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Lactobacillus plantarum IFPL935 Favors the Initial Metabolism of Red Wine Polyphenols When Added to a Colonic Microbiota

Elvira Barroso,[†] Fernando Sánchez-Patán,[†] Pedro J. Martín-Alvarez,[†] Begoña Bartolomé,[†] María Victoria Moreno-Arribas,[†] Carmen Peláez,[†] Teresa Requena,[†] Tom van de Wiele,[‡] and M. Carmen Martínez-Cuesta*[,†]

[†]Department of Biotechnology and Microbiology, Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM), Nicolás Cabrera 9, 28049 Madrid, Spain

[‡]LabMET, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium

ABSTRACT: This work aimed to unravel the role of *Lactobacillus plantarum* IFPL935 strain in the colonic metabolism of a polyphenolic red wine extract, when added to a complex human colonic microbiota from the dynamic simulator of the human intestinal microbial ecosystem (SHIME). The concentration of microbial-derived phenolic metabolites and microbial community changes along with fermentative and proteolytic activities were monitored. The results showed that *L. plantarum* IFPL935 significantly increased the concentration of the initial microbial ring-fission catabolite of catechins and procyanidins, diphenylpropanol, and, similarly, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid production. Overall, the addition of *L. plantarum* IFPL935 did not have an impact on the total concentration of phenolic metabolites, except for batches inoculated with colonic microbiota from the effluent compartment (EC), where the figures were significantly higher when *L. plantarum* IFPL935 was added (24 h). In summary, the data highlighted that *L. plantarum* IFPL935 may have an impact on the bioavailability of these dietary polyphenols. Some of the microbial-derived metabolites may play a key role in the protective effects that have been linked to a polyphenol-rich diet.

KEYWORDS: *Lactobacillus, polyphenols, metabolism, colonic microbiota, batch fermentations*

INTRODUCTION

There is increased epidemiological evidence that associates a moderate consumption of red wine to several health benefits in humans, such as protection against cardiovascular and neurodegenerative diseases and certain types of cancer, including colon, basal cell, ovarian, and prostate carcinoma.[1] This protective effect has been mainly linked to the presence of polyphenol compounds in wine. Polyphenols are secondary plant metabolites widely diverse and abundantly present in our diet in different food and beverages. Flavonoids are the most abundant, and among the most bioactive polyphenols present in red wine, mainly including flavan-3-ols (also known as flavanols), flavonols, and anthocyanins.[2] Unlike other classes of flavonoids, which exist in plants primarily in glucoside forms, flavanols are usually present in the aglycon form as monomers or oligomers (condensed tannins or proanthocyanidins) or esterified with gallic acid, giving rise to epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Research studies suggest that the health effects of dietary polyphenols are linked to its bioavailability, absorption, and metabolism.[3]

Colonic microbiota may play a crucial role in the potential health effects of the polyphenols.[3] Absorption of polyphenols from the upper intestinal tract is highly variable although it is established that many of them are poorly absorbed. The bioavailability of flavan-3-ols is largely influenced by their degree of polymerization: while monomers are readily absorbed in the small intestine, oligomers and polymers need to be biotransformed by the colonic microbiota before absorption.[4]

The human intestinal microbiota is a highly complex and dynamic ecosystem that harbors over a thousand different strains. In the colon, bacterial numbers can reach 100 trillion bacteria and such large numbers have enormous and diverse metabolic activity with significant implication for plant polyphenol metabolism.[5] This metabolic activity, which exceeds that of the liver in a factor of 100, is responsible for the major transformations of polyphenols in compounds that may have higher bioactivity and/or biological significance than their precursors.[6] Microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones and are able to break down the polyphenolic skeleton and perform reactions of reduction, decarboxylation, demethylation, and dehydroxylation.[7]

Parallel to this microbial metabolism, polyphenols could also modify the intestinal bacterial population composition and/or activity, thus establishing a bidirectional interaction between colonic microbiota and dietary polyphenols.[8,9] Related to this, batch fermentations performed with human feces inoculated with catechin have shown to have a positive effect on the growth of the *Clostridium cocccoides*−*Eubacterium rectale* group and *Bifidobacterium*, while inhibiting the growth of the *Clostridium histolyticum* group.[10] In vivo studies conducted in animals have shown an increase in *Lactobacillus* and

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Table 1. Primer Sets Used in this Study for Quantitative PCR

target gene/group	primer name	primer sequence 5′−3′	amplicon size	annealing temperature	standard	ref
total bacteria	968 F	AACGCGAAGAACCTTAC	489	55	*Escherichia coli* DH5α	23
	1401 R	CGGTGTGTACAAGACCC				23
Lactobacillus	LactoF	TGGAAACAGRTGCTAATACCG	192	55	*L. plantarum* IFPL 935	24
	LactoR	GTCCATTGTGGAAGATTCCC				24
Bifidobacterium	G Bifid F	CTCCTGGAAACGGGTGG	593	55	*Bifidobacterium breve* 29M2	25
	G Bifid R	GGTGTTCTTCCCGATATCTACA				25
Bacteroides	Bac303F	GAAGGTCCCCCACATTG	103	60	*Bacteroides fragilis* DSM2151	26
	Bfr-Fmrev	CGCKACTTGGCTGGTTCAG				27
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	513	55	clone	25
	g-Prevo-R	GGTCGGGTTGCAGACC				25
Enterobacteriaceae	F-Ent	ATGGCTGTCGTCAGCTCGT	385	58	clone	28
	R-Ent	CCTACTTCTTTTGCAACCCACTC				29
C. coccoides−*E. rectale* group	CcocErec-F	CGGTACCTGACTAAGAAGC	429	55	clone	30
(cluster XIVa)	CcocErec-R	AGTTTYATTCTTGCGAACG				30
Clostridium leptum subgroup-specific	sg-Clept F	GCACAAGCAGTGGAGT	239	55	clone	31
(cluster IV)	sg-Clept R3	CTTCCTCCGTTTTGTCAA				31
Ruminococcus	Rflbr730F	GGCGGCYTRCTGGGCTTT	157	60	clone	27
(cluster IV)	Clep866mR	CCAGGTGGATWACTTATTGTGTTAA				27
butyryl-CoA:acetate-CoA transferase gene	BCoATscrF	GCIGAICATTTCACITGGAAYWSITGGCAYATG	557	53	clone	32
	BCoATscrR	CCTGCCTTTGCAATRTCIACRAANGC				32

