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Abstract

The colonic microbiota plays an important role in the bioavailability 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

Moderate consumption of red wine and/or regular consumption of other polyphenol-rich beverages and foods has proved in epidemiological studies to reduce the incidence of certain chronic diseases (Arranz et al. 2012; Kishimoto et al. 2013). Although the health effects of food polyphenols have been repeatedly associated with their free radical scavenging and antioxidant activity, recent evidence has, however, indicated that the effects of antioxidants are less relevant than expected (Tomás-Barberán and Andrés-Lacueva 2012). The bioavailability, absorption and metabolism of polyphenolic compounds are, indeed, key issues required to explain their role in human health. Depending on their chemical structure and food matrix, a high percentage of dietary polyphenols are not absorbed in the small intestine, reaching the colon where they are metabolized by the gut microbiota before being absorbed. Likewise, polyphenols and their resultant metabolites may selectively modulate the gut microbial composition by their antimicrobial or eventually prebiotic-like properties (Queipo-Ortuño et al. 2012; Tzounis et al. 2008). In this sense, an increase in *Lactobacillus* and *Bifidobacterium* species following administration of red wine polyphenols has been reported (Dolara et al. 2005; Queipo-Ortuño et al. 2012). Moreover, a significant correlation of moderate wine consumption with human microbiome composition, but not with enterotype partitioning, has been established (Wu et al., 2011).

Flavonoids are the most abundant polyphenols present in red wine, mainly including flavan-3-ols and anthocyanins (Waterhouse 2002). There are several metabolic pathways proposed for the catabolism of monomeric flavan-3-ol and dimeric procyanidins by the intestinal microbiota (Monagas et al. 2010; Selma et al. 2009).

However, bacterial degradation of flavan-3-ols is hindered by the inherent antibacterial effects of these molecules. In previous studies, we have reported that *L. plantarum* IFPL935 was capable to metabolize galloylated flavan-3-ols leading to the formation of gallic acid, pyrogallol and catechol through galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase enzyme activities, respectively (Tabasco et al. 2011). The most remarkable feature of *L. plantarum* IFPL935 was its ability to cleave the heterocyclic ring of monomeric flavan-3-ols, giving rise to 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (i.e. diphenylpropanol), which is the first metabolite in the microbial catabolic pathway of flavan-3-ols (Sánchez-Patán et al. 2012b). This activity has only been reported in a few intestinal bacteria to date, such as *Eubacterium* sp. SDG-2, *Eggerthella lenta*, and *Flavonifractor plautii* (Jin and Hattori 2012; Kutschera et al. 2011; Wang et al. 2001). The capability of *L. plantarum* IFPL935 to initiate the catabolism of flavan-3-ols via the formation of diphenylpropanol has also been demonstrated when the strain was incubated in batches with a complex human intestinal microbiota (Barroso et al., 2013). It was also observed that the addition of this strain had an impact on the formation of butyric acid, probably through cross-feeding with colonic butyrate-producing bacteria (Barroso et al., 2013).

To get a deeper insight on the potential use of *L. plantarum* IFPL935 to benefit polyphenols metabolism we have monitored the effect on the gut microbiota upon addition of 10^{10} cfu of the strain to a daily intake of a polyphenolic extract (equivalent to two 125 ml-glasses of alcohol-free red wine) in a long-term feeding experiment. The study was conducted in the dynamic multireactor gastrointestinal Simulator of the Human Intestinal Microbial Ecosystem (SHIME).

96 **Materials and methods**

97

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

99 In this study, a Twin-SHIME[®] (UGent/ProDigest) setup was used by operating two
100 parallel SHIME systems, each one consisting of five consecutive reactors, simulating
101 the stomach, small intestine, ascending colon (AC), transverse colon (TC), and
102 descending colon (DC), as described by Van den Abbeele et al. (2010). At the
103 beginning of the experiment, the AC, TC and DC vessels from the Twin-SHIME
104 systems were all simultaneously inoculated with the same fecal sample from a healthy
105 human volunteer previously identified as a flavan-3-ol metabolizer (unpublished results)
106 to enable comparison of the results between the two parallel experiments. The colonic
107 microbiota was allowed for stabilization reaching the steady state after 3 weeks. The
108 inoculum preparation and the SHIME feed composition during the stabilization period
109 were essentially as described by De Boever et al. (2004). After stabilization of the
110 colonic microbiota, both Twin-SHIME systems were subjected to a 2-week experiment
111 by daily feeding the stomach compartments with 200 mg of a commercial red wine
112 extract, Provinols[™] (Safic-Alcan Especialidades, Barcelona, Spain), which corresponds
113 to a daily polyphenol intake of two small glasses of red wine (250 ml). The total
114 phenolic content of the red wine extract was 474 mg of gallic acid equivalents per g
115 (2.79 mmol/g) and its composition was reported before (Sánchez-Patán et al. 2012a),
116 being flavan-3-ols the main phenolic compounds. In addition, one of the Twin-SHIME
117 systems was simultaneous fed with a daily dose of 10¹⁰ cfu of *Lactobacillus plantarum*
118 IFPL935. Finally, a 1-week wash-out period was included at the end of the experiment
119 for both systems. During the whole study, samples were collected at regular time points

