

1 **INFLUENCE OF OMEGA-3 PUFAs ON THE METABOLISM OF**
2 **PROANTHOCYANIDINS IN RATS**

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25 bioavailability

| | |
|----|-----------------------------------|
| 26 | List of abbreviations |
| 27 | EC, (epi)catechin |
| 28 | EGC, (epi)gallocatechin |
| 29 | Gluc, glucuronyl group |
| 30 | GSE, grape seed extract |
| 31 | Me, methyl group |
| 32 | MRM, multiple reaction monitoring |
| 33 | PAs, proanthocyanidins |
| 34 | STD, standard diet |
| 35 | Sulf, sulfate group |
| 36 | SPE, solid-phase extraction |
| 37 | |

38 **Abstract**

39 Studies of the bioavailability of proanthocyanidins usually consider them independently
40 of other dietary constituents, while there is a tendency in the field of functional foods
41 towards the combination of different bioactive compounds in a single product. This
42 study examined the long-term effects of ω -3 polyunsaturated fatty acids of marine
43 origin on the metabolic fate of grape proanthocyanidins. For this, female adult Wistar-
44 Kyoto rats were fed (18 weeks) with a standard diet supplemented or not with
45 eicosapentaenoic acid/docosahexanoic acid (1:1, 16.6 g/kg feed), proanthocyanidin-rich
46 grape seed extract (0.8 g/kg feed) or both. A total of 39 microbial-derived metabolites
47 and 16 conjugated metabolites were detected by HPLC-MS/MS either in urine or in the
48 aqueous fraction of feces. An unexpected significant increase in many proanthocyanidin
49 metabolites in urine and feces was observed in the group supplemented with ω -3
50 polyunsaturated fatty acids group as compared to the animals fed a standard diet, which
51 contains a small amount of polyphenols. However, proanthocyanidin metabolites in rats
52 given ω -3 polyunsaturated fatty acids and grape seed extract did not significantly differ
53 from those in the group supplemented only with grape seed extract. It was concluded
54 that ω -3 polyunsaturated fatty acids collaborate in the metabolism of polyphenols when
55 present at low doses, while the capacity of ω -3 polyunsaturated fatty acids to induce
56 microbiota transformations when proanthocyanidins are present at high doses is not
57 relevant compared to that of polyphenols themselves.

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61 1. INTRODUCTION

62 Polyphenols are a large group of compounds found in plant foods that have been shown to
63 have health-related effects in relation to several chronic diseases (Scalbert et al., 2005).
64 Flavanols, included in the family of flavonoids, are among the most studied polyphenols.
65 They range in complexity from monomers, such as (+)-catechin or (-)-epicatechin, to
66 combinations of these structures via different linkages, which gives rise to the
67 corresponding oligomers or polymers- (proanthocyanidins, PAs). Flavanols are found in
68 many common foods, such as grapes, nuts or cocoa, and have been shown to have
69 beneficial effects in relation to different markers of cardiovascular disease. Indeed, the
70 European Food Safety Agency approved a health claim regarding the effects of cocoa
71 flavanols on endothelium-dependent vasodilatation (EFSA, 2006).

72 A key aspect of the study of PAs is the proper knowledge of their metabolic fate, since they
73 are extensively transformed after ingestion. Dimers and to a lesser extent trimers may be
74 absorbed in the small intestine; the former may be methylated while no post-absorption
75 transformation has been reported for trimers (Monagas et al., 2010). However, most
76 ingested PAs reach the colon, being either extensively depolymerized and absorbed as
77 monomers or metabolized by the gut microbiota (Touriño et al., 2011). Monomers are
78 extensively conjugated in the liver and then circulate in the body before being excreted in
79 urine or accumulated in tissues, or they return to the intestine via enterohepatic circulation.
80 While for those PA transformed by the microbiota, the resulting metabolites are mostly
81 phenolic acids that may be absorbed and follow the same routes as polyphenols absorbed
82 in the small intestine (Urpí-Sardá et al., 2009; Monagas et al., 2010; Mateos-Martín, Pérez-
83 Jiménez, Fuguet, & Torres, 2012a). Increasing evidence suggests that circulating
84 polyphenol-derived metabolites, especially those produced during colonic fermentation,
85 may be the compounds responsible for the health-related properties of these food

86 constituents (Williamson, & Clifford, 2010).

87 To elucidate the metabolic fate of polyphenols, they are administered alone and in acute
88 doses (Urpí-Sardá et al., 2009; Monagas et al., 2010; Touriño et al., 2011; Mateos-Martín
89 et al., 2012a). In a common diet, however, polyphenols are consumed in combination with
90 other food components, which may have either synergic or antagonistic effects on their
91 bioavailability. Moreover, there is currently increasing interest in the development of
92 functional foods with combinations of bioactive components (Peluso, Romanelli, &
93 Palmery, 2014), which may affect the bioavailability of polyphenols and the health effects
94 derived from them. Therefore, now that the general process of transformation of these
95 compounds has been reported, there is increasing interest in the effects that other food
96 constituents, such as carbohydrates, proteins or dietary fiber, may have on their
97 bioavailability (Bohn, 2004; Zhang et al., 2014).

98 In relation to dietary fat, studies with animal models (Lesser, Cermak, & Wollfram, 2008)
99 and humans (Tulipani et al., 2002; Guo et al., 2013) have reported that this food constituent
100 increases the bioaccessibility and absorption of certain flavonoids, through different
101 mechanisms. Differential effects of long-chain and medium-chain fatty acids on the
102 bioavailability of polyphenols are probably due to the different metabolic routes that these
103 compounds follow (Lesser, Cermak, & Wollfram, 2006; Murota, Cermak, Terao, &
104 Wollfram, 2013). In contrast, the effects of fatty acids with different degree of unsaturation
105 on the metabolic fate of polyphenols have not been explored. Indeed, studies in this area
106 have only evaluated the differential effects of saturated and monounsaturated fats (Tulipani
107 et al., 2002; Lesser, Cermak, & Wollfram, 2006; Lesser, Cermak, & Wollfram, 2008; Guo
108 et al., 2013; Murota et al., 2013), and to the best of our knowledge, only one *in vitro* study
109 has considered the effects of a polyunsaturated fat: hazelnut oil (Ortega, Macià, Romero,
110 Reguant, & Motilva, 2011).

111 Long-chain ω -3 PUFAs of marine origin are a class of bioactive dietary components that
112 have generated a great deal of interest due to their beneficial effects in both animal and
113 human studies, on parameters related to cardiovascular disease (Aguilera, Díaz, Barcelata,
114 Guerrero, & Ros, 2004; Lorente-Cebrián, Costa, Navas-Carretero, Zabala, Martínez, &
115 Moreno-Aliaga, 2013). In a common diet, and in supplements containing different
116 bioactive compounds, polyphenols may be consumed together with ω -3 PUFAs and
117 different interactions may take place (Peluso et al., 2014), which could also affect their
118 metabolic fate. Therefore, the aim of this study was to evaluate the effect that ω -3 PUFAs
119 had on the metabolic fate of grape PAs after long-term *in vivo* supplementation. To this
120 end, a pilot study was carried out in Wistar-Kyoto rats, and the profile of polyphenol
121 metabolites was measured by targeted HPLC-ESI-MS/MS analysis of urine and the
122 aqueous fraction of feces.