Bifidobacterium species following administration of red wine polyphenols, while a decrease in *Clostridium* and *Bacteroides* was reported.[11] Recently, a pilot human intervention study (n = 8) has shown a significant increase in the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *C. coccoides*−*E. rectale* groups in fecal samples after the consumption of red wine polyphenol for 4 weeks in comparison to baseline.[12]

In previous studies, we have reported that some strains of *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* were able to grow in the presence of a flavan-3-ol extract from grape seeds. In addition, *L. plantarum* IFPL935 was also capable of metabolizing these polyphenolic extracts through galloyl-esterase, decarboxylase, and benzyl alcohol dehydrogenase enzyme activities, leading to the formation of gallic acid, pyrogallol, and catechol, respectively.[13] Interestingly, *L. plantarum* IFPL935 showed the potential 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.[14] This activity has only been reported in a few intestinal bacteria to date.[15,16]

The aim of this work was to unravel the role of *L. plantarum* IFPL935 strain in the colonic metabolism of polyphenols by carrying out in vitro batch incubations containing a human microbiota from the colonic compartments of the dynamic simulator of the human intestinal microbial ecosystem (SHIME) and a commercial polyphenolic red wine extract. The study was undertaken by monitoring changes in the main microbial groups along with metabolic activity (short-chain fatty acids and ammonium production) and polyphenolic catabolism analyses.

MATERIALS AND METHODS

Bacterial Strains, Media and Phenolic Extract. *L. plantarum* IFPL935 isolated from raw milk cheeses[17] (CIAL Collection, CSIC, Spain) was grown at 37 °C in MRS broth (Pronadisa, Madrid, Spain). Growth of bacterial cultures was routinely monitored by measuring the optical density at 600 nm (OD_{600}).

A commercial alcohol-free red wine extract, Provinols, which contains at least 95% of polyphenols was used (Safic-Alcan Especialidades, Barcelona, Spain). As indicated by the supplier, 100 mg of Provinols corresponds to the polyphenol content of one glass of red wine (125 mL). The phenolic composition of this red wine extract was reported before,[18] flavan-3-ols and anthocyanins being the main phenolic compounds.

In Vitro Batch Incubations with Intestinal Bacteria. In vitro batch incubations were performed by sampling 25 mL of the colon ascendens, colon descendens, and effluent compartments (AC, DC and EC, respectively) (~8 log copy number/mL) of the SHIME.[19] This dynamic in vitro gastrointestinal model comprises a series of five double-jacketed fermentation vessels simulating the stomach, small intestine, and the three-stage large intestine conditions. The colon compartments contained in vitro cultured microbiota that were isolated from feces of a healthy adult volunteer and harbored a reproducible microbial community representative of the in vivo conditions both in composition and metabolic activity.[20] Detailed information about the SHIME system, the SHIME feed, and its in vivo validation can be found.[19,21,22] Following sampling, the colon microbial suspensions (25 mL) were placed into bottles containing Provinols (500 mg/L) or Provinols (500 mg/L) plus *L. plantarum* IFPL935 (10^7 ufc/mL) and were incubated for 48 h at 37 °C. To obtain anaerobic conditions, L-cystein (0.5 g/L) was added, and bottles were closed with butyl rubber stoppers and flushed with N_2 during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure. Before starting the incubation, bottles were placed at atmospheric pressure. Samples were taken at 0, 6, 24, and 48 h with a needle that extends beyond the butyl rubber stoppers that seal off the incubation bottles. Upon sampling, the mixture was flushed with N_2 to ensure anaerobic conditions. Samples were immediately stored at −20

Table 2. Quantitative-PCR Data (log copy number/mL) for the Microbial Groups Analyzed[a]