from the three colon vessels and stored at -20°C until further analysis, excepting for microbiological plate counts that were performed at the time of sampling.

Bacterial culture conditions and plate counts

Lactobacillus plantarum IFPL935 (CECT 4599) was routinely grown aerobically at 37°C in MRS broth. To reach the daily supplementation of 10^{10} cfu of IFPL935, 10 ml of *L. plantarum* IFPL953 grown to 10^9 cfu/ml were added daily to the stomach compartment in one of the SHIME systems, right after the addition of the red wine extract. Numbers of lactobacilli in the inoculum were counted using MRS-agar plates and in the AC, TC and DC vessels using LAMVAB agar (Hartemink et al. 1997), and plates were incubated aerobically at 37°C for 48 h. Total aerobes and total anaerobes were determined by plating on BHI agar and incubation at 37°C for 24 h aerobically or 72 h anaerobically, respectively. Total coliforms were enumerated in MacConkey agar incubated for 24 h aerobically at 37°C .

DNA extraction and analysis

Bacterial DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) buffer and phenol-chloroform-isoamyl alcohol and bead-beating, as described previously (Griffiths et al. 2000; Kowalchuk et al. 1998). The DNA was precipitated with polyethelene glycol (PEG-6000), washed in ice cold 70% ethanol and dried in a Speed-Vac, prior to resuspension in distilled water. The concentration and quality of the samples were assessed with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Quantitative PCR (qPCR)

Bacterial numbers in the AC, TC and DC vessels were quantified by qPCR using SYBR green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc., Hercules, CA, USA). The bacterial groups targeted for qPCR were total bacteria, *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae*, and the specific phylogenetic groups *Clostridium coccoides*-*Eubacterium rectale* Cluster XIVa, *Ruminococcus* Cluster IV, and *Clostridium leptum* subgroup specific cluster IV. Butyrate-producing bacteria were also estimated by quantifying the gene encoding butyryl-CoA:acetate CoA transferase (BcoAT). Primers, amplification conditions and calculation of copy numbers have been detailed previously (Barroso et al. 2013). DNA from *Escherichia coli* DH5 α , *L. plantarum* IFPL935, *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, respectively. For the rest of groups analyzed, samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as described previously (Barroso et al., 2013).

PCR-DGGE

The diversity of the *Lactobacillus* community in the AC, TC and DC vessels and the presence of *L. plantarum* were assessed by PCR-DGGE and using the primers Lab-159F and Uni-515-GC-R, as described by Heilig et al. (2002). DGGE was performed with a DCode system (Bio-Rad) using a 9% polyacrylamide gel with a 30–50% gradient 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.

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 × 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 °C, and a heated column

compartment (40 °C). The column employed was a BEH-C18, 2.1 x 100 mm and 1.7 µm particle size from Waters (Milford, MA, USA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as 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 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 ml/min and injection volume was 2 µl. The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 °C; desolvation temperature, 400 °C; desolvation gas (N₂) flow rate, 750 l/h; cone gas (N₂) flow rate, 60 l/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids and valerolactones) were previously reported (Jiménez-Girón et al. 2013). All metabolites were quantified using the calibration curves of their corresponding standards, except for 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric, and 4-hydroxy-5-(phenyl)-valeric acids) which were quantified using the calibration curves of 3-(3',4'-dihydroxyphenyl)-propionic, and propionic acids, respectively. Data acquisition and processing was realized with MassLynx 4.1 software.

