating cultured cells with 1 mM hydroxide peroxide for 2 h. CFS of grape marc samples fermented by *Lactobacillus plantarum* 12A, *L. plantarum* PU1, *Lactobacillus paracasei* 14A, and *Bifidobacterium breve* 15A and GMG un-inoculated and incubated (Control un-inoculated) were assayed at concentration ranging from 0.01 to 1 mg/mL. α-Tocopherol (α-tp) was used as the positive control at same concentrations. The viability of cell without samples and antioxidant compounds (Control) and H_2O_2-stressed cells incubated without antioxidant compounds (control + H_2O_2) was also included. Data are the means of three independent experiments twice analysed.

Figure 5. Expression of Superoxide dismutase 2 (*SOD-2*) gene in adenocarcinoma Caco-2 cells as determined by RT-PCR. Caco-2 cells were treated at 37°C for 16 h. under 5% CO_2 with: basal medium containing 2.5% fetal bovine serum alone (negative control); with: basal medium containing 2.5% fetal bovine serum added with H_2O_2 1mM; with: basal medium containing 2.5% fetal bovine serum added with H_2O_2 1mM and cell-free supernatant (CFS) of grape marc samples (*Lactobacillus plantarum* 12A, *L. plantarum* PU1, *Lactobacillus paracasei* 14A, and *Bifidobacterium breve* 15A) and GMG un-inoculated and incubated (Control un-inoculated) assayed at concentration ranging from 0.01 to 1 mg/mL. α-tocopherol at concentration 0.01-0.1 and 1mg/mL. Data are the means of three independent experiments twice analysed.

Figure S1. HPLC-DAD chromatograms detected at 280, 313 and 350 nm of cell-free supernatant of grape marc un-inoculated and incubated (Control). **1**, Gallic acid; **2**, uk1; **3**, uk2; **4**, Protocatechuic

acid; **5**, uk3; **6**, Procyanidin B1; 7, Caftaric acid; **8**, (+)-Catechin; **9**, Caffeic acid; **10**, Syringic acid; **11**, uk4; **12**, (-)-Epicatechin; **13**, uk5; **14**, uk6; **15**, Rutin; **16**, uk7.

Figure S2. HPLC-DAD chromatograms detected at 280, 313 and 350 nm of cell-free supernatant of grape marc fermented with *Lactobacillus plantarum* PU1. **1**, Gallic acid; **2**, uk1; **3**, uk2; **4**, Protocatechuic acid; **5**, uk3; **6**, Procyanidin B1; 7, Caftaric acid; **8**, (+)-Catechin; **9**, Caffeic acid; **10**, Syringic acid; **11**, uk4; **12**, (-)-Epicatechin; **13**, uk5; **14**, uk6; **15**, Rutin; **16**, uk7.

Figure 1.