bacterial group	assays	compartment	incubation time 0 h	24 h	48 h
total bacteria	Provinols	AC	8.53(0.32) B c	7.81(0.10) BC b	6.76(0.33) B a
		DC	7.80(0.48) A b	7.08(0.37) A b	5.68(0.67) A a
		EC	8.52(0.31) B b	7.95(0.31) C b	7.02(0.67) B a
	Provinols + *L. plantarum* IFPL935	AC	8.24(0.36) B c	7.36(0.41) AB b	6.49(0.02) B a
		DC	8.11(0.54) AB b	6.97(0.48) A a	6.46(0.59) AB a
		EC	8.48(0.38) B b	8.07(0.47) C b	6.90(0.64) B a
Lactobacillus	Provinols	AC	7.25(0.33) B c	6.63(0.01) CD b	5.65(0.37) B a
		DC	6.49(0.53) A b	5.79(0.40) A b	4.57(0.50) Aa
		EC	7.05(0.14) AB b	6.64(0.30) CD b	5.66(0.70) B a
	Provinols + *L. plantarum* IFPL935	AC	7.05(0.44) AB c	6.43(0.40) BC b	5.67(0.05) B a
		DC	6.88(0.58) AB b	5.90(0.51) AB a	5.52(0.51) B a
		EC	7.31(0.20) B b	7.12(0.41) D b	6.07(0.63) B a
Bifidobacterium	Provinols	AC	7.31(0.32) B b	7.09(0.10) B b	5.94(0.20) B a
		DC	6.59(0.71) A b	6.31(0.60) A b	4.94(0.69) A a
		EC	6.66(0.15) A b	6.37(0.27) AB b	5.46(0.75) AB a
	Provinols + *L. plantarum* IFPL935	AC	7.10(0.43) B b	6.71(0.57) AB b	5.70(0.03) B a
		DC	6.90(0.80) A b	6.19(0.79) A ab	5.73(0.53) Ba
		EC	6.59(0.27) A b	6.45(0.29) AB b	5.54(0.55) AB a
Bacteroides	Provinols	AC	7.58(0.29) C c	6.80(0.30) B b	5.81(0.35) B a
		DC	6.75(0.19) A b	6.21(0.10) A b	4.53(0.84) A a
		EC	7.18(0.16) BC b	6.69(0.30) Bb	5.70(0.62) B a
	Provinols + *L. plantarum* IFPL935	AC	7.29(0.28) BC c	6.54(0.40) AB b	5.68(0.05) B a
		DC	7.09(0.35) AB b	6.14(0.26) A a	5.48(0.87) B a
		EC	7.12(0.11) B b	6.71(0.35) B b	5.57(0.47) B a
Prevotella	Provinols	AC	7.40(0.33) C c	5.67(0.21) Cb	4.21(0.13) A a
		DC	5.61(0.46) A b	3.93(0.14) A a	3.40(1.61) A a
		EC	6.40(0.60) B c	5.04(0.17) B b	4.30(0.47) A a
	Provinols + *L. plantarum* IFPL935	AC	7.56(0.52) C c	5.24(0.40) BC b	3.94(0.10) A a
		DC	6.24(0.73) AB b	4.32(0.47) A a	3.66(0.62) A a
		EC	6.25(0.42) AB c	5.11(0.29) B b	4.18(0.61) A a
Enterobacteriaceae	Provinols	AC	8.40(0.41) C c	7.42(0.20) BC b	6.29(0.60) AB a
		DC	7.22(0.62) A b	6.28(0.32) A ab	5.10(1.31) A a
		EC	8.22(0.45) BC b	7.49(0.66) BC b	6.48(0.64) B a
	Provinols + *L. plantarum* IFPL935	AC	8.15(0.70) BC b	6.91(0.35) AB a	6.15(0.40) AB a
		DC	7.46(0.58) AB b	6.67(0.94) AB ab	5.70(0.97) AB a
		EC	8.43(0.66) C b	7.94(0.29) C b	6.29(0.66) ABa
C. coccoides–E. rectale group	Provinols	AC	7.08(0.27) B c	6.65(0.13) B b	5.53(0.29) B a
		DC	6.36(0.53) A b	5.82(0.30) A b	4.20(0.90) A a
		EC	7.05(0.14) B b	6.73(0.25) B b	5.28(1.07) B a
	Provinols + *L. plantarum* IFPL935	AC	6.75(0.41) AB b	6.33(0.26) B b	5.31(0.08) B a
		DC	6.74(0.64) AB b	5.76(0.45) A a	5.05(0.63) AB a
		EC	6.97(0.21) B b	6.75(0.42) B b	5.43(0.71) B a
C. leptum	Provinols	AC	4.80(0.71) A b	4.77(0.14) A b	3.81(0.31) A a
		DC	5.49(0.56) B b	4.95(0.38) A b	3.53(0.53) A a
		EC	6.35(0.18) D b	5.90(0.31) B b	4.73(0.91) B a
	Provinols + *L. plantarum* IFPL935	AC	4.49(0.30) A b	4.44(0.27) A b	3.68(0.06) A a
		DC	5.77(0.61) BC b	4.90(0.54) A ab	4.34(0.53) AB a
		EC	6.31(0.18) CD b	6.00(0.42) Bb	4.75(0.74) Ba
Ruminococcus	Provinols	AC	3.01(0.19) A c	2.44(0.14) A b	1.33(0.20) Aa
		DC	4.56(0.62) B b	4.14(0.32) B b	2.50(2.55) B a
		EC	5.54(0.13) D b	5.20(0.34) C b	3.90(1.01) C a
	Provinols + *L. plantarum* IFPL935	AC	2.77(0.22) A c	2.23(0.35) A b	1.44(0.14) Aa
		DC	4.91(0.69) BC b	4.11(0.65) B ab	3.44(0.50) Ca
		EC	5.37(0.20) CD b	5.39(0.58) C b	3.95(0.82) C a
butyryl-CoA:acetate-CoA transferase gene	Provinols	AC	6.33(0.04) A c	5.91(0.17) A b	5.42(0.11) A a
		DC	6.27(0.37) A b	5.89(0.27) A ab	5.50(0.43) A a
		EC	7.01(0.35) C b	6.40(0.25) B a	5.96(0.31) B a
	Provinols + *L. plantarum* IFPL935	AC	6.28(0.27) A c	5.83(0.20) A b	5.51(0.07) A a
		DC	6.46(0.52) AB b	5.85(0.39) A ab	5.55(0.24) AB a

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Table 2. continued

bacterial group	assays	compartment	incubation time 0 h	24 h	48 h
		EC	6.91(0.22) BC b	6.53(0.18) B b	5.76(0.31) AB a

[a]Data are expressed as means and standard deviation (SD). For a given microbial group analyzed, different capital letters denote significant differences ($P < 0.05$, from LSD test) between compartments in the presence/absence of *L. plantarum* IFPL935. For the same row, different lowercase letters denote significant differences ($P < 0.05$, from LSD test) along the incubation time (for a given compartment).

°C until further analysis. Two independent experiments, each of them analyzed in triplicate, were carried out.

Microbial Community Analyses. Quantitative PCR (qPCR) on total bacteria and different groups and genera of bacteria (Table 1) was performed to study the effect of the incubation of the intestinal bacteria with the wine phenolic extract. Genomic DNA was extracted from samples (1 mL) by a method based on the protocols described previously.[33,34] Extractions were performed by the addition of 0.5 mL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer (pH 8.0) and 0.5 mL of phenol−chloroform−isoamyl alcohol (25:24:1). Samples were lysed with glass beads (150−212 μm diameter) by using FastPrep equipment (BIO 101, Savant Instruments, Holbrook, NY) for 30 s (three times) at a machine speed setting of 4.5 m/s. The aqueous phase containing nucleic acids was separated by centrifugation (3000*g*) for 5 min at 4 °C and mixed with an equal volume of chloroform−isoamyl alcohol (24:1) followed by centrifugation (16 000*g*) for 5 min at 4 °C. Total nucleic acids were subsequently precipitated from the extracted aqueous layer with 2 volumes of polyethelene glycol PEG-6000 (Merck, Hohenbrunn, Germany) for 2 h at room temperature, followed by centrifugation (18 000*g*) at 4 °C for 10 min. Pelleted nucleic acids were washed in ice cold 70% (v/v) ethanol and dried in a Speed-Vac (SPD 111 V; Savant Instruments) prior to resuspension in 100 μL of distilled water.