123 **2. MATERIALS AND METHODS**

124 **2.1 Chemicals and reagents**

125 The standard diet was Teklad Global 2014 (Harlan Teklad Inc., Indianapolis, IN, USA).
126 Fine Grajfnol[®] powder, 98% grape seed, was obtained from JF-Natural Product (Tianjin,
127 China), with the following composition: total PAs (UV), $\geq 95\%$; oligomeric PAs, $\geq 60\%$;
128 procyanidin dimer B₂ (HPLC), $\geq 1.8\%$; ash, $\leq 1.5\%$; weight loss on drying, $\leq 5.0\%$.
129 Porcine gelatin type A 240/260 was from Juncà (Girona, Spain) and the soybean lecithin
130 Topcithin 50 from Cargill (Barcelona, Spain). Oil with an eicosapentaenoic
131 acid:docosahexanoic acid (EPA:DHA) ratio of 1:1 was obtained by mixing appropriate
132 quantities of the commercial fish oils AFAMPES 121 EPA (A.F.A.M.S.A., Vigo, Spain),
133 EnerZona Omega 3 RX (Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98,

134 S.L., Barcelona). Soybean oil, obtained from unrefined organic soybean oil (first cold
135 pressing), was from Clearspring Ltd. (London, UK).
136 Ketamine chlorhydrate was purchased from Merial Laboratorios (Barcelona, Spain) and
137 xylazine from Química Farmaceutica (Barcelona, Spain). Standards of (-)-epicatechin (\geq
138 98%), (-)-epigallocatechin (\geq 95%), 3-hydroxyphenylacetic acid (\geq 99%), 4-
139 hydroxyphenylacetic acid (\geq 98%), 3,4-dihydroxyphenylacetic acid (\geq 98%), 3-
140 hydroxybenzoic acid (\geq 99%), 4-hydroxybenzoic acid (\geq 99%), homovanillic acid (\geq
141 98%), vanillic acid (\geq 97%), caffeic acid (\geq 98%), 3,4-dihydroxyphenylpropionic acid (\geq
142 98%), 3-hydroxyphenylpropionic acid (\geq 98%), 4-hydroxyphenylpropionic acid (\geq 98%),
143 3,4-dihydroxybenzoic acid (\geq 97%), benzoic acid (\geq 99%), hippuric acid (\geq 98%), ferulic
144 acid (\geq 99%), isoferulic acid (\geq 97%), *p*-coumaric acid (\geq 98%), *m*-coumaric acid (\geq 98%),
145 gallic acid (\geq 97%), enterodiol (\geq 95%), phenylacetic acid (\geq 99%), taxifolin (\geq 85%), and
146 tert-butylhydroquinone and formic acid (analytical grade) were obtained from Sigma
147 Chemical (St Louis, MO, USA). Methanol (analytical grade) and hydrochloric acid (\geq
148 85%) were from Panreac (Castellar del Vallès, Barcelona, Spain). Acetonitrile (HPLC
149 grade) was obtained from Merck (Darmstadt, Germany). Water for the assay solutions was
150 obtained using a water Milli-Q purification system (Millipore Corporation, Billerica, MA,
151 USA).

152 **2.2 Diets**

153 Four diets were prepared: the standard diet; the standard diet supplemented with ω -3
154 PUFAs; the standard diet supplemented with grape seed extract; and the standard diet
155 supplemented with both ω -3 PUFAs and grape seed extract. The diets without ω -3 PUFAs
156 were enriched with soybean oil in order to make them isocaloric. All the diets were
157 prepared in-house and included tert-butylhydroquinone as an antioxidant, porcine gelatin
158 to promote gelatinization and soybean lecithin as an emulsifier. The mixtures were freeze-

159 dried to obtain pellets that were stored at 4°C to prevent oxidation and fungal
160 contamination. The composition of each diet, including the supplementations with ω -3
161 PUFAs and GSE, as well as both the macronutrient and micronutrient profile, is shown in
162 **Supplemental Table 1**. A mixture of EPA and DHA in a ratio of 1:1 was used, since it has
163 been suggested that each fatty acid may have different health effects (Lorente-Cebrián et
164 al. 2013). This ratio was previously reported as the most beneficial for cardiometabolic
165 risk factors (Lluís et al., 2013; Méndez et al., 2013). The fatty acid composition of the
166 soybean oil and ω -3 PUFA supplement were ascertained by gas chromatography after
167 methylation (Lepage, & Roy, 1986) and are provided in **Supplemental Table 2**. Because
168 PUFAs are extremely susceptible to oxidation and due to the potential toxic effects of lipid
169 oxidation byproducts, the lipid oxidation level was checked throughout the dietary
170 interventional experiment (peroxide values < 5 meq. oxygen per kg of oil).

171 The doses of ω -3 PUFAs (16.6 g/kg feed) and grape PAs (0.8 g/kg feed) were chosen on
172 the basis of previous studies where similar doses showed beneficial effects (Masson et al.,
173 2008; Castell-Auví, Cedó, Pallarés, Blay, Pinent, & Ardévol., 2013) and as realistic doses,
174 i.e., doses which could easily be incorporated into a typical common diet.

175 **2.3 Animals and sample collection**

176 Twenty female, 8- to 9-week-old, Wistar-Kyoto rats (Charles River Laboratories,
177 Wilmington, MA, USA) were housed in cages ($n = 2$ -3/cage) under controlled conditions
178 of a 12 h light/12 h dark cycle, temperature of 22°C \pm 2°C and relative humidity of 50% \pm
179 10%. They had free access to water and pelleted feed (**Supplemental Table 1**) for 18
180 weeks after being randomly divided into the four dietary groups: STD group ($n = 5$), given
181 the standard diet supplemented with soybean oil; ω -3 group ($n = 5$), given the standard diet
182 supplemented with ω -3 PUFAs; GSE group ($n = 5$), given the standard diet supplemented

183 with grape seed extract and soybean oil; and ω -3+GSE group ($n = 5$), given the standard
184 diet supplemented with both ω -3 PUFAs and grape seed extract. For urine and feces
185 collection, the rats were individually placed in metabolic cages and deprived of food for 24
186 h. At the end of the experiment, the rats were fasted overnight and anesthetized by an
187 intraperitoneal injection of 80 mg/kg of ketamine (Imalgene 1000, Merial Laboratorios
188 S.A., Barcelona, Spain) and 10 mg/kg of xylazine (Rompun 2%, Química Farmacéutica
189 S.A., Barcelona, Spain). Female rats were chosen because they had previously been
190 demonstrated to be as valid model for studying the metabolic fate of PAs (Mateos-Martín
191 et al., 2012a; Touriño et al., 2011; Molinar-Toribio et al., *in press*)

192 The handling and killing of the animals were in full accordance with the European Union
193 guidelines on the care and management of laboratory animals, and the pertinent permission
194 was obtained from the CSIC Subcommittee for Bioethical Issues (ref. AGL2009-12 374-
195 C03-03).