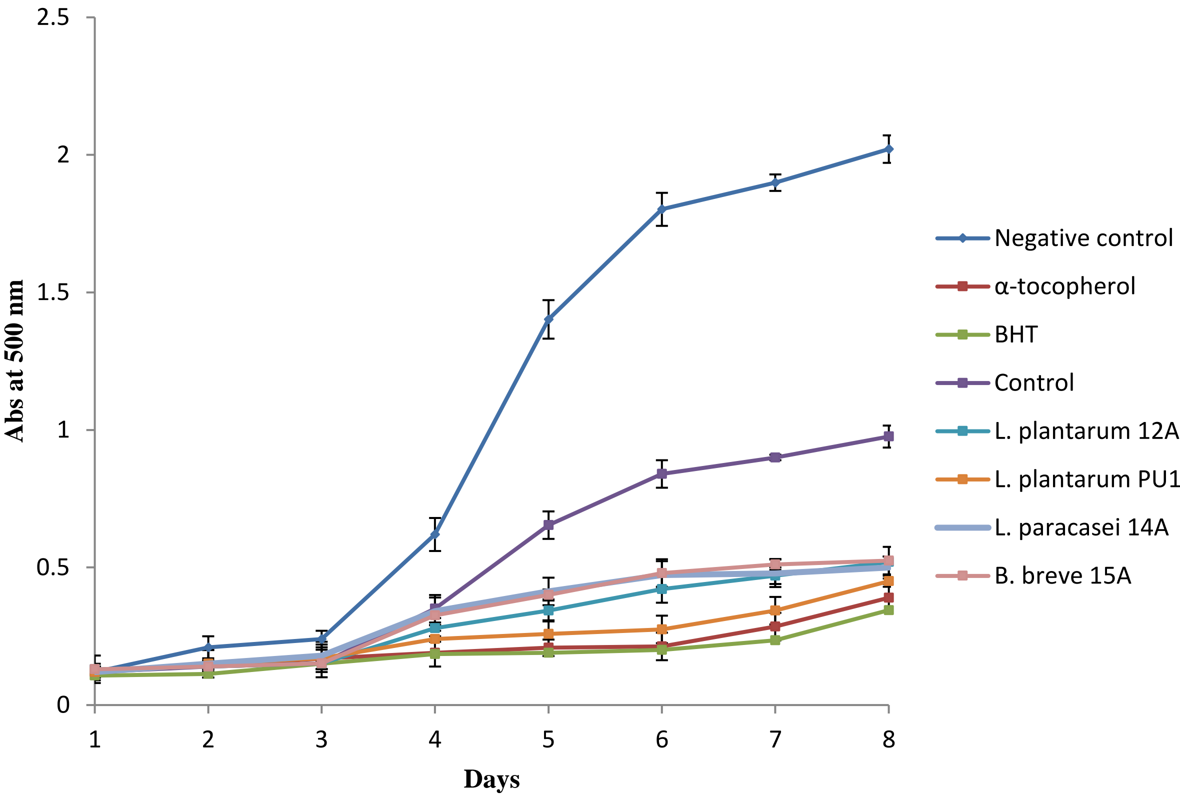


Figure 2.

