1   *Lactobacillus plantarum* **IFPL935 impacts colonic metabolism in a simulator of the**

2   **human gut microbiota during feeding with red wine polyphenols**

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Abstract

The colonic microbiota plays an important role in the bioavailibility of dietary polyphenols. This work has evaluated the impact on the gut microbiota of long-term feeding with both a red wine polyphenolic extract and the flavan-3-ol metabolizer strain *Lactobacillus plantarum* IFPL935. The study was conducted in the dynamic Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The feeding of the gut microbiota model with red wine polyphenols caused an initial decrease in the counts of total bacteria in the ascending colon (AC), being *Bacteroides*, *Clostridium coccoides*/*Eubacterium rectale* and *Bifidobacterium* the most affected bacterial groups. The bacterial counts recovered to initial numbers faster than the overall microbial fermentation and proteolysis, which seemed to be longer affected by polyphenols. Addition of *L. plantarum* IFPL935 helped to promptly recover total counts, *Lactobacillus* and *Enterobacteriaceae* and led to an increase in lactic acid formation in the AC vessel at the start of the polyphenol treatment as well as butyric acid in the transverse (TC) and descending (DC) vessels after 5 days. Besides, *L. plantarum* IFPL935 favoured the conversion in the DC vessel of monomeric flavan-3-ols and their intermediate metabolites into phenylpropionic acids and in particular 3-(3´-hydroxyphenyl)propionic acid. The results open possibilities of using *L. plantarum* IFPL935 as a food ingredient for helping individuals showing a low polyphenol-fermenting metabotype to increase their colonic microbial capacities of metabolizing dietary polyphenols.

Keywords *Lactobacillus*, polyphenol, colonic metabolism, probiotic, intestinal microbiota

Introduction

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50 Moderate consumption of red wine and/or regular consumption of other polyphenol-rich

51 beverages and foods has proved in epidemiological studies to reduce the incidence of

52 certain chronic diseases (Arranz et al. 2012; Kishimoto et al. 2013). Although the health

53 effects of food polyphenols have been repeatedly associated with their free radical

54 scavenging and antioxidant activity, recent evidence has, however, indicated that the

55 effects of antioxidants are less relevant than expected (Tomás-Barberán and Andrés-

56 Lacueva 2012). The bioavailability, absorption and metabolism of polyphenolic

57 compounds are, indeed, key issues required to explain their role in human health.

58 Depending on their chemical structure and food matrix, a high percentage of dietary

59 polyphenols are not absorbed in the small intestine, reaching the colon where they are

60 metabolized by the gut microbiota before being absorbed. Likewise, polyphenols and

61 their resultant metabolites may selectively modulate the gut microbial composition by

62 their antimicrobial or eventually prebiotic-like properties (Queipo-Ortuño et al. 2012;

63 Tzounis et al. 2008). In this sense, an increase in *Lactobacillus* and *Bifidobacterium*

64 species following administration of red wine polyphenols has been reported (Dolara et

65 al. 2005; Queipo-Ortuño et al. 2012). Moreover, a significant correlation of moderate

66 wine consumption with human microbiome composition, but not with enterotype

67 partitioning, has been established (Wu et al., 2011).

68　　　　Flavonoids are the most abundant polyphenols present in red wine, mainly

69 including flavan-3-ols and anthocyanins (Waterhouse 2002). There are several

70 metabolic pathways proposed for the catabolism of monomeric flavan-3-ol and dimeric

71 procyanidins by the intestinal microbiota (Monagas et al. 2010; Selma et al. 2009).

72 However, bacterial degradation of flavan-3-ols is hindered by the inherent antibacterial

73 effects of these molecules. In previous studies, we have reported that *L. plantarum*

74 IFPL935 was capable to metabolize galloylated flavan-3-ols leading to the formation of

75 gallic acid, pyrogallol and catechol through galloyl-esterase, decarboxylase and benzyl

76 alcohol dehydrogenase enzyme activities, respectively (Tabasco et al. 2011). The most

77 remarkable feature of *L. plantarum* IFPL935 was its ability to cleave the heterocyclic

78 ring of monomeric flavan-3-ols, giving rise to 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-

79 trihydroxyphenyl)-propan-2-ol (i.e. diphenylpropanol), which is the first metabolite in

80 the microbial catabolic pathway of flavan-3-ols (Sánchez-Patán et al. 2012b). This

81 activity has only been reported in a few intestinal bacteria to date, such as *Eubacterium*

82 sp. SDG-2, *Eggerthella lenta*, and *Flavonifractor plautii* (Jin and Hattori 2012;

83 Kutschera et al. 2011; Wang et al. 2001). The capability of *L. plantarum* IFPL935 to

84 initiate the catabolism of flavan-3-ols via the formation of diphenylpropanol has also

85 been demonstrated when the strain was incubated in batches with a complex human

86 intestinal microbiota (Barroso et al., 2013). It was also observed that the addition of this

87 strain had an impact on the formation of butyric acid, probably through cross-feeding

88 with colonic butyrate-producing bacteria (Barroso et al., 2013).

89 To get a deeper insight on the potential use of *L. plantarum* IFPL935 to benefit

90 polyphenols metabolism we have monitored the effect on the gut microbiota upon

91 addition of 10^{10} cfu of the strain to a daily intake of a polyphenolic extract (equivalent

92 to two 125 ml-glasses of alcohol-free red wine) in a long-term feeding experiment. The

93 study was conducted in the dynamic multireactor gastrointestinal Simulator of the

94 Human Intestinal Microbial Ecosystem (SHIME).

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Materials and methods

Simulator of the Human Intestinal Microbial Ecosystem (Twin-SHIME)

In this study, a Twin-SHIME® (UGent/ProDigest) setup was used by operating two parallel SHIME systems, each one consisting of five consecutive reactors, simulating the stomach, small intestine, ascending colon (AC), transverse colon (TC), and descending colon (DC), as described by Van den Abbeele et al. (2010). At the beginning of the experiment, the AC, TC and DC vessels from the Twin-SHIME systems were all simultaneously inoculated with the same fecal sample from a healthy human volunteer previously identified as a flavan-3-ol metabolizer (unpublished results) to enable comparison of the results between the two parallel experiments. The colonic microbiota was allowed for stabilization reaching the steady state after 3 weeks. The inoculum preparation and the SHIME feed composition during the stabilization period were essentially as described by De Boever et al. (2004). After stabilization of the colonic microbiota, both Twin-SHIME systems were subjected to a 2-week experiment by daily feeding the stomach compartments with 200 mg of a commercial red wine extract, Provinols^{TM} (Safic-Alcan Especialidades, Barcelona, Spain), which corresponds to a daily polyphenol intake of two small glasses of red wine (250 ml). The total phenolic content of the red wine extract was 474 mg of gallic acid equivalents per g (2.79 mmol/g) and its composition was reported before (Sánchez-Patán et al. 2012a), being flavan-3-ols the main phenolic compounds. In addition, one of the Twin-SHIME systems was simultaneous fed with a daily dose of 10^{10} cfu of *Lactobacillus plantarum* IFPL935. Finally, a 1-week wash-out period was included at the end of the experiment for both systems. During the whole study, samples were collected at regular time points

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120 from the three colon vessels and stored at −20 °C until further analysis, excepting for

121 microbiological plate counts that were performed at the time of sampling.