Triplicate samples of 10-fold diluted genomic DNA were analyzed for total bacteria, *Enterobacteriaceae*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, the specific phylogenetic groups *Clostridium coccoides−Eubacterium rectale* cluster XIVa, *Ruminococcus* cluster IV, *Clostridium leptum* subgroup specific cluster IV, and the gene encoding butyryl-CoA:acetate CoA transferase (BcoAT). Assays were performed using SYBR green methodology (Kappa Biosystems, Woburn, MA) with the IQ5Multicolor real-time PCR detection system (Bio-Rad), and data analyses were performed with iQ5 Optical System Software Version 1.1. Target microbial groups and functional genes, primers, amplicon size, annealing temperature, pure bacteria culture or clone for standard curves, and references are listed in Table 1. When samples were quantified using standards derived from one clone, each specific target sequence was cloned separately using the pGEM-T cloning vector system kit (Promega, Madison, WI) according to the manufacturer's instructions. The recombinant vector was transformed into chemically competent *Escherichia coli* DH5α cells. Transformed colonies were picked and processed for plasmid isolation. Plasmid purification was done using a Plasmid Mini kit (Qiagen, Hilden, Germany). The presence of the insert in the recombinant clones was confirmed by sequence analysis. Linearized plasmid was quantified using a spectrophotometer and copy numbers were calculated for all standards by the following formula:[35]

$$\text{number of copies}/\mu\text{L} = \frac{(6.022 \times 10^{23}\ \text{molecules/mol}) \times \text{plasmid concn (g}/\mu\text{L)}}{(\text{number of base pairs}) \times (660\ \text{Da/base pair})}$$

Short-Chain Fatty Acids (SCFAs) and Ammonium Determination Analyses. The SCFAs were extracted from the samples with diethyl ether, after the addition of 2-methylhexanoic acid as an internal standard, and extracts were analyzed as described previously.[36] Briefly, 1 μL of the diethyl ether layer was injected and measured in a Di200 gas chromatograph (GC) (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a capillary free fatty acid packed column [EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 m × 0.53 mm; film thickness 1.2 μm], a flame ionization detector, and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL/min. The column temperature and the temperature of the injector and detector were set at 130 and 195 °C respectively. The concentration of SCFAs was calculated in milligrams/liter. Total SCFAs were calculated on the basis of the amounts of acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and caproate.

Ammonium determination was performed as described.[37] Chiefly, ammonium from the samples (1 mL) was released as ammonia by addition of magnesium oxide (MgO) using an autodistillation Vapodest 30′ (Gerhardt Analytical Systems, Brackley Northants, UK); thus, ammonia was separated by steam distillation, collected in boric acid-indicator solution, and determined by titration with standard acid using a 685 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium). Ammonium ion concentration was expressed as millimoles/liter.

Targeted Analysis of Phenolic Metabolites. Phenolic metabolites were analyzed by a previously reported UPLC−ESI-MS/MS method.[38] The liquid chromatographic system was a Waters Aquity UPLC (Milford, MA) equipped with a binary pump, autosampler thermostated at 10 °C, and a heated column compartment (40 °C). The column employed was a BEH-C18, 2.1 × 100 mm and 1.7 μm particle size from Waters (Milford, MA). 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 run time of 18 min. The flow rate was set constant at 0.5 mL min^{-1} 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 (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound and using external calibration curves. Data of the MS/MS parameters (MRM transitions, cone voltages and collision energies) for phenolic metabolites (phenols, mandelic acids, benzoic acids, hippuric acids, phenylacetic acids, phenylpropionic acids, valeric acids, cinnamic acids, valerolactones, and other metabolites) ($n = 60$) and for flavan-3-ols (monomers, procyanidin dimers, procyanidin trimers, and gallates) were previously optimized.[39] Quantification was made on the basis of pure standards [from Sigma-Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany), and Extrasynthèse (Genay, France)] except for valeric acids [4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric, 4-hydroxy-5-(3′-hydroxyphenyl)valeric, and 4-hydroxy-5-phenylvaleric acids] that were quantified using the calibration curves of 3-(3,4-dihydroxyphenyl)propionic, 3-(3-hydroxyphenyl)propionic, and propionic acids, respectively. Injections were carried out in duplicate. Data acquisition and processing were 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). Three-way analysis of variance (ANOVA) was used to test the main effects of three factors studied (time, compartments, and

addition of *L. plantarum* IFPL935); and the least significant difference (LSD) test was applied for means comparisons ($P < 0.05$) between batches including or not *L. plantarum* IFPL935, at a certain time of incubation. Principal component analysis (PCA) from matrix correlation (where variables were previously standardized using all samples) was used to summarize changes in the concentration of microbial-derived phenolic metabolites. All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006, www.statsoft.com).

RESULTS

Microbial Community Analyses. Quantitative PCR (qPCR) was used to analyze the microbial community composition by targeting general bacteria and specific phylogenetic and functional groups (Table 2). The microbiological results of the batches at the incubation onset reproduced the variations in bacterial populations of the SHIME colonic compartments. *Bacteroides*, *Bifidobacterium*, and *Prevotella* were predominant in the AC (ascending colon) batches, whereas *C. leptum* and *Ruminococcus* prevailed in DC (descending colon) incubations. Microbiota found in EC (effluent compartment) batches, more representative of the fecal microbiota, was more similar to the DC batches than to the AC ones (Table 2). Besides, qPCR data showed that counts of all microbial groups assessed decreased along the incubation with the polyphenolic extract (Table 2). Reduction of microbial counts was also associated with the fact that batch culture incubations used in this study were static and closed systems in which the substrate was limited. These in vitro systems, however, have been stated as useful for short time course experiments.[40]

Inoculation of the colonic microbial batches with *L. plantarum* IFPL935 did not increase the total bacteria nor the *Lactobacillus* counts, although a slightly increase of *Lactobacillus* numbers was observed during incubation of the DC and EC batches inoculated with *L. plantarum* IFPL935 (Table 2). Similarly, DC batches inoculated with *L. plantarum* IFPL935 showed significantly higher counts of *Bifidobacterium*, *Bacteroides*, and *Ruminococcus* after 48 h incubation than DC batches without IFPL935. Nevertheless, for the other microbial groups analyzed, no significant differences were found in the batches inoculated with a colon-region-specific microbiota when IFPL935 was added.