196 **2.4 Sample processing**

197 Urine samples were processed following a previously described solid-phase extraction
198 (SPE) procedure (Touriño, Fuguet, Vinardell, Cascante, & Torres, 2009) for the extraction
199 of phenolic metabolites. Briefly, Oasis HLB (60 mg) cartridges from Waters Corporation
200 (Milford, MA, USA) were activated with 1 mL of methanol and 2 mL of water acidified to
201 pH 3 with formic acid (acid water). The samples (total volume of collected urine) were
202 loaded, interfering components were removed with 9 mL of acid water and then the
203 phenolic compounds were eluted with 1 mL of methanol. Taxifolin (final concentration of
204 5 mg L⁻¹) was used as internal standard.

205 Fecal samples collected over 24 h were re-suspended in acid water and homogenized on a
206 vortex. Then, after adding the internal standard (taxifolin, 5 mg L⁻¹) the mixtures were
207 centrifuged (10000 g, 10 min at 4°C) to obtain a supernatant containing the aqueous

208 fraction of the feces. This supernatant was freeze-dried and re-suspended in 1 mL of acid
209 water, homogenized on a vortex, and then subjected to SPE and the work-up process
210 described for the urine samples.

211

212 **2.5 HPLC-ESI-MS/MS analysis of polyphenol metabolites**

213 An Applied Biosystems (PE Sciex, Concord, Ontario, Canada) API 3000 triple quadrupole
214 mass spectrometer with a TurboIon spray source was used in negative mode to obtain MS
215 and MS/MS data. HPLC separations were performed on an Agilent 1100 series (Agilent,
216 Waldbronn, Germany) liquid chromatograph equipped with a Phenomenex (Torrance, CA,
217 USA) Luna C18 (50 x 2.0 mm i.d.) 3.0 μm particle size column and a Phenomenex
218 Securityguard C18 (4 x 2.0 mm i.d.) column. Gradient elution was performed with a binary
219 system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in CH_3CN .
220 The following increasing linear gradient (v/v) of [B] was used, (t (min), % B): 0,8; 10,23;
221 15,50; 20,50; 21,100 followed by a re-equilibration step. MS conditions were as previously
222 described (Tourino et al., 2009). Each metabolite in the urine samples was first identified
223 by MRM (multiple reaction monitoring) transition of the putative metabolites using a dwell
224 time of 100 ms and then confirmed either by comparison with a standard when available,
225 second MRM, or neutral-loss and product ion scan experiments. Identification details are
226 published elsewhere (Molinar-Toribio et al., *in press*). The MS conditions for each MRM
227 transition were optimized by direct injection of metabolite standards, when available; for
228 other metabolites, the conditions of the most structurally similar standard were used.
229 Analyst 1.4.2 software from AB Sciex was used for data acquisition and processing.
230 Standard calibrations curves were made for each metabolite using between 4 and 11
231 different concentrations for each of them, between 0.001 and 60 mg L^{-1} , and they were
232 used to determine the concentration of each metabolite in the samples, after correction by

233 the internal standard concentration. When no commercial standard was available, the
234 metabolites were quantified using a structurally related commercial standard. For details of
235 the calibration curves used, see **Supplemental Table 3**. The structurally related
236 commercial standard may still show a different response from that of the metabolite, so this
237 method should be used mainly for comparative purposes.

238 **2.6 Statistics**

239 Results are expressed as mean concentrations \pm standard error of the mean (SEM),
240 expressed in $\mu\text{mol/L}$ urine or $\mu\text{mol/g}$ dried feces, adjusted in both cases per kg feed/kg
241 body weight. Mean body weight and feed consumption data per group were used for this
242 adjustment. Since the data did not follow a normal distribution, the non-parametric
243 Kruskal-Wallis and Mann-Whitney U tests were applied to analyze the significant
244 differences ($P < 0.05$) comparing the four groups to one another. The Kruskal-Wallis test
245 was applied to determine any significant difference between the treatments and, if any
246 were detected, the Mann-Whitney U test was used to compare all the different pairs of the
247 treatments. The SPSS IBM 19 package for Windows was used throughout.

248 **3. RESULTS**

249 **3.1 Feed intake**

250 Feed intake was monitored throughout the study. Mean values standardized by rat weight
251 (g/kg rat/day) were: STD group, 59.4 (SEM 2.6); ω -3 group, 41.4 (SEM 4.5); GSE group,
252 56.4 (SEM 4.2); ω -3+GSE group, 40.0 (SEM 3.6). The intakes in ω -3 group and in the ω -
253 3+GSE group were significantly lower than in the STD group (P 0.020 and 0.0058,
254 respectively). The mean values of caloric intake (kcal/100 g rat/day) throughout the study
255 were: STD group, 184.0 (SEM 38.9); ω -3 group, 128.4 (SEM 44.5); GSE group, 174.8
256 (SEM 44.5); ω -3+GSE group, 124.9 (SEM 39.7). Similarly, the energy intakes in ω -3

257 groups were significantly lower than in the STD and in the GSE groups. Due to these
258 differences in the intakes, the results were adjusted per feed intake and body weight.

259 **3.2 Conjugated metabolites of (epi)catechin and (epi)gallo catechin in urine**

260 A total of 39 transitions were searched for in urine, corresponding to mono-, di- and tri-
261 conjugated metabolites of (epi)catechin (EC) and (epi)gallo catechin (EGC) (derived from
262 the combinations of the methylated or Me, sulfated or Sulf and glucuronidated or Gluc
263 forms). The fragmentation patterns obtained for the different compounds were compared
264 with those reported in the literature for PA-derived conjugated metabolites (Urpí-Sardá et
265 al., 2009; Mateos-Martín et al., 2012a).

266 A total of 8 transitions were detected in the samples, corresponding to 12 metabolites from
267 EC and 2 from EGC. Different metabolites may be identified by the same transition, since
268 a substituent may be attached at different positions of the phenolic structure. This is the
269 case, for example, of Gluc-EC, for which 5 different positional isomers were separated and
270 identified by HPLC-MS/MS. The identification spectra for Gluc-EC is shown in
271 **Supplemental Figure 1**. Characteristic fragments of this metabolite were detected at m/z
272 289, indicating the loss of the glucuronide moiety, as well as at m/z 175 and 113,
273 corresponding to the degradation of this moiety.