122

123 Bacterial culture conditions and plate counts

124 *Lactobacillus plantarum* IFPL935 (CECT 4599) was routinely grown aerobically at 37

125 °C in MRS broth. To reach the daily supplementation of 10^{10} cfu of IFPL935, 10 ml of

126 *L. plantarum* IFPL953 grown to 10^9 cfu/ml were added daily to the stomach

127 compartment in one of the SHIME systems, right after the addition of the red wine

128 extract. Numbers of lactobacilli in the inoculum were counted using MRS-agar plates

129 and in the AC, TC and DC vessels using LAMVAB agar (Hartemink et al. 1997), and

130 plates were incubated aerobically at 37 °C for 48 h. Total aerobes and total anaerobes

131 were determined by plating on BHI agar and incubation at 37 °C for 24 h aerobically or

132 72 h anaerobically, respectively. Total coliforms were enumerated in MacConkey agar

133 incubated for 24 h aerobically at 37 °C.

134

135 *DNA extraction and analysis*

136 Bacterial DNA was extracted using hexadecyltrimethylammonium bromide (CTAB)

137 buffer and phenol-chloroform-isoamyl alcohol and bead-beating, as described

138 previously (Griffiths et al. 2000; Kowalchuk et al. 1998). The DNA was precipitated

139 with polyethelene glycol (PEG-6000), washed in ice cold 70% ethanol and dried in a

140 Speed-Vac, prior to resuspension in distilled water. The concentration and quality of the

141 samples were assessed with a Nanodrop spectrophotometer (Thermo Fisher Scientific,

142 Wilmington, DE, USA).

143 *Quantitative PCR (qPCR)*

144 Bacterial numbers in the AC, TC and DC vessels were quantified by qPCR using SYBR

145 green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor

146 Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc.,

147 Hercules, CA, USA). The bacterial groups targeted for qPCR were total bacteria,

148 *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae*, and the specific

149 phylogenetic groups *Clostridium coccoides-Eubacterium rectale* Cluster XIVa,

150 *Ruminococcus* Cluster IV, and *Clostridium leptum* subgroup specific cluster IV.

151 Butyrate-producing bacteria were also estimated by quantifying the gene encoding

152 butyryl-CoA:acetate CoA transferase (BcoAT). Primers, amplification conditions and

153 calculation of copy numbers have been detailed previously (Barroso et al. 2013). DNA

154 from *Escherichia coli* DH5α, *L. plantarum* IFPL935, *Bifidobacterium breve* 29M2 and

155 *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria,

156 *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, respectively. For the rest of groups

157 analyzed, samples were quantified using standards derived from targeted cloned genes

158 using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as

159 described previously (Barroso et al., 2013).

160 *PCR-DGGE*

161 The diversity of the *Lactobacillus* community in the AC, TC and DC vessels and the

162 presence of *L. plantarum* were assessed by PCR-DGGE and using the primers Lab-

163 159F and Uni-515-GC-R, as described by Heilig et al. (2002). DGGE was performed

164 with a DCode system (Bio-Rad) using a 9% polyacrylamide gel with a 30–50% gradient

165 of 7 M urea and 40% formamide. The obtained band patterns were analyzed using

166 InfoQuest FP software version 5.1 (Bio-Rad). Clustering was performed with Pearson

167 correlation and the UPGMA method.

168

169 Bacterial metabolism

170 *Analysis of short and branched-chain fatty acids (SCFA and BCFA), lactic acid and*

171 *ammonium*

172 The SCFA and BCFA were extracted from the samples with diethyl ether, after the

173 addition of 2-methyl hexanoic acid as an internal standard and extracts were analysed

174 by GC as described previously (Possemiers et al. 2004). SCFA were separated using a

175 capillary free fatty acid packed column (EC-1000 Econo-Cap, 25 m $\times$ 0.53 mm x 1.2

176 µm) and detected with a flame ionization detector. Nitrogen was used as carrier gas.

177 Lactic acid was measured spectrometrically with an enzymatic D-/L-lactic acid Kit (R-

178 Biopharm, Darmstadt, Germany), according to the manufacturer's instructions.

179　　　　Ammonium was released from samples as ammonia by addition of MgO and

180 distillation into boric acid-indicator solution using an autodistillation Vapodest 30´

181 (Gerhardt Analytical Systems, Brackley Northants, UK), as earlier described by

182 Bremner and Keeney (1965). Ammonia was determined by titration with standard HCl

183 using a 685 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium).

184 *Analysis of phenolic metabolites*

185 Phenolic metabolites were analyzed by a previous UPLC-ESI-MS/MS method

186 (Sánchez-Patán et al. 2011) further implemented (Jiménez-Girón et al. 2013). The liquid

187 chromatographic system was a Waters Acquity UPLC (Milford, MA, USA) equipped

188 with a binary pump, an autosampler thermostated at 10 ºC, and a heated column

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189 compartment (40 ºC). The column employed was a BEH-C18, 2.1 x 100 mm and 1.7

190 μm particle size from Waters (Milford, MA, USA). The mobile phases were 2% acetic

191 acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as

192 follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14

193 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium

194 time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set constant

195 at 0.5 ml/min and injection volume was 2 μl. The LC effluent was pumped to an

196 Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray

197 electrospray ionization (ESI) source operated in negative polarity mode. The ESI

198 parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 ºC;

199 desolvation temperature, 400 ºC; desolvation gas (N_2) flow rate, 750 l/h; cone gas (N_2)

200 flow rate, 60 l/h. The ESI was operated in negative ionization mode. For quantification

201 purposes, data were collected in the multiple reaction monitoring (MRM) mode,

202 tracking the transition of parent and product ions specific to each compound. The

203 MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60

204 phenolic compounds targeted in the present study (mandelic acids, benzoic acids,

205 phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-

206 hydroxyvaleric acids and valerolactones) were previously reported (Jiménez-Girón et al.

207 2013). All metabolites were quantified using the calibration curves of their

208 corresponding standards, except for 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric, and

209 4-hydroxy-5-(phenyl)-valeric acids) which were quantified using the calibration curves

210 of 3-(3',4'-dihydroxyphenyl)-propionic, and propionic acids, respectively. Data

211 acquisition and processing was realized with MassLynx 4.1 software.

212

213 *Statistical analysis*

214   Mean values, standard deviations and correlation coefficients were calculated on the

215   basis of the values for the different variables during the incubation period (microbial

216   groups, SCFAs, acetate, propionate, butyrate, ammonium and phenolic metabolites).