In addition to *Clostridium* clusters IV and XIVa counts, a molecular approach based on the enumeration of the butyryl-CoA:acetate CoA transferase (BCoAT) gene was used for estimating the number of butyrate-producing bacteria. Data analysis showed that BCoAT gene copy number decreased along the incubation in all batches representing the distinct colon region microbiota, along with the reduction of bacteria populations also found at 48 h (Table 2). Relating these findings to the microbial groups analyzed, a significant ($P < 0.05$) correlation was found between this functional gene and the presence of *C. leptum* ($r = 0.91$), *C. coccoides*−*E. rectale* group ($r = 0.84$) and to a lesser extent to *Ruminococcus* ($r = 0.79$).

Fermentative and Proteolytic Activities. An important metabolic activity of the colonic microbiota is the formation of short-chain fatty acids (SCFAs). The initial SCFAs content was higher in the batches containing colonic microbiota from the DC and EC vessels (Figure 1a). During incubation, the higher increase in SCFAs production was measured in the AC vessels, although SCFAs increased in all batches, indicating that microbiota remained metabolically active. The increase in

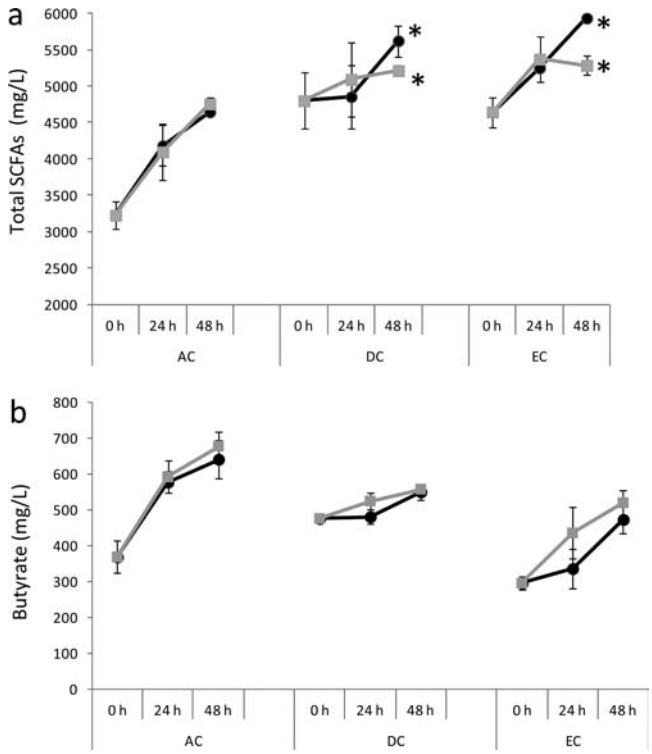

Figure 1. Average total SCFAs (a) and butyrate (b) production during the incubation of the microbiota representing the different colon compartments (ascendens, AC; descendens DC; effluent, EC) with Provinols (black circles) and Provinols plus *L. plantarum* IFPL935 (gray squares). For the batches containing microbiota for the same colon compartment, an asterisk indicates significant differences when *L. plantarum* IFPL935 was added.

SCFAs production was attributed mainly to an increase in acetate ($r = 0.95$), propionate ($r = 0.86$), and butyrate ($r = 0.23$). In general, acetate content was lower in the batches incubated with *L. plantarum* IFPL935, and this was found to be significant in the DC and EC batches (data not shown). This remark correlates with the significantly lower levels of SCFAs found in the DC and EC batches (48 h) when *L. plantarum* IFPL935 was added (Figure 1a). In contrast, and although the differences were not significant, a higher butyrate production (up to 29%) was found in all the batches incubated with *L. plantarum* IFPL935. Also, the higher increase in the butyrate production was detected in the AC batches (Figure 1b).

Ammonium concentration, a marker for proteolytic activity, was higher in the batches inoculated with the DC and EC microbiota (average 20.62 ± 1.12 mmol/L) than in the AC batches (average 12.14 ± 2.21 mmol/L) at the onset of the incubation. However, a marked increase in ammonium production was noticed in the course of the incubation (48 h) in the batches containing the AC microbiota, in contrast to that of DC and EC batches, where the figures remained stable (data not shown). Furthermore, ammonium concentration was not found to be significantly different in the batches incubated with IFPL935, except for EC batches (24 h), where this concentration was lower ($P < 0.05$) in batches with IFPL935 (16.08 ± 1.49 mmol/L) than in the control ones (21.55 ± 0.43 mmol/L) (results not shown).

Microbial Metabolism of Phenolic Compounds. A total of 28 microbial-derived phenolic metabolites, including benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, valeric

Table 3. Concentration of the Phenolic Metabolites Measured[a] and Main Effects of the Factors[b]