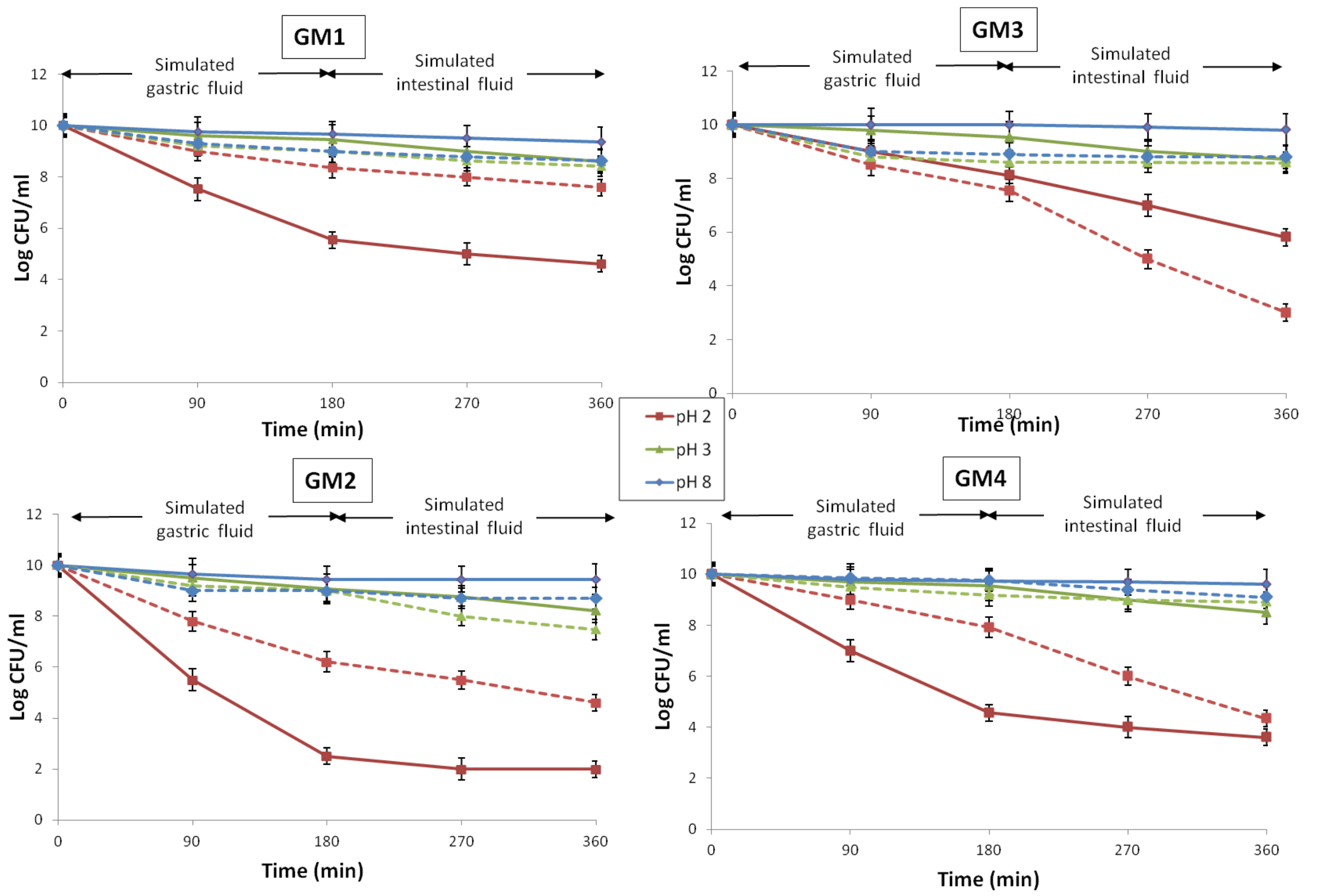


Figure 3.

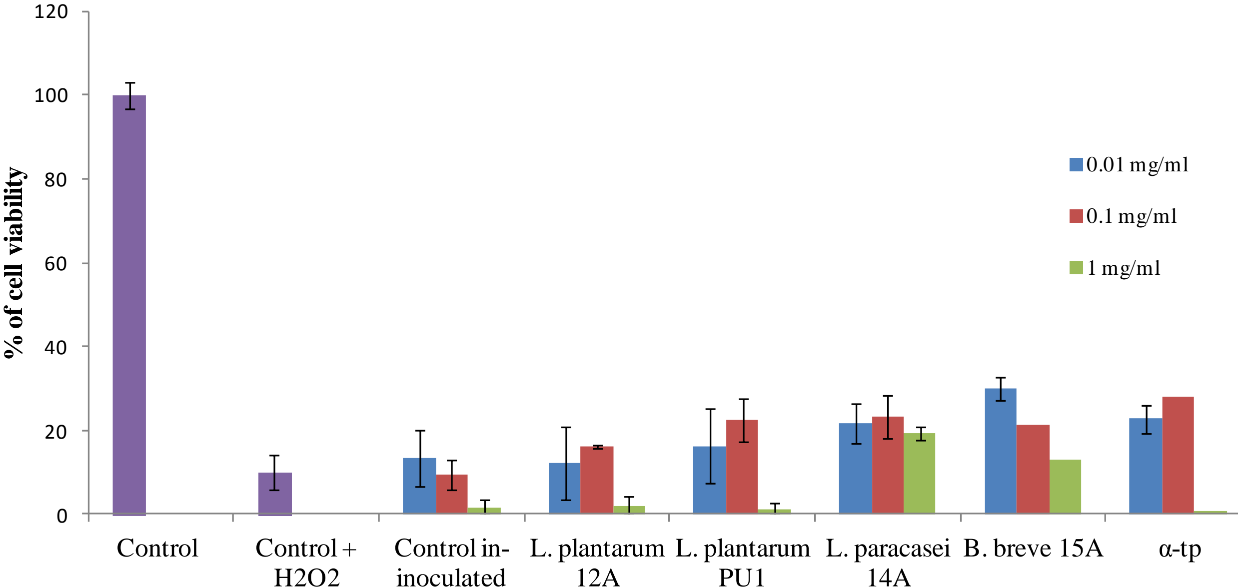


Figure 4.