Statistical analysis

Mean values, standard deviations and correlation coefficients were calculated on the basis of the values for the different variables during the incubation period (microbial groups, SCFAs, acetate, propionate, butyrate, ammonium and phenolic metabolites). Analysis of variance (ANOVA) was used for multiple comparisons of the different variables taken into account different factors (time, compartments, and addition of *L. plantarum* IFPL935) ($P < 0.05$). All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984–2006, www.statsoft.com).

Results

Microbiological changes

The colonic microbiota was allowed to reach the steady-state conditions during the 3-weeks period of stabilization in each of the three colon vessels of the Twin-SHIME systems (Day 0, Table 1). After this stabilization period, impact of daily feeding with either the phenolic extract or the phenolic extract and *L. plantarum* IFPL935 on log numbers of total bacteria, *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Enterobacteriaceae*, is shown in Table 1. Figure 1 shows the log numbers for the butyrate- producing phylogenetic groups *C. coccoides*-*E. rectale* Cluster XIVa, *C. leptum* subgroup specific Cluster IV, and *Ruminococcus* Cluster IV. Feeding the SHIME with the phenolic extract caused a decrease of approx. 1 log numbers of total bacteria in the AC vessels after 24 h treatment (Table 1). This initial decrease of numbers affected mostly to the counts of *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae* (Table 1) and *C. coccoides*/*E. rectale* (Fig. 1). In most cases,

bacterial numbers were readily recovered after the first week of treatment. The largest
 reduction in numbers was recorded for *Bacteroides*, showing a decrease of 2 log
 numbers in the AC vessels at the first day of treatment. The inhibitory effect of the red
 wine extract was less pronounced but more persistent for *Bifidobacterium*, which
 numbers continuously fell down during the first week of treatment and did not recover
 until the second week. Addition of *L. plantarum* IFPL935 to one of the Twin-SHIME
 systems together with the phenolic extract resulted in relative stable numbers
 throughout the treatment of total bacteria, *Lactobacillus* and *Enterobacteriaceae* (Table
 1). In these bacterial groups, no impact of phenolic extract on the log numbers after 1
 day of treatment was observed with the presence of *L. plantarum* IFPL935. The
 diversity of lactobacilli in the AC, TC and DC vessels was followed by PCR-DGGE
 targeting the *Lactobacillus* genus. The cluster analysis of the DGGE profiles grouped
 the samples during the treatment period mainly based on the addition of *L. plantarum*
 IFPL935 to the SHIME feeding medium (Fig. S1 in the supplemental material).
 Additionally, agar plate counts revealed higher lactobacilli values during the treatment
 and first days of wash out in the colonic vessels of the SHIME unit supplemented with
L. plantarum IFPL935 (results not shown).

The numbers of the butyrate-producing bacteria group *C. coccoides*-*E. rectale*
 was equally represented in the three colonic vessels at the start of the treatment (about 7
 log), whereas *C. leptum* and *Ruminococcus* prevailed in TC and DC vessels (about 6-7
 log) (Fig. 1), being almost under the detection limit in the AC vessels. Impact of feeding
 the Twin-SHIME units with the phenolic extract on these three bacterial groups was
 more pronounced in the AC vessels for *C. coccoides*-*E. rectale* and in the TC vessels
 for *C. leptum* and *Ruminococcus*, where the effects appeared earlier in time. The
 BCoAT gene numbers corresponded with the results of butyrate producing bacteria

counts, showing higher values in the TC and DC vessels than in the AC ones and, particularly, in the DC from the SHIME unit supplemented with *L. plantarum* IFPL935 (results not shown).

Wine polyphenols supplementation affects microbial metabolism

Evolution of the microbial metabolism (fermentation and proteolysis) expressed as the content in SCFA, lactic acid, BCFA and ammonium is shown in Fig. 2. Daily intake of the polyphenolic extract caused a sharp decrease in both fermentation and proteolysis measured during the first days of treatment in all the AC, TC and DC vessels.