217   Analysis of variance (ANOVA) was used for multiple comparisons of the different

218   variables taken into account different factors (time, compartments, and addition of *L.*

219   *plantarum* IFPL935) ($P < 0.05$). All statistical analyses were carried out using the

220   STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006,

221   www.statsoft.com).

222

223

224   **Results**

225   Microbiological changes

226   The colonic microbiota was allowed to reach the steady-state conditions during the 3-

227   weeks period of stabilization in each of the three colon vessels of the Twin-SHIME

228   systems (Day 0, Table 1). After this stabilization period, impact of daily feeding with

229   either the phenolic extract or the phenolic extract and *L. plantarum* IFPL935 on log

230   numbers of total bacteria, *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and

231   *Enterobacteriaceae*, is shown in Table 1. Figure 1 shows the log numbers for the

232   butyrate- producing phylogenetic groups *C. coccoides-E. rectale* Cluster XIVa, *C.*

233   *leptum* subgroup specific Cluster IV, and *Ruminococcus* Cluster IV. Feeding the

234   SHIME with the phenolic extract caused a decrease of approx. 1 log numbers of total

235   bacteria in the AC vessels after 24 h treatment (Table 1). This initial decrease of

236   numbers affected mostly to the counts of *Lactobacillus*, *Bifidobacterium*, *Bacteroides,*

237   *Enterobacteriaceae* (Table 1) and *C. coccoides/E. rectale* (Fig. 1). In most cases,

10

238 bacterial numbers were readily recovered after the first week of treatment. The largest

239 reduction in numbers was recorded for *Bacteroides*, showing a decrease of 2 log

240 numbers in the AC vessels at the first day of treatment. The inhibitory effect of the red

241 wine extract was less pronounced but more persistent for *Bifidobacterium*, which

242 numbers continuously fell down during the first week of treatment and did not recover

243 until the second week. Addition of *L. plantarum* IFPL935 to one of the Twin-SHIME

244 systems together with the phenolic extract resulted in relative stable numbers

245 throughout the treatment of total bacteria, *Lactobacillus* and *Enterobacteriaceae* (Table

246 1). In these bacterial groups, no impact of phenolic extract on the log numbers after 1

247 day of treatment was observed with the presence of *L. plantarum* IFPL935. The

248 diversity of lactobacilli in the AC, TC and DC vessels was followed by PCR-DGGE

249 targeting the *Lactobacillus* genus. The cluster analysis of the DGGE profiles grouped

250 the samples during the treatment period mainly based on the addition of *L. plantarum*

251 IFPL935 to the SHIME feeding medium (Fig. S1 in the supplemental material).

252 Additionally, agar plate counts revealed higher lactobacilli values during the treatment

253 and first days of wash out in the colonic vessels of the SHIME unit supplemented with

254 *L. plantarum* IFPL935 (results not shown).

255 The numbers of the butyrate-producing bacteria group *C. coccoides-E. rectale*

256 was equally represented in the three colonic vessels at the start of the treatment (about 7

257 log), whereas *C. leptum* and *Ruminococcus* prevailed in TC and DC vessels (about 6-7

258 log) (Fig. 1), being almost under the detection limit in the AC vessels. Impact of feeding

259 the Twin-SHIME units with the phenolic extract on these three bacterial groups was

260 more pronounced in the AC vessels for *C. coccoides-E. rectale* and in the TC vessels

261 for *C. leptum* and *Ruminococcus*, where the effects appeared earlier in time. The

262 BCoAT gene numbers corresponded with the results of butyrate producing bacteria

11

263 counts, showing higher values in the TC and DC vessels than in the AC ones and,

264 particularly, in the DC from the SHIME unit supplemented with *L. plantarum* IFPL935

265 (results not shown).

266

267 Wine polyphenols supplementation affects microbial metabolism

268 Evolution of the microbial metabolism (fermentation and proteolysis) expressed as the

269 content in SCFA, lactic acid, BCFA and ammonium is shown in Fig. 2. Daily intake of

270 the polyphenolic extract caused a sharp decrease in both fermentation and proteolysis

271 measured during the first days of treatment in all the AC, TC and DC vessels.

272 Proteolysis rate measured by ammonium and BCFA content showed the minimum

273 values in all vessels during the first week of treatment, particularly for ammonium. No

274 differences were observed between both treatments with and without *L. plantarum*

275 IFPL935 (Fig. 2A). Regarding the fermentation profile, acetic and propionic acid values

276 decreased similarly, reaching minimum values in all three vessels during the first three

277 days for both treatments (Fig. 2B). Butyric acid was lessened to no detectable levels

278 during the first two days of treatment in all the vessels excepting the DC and TC vessels

279 added with phenolic extract and *L. plantarum* IFPL935 (Fig. 2C). On the other hand, the

280 lactic acid content was not negatively affected by any of the treatments. Moreover, the

281 AC vessel of the SHIME supplemented with the phenolic extract and *L. plantarum*

282 IFPL935 showed an increase of lactic acid at the starting of the intervention period and

283 a decrease associated to the recovery of butyrate content to initial levels (Fig. 2C). The

284 addition of *L. plantarum* IFPL935 also caused differences in the production of butyric

285 acid in the DC vessels where the highest values for this acid were found. In general, the

286 microbial metabolic activity present at the start of the feeding was recovered in all

12

287   vessels for both treatments after 5 days of the experiments, except for the production of

288   butyric acid in the DC vessel without *L. plantarum* IFPL935, which only returned to

289   initial values at the end of the wash-out period (Fig. 2C).

290

291   Changes in phenolic metabolism

292   Within the 60 phenolic metabolites targeted, a total of 26 compounds including benzoic

293   acids, phenols, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones

294   and cinnamic acids, exhibiting different substitutions in the aromatic ring, were

295   quantified in the AC, DC and TC contents during the 2-weeks period of treatments and

296   the wash-out period (Table S1 in the supplemental material). Formation of these

297   metabolites was in accordance with disappearance of wine phenolic precursors (Table

298   S2 in the supplemental material), mainly flavan-3ols (monomers and dimeric

299   procyanidins), but also anthocyanins, flavonols and stilbenes. To better summarize these

300   changes, Fig. 3 displays the sum of concentrations of the precursor monomers (+)-

301   catechin and (-)-epicatechin, and procyanidins B1, B2, B3, B4 (Fig. 3A), the

302   intermediate metabolites phenyl-γ-valerolactones and phenylvaleric acid derivatives

303   (Fig. 3B) and the phenylpropionic and phenylacetic acid derivatives (Fig. 3C) during

304   the continuous feeding of the Twin-SHIME with the red wine polyphenolic extract.