	mean (μg/mL)	minimum (μg/mL)	maximum (μg/mL)	factor's effect		
				time	microbiota	*L. plantarum* IFPL935
Benzoic Acids						
gallic acid	0.85(0.53)	0.00	2.27		*	
protocatechuic acid	0.60(0.21)	0.23	1.07	*	*	
4-hydroxybenzoic acid	0.23(0.04)	0.15	0.33	*	*	
vanillic acid	0.16(0.08)	0.04	0.33	*	*	
syringic acid	0.32(0.15)	0.04	0.72	*	*	
benzoic acid	0.39(0.08)	0.23	0.61	*	*	
salicylic acid	0.16(0.05)	0.09	0.30	*	*	
Phenols						
phloroglucinol	0.04(0.10)	0.00	0.60	*		
pyrogallol	0.08(0.20)	0.00	0.90	*	*	
catechol/pyrocatechol	0.13(0.11)	0.00	0.39	*	*	
Phenylacetic Acids						
3,4-dihydroxyphenylacetic acid	0.43(0.67)	0.00	5.01	*		
4-hydroxyphenylacetic acid	4.18(1.73)	0.63	7.79		*	
3-hydroxyphenylacetic acid	0.09(0.05)	0.00	0.19	*	*	
4-hydroxy-3-methoxyphenylacetic acid	0.01(0.02)	0.00	0.06	*	*	
phenylacetic acid	14.66(4.11)	5.10	21.08	*	*	
Phenylpropionic Acids						
3-(3,4-dihydroxyphenyl)propionic acid	1.97(1.52)	0.00	5.22	*	*	
3-(4-hydroxyphenyl)propionic acid	2.34(1.45)	0.22	5.33	*	*	
3-(3-hydroxyphenyl)propionic acid	0.20(0.54)	0.00	2.75	*	*	
phenylpropionic acid	0.66(0.39)	0.00	1.47	*	*	
Valeric Acids						
4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	0.50(1.06)	0.00	4.08	*		
4-hydroxy-5-(3′-hydroxyphenyl)valeric acid	0.06(0.19)	0.00	1.04	*		*
4-hydroxy-5-phenylvaleric acid	17.03(34.31)	0.00	119.27	*	*	
Valerolactones						
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone	0.45(0.89)	0.00	3.37	*		
Cinnamic Acids						
caffeic acid	0.27(0.43)	0.00	1.29	*		
p-coumaric acid	0.16(0.23)	0.00	0.78	*		
ferulic acid	0.02(0.03)	0.00	0.11	*		
isoferulic acid	0.02(0.03)	0.00	0.11	*	*	
Other Metabolites						
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	0.07(0.19)	0.00	0.99	*	*	*
∑ phenolic metabolites	43.90(39.33)	7.52	153.95	*	*	

[a]Mean, standard deviation (SD), and range of variation (minimum and maximum) of the phenolic metabolites concentration measured after incubation of the wine extract with the different colon region microbiota. [b]Statistical significance (*) ($P < 0.05$) of the main effects of the factors: time (0, 6, 24, and 48 h), microbiota (AC, DC and EC), and addition of *L. plantarum* IFPL935 is also shown.

acids, valerolactones, and cinnamic acids, were found when incubating the commercial wine extract with the microbiota from the three different colonic compartments (AC, DC, and EC) (Table 3). The total concentration of phenolic metabolites (sum of individual metabolite concentration) increased along the incubation period and turned out to be significantly higher in the DC and EC batches with regard to the AC batches (Figure 2a). Overall, the addition of *L. plantarum* IFPL935 to the colonic microbiota did not have an impact on the total concentration of phenolic metabolites, except for the EC batches, where the figures were significantly higher when IFPL935 was added (24 h). In this regard, a three-way ANOVA analysis showed that the main factors that have a significant effect ($P < 0.05$) on the total concentration of phenolic metabolites were the incubation time and the colon-region-specific microbiota (AC, DC, and EC) (Table 3).

When analyzing specific phenolic metabolites, the highest concentrations were found for phenylacetic and 4-hydroxy-5-phenylvaleric acids (21 and 119 μg/mL, respectively), followed by other phenylacetic (3,4-dihydroxy and 4-hydroxy) and phenylpropionic (3,4-dihydroxy and 4-hydroxy) acids (<8 μg/mL) (Table 3). Furthermore, when the data was analyzed using a three-way analysis of variance (ANOVA), only concentrations of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol (i.e., diphenylpropanol) and 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid showed significant main effects for the addition of *L. plantarum* IFPL935 (Table 3). As example, Figure 2b depicts the production of the intermediate microbial ring-fission catabolite of catechins and procyanidins, diphenylpropanol, in the three batches, up to 48 h. Although addition of *L. plantarum* IFPL935 significantly increased the concentration of this metabolite in the batches containing DC and EC