Proteolysis rate measured by ammonium and BCFA content showed the minimum values in all vessels during the first week of treatment, particularly for ammonium. No differences were observed between both treatments with and without *L. plantarum* IFPL935 (Fig. 2A). Regarding the fermentation profile, acetic and propionic acid values decreased similarly, reaching minimum values in all three vessels during the first three days for both treatments (Fig. 2B). Butyric acid was lessened to no detectable levels during the first two days of treatment in all the vessels excepting the DC and TC vessels added with phenolic extract and *L. plantarum* IFPL935 (Fig. 2C). On the other hand, the lactic acid content was not negatively affected by any of the treatments. Moreover, the AC vessel of the SHIME supplemented with the phenolic extract and *L. plantarum* IFPL935 showed an increase of lactic acid at the starting of the intervention period and a decrease associated to the recovery of butyrate content to initial levels (Fig. 2C). The addition of *L. plantarum* IFPL935 also caused differences in the production of butyric acid in the DC vessels where the highest values for this acid were found. In general, the microbial metabolic activity present at the start of the feeding was recovered in all

vessels for both treatments after 5 days of the experiments, except for the production of butyric acid in the DC vessel without *L. plantarum* IFPL935, which only returned to initial values at the end of the wash-out period (Fig. 2C).

Changes in phenolic metabolism

Within the 60 phenolic metabolites targeted, a total of 26 compounds including benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones and cinnamic acids, exhibiting different substitutions in the aromatic ring, were quantified in the AC, DC and TC contents during the 2-weeks period of treatments and the wash-out period (Table S1 in the supplemental material). Formation of these metabolites was in accordance with disappearance of wine phenolic precursors (Table S2 in the supplemental material), mainly flavan-3-ols (monomers and dimeric procyanidins), but also anthocyanins, flavonols and stilbenes. To better summarize these changes, Fig. 3 displays the sum of concentrations of the precursor monomers (+)-catechin and (-)-epicatechin, and procyanidins B1, B2, B3, B4 (Fig. 3A), the intermediate metabolites phenyl- γ -valerolactones and phenylvaleric acid derivatives (Fig. 3B) and the phenylpropionic and phenylacetic acid derivatives (Fig. 3C) during the continuous feeding of the Twin-SHIME with the red wine polyphenolic extract.

Flavan-3-ol monomers and procyanidins were mainly detected in AC vessels and starting from day 2 of the wine polyphenol treatment. The concentration of these precursor compounds in the AC vessels increased during the intervention, followed by a return to baseline during the wash-out period (Figure 3A). Precursors were also detected in the TC compartments, but their concentration diminished earlier than in the AC vessels. Interestingly, the content of these flavan-3-ol monomers and procyanidins in

TC and DC vessels were lower in the SHIME unit supplemented with *L. plantarum* IFPL935.

The formation of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone and 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid, intermediate metabolites arising from first steps in the microbial degradation of flavan-3-ols, was mainly observed in the TC vessels and their concentration increased after day 4 of both treatments (Fig. 3B). These compounds were also present in the DC vessels but to a much lesser extent and only in those with no addition of *L. plantarum* IFPL935. In accordance with these results, 4-hydroxy-5-(phenyl)-valeric acid content increased with time and was mainly detected in the DC vessels (Table S1 in the supplemental material).

Also, and as derived from galloylated flavan-3-ols, an increase in the content of gallic acid was only detected in the AC vessels, whereas 3-*O*-methylgallic acid was also measured in the TC vessels (Table S1 and Fig S2 in the supplemental material). Likewise, pyrogallol formation was only observed in the AC vessels whereas pyrocatechol was measured mainly in the AC and TC compartments. Supplementation with *L. plantarum* IFPL935 did not cause differences in the metabolism of these compounds.

The evolution of 3-(4'-hydroxyphenyl) propionic acid, 4-hydroxyphenylacetic acid and phenylacetic acid, which can also be derived from aromatic amino acid catabolism, followed the same trends as the microbial metabolites released from proteolysis depicted in Fig. 2A (Table S1 in the supplemental material). The extent of formation of microbial phenolic metabolites from the flavan-3-ol compounds was estimated in the SHIME vessels during the treatment and wash-out periods by computing the formation of phenylpropionic acids [3-(3',4'-dihydroxyphenyl)-

propionic, 3-(3'-hydroxyphenyl)-propionic, and 3-phenylpropionic] and phenylacetic acids [3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic] derivatives (Fig. 3C). The highest values of these metabolites were observed in the DC vessels, being still detected during the wash-out period. In the SHIME unit supplemented with *L. plantarum* IFPL935 the phenylpropionic acid derivatives, mainly represented by 3-(3'-hydroxyphenyl)propionic acid, appeared at earlier stages than the phenylacetic acid metabolites and their concentration was the highest when comparing values from the two SHIME units.