305   Flavan-3-ol monomers and procyanidins were mainly detected in AC vessels

306   and starting from day 2 of the wine polyphenol treatment. The concentration of these

307   precursor compounds in the AC vessels increased during the intervention, followed by a

308   return to baseline during the wash-out period (Figure 3A). Precursors were also detected

309   in the TC compartments, but their concentration diminished earlier than in the AC

310   vessels. Interestingly, the content of these flavan-3-ol monomers and procyanidins in

13

311 TC and DC vessels were lower in the SHIME unit supplemented with *L. plantarum*

312 IFPL935.

313   The formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-

314 (3′,4′-dihydroxyphenyl)-valeric acid, intermediate metabolites arising from first steps in

315 the microbial degradation of flavan-3-ols, was mainly observed in the TC vessels and

316 their concentration increased after day 4 of both treatments (Fig. 3B). These compounds

317 were also present in the DC vessels but to a much lesser extent and only in those with

318 no addition of *L. plantarum* IFPL935. In accordance with these results, 4-hydroxy-5-

319 (phenyl)-valeric acid content increased with time and was mainly detected in the DC

320 vessels (Table S1 in the supplemental material).

321   Also, and as derived from galloylated flavan-3-ols, an increase in the content of

322 gallic acid was only detected in the AC vessels, whereas 3-*O*-methylgallic acid was also

323 measured in the TC vessels (Table S1 and Fig S2 in the supplemental material).

324 Likewise, pyrogallol formation was only observed in the AC vessels whereas

325 pyrocatechol was measured mainly in the AC and TC compartments. Supplementation

326 with *L. plantarum* IFPL935 did not cause differences in the metabolism of these

327 compounds.

328   The evolution of 3-(4´-hydroxyphenyl) propionic acid, 4-hydroxyphenylacetic

329 acid and phenylacetic acid, which can also be derived from aromatic amino acid

330 catabolism, followed the same trends as the microbial metabolites released from

331 proteolysis depicted in Fig. 2A (Table S1 in the supplemental material). The extent of

332 formation of microbial phenolic metabolites from the flavan-3-ol compounds was

333 estimated in the SHIME vessels during the treatment and wash-out periods by

334 computing the formation of phenylpropionic acids [3-(3´,4´-dihydroxyphenyl)-

335 propionic, 3-(3´-hydroxyphenyl)-propionic, and 3-phenylpropionic] and phenylacetic

336 acids [3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic] derivatives (Fig. 3C).

337 The highest values of these metabolites were observed in the DC vessels, being still

338 detected during the wash-out period. In the SHIME unit supplemented with *L.*

339 *plantarum* IFPL935 the phenylpropionic acid derivatives, mainly represented by 3-(3´-

340 hydroxyphenyl)propionic acid, appeared at earlier stages than the phenylacetic acid

341 metabolites and their concentration was the highest when comparing values from the

342 two SHIME units.

343

Discussion

345 The human gut microbiota contributes to many of the important host metabolic

346 functions, including the increase of bioavailabilityof dietary polyphenols (van

347 Duynhoven et al. 2011). Conversely, polyphenols and their resultant metabolites can

348 modify the gut bacterial population composition and activity (Requena et al. 2010).

349 Most of the studies that have examined the antimicrobial effect of dietary polyphenols

350 have focused on single polyphenol molecules and/or single strains. In this work, we

351 have studied the effect on the gut microbiota of the long-term feeding with a red wine

352 polyphenolic extract and the impact of the supplementation of *L. plantarum* IFPL935,

353 previously characterized as a flavan-3-ol metabolizer (Barroso et al. 2013; Sánchez-

354 Patán et al. 2012b). We have conducted the study in the *in vitro* model of the human

355 intestinal microbiota SHIME, which has demonstrated to allow nutritional interventions

356 with repeated doses to evaluate long-term effect of food ingredients on the microbiota

357 allocated in three consecutive colon regions (Grootaert et al. 2009; Marzorati et al.

358 2010; Van de Wiele et al. 2007; Van den Abbeele et al. 2013).

359   The present study shows that the initial addition of red wine polyphenols exerted

360 an overall antimicrobial effect on the gut microbiota in the AC, but the effect

361 disappeared during continuous feeding with polyphenols, probably due to the onset of

362 microbial metabolism of polyphenols. The antimicrobial effect of red wine and grape

363 polyphenolic extracts following either a single dose or a continuous feeding of the

364 SHIME has recently been described (Kemperman et al. 2013). These authors reported a

365 more pronounced antimicrobial effect on the colonic microbiota, which did not fully

366 recover until the wash-out period. Differences between studies could be due to the fact

367 that the amount of polyphenolic compounds daily supplemented by Kemperman et al.

368 (2013) was 5-times higher than in the present study. Besides, grape extracts have

369 demonstrated higher antimicrobial capacity than extracts obtained from red wine (Cueva

370 et al. 2013), probably because of a higher content of proanthocyanidins and gallate

371 derivatives (Xia et al. 2010).

372   Among polyphenols, flavan-3-ols and flavonols have received most attention

373 due to their wide spectrum and higher antimicrobial activity in comparison with other

374 polyphenols (Daglia 2012). In our study, all bacterial groups analyzed in the AC vessels

375 were initially affected by the supplementation with red wine polyphenols. Among them,

376 *Bacteroides* and the butyrate-producers *C. coccoides*/*E. rectale* were the most affected

377 groups, whereas *Bifidobacterium* showed a more persistent effect. *Bacteroides* and

378 *Bifidobacterium* have been described previously (Kemperman et al. 2013) as the genera

379 most affected after feeding the SHIME colonic microbiota with black tea and red wine

380 polyphenols. Additionally, intake of flavan-3-ol-rich sources for prolonged periods of

381 time has generally correlated with the decrease of *Clostridium* groups (Dolara et al.

382 2005; Tzounis et al. 2011; Wu et al. 2011). On the other hand, counts of lactobacilli are

383 generally unaffected or moderately increase after intake of diets enriched in flavanols

16

384 (Tzounis et al. 2011). Nevertheless, a different sensitivity to the presence of flavan-3-ols

385 in the growth medium has been described for several *Lactobacillus* species (Tabasco et

386 al. 2011), ranging from the optimal growth observed for *L. plantarum*, *L. casei*, and *L.*

387 *bulgaricus* strains and the growth inhibition of *L. fermentum*, *L. acidophilus* and *L.*

388 *vaginalis*. Among the strains assayed, however, only *L. plantarum* IFPL935 was able to

389 metabolize flavan-3-ol compounds (Fernández-Patán, 2012b; Tabasco et al. 2011).

390   Although the antimicrobial effect caused by daily feeding the SHIME with red

391 wine polyphenols was predominantly observed during the first 1-2 days of treatment

392 and it was more distinguished in the AC vessels, the inhibitory effect on the overall

393 microbial metabolism, however, was similar in the three vessels and it lasted for the

394 first 5 days of treatment (Fig. 2). These results indicate a more pronounced effect of the

395 polyphenol compounds in the microbial functionality than in its viability. The initial

396 exposure to polyphenols may up-regulate polyphenol-induced stress responses related to

397 defensive mechanisms while simultaneously down-regulating various metabolic

398 functions, for example, carbon and energy metabolism (Stevenson and Hurst 2007).