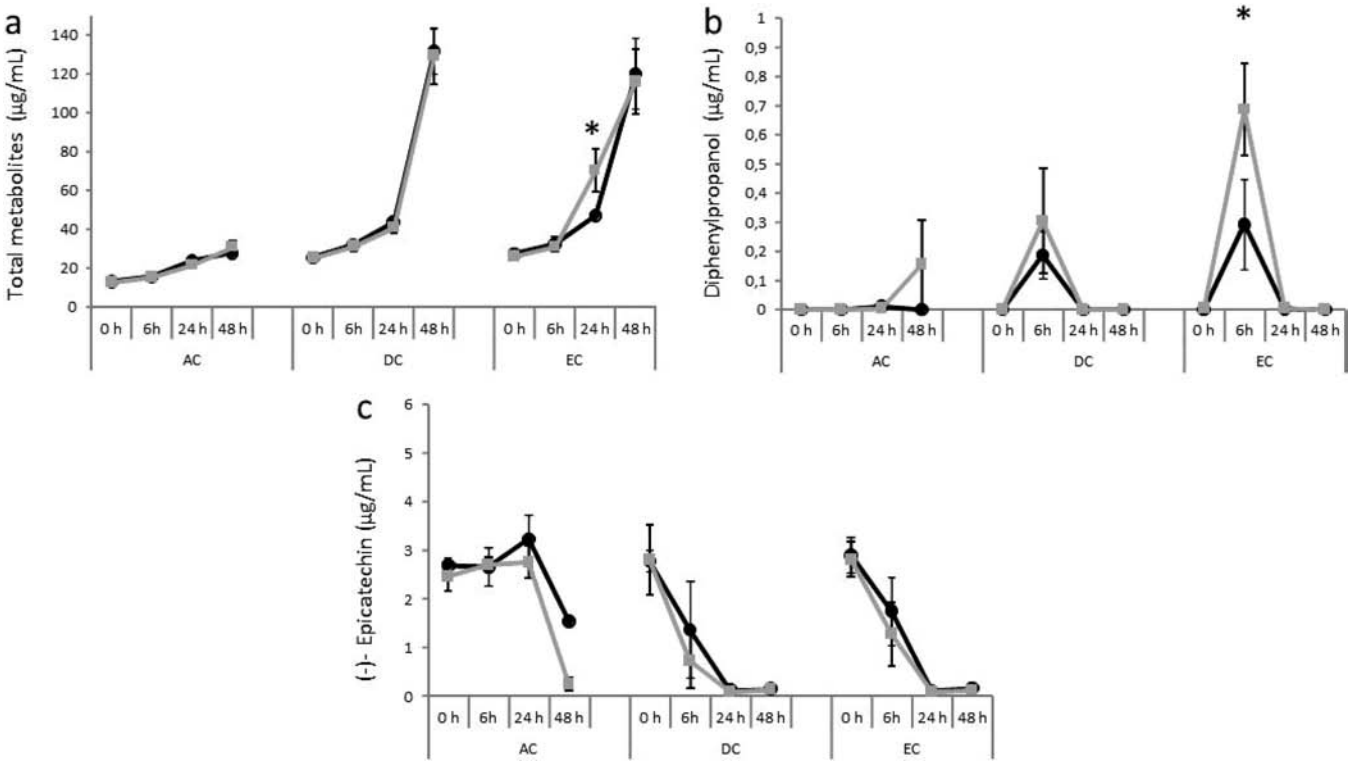

Figure 2. Production of total microbial-derived phenolic metabolites (a) and 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (b) and the disappearance of (−)-epicatechin (c) during the incubation of the wine extract with the three different colon region microbiota (AC, DC, and EC) with Provinols (black circles) and Provinols plus *L. plantarum* IFPL935 (gray squares). For the batches containing microbiota for the same colon compartment, an asterisk denotes significant differences with regard to the batches where *L. plantarum* IFPL935 was added.

microbiota (6 h), in those harboring AC microbiota (48 h), the LSD test for means comparisons confirmed significant differences ($P < 0.05$) in the batch containing EC microbiota. Similarly, the production of 4-hydroxy-5-(3′-hydroxyphenyl)-valeric acid, an intermediate metabolite derived from 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid which in turn comes out from the diphenylpropanol,[41] followed the same trend (results not shown). Moreover, (−)-epicatechin, one of the main flavan-3-ols present in the wine extract, was completely degraded by the DC and EC microbiota at 24 h of incubation, while degradation of (−)-epicatechin was only noticeable at 48 h in the AC batches, being greater in the presence of *L. plantarum* IFPL935 (Figure 2c); however, no significant differences between batches including *L. plantarum* IFPL935 or not were found at any time of incubation for any microbiota (AC, DC, and EC) (Figure 2c). Similar trends were observed for other compounds present in the wine extract such as (+)-catechin and procyanidin dimers and trimers (results not shown).

Principal component analysis (PCA) was performed to obtain a simplified view of the changes in phenolic microbial metabolism under the different conditions. Two principal components, PC1 and PC2, which explained 49% of the total variance, were found. For a better understanding of the data, mean values of the scores of the triplicate assays in the different time periods (0, 6, 24, and 48 h), considering the distinct colon-region-specific microbiota and the addition of *L. plantarum* IFPL935, were plotted in the plane delimited by the first two principal components (Figure 3). PC1, which explained 36.1% of the variance, reflected overall changes occurring during the time-course of the microbial catabolism of

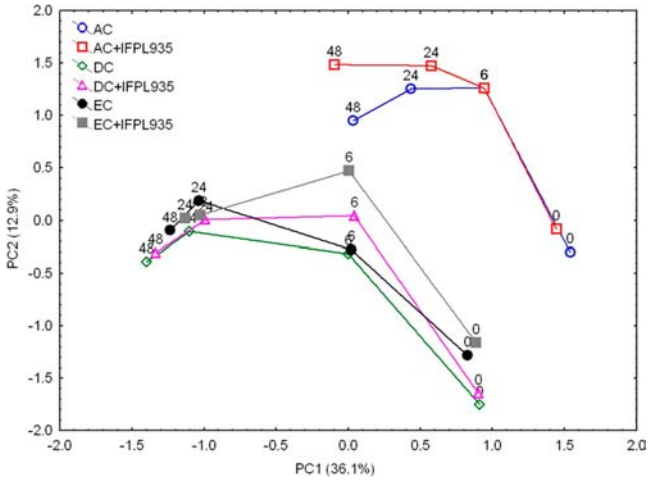

Figure 3. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulted from a PCA of microbial-derived phenolic metabolites for the different colon-region-specific microbiota (AC, DC, and EC) and considering the addition of *L. plantarum* IFPL935 at different incubation times (0, 6, 24, and 48 h).

the wine polyphenols. This component was negatively correlated (loadings >0.72) with 3-(4-dihydroxyphenyl)-propionic acid (−0.85), phenylpropionic acid (−0.85), salicylic acid (−0.82), protocatechuic acid (−0.79), 3-(3,4-dihydroxyphenyl)propionic acid (−0.79), catechol/pyrocatechol (−0.76), and phenylacetic acid (−0.73). Therefore, lower values of PC1 corresponded to higher concentrations of the compounds, which were measured at longer incubation times,

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mainly in the vessels containing the DC and EC microbiota. Interestingly, PC2, which explained 12.9% of the variance, showed differences in the phenolic metabolic profile of the batches containing the same colon-region-specific microbiota when *L. plantarum* IFPL935 was added. These differences in the metabolites profiles were detected at 6 h for DC and EC batches and for the AC batches at 24−48 h of incubation. In all cases, higher values of PC2 were related to the addition of IFPL935.

DISCUSSION

Bearing in mind that the composition and metabolic activity of the microbiota is colon-region-dependent, this study was aimed to evaluate the contribution of *L. plantarum* IFPL935 to the colonic metabolism of wine polyphenols using in vitro batch fermentations inoculated with colonic microbiota developed in different colonic compartments of the SHIME. The SHIME has been reported to be able to simulate reproducible and highly diverse microbial communities that are colon-region-specific.[20]

The colonic microbiota plays an important role in the microbial catabolism of dietary polyphenols. It has been estimated that 90−95% of dietary polyphenols are not absorbed in the small intestine and therefore accumulate in the colon.[42] Therefore, the bioactivity of these compounds is largely dependent on the microbiota activity. In this study, a wide range of potential phenolic metabolites arising from flavan-3-ols catabolism, including first and intermediate metabolites [diphenylpropanol, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid] and end products (hydroxypropionic acid, hydroxyacetic acids, cinnamic acids) were targeted during the course of the batch fermentations. Besides, other metabolites detected, such as *O*-methylated benzoic acids including syringic and vanillic acids, could also arise from the catabolism of anthocyanins and other flavonoids also present in the red wine extract, as it has been previously reported.[18] Furthermore, the microbial metabolic activity on the red wine extract polyphenols appears to vary according the specific colon-region microbiota, as different phenolic metabolite profiles were detected in the distinct batch fermentations. Thus, production of phenolic intermediate metabolites and phenolic acids were detected earlier during the incubation and reached higher concentrations in the batches containing colonic microbiota simulating the distal colon regions (DC and EC) when compared to the batches inoculated with microbiota from the ascending colon region (AC) (Figure 2), suggesting that bacterial conversion of wine polyphenols was more favorable in the distal colon. This colon site specificity of the microbial transformations is in agreement with previous studies where a higher microbial conversion of phenolic compounds was found in the distal compartments.[22,43]