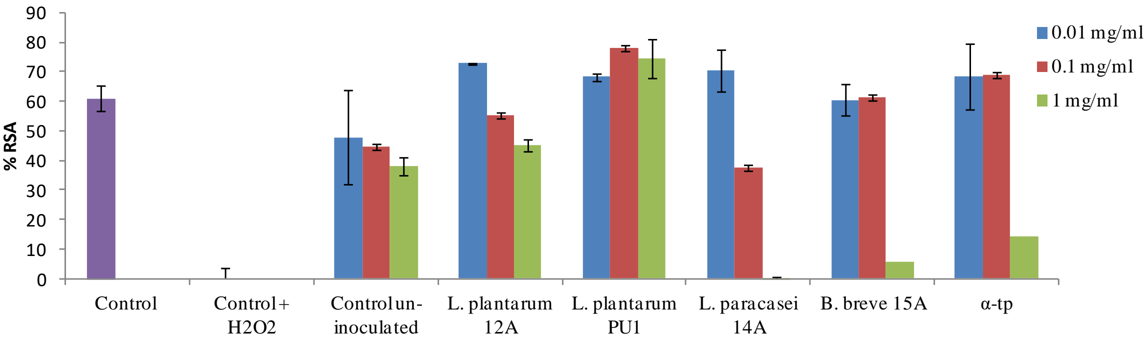


Figure 5.

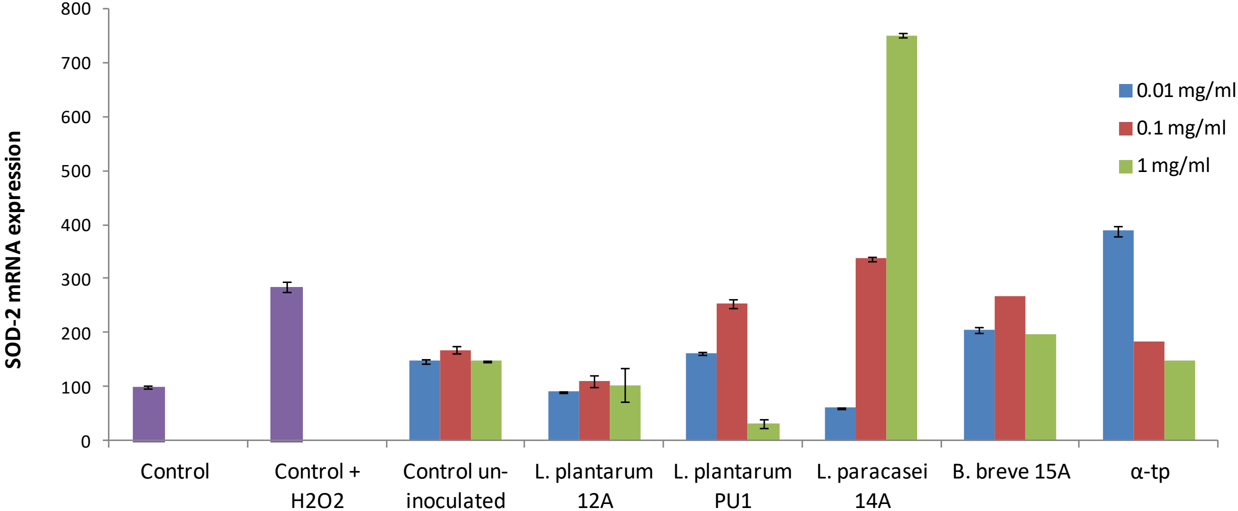


Table 1. Final pH and cellular density (log CFU/mL) of grape marc (GM) alone or added of 1% of glucose (GMG) samples inoculated (ca. 7 log CFU/mL) by probiotic bacteria and fermented for 24 h at 30°C (*Lactobacillus*) or 37°C (*Bifidobacterium*).

	GM		GMG	
	pH	**Cell density**	**pH**	**Cell density**
Un-inoculated and incubated GM and GMG (Controls)	5.41±007[a]	nd	5.32±0.09[a]	nd
L. rossiae DSM15814	5.40±0.06[a]	7.11±0.14[c]	5.45±0.08[a]	7.25±0.12[c]
L. reuteri DSM20016	5.69±0.11[a]	7.22±0.11[c]	5.44±0.06[a]	7.12±0.15[c]
L. plantarum 12A	4.61±0.08[b]	8.83±0.15 [b]	3.62±0.11[b]	9.07±0.20[ab]
L. plantarum PU1	4.51±0.06[b]	9.16±0.12[a]	3.76±0.10 [b]	9.41±0.07[a]
L. rhamnosus SP1	5.50±0.09[a]	7.13±0.15[c]	5.23±0.23[a]	7.50±0.12[c]
L. casei FC1-13	5.56±0.08[a]	7.15±0.22[c]	5.36±0.17[a]	7.60±0.17[c]
L. paracasei 14A	4.65±0.10[b]	8.80±0.15[b]	3.65±0.05 [b]	9.38±0.14[a]
B. breve 15A	4.56±0.06[b]	8.95±0.09[ab]	3.63±0.07 [b]	9.55±0.14[a]
B. animalis 13A	5.45±0.07[a]	7.19±0.15[c]	5.40±0.22[a]	7.23±0.20[c]