399 Decreased rate of microbial fermentation and proteolysis, determined as reduction in the

400 formation of SCFA and BCFA, is associated with high tannin-rich feeds intake in

401 ruminant livestock (Bodas et al. 2012). The effects of some plant extracts have been

402 described to last only a few days, indicating that ruminal microorganisms adapt to the

403 compounds with time (Busquet et al. 2005). Similarly, the regular feeding of the

404 SHIME with a red wine polyphenolic extract was able to introduce microbial and

405 metabolic changes until the microbiota adapted its metabolism and returned to steady

406 state similar conditions during the treatment.

407   In terms of microbial metabolism, the addition of *L. plantarum* IFPL935 caused

408 an increase in the formation of lactic acid at the start of the polyphenol treatment in the

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409 AC vessel and of butyric acid in the TC and DC vessels after the day 5 of treatment

410 (Fig. 2). The increased formation of butyric acid in the DC vessel supplemented with *L.*

411 *plantarum* IFPL935 was also associated with a lower content of lactic acid. Both

412 phenomena can partially be explained by the recovery at this point of the counts of

413 butyrate-producing bacteria from clostridial cluster XIVa and eventually by bacterial

414 cross-feeding production of butyric acid from lactic acid. In fact, the only vessels where

415 the content of butyric acid did not decrease to zero values at the start of the polyphenol

416 treatment were the TC and DC vessels of the SHIME supplemented with *L. plantarum*

417 IFPL 935 (Fig. 2). Duncan et al. (2004) reported that most of the faecal species that

418 utilize lactate to produce butyrate via the butyryl CoA:acetate CoA transferase route,

419 belong to the clostridial cluster XIVa (*Lachnospiraceae*). The results obtained in this

420 study confirm the previously observed effect of *L. plantarum* IFPL935 to increase the

421 formation of butyric acid during batch incubations with different colonic microbiota

422 (Barroso et al. 2013). This compound has been described as beneficial for human health

423 since low prevalence in the gut has been associated with chronic immune or metabolic-

424 related diseases including inflammatory bowel disease (Frank et al. 2007) and obesity

425 (Le Chatelier et al. 2013).The capability of *L. plantarum* IFPL935 to initiate the

426 catabolism of polyphenols either via galloyl-esterase, decarboxylase and benzyl alcohol

427 dehydrogenase activities (Tabasco et al. 2011) or mostly by the cleavage of the

428 heterocyclic ring of monomeric flavan-3-ols (Sanchez-Patán et al. 2012b), might have

429 contributed to diminish the inhibitory effect of the red wine extract when both were

430 added to the SHIME units. So far and besides this strain, only a few bacterial strains,

431 such as *Eubacterium* sp. SDG-2, *Eggerthella* sp. CAT-1, *Eggerthella lenta* rK3, and

432 *Flavonifractor plautii* aK2 have been reported to be able to initiate the metabolism of

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433 flavanol-3-ols by cleavage of the heterocyclic C-ring (Jin and Hattori 2012; Kutschera

434 et al. 2011; Wang et al. 2001).

435   A lower concentration of monomeric flavan-3-ols and first and intermediate

436 metabolites (5-(3′-hydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′-

437 hydroxyphenyl)valeric acid), mainly represented in the AC and TC vessels, was

438 observed in the vessels of the SHIME unit supplemented with *L. plantarum* IFPL935. In

439 addition, further metabolism of the polyphenol intermediate compounds to the

440 formation of phenylpropionic acids was detected earlier and reached higher

441 concentrations in the DC vessel containing the strain when compared to the other

442 colonic vessels (Fig. 3C). For both treatments, bacterial conversion of red wine

443 polyphenols was more favorable in the distal colon, which is in agreement with

444 previous studies where a higher microbial conversion of phenolic compounds was found

445 in the distal compartments (Barroso et al. 2013; Van Dorsten et al. 2012).

446   Phenyl propionic acid and 3-(3´-hydroxyphenyl)propionic acid were the

447 microbial metabolites that accounted most for the differences between treatments.

448 Phenylpropionic acid derivatives are considered to arise from β-oxidation of

449 phenylvaleric acid derivatives (Monagas et al. 2010), except for 3-(4´-

450 hydroxyphenyl)propionic acid that can result from the microbial catabolism of tyrosine

451 (Russell et al. 2013). The evolution of this compound during the SHIME treatment with

452 red wine polyphenols indicated that 3-(4´-hydroxyphenyl)propionic acid aroused mostly

453 from amino acid catabolism (Table S1 in the supplemental material). The phenyl

454 propionic metabolite produced at a higher concentration was 3-(3´-

455 hydroxyphenyl)propionic acid, which has been identified as a strong urinary marker of

456 red wine and grape extracts intake (Jacobs et al. 2012; Ward et al. 2004). This microbial

457 metabolite has been shown to reduce the inflammatory response of human peripheral

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458 blood mononuclear cells stimulated with lipopolysaccharide (Monagas et al. 2009) and

459 to have anti-thrombotic activity (Rechner and Kroner 2005). In conclusion, this study

460 provides data supporting the potential use of *L. plantarum* IFPL935 to increase gut

461 butyrate formation and favour polyphenol metabolism in an *in vitro* gastro intestinal

462 system. This opens possibilities of using the strain as a food ingredient for helping

463 individuals showing a low polyphenol-fermenting metabotype (Bolca et al. 2013) to

464 increase their gut capacities of metabolizing dietary polyphenols.

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649 **Legend to figures.**

650 Fig. 1. Changes in q-PCR counts (log copy number/ml) of butyrate-producing groups

651 belonging to *Clostridium* clusters IV and XIVa in the ascending (AC), transverse (TC)

652 and descending colon (DC) of the Twin-SHIME during the treatment with the red wine

653 phenolic extract (grey squares) and the extract supplemented with *L. plantarum*

654 IFPL935 (black circles) and the was out period. The dotted line indicates the end of the

655 polyphenol treatment.

656 Fig. 2. Changes in concentration (mM) of ammonium (circles), BCFA (squares), acetic

657 acid (diamonds), propionic acid (triangles), butyric acid (dots) and lactic acid (asterisks)

658 in the ascending (AC; A), transverse (TC; B) and descending colon (DC; C) of the

659 Twin-SHIME during the treatment with the red wine phenolic extract (grey symbols)

660 and the extract supplemented with *L. plantarum* IFPL935 (black symbols) and the was

661 out period. The vertical dotted line indicates the end of the polyphenol treatment.