274 The concentrations of the conjugated metabolites detected in urine are provided in **Table 1**.
275 For the individual metabolites identified, urine concentrations of Gluc-EC-1, Me-Gluc-EC-
276 1, Me-Gluc-EC-2 and Me-Gluc-EC-3 were significantly increased in both the GSE and ω -
277 3+GSE groups, compared to the STD and ω -3 groups (Me-Gluc-EC-2, **Supplemental**
278 **Figure 2**). Gluc-EC-2 was also significantly increased in the GSE group but not in the ω -
279 3+GSE group, compared to the STD group. In the ω -3 group, the urinary excretion of three
280 glucuronidated EC forms was significantly higher than in the STD group.

281 Overall, the levels of conjugated metabolites of the monomers of PAs excreted in urine

282 were increased in both the GSE and the ω -3+GSE groups as compared to the STD group.

283 **3.3 Microbial-derived metabolites in urine**

284 We searched for 48 transitions, corresponding to the microbial metabolites formed in the 8
285 different steps of the microbial fermentation of PAs (valerolactones, lignans, phenylvaleric
286 acids, phenylpropionic acids, phenylacetic acids, benzoic acids, cinnamic acids and
287 glycinated benzoic acids) in the samples.

288 A total of 31 transitions were detected in the samples, corresponding to 39 metabolites,
289 since it is known that the microbial metabolites of some PAs may present structural
290 isomers—e.g., 3- or 4-hydroxybenzoic acid—and some of them may later be conjugated at
291 different positions (Redeuil et al., 2011). For instance, the identification of *m*-coumaric and
292 *p*-coumaric acid was based on standard retention times (**Supplemental Figure 3**).
293 Metabolite concentrations are shown in **Table 2**.

294 In the GSE group, 24 metabolites occurred at concentrations significantly greater than in
295 the STD group. In the case of the ω -3+GSE group, this affected 19 metabolites. No
296 significant differences were observed between the two groups supplemented with GSE. In
297 the ω -3 group, the concentration of 18 metabolites significantly increased with respect to
298 the STD group- in most of the cases, the concentrations found in this group were lower
299 than those found in the GSE and in the ω -3+GSE group.

300 **3.4 Microbial-derived metabolites in feces**

301 None of the EC or EGC conjugated metabolites found in urine were detected in feces.
302 However, 11 microbial-derived metabolites were identified in the fecal samples (**Table 3**).
303 In the GSE and ω -3+GSE groups, 7 metabolites were significantly greater than in the STD
304 group. 4-Hydroxyphenylpropionic acid was also significantly higher in the ω -3+GSE
305 group as compared to the STD group. In the ω -3 group, 5 metabolites were at significantly

306 greater concentrations than in the STD group. The concentrations in the ω -3 and the ω -
307 3+GSE groups were within the same range for some compounds, while 4-
308 hydroxyphenylpropionic acid showed the highest concentration in the ω -3+GSE group,
309 and 3-hydroxyphenylpropionic acid, Me-hippuric acid-1 and Me-hippuric acid-2 showed the
310 highest concentrations in the GSE group, although for Me-hippuric acid-1 the increment
311 was not statistically significant .

312 **4. DISCUSSION**

313 Here, we carried out a pilot study on the effects of ω -3 PUFAs on the metabolic fate of
314 PAs; an aspect that had not previously been explored. To mimic a human dietary situation,
315 this was evaluated in rats after long-term exposure to diets that incorporated both
316 components. PA-derived conjugated EC and microbially generated metabolites were
317 measured in urine; the biological fluid considered the most appropriate for evaluating the
318 bioavailability of polyphenols (Pérez-Jiménez et al., 2010), given the short half-life in
319 plasma of their metabolites. These metabolites were also measured in fecal water, i.e., the
320 fraction of the feces closest to the colonic epithelium (Gill et al., 2010) that could
321 potentially be involved in the reported effects of PAs on colonic health (Rossi, Bossetti,
322 Negri, Lagiou, & La Vecchia, 2010; Sánchez-Tena et al., 2013). Since intakes were
323 significantly different between the groups- i.e., the lowest intakes were found in the ω -3
324 groups- metabolite concentrations were adjusted per feed intake. Nevertheless, metabolite
325 concentrations in the GSE groups were in the same range as those reported in studies with
326 similar supplementations for shorter periods (Tsang et al., 2005; Choy et al., 2014). Also,
327 the nature of the metabolites found was the same as that of those previously reported in
328 studies of the metabolic fate of PAs, a process described in detail elsewhere (Mosele,
329 Macià, & Motilva, 2015). This shows that the same tendencies are maintained for long-
330 term supplementation.

331 We obtained an unexpected result, in that microbial-derived PA metabolites increased (in
332 urine and in feces), as did some EC conjugates, in the ω -3 group (without GSE
333 supplementation). Since these metabolites are not present in the metabolic routes of ω -3
334 PUFAs, it seems that ω -3 PUFAs collaborated in the transformation of polyphenols already
335 present in the basic STD diet (and responsible for the values obtained in the STD group).
336 The STD diet contained wheat middlings, ground wheat and ground corn; cereals that
337 include PAs among their phenolic compounds (McCallum, & Walker, 1990; Hichem,
338 Mounir, & Naceur, 2009; Arranz, & Saura-Calixto, 2010). Indeed, the STD diet contains
339 not only the PAs that occur free in the food matrix (known as extractable PAs), but also the
340 fraction associated with the dietary matrix, the so-called non-extractable PAs (Arranz, &
341 Saura-Calixto, 2010) which are also extensively metabolized by the colonic microbiota
342 after being released from the food matrix (Mateos-Martín, Pérez-Jiménez, Fuguet, &
343 Torres, 2012b). Our results suggest that ω -3 PUFAs may promote PA metabolism through
344 interactions with the gut microbiota. This point needs to be addressed further, as the
345 information available to date is quite limited. It seems that fish oil supplementation
346 increases the proportion of *Lacotobacilliales*, as observed in rats with chronic intestinal
347 rejection (Li, Zhang, Wang, Tang, Zhang, & Li, 2011), in a murine model of colorectal
348 cancer (Piazzi et al., 2014) and in gnotobiotic piglets, in this last case with an increase in
349 *Lactobacillus paracasei* adhesion to the jejunal mucosa (Bomba, Nemcová, Gankarciková,
350 Herich, Guba, & Mudronová, 2002). In contrast, PUFA supplementation of animal models
351 resulted in a decrease in *Escherichia coli*, *Bacteroides* spp. and *Clostridiales* spp., among
352 others (Li et al., 2011; Yu, Zhu, Pan, Shen, Shan, & Das, 2014). Interestingly,
353 *Lactobacillus plantarum* has been reported to stimulate the colonic fermentation of red
354 wine polyphenols (Barroso et al., 2014), which are quite similar to the PAs included in the
355 GSE tested here.