L., *Lactobacillus*; *B.*, *Bifidobacterium*.
[a-c]Values with different superscript letters, in the same column, differ significantly ($P < 0.05$).
nd, not detected.

Table 2. Chemical and nutritional characteristics of grape marc added of 1% of glucose (GMG) inoculated by selected probiotic bacteria (*Lactobacillus plantarum* 12A, *L. plantarum* PU1, *Lactobacillus paracasei* 14A and *Bifidobacterium breve* 15A) or un-inoculated (control) and incubated for 24 h at 30°C (*Lactobacillus*) or 37°C (*Bifidobacterium*).

	Control	***L. plantarum* 12A**	***L. plantarum* PU1**	***L. paracasei* 14A**	***B. breve* 15A**
Total titratable acidity (g/L)	1.24±0.01^{d}	4.51±0.04^{c}	6.19±0.00^{a}	5.85±0.02^{b}	6.24±0.05^{a}
Volatile acidity (mg/L)	nd	nd	0.06±0.01^{a}	nd	0.10±0.01^{a}
Carbohydrates (mg/L)	6.13±0.01^{a}	1.22±0.35^{b}	nd	nd	nd
Lactic acid (g/L)	0	2.88±0.06^{d}	4.73±0.03^{a}	3.85±0.02^{c}	4.60±0.02^{b}
Citric acid (g/L)	0.45±0.02^{a}	0.18±0.02^{b}	0.12±0.01^{c}	0.20±0.01^{b}	0.09±0.05^{c}
Glycerol (g/L)	4.32±0.01^{a}	3.21±0.09^{c}	2.91±0.02^{d}	3.56±0.01^{b}	3.26±0.06^{c}
Minerals (mg/Kg)					
Phosphorus (P)	359±11^{a}	328±9^{b}	286±8^{c}	221±7^{d}	348±18^{a}
Calcium (Ca)	200±14^{ab}	149±12^{d}	159±7^{cd}	232±12^{a}	171±5^{c}
Magnesium (Mg)	80±8^{a}	91±9^{a}	77±8^{a}	63±6^{ab}	72±7^{ab}
Potassium (K)	2361±236^{a}	2186±219^{a}	2148±215^{a}	1922±192^{ab}	1927±193^{ab}
Sodium (Na)	916±92^{a}	1058±106^{a}	1046±105^{a}	1039±104^{a}	1062±106^{a}
Iron (Fe)	5.8±0.60^{a}	6.7±0.70^{a}	nd	nd	7.1±0.70^{a}
Fiber (%)					
Insoluble fiber	0.07±0.01^{d}	0.36±0.07^{c}	2.15±0.43^{a}	0.63±0.12^{b}	0.85±0.17^{b}
Soluble fiber	0.60±0.10^{a}	0.80±0.20^{a}	0.80±0.20^{a}	0.70±0.10^{a}	0.80±0.20^{a}
Vitamins (mg/Kg)					
Vitamin B1 (Tiamin)	0.18±0.07^{a}	0.14±0.07^{a}	0.10±0.03^{a}	0.17±0.07^{a}	0.10±0.02^{a}
Vitamin PP (Niacin)	10.30±1.60^{a}	10.90±1.70^{a}	10.10±1.40^{a}	10.10±1.60^{a}	9.80±0.83^{a}

Data are the mean of three independent analyses.
$^{a\text{-}c}$Values with different superscript letters, in the same column, differ significantly (P < 0.05).
nd, not detected.

Table 3. Means of free amino acids (FAA) (mg/kg) of grape marc added of 1% of glucose (GMG) inoculated by selected probiotic bacteria (*Lactobacillus plantarum* 12A, *L. plantarum* PU1, *Lactobacillus paracasei* 14A and *Bifidobacterium breve* 15A) or un-inoculated (control) and incubated for 24 h at 30 °C (*Lactobacillus*) or 37°C (*Bifidobacterium*).