662 Fig. 3. Changes in concentration (μg/ml) of the sum of (A) precursor monomers (+)-

663 catechin and (-)-epicatechin (circles) and procyanidins B1, B2, B3, B4 (squares), (B) the

664 intermediate metabolites phenyl-γ-valerolactones (diamonds) and phenylvaleric acid

665 derivatives (triangles) and (C) the phenylpropionic (dots) and phenylacetic acid

666 (asterisks) derivatives in the ascending (AC), transverse (TC) and descending colon

667 (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey

668 symbols) and the extract supplemented with *L. plantarum* IFPL935 (black symbols) and

669 the was out period. The vertical dotted line indicates the end of the polyphenol

670 treatment.

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Table 1. Mean (SD) q-PCR counts (log copy number/ml) of bacterial groups in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (Provin) and the extract supplemented with *L. plantarum* IFPL935 (Provin+Lp935) and the was out period.

| Bacteria	Colonic vessel	Treatment	Intervention period				
			Day 0 (steady state)	Day 1	Week 1	Week 2	Week 3 (wash out)
Total counts	AC	Provin	8.28 (0.32)	7.20 (0.08)	8.28 (0.34)	7.86 (0.29)	8.03 (0.21)
		Provin+Lp935	8.17 (0.06)	8.66 (0.07)	8.26 (0.38)	8.00 (0.31)	7.99 (0.14)
	TC	Provin	8.60 (0.12)	8.49 (0.03)	8.20 (0.38)	8.06 (0.50)	8.22 (0.30)
		Provin+Lp935	8.42 (0.30)	8.00 (0.10)	8.23 (0.43)	8.19 (0.11)	8.06 (0.15)
	DC	Provin	8.43 (0.13)	8.33 (0.07)	8.42 (0.30)	8.34 (0.50)	8.37 (0.44)
		Provin+Lp935	8.34 (0.25)	8.08 (0.05)	8.57 (0.14)	8.33 (0.06)	8.14 (0.42)
Lactobacillus	AC	Provin	7.79 (0.41)	6.30 (0.16)	7.09 (0.29)	7.12 (0.67)	7.29 (0.30)
		Provin+Lp935	7.57 (0.62)	7.62 (0.34)	7.40 (0.13)	7.16 (0.57)	7.69 (0.15)
	TC	Provin	7.63 (0.11)	7.16 (0.16)	6.76 (0.20)	7.29 (0.25)	7.31 (0.18)
		Provin+Lp935	7.51 (0.13)	6.94 (0.37)	7.44 (0.05)	7.74 (0.24)	7.54 (0.29)
	DC	Provin	7.32 (0.06)	6.89 (0.02)	6.87 (0.10)	7.21 (0.41)	7.33 (0.35)
		Provin+Lp935	7.44 (0.12)	7.03 (0.31)	7.11 (0.12)	7.55 (0.16)	7.42 (0.41)
Bifidobacterium	AC	Provin	6.74 (0.40)	6.04 (0.20)	5.91 (0.68)	6.94 (0.21)	6.50 (0.80)
		Provin+Lp935	6.73 (0.60)	6.36 (0.54)	5.44 (0.91)	6.74 (0.67)	5.96 (0.63)
	TC	Provin	6.97 (0.17)	7.01 (0.30)	6.57 (0.22)	7.31 (0.58)	7.17 (0.87)
		Provin+Lp935	6.72 (0.15)	6.75 (0.23)	6.07 (0.17)	7.23 (0.26)	6.86 (0.70)
	DC	Provin	7.03 (0.45)	6.69 (0.39)	6.43 (0.17)	7.23 (0.43)	6.92 (0.82)
		Provin+Lp935	6.69 (0.29)	6.52 (0.65)	6.47 (0.10)	7.13 (0.46)	6.92 (0.73)
Bacteroides	AC	Provin	8.23 (0.07)	6.20 (0.05)	8.09 (0.52)	8.28 (0.08)	8.33 (0.32)
		Provin+Lp935	8.20 (0.46)	6.23 (0.01)	8.27 (0.32)	8.11 (0.01)	8.46 (0.26)
	TC	Provin	8.47 (0.12)	7.95 (0.02)	8.28 (0.52)	8.27 (0.35)	8.37 (0.24)
		Provin+Lp935	8.32 (0.23)	7.19 (0.07)	8.35 (0.53)	8.32 (0.08)	8.39 (0.49)
	DC	Provin	8.31 (0.14)	7.98 (0.03)	8.49 (0.24)	8.04 (0.40)	8.41 (0.25)
		Provin+Lp935	8.37 (0.13)	7.49 (0.01)	8.68 (0.34)	8.04 (0.47)	8.00 (0.28)
Enterobacteriaceae	AC	Provin	7.35 (0.21)	6.37 (0.21)	7.34 (0.33)	6.59 (0.73)	7.28 (0.19)
		Provin+Lp935	7.15 (0.20)	7.06 (0.23)	7.52 (0.55)	6.44 (0.35)	7.25 (0.10)
	TC	Provin	7.63 (0.09)	7.40 (0.02)	7.31 (0.23)	7.15 (0.51)	7.35 (0.12)
		Provin+Lp935	7.54 (0.26)	6.84 (0.24)	7.09 (0.29)	7.16 (0.29)	7.26 (0.27)
	DC	Provin	7.44 (0.17)	7.43 (0.23)	7.04 (0.54)	7.31 (0.34)	7.35 (0.09)
		Provin+Lp935	7.40 (0.29)	7.01 (0.22)	6.69 (0.76)	7.25(0.28)	7.13 (0.42)