356 In the present study, the STD diet had a soybean oil content similar to that of PUFAs in the
357 ω -3 diet, in order to make them isocaloric. Since the main constituents of soybean oil are
358 monounsaturated fatty acids (followed by PUFAs), the changes observed in the microbial
359 metabolism of PAs also indicate differences in the microbiota profile when consuming
360 monounsaturated or polyunsaturated fats. Indeed, when comparing the effects on the
361 human gut microbiome of three kinds of monounsaturated fats with those of two kinds of
362 polyunsaturated fats, the modifications in the bacteria profile that resulted from the
363 consumption of monounsaturated fats were observed to be consistent with those derived
364 from PUFA consumption (Pu, Khazenehei, Jones, & Khapifaur, 2016).

365 Additionally, since cereals contain phenolic acids as major phenolic compounds, and these
366 generate several metabolites in common with those of PAs (Rodríguez-Mateos et al.,
367 2014), it seems plausible that ω -3 PUFAs would also stimulate the release and
368 transformation of such compounds. Additionally, some metabolites, such as hippuric or
369 valerolactones, may originate from other food components (Pero, 2010; Molinar-Toribio et
370 al., *in press*), so their increase in the ω -3 group might be due to an effect on other
371 metabolic routes, becoming particularly evident in a long-term study such as this one.

372 Another interesting result of this study was that, while GSE contained mostly (95%)
373 oligomeric PAs, in the groups supplemented with it there was an increase in the monomeric
374 conjugated metabolites of EC. This agrees with our previous suggestion (Pérez-Jiménez et
375 al., 2010) of a depolymerization of PAs by bacteria, releasing free EC which would then be
376 subjected to further absorption and conjugation. ω -3 PUFAs also seem to affect the activity
377 of the bacteria responsible for this, since in the ω -3 groups there was also a tendency
378 towards an increase in these compounds, as compared to the STD group.

379 When GSE and ω -3 PUFAs were administered together, the concentrations of the detected
380 metabolites were in the same range than those found in the GSE group, without significant

381 differences. Therefore, ω -3 PUFAs did not have either an enhancing or inhibitory effect on
382 a diet supplemented with grape PA, despite the enhancing effects they showed towards the
383 transformation of polyphenols already present in the basal diet. It is known that polyphenol
384 supplementation causes a shift in microbial communities towards those species indeed able
385 to transform them, e.g. the *Eubacterium rectale* group (Selma, Espín, & Tomás-Barberán,
386 2009; Queipo-Ortuño et al., 2012). We hypothesize that the changes that ω -3 PUFAs may
387 cause in the microbiota, and that had an effect in the transformation of polyphenols already
388 present in the basal diet, may not be relevant against the modifications that polyphenols
389 themselves cause in the microbiota when provided at high doses.

390 Anyway, the concentration values for the pool of putatively beneficial circulating PA-
391 derived metabolites in the ω -3+GSE group were in the same range than in the GSE group.
392 So, PA metabolites from GSE are bioavailable for possible collaborative functional effects
393 with ω -3 PUFAs. Indeed, when evaluating the effects of ω -3 PUFAs and GSE on the
394 metabolic alterations induced by a high-fat high-sucrose diet, it was observed that the
395 combination was more efficient than the separate supplements at averting metabolic
396 alterations (Ramos-Romero et al., 2016).

397 The main limitations of this study are intrinsic to experiments on the metabolic fate of
398 polyphenols. First, there is a lack of commercial standards for many metabolites (Kay,
399 2010), which forced us to express the results as equivalents of the most closely related
400 compound, with an associated error. Secondly, we had to deal with the high inter-
401 individual variability of results; an aspect widely reported for the metabolic fate of
402 polyphenols in both animals and humans (Choy et al., 2014; Muñoz-González et al., 2014).
403 This latter aspect may have been exaggerated in this study, given that it involved long-term
404 supplementation, where the measured concentrations for each metabolite did not
405 correspond to the maxima and it is not known when each animal received the last dose of

406 polyphenols before fasting, since they were fed *ad libitum*. Also, more animals would have
407 increased the statistical power of the test thereby increasing the likelihood of detecting
408 statistically significant differences in the metabolites. Be that as it may, this did not
409 preclude us from observing the emergence of some general tendencies, as discussed above;
410 while at the same time, it has the advantage of reflecting a situation closer to a genuine
411 human dietary situation.

412 **5. CONCLUSIONS**

413 This study shows that combined long-term supplementation with ω -3 PUFAs and PAs from
414 GSE to healthy rats did not significantly affect the levels of urinary and fecal PA
415 metabolites, compared to supplementation with GSE alone. Meanwhile, ω -3 fatty acids
416 seem to enhance the metabolism of the polyphenols present in the STD feed. ω -3 PUFAs
417 appear to collaborate in the release and metabolism of polyphenols when they are present
418 at low doses in the feed matrix, while their capacity to induce transformations when
419 polyphenols are added at high doses does not seem to be relevant.

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429 **AUTHORS' CONTRIBUTIONS**

430 I.M., J.L.T. and J.P.-J. designed the research. E.M.-T., S.R.-R., N.T., M.R., L.M., and J. P.-
431 J. conducted the research. E.M.-T., S.R.-R., E.F. and J.P.-J. analyzed the data. J.P.-J. and
432 J.L.T. wrote the first draft of the manuscript. All the authors contributed to writing the
433 manuscript and approved the final version. J.P.-J. and J.L.T. had primary responsibility for
434 final content.

435

436 **CONFLICT OF INTEREST**

437 None of the authors declare any conflict of interest.

438

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TABLES

Table 1. (Epi)catechin and (epi)gallocatechin conjugated metabolites in urine from rats fed a standard diet without supplementation (STD) or supplemented with ω -3 PUFAs (ω -3), grape seed extract (GSE) or ω -3 PUFAs and grape seed extract (ω -3 + GSE)¹. Results expressed as μ M, adjusted per kg feed intake/kg body weight, after quantification with structurally similar commercial standards (see Table S3).