FAA	Control	*L. plantarum* 12A	*L. plantarum* PU1	*L. paracasei* 14A	*B. breve* 15A
Asp	161.91±3.82^{a}	106.77±0.09^{b}	106.83±7.46^{b}	105.86±7.96^{b}	89.35±2.54^{c}
Thr	132.92±5.22^{a}	82.78±3.76^{b}	85.78±5.32^{b}	78.75±5.69^{b}	88.82±2.20^{b}
Ser	171.75±9.71^{a}	59.21±2.80^{c}	51.56±19.47^{c}	54.79±0.06^{c}	78.53±4.12^{b}
Glu	391.51±19.03^{a}	260.49±20.32^{b}	283.52±6.61^{b}	261.76±0.05^{bc}	271.12±8.52^{b}
Gly	126.16±5.60^{a}	105.36±6.37^{b}	104.02±0.51^{b}	106.59±13.26^{b}	106.24±1.08^{b}
Ala+Cys	416.63±15.83^{a}	267.56±13.21^{b}	290.55±17.48^{b}	254.87±8.94^{bc}	213.73±9.33^{c}
Val	186.00±9.70^{a}	135.68±13.97^{bc}	143.10±10.18^{b}	143.00±3.97^{b}	148.04±2.65^{b}
Met	61.96±1.76^{a}	42.24±3.02^{b}	45.70±0.84^{b}	44.71±2.68^{b}	47.67±2.45^{b}
Ile	147.42±4.64^{a}	97.11±8.79^{b}	103.11±7.29^{b}	103.27±3.16^{b}	103.62±3.36^{b}
Leu	262.89±10.05^{a}	182.82±13.16^{b}	194.09±13.24^{b}	187.34±1.99^{bc}	199.50±3.80^{b}
Tyr	42.10±1.65^{a}	15.90±0.23^{bc}	18.39±0.22^{b}	16.69±1.69^{b}	18.11±0.35^{b}
Phe	151.03±6.16^{a}	77.42±4.22^{d}	85.31±0.30^{c}	79.92±1.07^{d}	92.67±1.79^{b}
GABA	41.27±2.29^{a}	22.71±1.41^{c}	23.53±0.91^{c}	22.90±0.52^{c}	25.18±0.34^{b}
His	112.58±3.81^{a}	83.03±4.92^{b}	85.34±4.86^{b}	80.97±1.20^{b}	78.12±1.12b^{c}
Trp	40.91±3.38^{a}	49.33±3.93^{b}	46.17±2.66^{ab}	52.62±9.77^{ab}	46.75±2.33^{ab}
Orn	9.13±0.53^{a}	11.95±6.43^{a}	8.19±0.72^{a}	8.34±0.20^{a}	8.69±0.63^{a}
Lys	148.82±2.35^{a}	113.34±1.52^{b}	116.13±2.04^{b}	113.50±8.35^{b}	114.99±2.28^{b}
Arg	169.54±4.38^{a}	131.83±2.51^{b}	140.81±6.74^{b}	138.82±0.98^{b}	144.32±3.86^{b}
Pro	276.77±7.70^{a}	172.71±21.67^{b}	160.54±8.07^{bc}	175.27±16.53^{b}	192.98±3.04^{b}
Total	3051.30±144.627^{a}	2018.25±103.02^{b}	2092.66±113.47^{b}	2029.95±37.73^{b}	2068.41±34.96^{b}

Data are the mean of three independent analyses.

$^{a-c}$Values with different superscript letters, in the same column, differ significantly ($P < 0.05$).