Discussion

The human gut microbiota contributes to many of the important host metabolic functions, including the increase of bioavailability of dietary polyphenols (van Duynhoven et al. 2011). Conversely, polyphenols and their resultant metabolites can modify the gut bacterial population composition and activity (Requena et al. 2010). Most of the studies that have examined the antimicrobial effect of dietary polyphenols have focused on single polyphenol molecules and/or single strains. In this work, we have studied the effect on the gut microbiota of the long-term feeding with a red wine polyphenolic extract and the impact of the supplementation of *L. plantarum* IFPL935, previously characterized as a flavan-3-ol metabolizer (Barroso et al. 2013; Sánchez-Patán et al. 2012b). We have conducted the study in the *in vitro* model of the human intestinal microbiota SHIME, which has demonstrated to allow nutritional interventions with repeated doses to evaluate long-term effect of food ingredients on the microbiota allocated in three consecutive colon regions (Grootaert et al. 2009; Marzorati et al. 2010; Van de Wiele et al. 2007; Van den Abbeele et al. 2013).

The present study shows that the initial addition of red wine polyphenols exerted an overall antimicrobial effect on the gut microbiota in the AC, but the effect disappeared during continuous feeding with polyphenols, probably due to the onset of microbial metabolism of polyphenols. The antimicrobial effect of red wine and grape polyphenolic extracts following either a single dose or a continuous feeding of the SHIME has recently been described (Kemperman et al. 2013). These authors reported a more pronounced antimicrobial effect on the colonic microbiota, which did not fully recover until the wash-out period. Differences between studies could be due to the fact that the amount of polyphenolic compounds daily supplemented by Kemperman et al. (2013) was 5-times higher than in the present study. Besides, grape extracts have demonstrated higher antimicrobial capacity than extracts obtained from red wine (Cueva et al. 2013), probably because of a higher content of proanthocyanidins and gallate derivatives (Xia et al. 2010).

Among polyphenols, flavan-3-ols and flavonols have received most attention due to their wide spectrum and higher antimicrobial activity in comparison with other polyphenols (Daglia 2012). In our study, all bacterial groups analyzed in the AC vessels were initially affected by the supplementation with red wine polyphenols. Among them, *Bacteroides* and the butyrate-producers *C. coccoides*/*E. rectale* were the most affected groups, whereas *Bifidobacterium* showed a more persistent effect. *Bacteroides* and *Bifidobacterium* have been described previously (Kemperman et al. 2013) as the genera most affected after feeding the SHIME colonic microbiota with black tea and red wine polyphenols. Additionally, intake of flavan-3-ol-rich sources for prolonged periods of time has generally correlated with the decrease of *Clostridium* groups (Dolara et al. 2005; Tzounis et al. 2011; Wu et al. 2011). On the other hand, counts of lactobacilli are generally unaffected or moderately increase after intake of diets enriched in flavanols

(Tzounis et al. 2011). Nevertheless, a different sensitivity to the presence of flavan-3-ols in the growth medium has been described for several *Lactobacillus* species (Tabasco et al. 2011), ranging from the optimal growth observed for *L. plantarum*, *L. casei*, and *L. bulgaricus* strains and the growth inhibition of *L. fermentum*, *L. acidophilus* and *L. vaginalis*. Among the strains assayed, however, only *L. plantarum* IFPL935 was able to metabolize flavan-3-ol compounds (Fernández-Patán, 2012b; Tabasco et al. 2011).

Although the antimicrobial effect caused by daily feeding the SHIME with red wine polyphenols was predominantly observed during the first 1-2 days of treatment and it was more distinguished in the AC vessels, the inhibitory effect on the overall microbial metabolism, however, was similar in the three vessels and it lasted for the first 5 days of treatment (Fig. 2). These results indicate a more pronounced effect of the polyphenol compounds in the microbial functionality than in its viability. The initial exposure to polyphenols may up-regulate polyphenol-induced stress responses related to defensive mechanisms while simultaneously down-regulating various metabolic functions, for example, carbon and energy metabolism (Stevenson and Hurst 2007). Decreased rate of microbial fermentation and proteolysis, determined as reduction in the formation of SCFA and BCFA, is associated with high tannin-rich feeds intake in ruminant livestock (Bodas et al. 2012). The effects of some plant extracts have been described to last only a few days, indicating that ruminal microorganisms adapt to the compounds with time (Busquet et al. 2005). Similarly, the regular feeding of the SHIME with a red wine polyphenolic extract was able to introduce microbial and metabolic changes until the microbiota adapted its metabolism and returned to steady state similar conditions during the treatment.