| Metabolite | STD ² | | ω -3 | | | GSE ² | | | ω -3 + GSE | | |
|--------------------------|------------------|------|-------------|------|---------------------|------------------|------|------------------------|-------------------|------|--|
| | Mean | SEM | Mean | SEM | <i>P</i> | Mean | SEM | <i>P</i> | Mean | SEM | <i>P</i> |
| <i>EC monoconjugated</i> | | | | | | | | | | | |
| Gluc-EC-1 | n.d. | | n.d. | | | 5.68 | 2.87 | 0.0081 ^{a, b} | 6.04 | 5.21 | 0.0081 ^{a, b} |
| Gluc-EC-2 | n.d. | | n.d. | | | 3.84 | 1.93 | 0.0321 ^{a, b} | 3.29 | 3.18 | |
| Gluc-EC-3 | 0.64 | 0.32 | 8.00 | 1.40 | 0.0081 ^a | 3.09 | 1.21 | | 15.55 | 5.37 | 0.0081 ^a 0.0321 ^b |
| Gluc-EC-4 | 0.32 | 0.09 | 2.83 | 0.75 | 0.0081 ^a | 0.95 | 0.47 | | 4.49 | 1.60 | 0.0081 ^a 0.0321 ^c |
| Gluc-EC-5 | 0.88 | 0.44 | 3.67 | 0.80 | 0.0321 ^a | 1.96 | 1.03 | | 6.34 | 1.86 | 0.0161 ^a |

| | | | | | | | | | | | |
|-------------------------|------|------|-------|------|---------------------|-------|------|-----------------------|-------|-------|-----------------------|
| Total | 1.84 | 0.61 | 14.50 | 2.86 | 0.0081 ^a | 15.52 | 6.24 | 0.0161 ^a | 35.72 | 16.64 | 0.0081 ^a |
| <i>EC diconjugated</i> | | | | | | | | | | | |
| Gluc-Sulf-EC | 0.97 | 0.40 | 1.05 | 0.20 | | 3.54 | 1.62 | 0.0321 ^b | 2.99 | 1.17 | |
| Me-Gluc-EC-1 | n.d. | | n.d. | | | 0.85 | 0.28 | 0.0081 ^{a,b} | 1.39 | 0.98 | 0.0081 ^{a,b} |
| Me-Gluc-EC-2 | n.d. | | n.d. | | | 14.79 | 4.88 | 0.0081 ^{a,b} | 13.61 | 9.21 | 0.0081 ^{a,b} |
| Me-Gluc-EC-3 | n.d. | | n.d. | | | 5.26 | 1.68 | 0.0081 ^{a,b} | 5.79 | 4.10 | 0.0081 ^{a,b} |
| Me-Sulf-EC | 2.46 | 0.84 | 2.00 | 0.32 | | 3.03 | 0.58 | 0.0081 ^{a,b} | 3.85 | 1.27 | 0.0161 ^a |
| | | | | | | | | | | | 0.0081 ^b |
| Total | 3.43 | 1.23 | 3.05 | 0.47 | | 27.47 | 8.09 | 0.0081 ^{a,b} | 27.64 | 16.33 | 0.0161 ^a |
| | | | | | | | | | | | 0.0081 ^b |
| <i>EC triconjugated</i> | | | | | | | | | | | |
| 3Me-EC | 1.09 | 0.27 | 0.94 | 0.17 | | 1.07 | 0.30 | | 1.65 | 0.48 | |
| 2Me-Gluc-EC | 0.63 | 0.23 | 0.86 | 0.29 | | 1.51 | 0.37 | | 1.73 | 0.75 | |
| Total | 1.72 | 0.47 | 1.80 | 0.46 | | 2.58 | 0.58 | | 3.38 | 1.17 | |

| | | | | | | | | | | |
|--------------------------|-------|------|-------|------|--|-------|------|--|-------|------|
| <i>EGC diconjugated</i> | | | | | | | | | | |
| 2Sulf-EGC | 14.16 | 2.81 | 15.55 | 2.71 | | 21.69 | 4.18 | | 27.72 | 8.75 |
| <i>EGC triconjugated</i> | | | | | | | | | | |
| Me-Gluc-Sulf-EGC | 3.95 | 1.23 | 8.71 | 1.29 | | 8.17 | 0.98 | | 16.17 | 5.95 |

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

¹ Values are mean \pm SEM , $n=5$

² Molinar-Toribio et al., *in press*.

^a differences with respect to STD group; ^b differences with respect to ω -3 group; ^c differences with respect to GSE group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests; pairs comparisons were performed between all the groups.

Table 2. Microbial-derived proanthocyanidin metabolites in urine from rats fed a standard diet without supplementation (STD) or supplemented with ω -3 PUFAs (ω -3), grape seed extract (GSE) or ω -3 PUFAs and grape seed extract (ω -3 + GSE) ¹. Results expressed as μ M, adjusted per kg feed intake/kg body weight, after quantification with structurally similar commercial standards (see Table S3).

| Metabolite | STD ² | | ω -3 | | | GSE ² | | | ω -3 + GSE | | |
|---|------------------|--------|-------------|--------|---------------------|------------------|--------|-----------------------|-------------------|---------|---------------------|
| | Mean | SEM | Mean | SEM | <i>P</i> | Mean | SEM | <i>P</i> | Mean | SEM | <i>P</i> |
| <i>Valerolactones</i> | | | | | | | | | | | |
| 3- or 4-Hydroxyphenylvalerolactone | 24.17 | 7.33 | 205.81 | 15.67 | 0.0081 ^a | 379.24 | 129.69 | 0.0081 ^a | 604.60 | 268.55 | 0.0081 ^a |
| 3,4-Dihydroxyphenylvalerolactone | 5.97 | 4.09 | 89.81 | 26.91 | 0.0081 ^a | 219.53 | 73.37 | 0.0081 ^a | 595.09 | 281.69 | 0.0081 ^a |
| Gluc-3,4-dihydroxyphenylvalerolactone | 51.48 | 15.61 | 133.16 | 41.14 | | 164.87 | 29.47 | 0.0081 ^a | 318.25 | 115.12 | 0.0321 ^a |
| Sulf-3,4-dihydroxyphenylvalerolactone | 15.92 | 9.76 | 535.46 | 98.76 | 0.0081 ^a | 1373.86 | 117.59 | 0.0081 ^{a,b} | 1638.69 | 604.44 | 0.0081 ^a |
| 3-Hydroxyphenylmethylvalerolactone | 24.17 | 7.20 | 32.31 | 4.5 | | 93.35 | 26.46 | 0.0081 ^a | 56.64 | 16.46 | |
| | | | | | | | | 0.0321 ^b | | | |
| 4-Hydroxyphenylmethylvalerolactone | 214.92 | 73.50 | 341.12 | 59.26 | | 628.27 | 174.41 | 0.0321 ^a | 481.06 | 125.84 | |
| Gluc-3-hydroxymethylphenylvalerolactone | 112.73 | 42.56 | 128.90 | 58.41 | | 171.21 | 23.38 | 0.0321 ^b | 170.88 | 57.94 | |
| Sulf-3- or 4-hydroxymethylphenylvalerolactone | 69.40 | 23.06 | 37.77 | 7.81 | | 91.63 | 16.42 | | 77.34 | 22.49 | |
| Total | 517.06 | 103.86 | 1504.34 | 305.22 | 0.0081 ^a | 3121.97 | 446.61 | 0.0081 ^a | 3987.54 | 1393.77 | 0.0081 ^a |
| | | | | | | | | 0.0321 ^b | | | |