1 **Table 4.** Individual (mg/L) and total phenol content (gallic acid equivalents per liter, mg GAE/L). on
2 cell free supernatants (CFS) of grape marc added of 1% of glucose (GMG) inoculated by selected
3 probiotic bacteria (*Lactobacillus plantarum* 12A, *L. plantarum* PU1, *Lactobacillus paracasei* 14A
4 and *Bifidobacterium breve* 15A) or un-inoculated (control) and incubated for 24 h at 30°C
5 (*Lactobacillus*) or 37°C (*Bifidobacterium*).
6

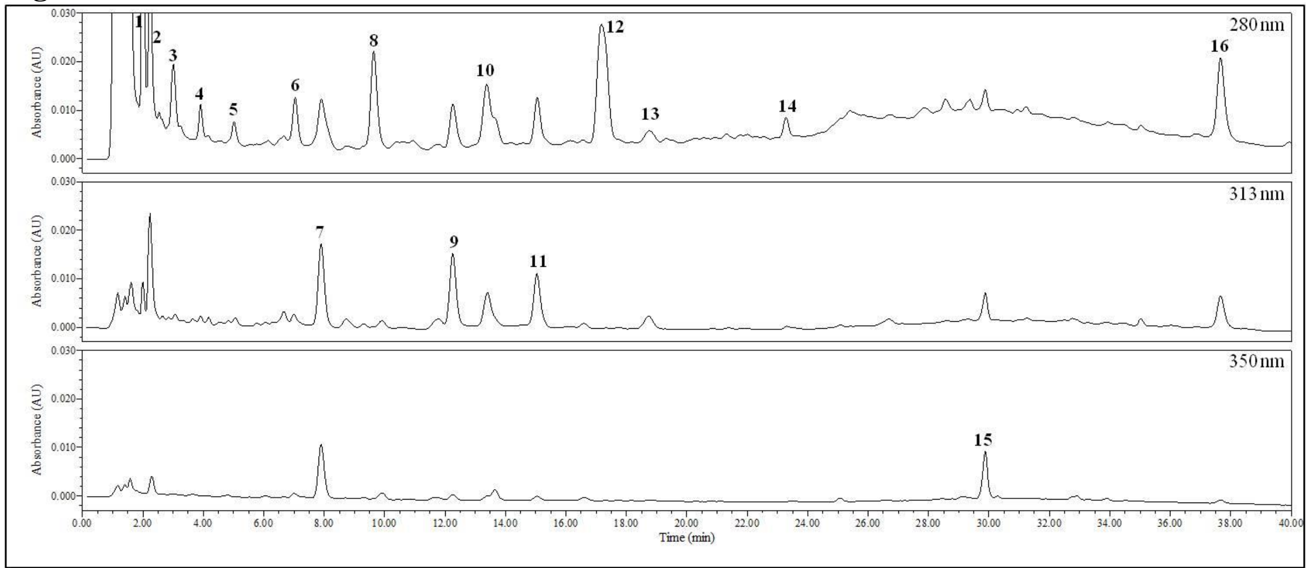
Phenolic compounds (mg/L)	GMG un-inoculated	*L. plantarum* 12A	*L. plantarum* PU1	*L. paracasei* 14A	*B. breve* 15A
Total phenol content	1123 ± 54^{a}	1028 ± 55^{a}	932 ± 70^{b}	1000 ± 78^{a}	977 ± 73^{b}
Gallic acid †	68.0±6.6^{a}	12.6±0.3^{b}	10.1±1.5^{c}	10.8±1.9^{bc}	9.4±2.3^{c}
uk1†	52.4±2.9^{a}	41.9±3.1^{b}	39.1±3.0^{b}	41.1±4.6^{b}	38.6±2.9^{b}
uk2†	34.6±5.4^{a}	21.5±2.2^{b}	20.3±0.8^{b}	21.5±1.1^{b}	22.5±1.5^{b}
Protocatechuic acid †	9.8±1.7^{ab}	7.1±1.1^{b}	4.5±0.9^{c}	7.5±2.1^{b}	6.0±0.6^{bc}
uk3†	8.6±0.6^{c}	9.4±0.6^{bc}	9.6±0.9^{bc}	11.3±1.2^{ab}	12.7±0.6^{a}
Procyanidin B1†	14.9±1.3^{a}	10.5±1.2^{c}	13.5±1.5^{b}	10.9±1.4^{bc}	14.6±3.6^{abc}
Caftaric acid §	3.5±0.1^{a}	3.4±0.3^{a}	3.2±0.2^{a}	3.2±0.3^{a}	3.3±0.3^{a}
(+)-Catechin †	40.3±6.1^{a}	39.6±3.5^{a}	35.9±5.9^{ab}	35.9±1.5^{ab}	38.7±7.9^{ab}
Caffeic acid §	4.1±0.2^{b}	4.6±0.4^{a}	4.3±0.2^{b}	4.3±0.8^{ab}	4.3±0.6^{ab}
Syringic acid †	23.8±0.9^{a}	19.5±1.3^{b}	20.1±0.3^{b}	20.3±1.0^{b}	18.7±2.8^{b}
uk4 §	2.9±0.1^{b}	3.1±0.3^{a}	2.9±0.2^{ab}	3.0±0.6^{a}	2.9±0.4^{a}
(-)-Epicatechin †	71.4±3.2^{a}	61.7±5.9^{b}	55.5±3.7^{bc}	55.2±3.0^{bc}	54.3±0.8^{c}
uk5 †	6.0±2.0^{c}	8.3±0.4^{a}	8.3±1.2^{ab}	7.2±0.6^{bc}	8.4±0.4^{a}
uk6 †	7.0±0.8^{b}	7.5±0.9^{ab}	7.1±1.7^{ab}	7.2±0.4^{b}	8.3±0.4^{a}
Rutin ¥	3.7±0.1^{a}	3.6±0.3^{a}	3.7±0.1^{a}	3.4±0.4^{a}	3.4±0.5^{a}
uk7 †	2.1±0.2^{a}	1.2±0.4^{b}	1.8±0.7^{ab}	1.6±0.5^{ab}	2.6±1.1^{ab}