Figure 1

C. coccoides/E. rectale   *C. leptum*   *Ruminococcus*

AC   TC   TC

DC   DC   DC

Figure 2

A   AC

B   TC

C   DC
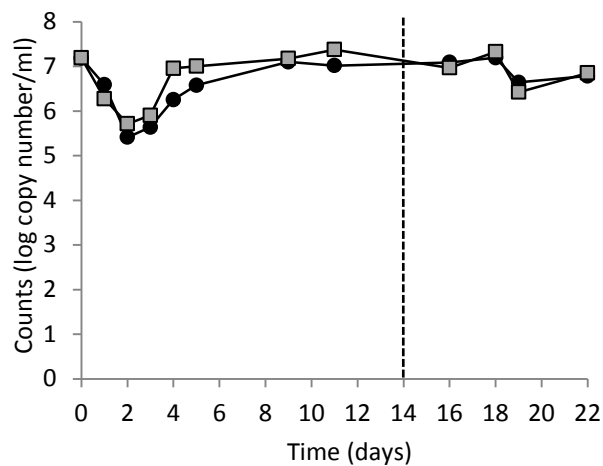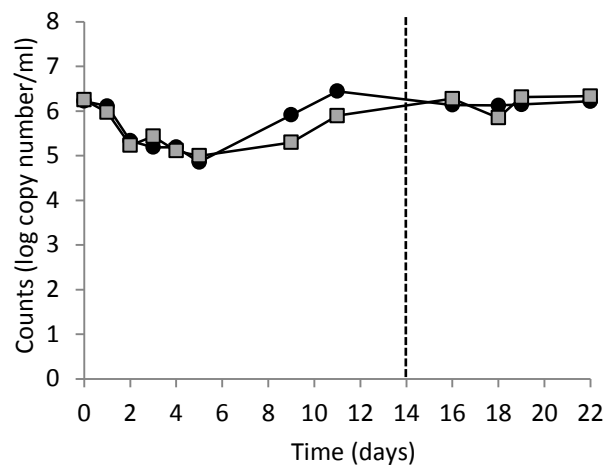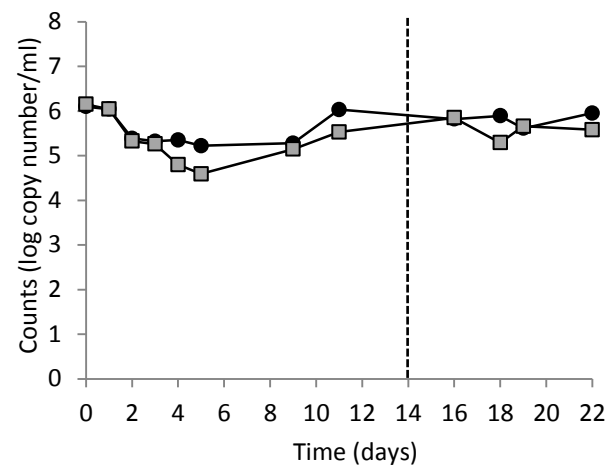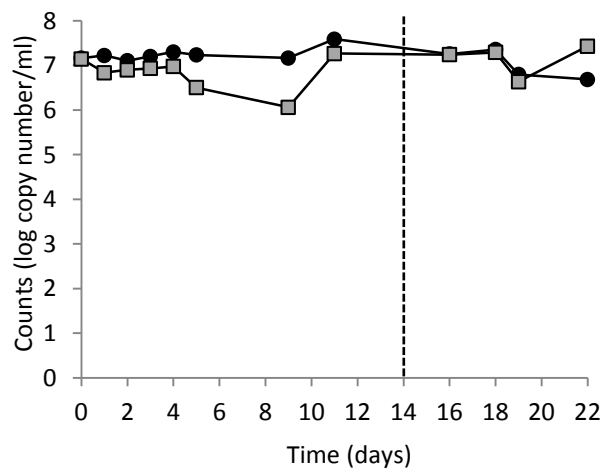