| | | | | | | | | | | | |
|---|---------|---------|---------|--------|---------------------|----------|---------|--|---------|---------|---------------------|
| <i>Lignans</i> | | | | | | | | | | | |
| Enterolactone | >60 | | >60 | | | >60 | | | >60 | | |
| Sulf-enterolactone | >60 | | >60 | | | >60 | | | >60 | | |
| <i>Phenylvaleric acids</i> | | | | | | | | | | | |
| 3-Hydroxyphenylvaleric acid | 31.10 | 13.42 | 20.63 | 5.25 | | 102.08 | 15.47 | 0.0161 ^a 0.0081 ^b | 157.89 | 73.49 | 0.0321 ^b |
| 4-Hydroxyphenylvaleric acid | 4.63 | 1.71 | 17.37 | 3.74 | 0.0321 ^a | 47.35 | 15.97 | 0.0081 ^a | 28.53 | 4.43 | 0.0081 ^a |
| 3,4-Dihydroxyphenylvaleric acid | 8.74 | 2.49 | 70.33 | 18.25 | 0.0081 ^a | 47.14 | 12.24 | 0.0161 ^a | 86.66 | 22.09 | 0.0081 ^a |
| Sulf-3,4-dihydroxyphenylvaleric acid | 28.02 | 11.34 | 530.70 | 160.71 | 0.0081 ^a | 1053.66 | 220.98 | 0.0081 ^a | 1061.01 | 403.49 | 0.0081 ^a |
| Total | 72.49 | 23.05 | 639.03 | 182.16 | 0.0081 ^a | 1250.23 | 223.86 | 0.0081 ^a | 1334.09 | 482.74 | 0.0081 ^a |
| <i>Phenylpropionic acids</i> | | | | | | | | | | | |
| 3-Hydroxyphenylpropionic acid | 6638.51 | 2746.72 | 4097.84 | 597.18 | | 14257.84 | 9274.35 | | 8957.36 | 2307.88 | |
| 4-Hydroxyphenylpropionic acid | >60 | | >60 | | | >60 | | | >60 | | |
| Gluc-3- or- 4hydroxyphenylpropionic acid | 21.34 | 12.93 | 13.93 | 6.64 | | 17.10 | 2.09 | | 28.36 | 11.52 | |
| Dihydrocaffeic acid (3,4- Dihydroxyphenylpropionic acid) | 3.65 | 1.60 | 10.49 | 2.65 | | 56.85 | 45.95 | 0.0321 ^a | 20.82 | 5.86 | |
| Sulf-3,4-dihydrocaffeic acid | 39.65 | 15.31 | 41.14 | 10.75 | | 120.60 | 76.86 | | 60.74 | 15.85 | |
| Total ² | 670.47 | 2751.81 | 4163.41 | 610.83 | | 14452.40 | 9397.29 | | 9067.28 | 2334.52 | |

Phenylacetic acids

| | | | | | | | | | | | |
|-------------------------------------|--------|-------|--------|--------|---------------------|---------|--------|---------------------|---------|--------|---------------------|
| 3-Hydroxyphenylacetic acid | 63.14 | 26.56 | 324.13 | 67.53 | 0.0081 ^a | 427.27 | 67.29 | 0.0081 ^a | 838.83 | 284.14 | 0.0321 ^a |
| 4-Hydroxyphenylacetic acid | 60.97 | 23.56 | 532.53 | 142.90 | 0.0081 ^a | 1816.39 | 312.24 | 0.0081 ^a | 1859.17 | 585.26 | 0.0081 ^a |
| | | | | | | | | 0.0321 ^b | | | |
| 3,4-Dihydroxyphenylacetic acid | 0.86 | 0.36 | 3.09 | 0.61 | 0.0161 ^a | 10.27 | 5.04 | 0.0081 ^a | 16.65 | 4.87 | 0.0161 ^a |
| Sulf-3,4-dihydroxyphenylacetic acid | 7.79 | 4.41 | 8.91 | 1.48 | | 8.41 | 1.90 | | 14.19 | 4.19 | |
| Total | 132.77 | 46.12 | 868.66 | 170.81 | 0.0081 ^a | 2262.34 | 292.60 | 0.0081 ^a | 2728.84 | 745.56 | 0.0081 ^a |
| | | | | | | | | 0.0161 ^b | | | |

Benzoic acids

| | | | | | | | | | | | |
|--------------------------------|--------|-------|--------|--------|---------------------|--------|--------|---------------------|---------|--------|---------------------|
| 4-Hydroxybenzoic acid | 13.88 | 5.56 | 78.01 | 23.75 | 0.0081 ^a | 63.86 | 20.04 | 0.0321 ^a | 52.31 | 13.40 | |
| 3,4-Dihydroxybenzoic acid | 0.36 | 0.22 | 7.16 | 2.72 | 0.0161 ^a | 21.35 | 9.80 | 0.0081 ^a | 19.36 | 11.58 | 0.0081 ^a |
| Gluc-3-hydroxybenzoic acid | 0.24 | 0.13 | 1.04 | 0.34 | | 2.73 | 1.17 | 0.0081 ^a | 1.77 | 0.50 | 0.0321 ^a |
| Gluc-4-hydroxybenzoic acid | 0.02 | 0.01 | 0.14 | 0.03 | 0.0321 ^a | 0.31 | 0.09 | 0.0081 ^a | 0.26 | 0.17 | 0.0321 ^a |
| Sulf-3,4-dihydroxybenzoic acid | 6.52 | 2.08 | 20.11 | 3.45 | 0.0081 ^a | 69.10 | 37.61 | 0.0081 ^a | 34.97 | 9.90 | 0.0321 ^a |
| Sulf-vanillic-acid | 327.72 | 64.53 | 681.32 | 200.67 | | 459.50 | 78.29 | | 1432.01 | 507.86 | |
| Total | 348.74 | 60.23 | 787.78 | 228.73 | | 616.85 | 129.27 | 0.0161 ^a | 1540.69 | 539.51 | |

Cinnamic acids

| | | | | | | | | | | | |
|--------------|------|------|------|------|--|------|------|--|------|------|--|
| Caffeic acid | 0.95 | 0.44 | 1.18 | 0.74 | | 3.07 | 1.85 | | 1.39 | 0.45 | |
|--------------|------|------|------|------|--|------|------|--|------|------|--|