7 The detection was carried out at different wavelengths: 280 nm (†), 313 nm (§) and 350 nm (¥).
8 Data are the mean of three independent analyses.
9 $^{a-c}$Values with different superscript letters, in the same column, differ significantly ($P < 0.05$).
10

11 **Supplementary material**

12

13 **Figure S1**



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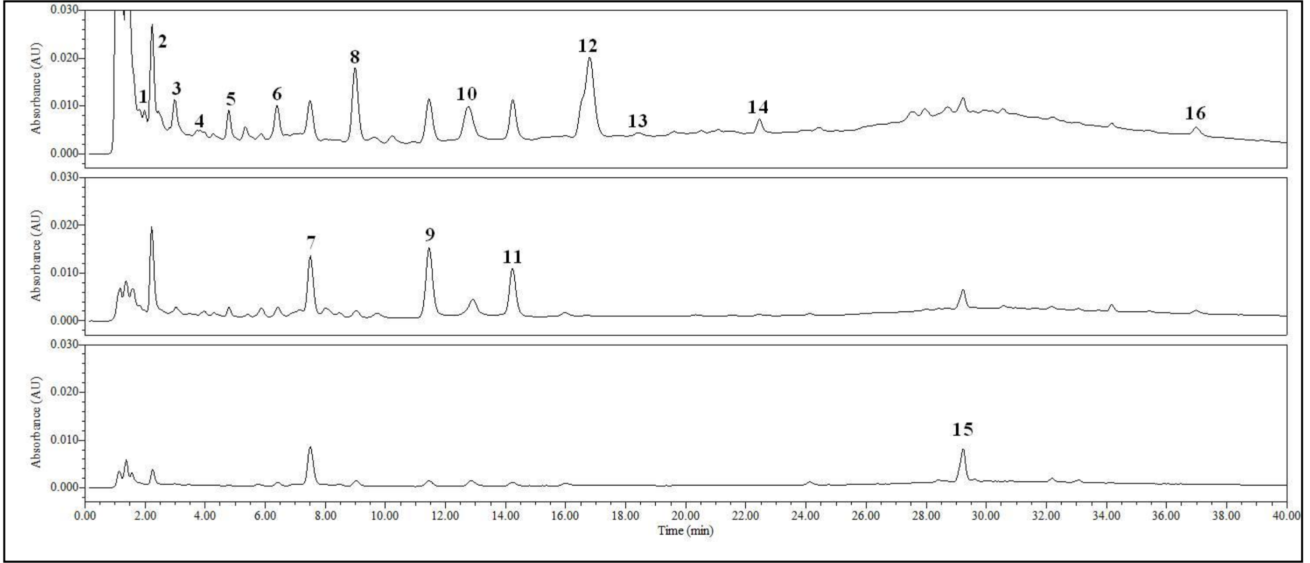
17 **Figure S2**



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