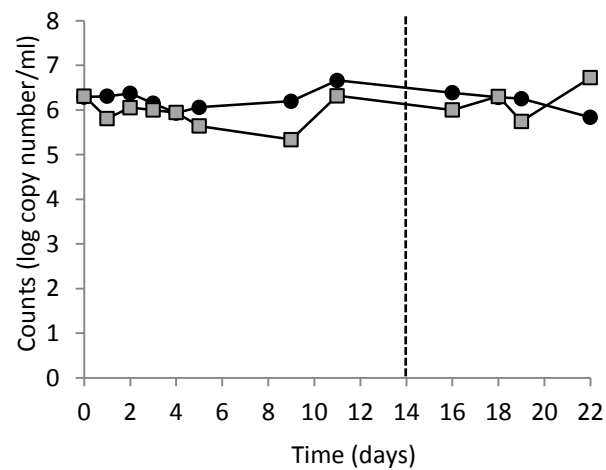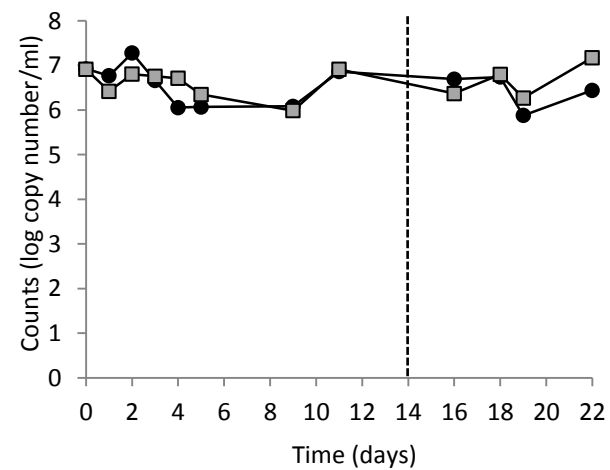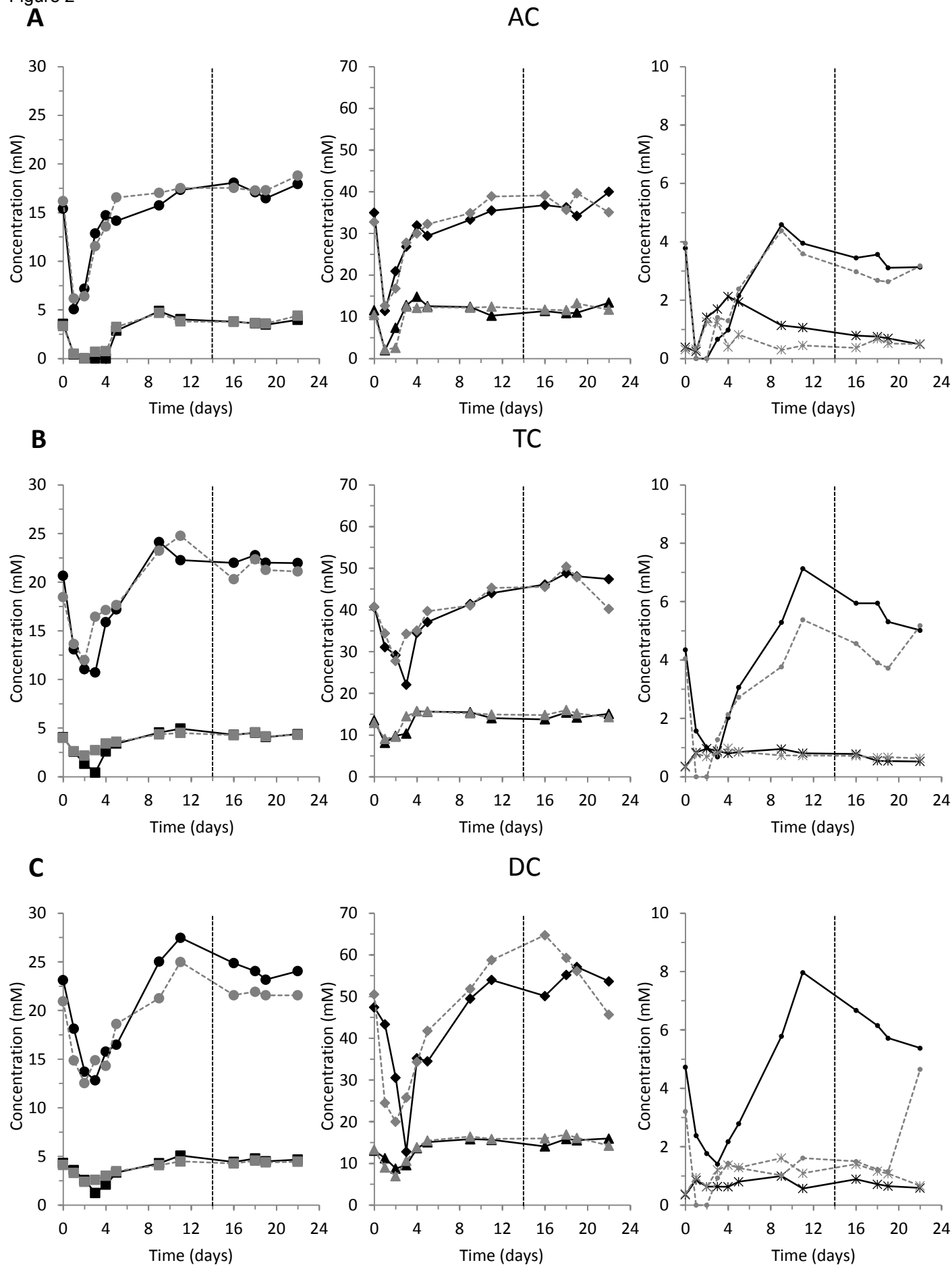
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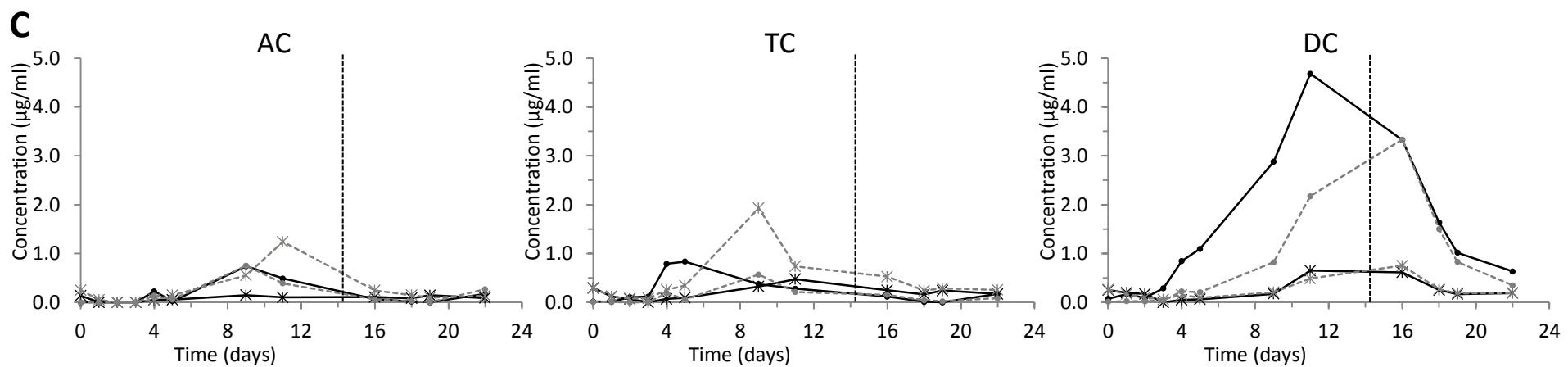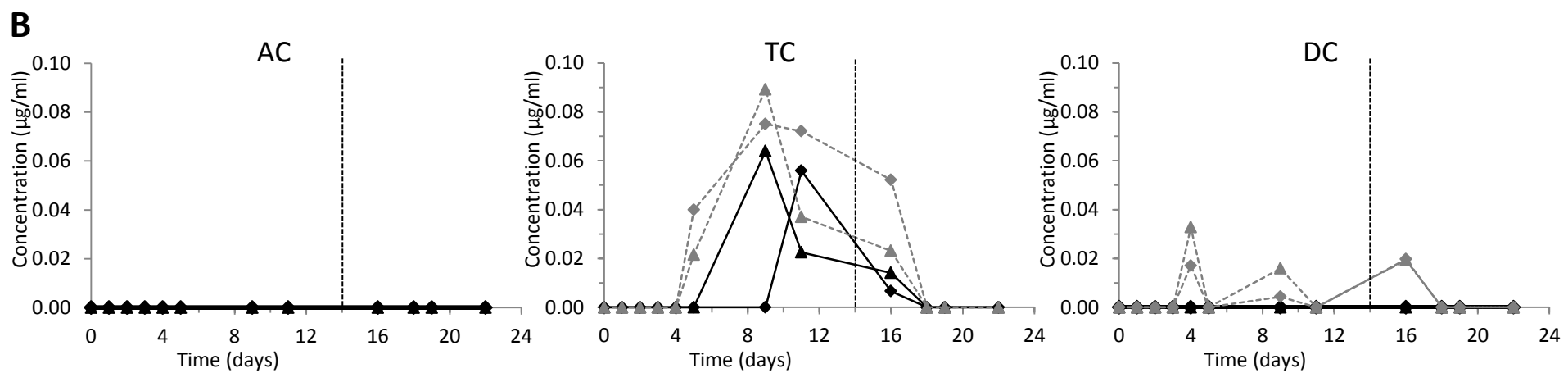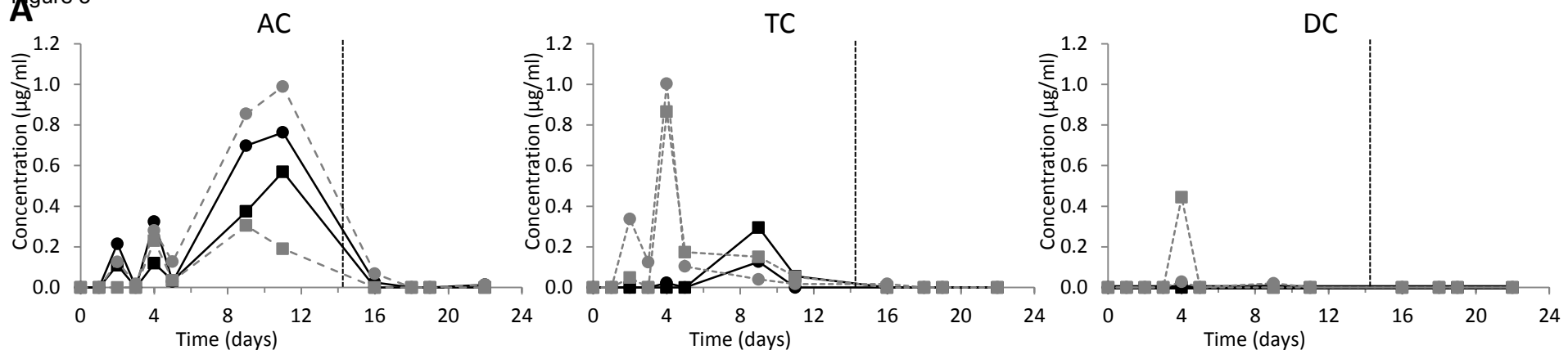
Supplementary Material