| | | | | | | | | | | | |
|---------------------------------|--------|-------|---------|--------|---------------------|---------|---------|---------------------|---------|--------|---------------------|
| <i>m</i> -Coumaric acid | 116.69 | 54.97 | 102.91 | 29.16 | | 247.53 | 30.90 | | 140.64 | 47.78 | |
| <i>p</i> -Coumaric acid | 24.03 | 8.90 | 20.41 | 10.93 | | 32.90 | 9.37 | | 46.87 | 31.48 | |
| Sulf-coumaric acid-1 | n.d. | | 6.53 | 4.78 | 0.0081 ^a | 14.05 | 4.77 | 0.0081 ^a | 7.67 | 5.87 | 0.0081 ^a |
| Sulf-coumaric acid-2 | 0.04 | 0.01 | 7.00 | 5.09 | 0.0321 ^a | 13.37 | 4.17 | 0.0081 ^a | 7.53 | 5.76 | 0.0081 ^a |
| Ferulic acid | 15.31 | 6.21 | 18.23 | 15.20 | | 20.30 | 7.54 | | 29.46 | 14.32 | |
| Total | 157.02 | 65.75 | 156.26 | 65.01 | | 331.22 | 44.11 | | 235.56 | 101.24 | |
| <i>Glycinated benzoic acids</i> | | | | | | | | | | | |
| Hippuric acid | 48.02 | 17.74 | 1385.71 | 396.40 | 0.0081 ^a | 2219.53 | 1311.56 | 0.0081 ^a | 1494.77 | 317.90 | 0.0081 ^a |
| Hydroxyhippuric acid | 0.29 | 0.26 | 14.31 | 5.03 | 0.0081 ^a | 16.99 | 3.57 | 0.0081 ^a | 18.23 | 5.35 | 0.0081 ^a |
| Me-hippuric acid-1 | 0.15 | 0.15 | 67.03 | 54.54 | 0.0081 ^a | 94.37 | 35.88 | 0.0081 ^a | 73.06 | 57.30 | 0.0081 ^a |
| Me-hippuric acid-2 | 2.91 | 1.22 | 5.49 | 4.84 | | 14.05 | 7.41 | | 16.62 | 13.76 | |
| Total | 51.37 | 18.50 | 1472.53 | 443.46 | 0.0081 ^a | 2344.94 | 1313.91 | 0.0081 ^a | 1602.68 | 328.88 | 0.0081 ^a |

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

Compounds detected in all the groups above the highest concentration of the calibration: Enterolactone, Sulf-Enterolactone and 4-Hydroxyphenylpropionic acid

¹ Values are mean \pm SEM, $n=5$

² Molinar-Toribio et al., *in press*.

^a differences with respect to STD group; ^b differences with respect to ω -3 group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests; pairs

comparisons were performed between all the groups.

Table 3. Microbial-derived proanthocyanidin metabolites in feces from rats fed a standard diet without supplementation (STD) or supplemented with ω -3 PUFAs (ω -3), grape seed extract (GSE) or ω -3 PUFAs and grape seed extract (ω -3 + GSE)¹. Results expressed as $\mu\text{mol/g}$ dried feces, adjusted per kg feed intake/kg body weight, after quantification with structurally similar commercial standards (see Table S3).

| Metabolite | STD ² | | ω -3 | | | GSE ² | | | ω -3 + GSE | | |
|-------------------------------|------------------|-------|-------------|--------|---------------------|------------------|--------|---------------------|-------------------|---------|---------------------|
| | Mean | SEM | Mean | SEM | <i>P</i> | Mean | SEM | <i>P</i> | Mean | SEM | <i>P</i> |
| <i>Lignans</i> | | | | | | | | | | | |
| Enterolactone | > 60 | | > 60 | | | > 60 | | | > 60 | | |
| <i>Phenylvaleric acids</i> | | | | | | | | | | | |
| 3-Hydroxyphenylvaleric acid | 3.14 | 1.47 | 78.48 | 36.55 | 0.0161 ^a | 985.26 | 872.17 | 0.0081 ^a | 975.65 | 485.91 | 0.0081 ^a |
| <i>Phenylpropionic acids</i> | | | | | | | | | | | |
| 3-Hydroxyphenylpropionic acid | 45.05 | 3.84 | 35.68 | 23.50 | 0.0161 ^a | 101.96 | 93.02 | 0.0081 ^a | 12.60 | 0.00 | 0.0081 ^a |
| 4-Hydroxyphenylpropionic acid | 10.91 | 2.77 | 381.06 | 242.99 | 0.0321 ^a | 41.15 | 32.21 | | 2033.79 | 1653.65 | 0.0321 ^a |
| Total | 55.96 | 41.61 | 416.73 | 246.22 | | 143.10 | 90.51 | | 2046.39 | 1653.65 | |
| <i>Benzoic acids</i> | | | | | | | | | | | |

| | | | | | | | | | | | |
|---------------------------------|------|------|--------|--------|---------------------|----------|----------|-----------------------|---------|--------|-----------------------|
| 4-hydroxybenzoic acid | n.d. | | 0.15 | 0.10 | | 1.06 | 0.75 | 0.0081 ^a | 3.94 | 1.68 | 0.0081 ^a |
| 3,4-Dihydroxybenzoic acid | n.d. | | n.d. | | | 0.22 | 0.16 | 0.0081 ^{a,b} | 0.26 | 0.14 | 0.0321 ^{a,b} |
| Total | n.d. | | 0.15 | 0.10 | | 1.27 | 0.91 | 0.0081 ^a | 4.20 | 1.72 | 0.0081 ^a |
| <i>Cinnamic acids</i> | | | | | | | | | | | |
| Caffeic acid | 0.01 | 0.01 | 0.51 | 0.29 | 0.0081 ^a | 0.53 | 0.41 | 0.0081 ^a | 1.55 | 0.67 | 0.0081 ^a |
| <i>p</i> -coumaric acid | 0.04 | 0.02 | 1.66 | 1.02 | | 2.72 | 2.06 | 0.0321 ^a | 4.83 | 1.76 | 0.0321 ^a |
| Total | 0.05 | 0.03 | 2.16 | 1.31 | 0.0321 ^a | 3.25 | 2.47 | 0.0321 ^a | 6.38 | 2.41 | 0.0161 ^a |
| <i>Glycinated benzoic acids</i> | | | | | | | | | | | |
| Hippuric acid | 0.05 | 0.03 | 0.75 | 0.75 | | 0.04 | 0.03 | | 1.50 | 0.76 | |
| Me-hippuric acid-1 | 6.54 | 5.23 | 486.95 | 453.35 | | 12279.53 | 11463.14 | | 1023.35 | 584.48 | |
| Me-hippuric acid-2 | n.d. | | 269.32 | 165.44 | 0.0081 ^a | 6283.37 | 5735.59 | 0.0081 ^a | 137.49 | 119.35 | 0.0081 ^a |

| | | | | | | | | | | |
|-------|------|------|--------|--------|----------|----------|---------------------|---------|--------|---------------------|
| Total | 6.59 | 5.24 | 757.01 | 567.91 | 18562.94 | 17211.60 | 0.0081 ^a | 1162.33 | 554.38 | 0.0081 ^a |
|-------|------|------|--------|--------|----------|----------|---------------------|---------|--------|---------------------|

n.d., non-detected; Gluc, glucuronide; Me, methyl; Sulf, sulfated

¹ Values are mean \pm SEM , $n=5$

² Molinar-Toribio et al., *in press*.

^a differences with respect to STD group; ^b differences with respect to ω -3 group. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney U tests; pairs comparisons were performed between all the groups.