In terms of microbial metabolism, the addition of *L. plantarum* IFPL935 caused an increase in the formation of lactic acid at the start of the polyphenol treatment in the

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

flavanol-3-ols by cleavage of the heterocyclic C-ring (Jin and Hattori 2012; Kutschera et al. 2011; Wang et al. 2001).

A lower concentration of monomeric flavan-3-ols and first and intermediate metabolites (5-(3'-hydroxyphenyl)- γ -valerolactone and 4-hydroxy-5-(3'-hydroxyphenyl)valeric acid), mainly represented in the AC and TC vessels, was observed in the vessels of the SHIME unit supplemented with *L. plantarum* IFPL935. In addition, further metabolism of the polyphenol intermediate compounds to the formation of phenylpropionic acids was detected earlier and reached higher concentrations in the DC vessel containing the strain when compared to the other colonic vessels (Fig. 3C). For both treatments, bacterial conversion of red wine polyphenols was more favorable in the distal colon, which is in agreement with previous studies where a higher microbial conversion of phenolic compounds was found in the distal compartments (Barroso et al. 2013; Van Dorsten et al. 2012).

Phenyl propionic acid and 3-(3'-hydroxyphenyl)propionic acid were the microbial metabolites that accounted most for the differences between treatments. Phenylpropionic acid derivatives are considered to arise from β -oxidation of phenylvaleric acid derivatives (Monagas et al. 2010), except for 3-(4'-hydroxyphenyl)propionic acid that can result from the microbial catabolism of tyrosine (Russell et al. 2013). The evolution of this compound during the SHIME treatment with red wine polyphenols indicated that 3-(4'-hydroxyphenyl)propionic acid arose mostly from amino acid catabolism (Table S1 in the supplemental material). The phenyl propionic metabolite produced at a higher concentration was 3-(3'-hydroxyphenyl)propionic acid, which has been identified as a strong urinary marker of red wine and grape extracts intake (Jacobs et al. 2012; Ward et al. 2004). This microbial metabolite has been shown to reduce the inflammatory response of human peripheral

blood mononuclear cells stimulated with lipopolysaccharide (Monagas et al. 2009) and to have anti-thrombotic activity (Rechner and Kroner 2005). In conclusion, this study provides data supporting the potential use of *L. plantarum* IFPL935 to increase gut butyrate formation and favour polyphenol metabolism in an *in vitro* gastro intestinal system. This opens possibilities of using the strain as a food ingredient for helping individuals showing a low polyphenol-fermenting metabotype (Bolca et al. 2013) to increase their gut capacities of metabolizing dietary polyphenols.

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Legend to figures.

Fig. 1. Changes in q-PCR counts (log copy number/ml) of butyrate-producing groups belonging to *Clostridium* clusters IV and XIVa in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey squares) and the extract supplemented with *L. plantarum* IFPL935 (black circles) and the was out period. The dotted line indicates the end of the polyphenol treatment.

Fig. 2. Changes in concentration (mM) of ammonium (circles), BCFA (squares), acetic acid (diamonds), propionic acid (triangles), butyric acid (dots) and lactic acid (asterisks) in the ascending (AC; A), transverse (TC; B) and descending colon (DC; C) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey symbols) and the extract supplemented with *L. plantarum* IFPL935 (black symbols) and the was out period. The vertical dotted line indicates the end of the polyphenol treatment.

Fig. 3. Changes in concentration ($\mu\text{g/ml}$) of the sum of (A) precursor monomers (+)-catechin and (-)-epicatechin (circles) and procyanidins B1, B2, B3, B4 (squares), (B) the intermediate metabolites phenyl- γ -valerolactones (diamonds) and phenylvaleric acid derivatives (triangles) and (C) the phenylpropionic (dots) and phenylacetic acid (asterisks) derivatives in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey symbols) and the extract supplemented with *L. plantarum* IFPL935 (black symbols) and the was out period. The vertical dotted line indicates the end of the polyphenol treatment.

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)
<i>Lactobacillus</i>	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)
<i>Bifidobacterium</i>	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)
<i>Bacteroides</i>	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)
<i>Enterobacteriaceae</i>	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

Figure 2

Figure 3

Supplementary Material

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