Microbial catabolism of polyphenols comprises a series of chain reactions, leading to numerous intermediate metabolites and end products.[7,9,41] The catabolism of flavan-3-ols starts with the reductive cleavage of the heterocyclic C ring, resulting in the formation of diphenylpropan-2-ol, followed by the breakdown of the A ring and further lactonization into phenylvalerolactones derivatives, specifically into 5-(3,4-dihydroxyphenyl)-γ-valerolactone. However, knowledge about intestinal bacteria involved in the cleavage of the C-ring conversion is limited to only a few strains isolated from human feces such as *Eubacterium* sp. strain SDG-2 and *Eggerthella lenta* rK3.[15,16] Previous findings showed that besides other polyphenol metabolic activities, *L. plantarum* IFPL935 was capable in pure culture of cleaving the heterocyclic ring of monomeric flavan-3-ols, giving rise to the first metabolite of the microbial catabolic pathway.[13,14] Hence, the results here presented demonstrate the capability of *L. plantarum* IFPL935 of initiating wine polyphenol catabolism in the complex environment of colonic microbiota, as seen for the quicker disappearance of flavan-3-ols [i.e., (−)-epicatechin] and production of diphenylpropanol and other intermediate metabolites immediately derived from it in the batches incubated with *L. plantarum* IFPL935 (Figure 2). Moreover, changes in the profile of these compounds observed at the first stages of phenolic degradation (after 6 h of incubation in the DC and EC batches and at 24−48 h of incubation in the AC batches) (Figure 2) might be associated with the differences observed in microbial communities between the colon-region-specific compartments (Table 2).

In this regard, the analysis of the microbial communities in the batches representing the AC, DC, and EC microbiota further indicate higher variations between in vitro batches harboring a different colon-region microbiota than those found when *L. plantarum* IFPL935 was added. Thus, AC batches, where the fermentation is very intense with a high production of short chain fatty acids (SCFAs), were shown to harbor the major proportion of saccharolytic bacteria (*Bacteroides*, *Bifidobacterium*, *Prevotella*). Therefore, most of the oligosaccharides used as prebiotics in the functional food industry are predominantly fermented in the proximal colon.[44] By contrast, the microbial community, represented by *Clostridium* groups was found in major numbers in the batches inoculated with microbiota simulating the distal regions (DC and EC vessels), where the putrefactive processes become quantitatively more important, although proteolysis occurs along the entire colon.

Although knowledge about specific gut bacteria capable of degrading wine polyphenols such as flavan-3-ols is still scarce, it may be difficult to identify a single bacterium capable of exhibiting the whole catabolic pathway, but rather the catabolism may be carried out by different bacteria species acting at some steps of the different degradation pathways. The formation of diphenylpropanol and other intermediate metabolites and its further transformation into phenylpropionic, phenylacetic, and benzoic acids could be critical steps delimiting the rate and extent of red wine polyphenols catabolism, thus influencing the bioavailability and bioactivity of these compounds in vivo. However, total production of phenolic metabolites seemed not to be affected by the presence of *L. plantarum* IFPL935 at further degradation steps or longer incubation times (Figure 2a), suggesting that although the initial cleavage of the heterocyclic ring of monomeric flavan-3-ols could be a limited step, once the metabolite 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol is formed, the colonic microbiota can proceed in further degradation steps. Further studies will be performed using gastrointestinal dynamic models where microbial populations and microbe-mediated metabolic effects can be dynamically monitored in the different colon regions and along the incubation time, avoiding the limitation of nutrients and phenolic substrates.

Besides polyphenol catabolism, special attention was given to the butyrate-producing bacteria, as they play an important role in the human colon, supplying energy to the gut epithelium and regulating host cell responses.[45] Butyrate-producing bacteria represent a functional group rather than a phylogenetic group, as their distribution within bacterial clusters is uneven. A

molecular approach based on the enumeration of the butyryl-CoA:acetate CoA transferase gene (BCoAT) was used for estimating the number of butyrate-producing bacteria in samples containing a complex microbiota.[46] BCoAT gene copy numbers significantly decreased along the incubation (48 h) in all batches representing the distinct colon-region-specific microbiota, in agreement with the decrease in bacterial population numbers along the incubation (Table 2). More meaningfully, data analysis showed a significant correlation ($P < 0.05$) between counts of this functional gene and the presence of *C. leptum* and *C. coccoides*−*E. rectale* group and to a lesser extent to *Ruminococcus*, confirming previous studies in which bacteria belonging to *Clostridium* (clusters IV and XIVa) were pointed out as important butyrate producers playing a key role in butyric acid production.[46]

Despite the decrease of butyrate producers, a higher butyrate production was detected in the batches containing the ascending colon microbiota (AC) at the end of the incubation compared to DC and EC batches (Figure 1 b). This is in agreement with the fact that most of the fermentative metabolism takes place in the AC, but the results also showed that the presence of *L. plantarum* IFPL935 tended to increase butyrate production while in turn decreasing acetate and in general total SCFAs concentration (Figure 1a). In this regard, when analyzing the SCFAs production, cross-feeding interactions between colon bacteria should be taken into account. On one hand, bifidobacteria and lactobacilli strains are potentially able to produce lactate that can be further turned into butyrate and propionate through cross-feeding by other bacteria such as *Eubacterium hallii* within *C. coccoides* cluster.[47] This could explain the favorable effect of the presence of *L. plantarum* IFPL935 on butyric acid production. On the other hand, bacteria of the *C. coccoides* cluster can also convert acetate produced by the main acetate producers such as *Bacteroides* and *Prevotella*[45] into butyric acid.[47,48]

In summary, the results highlighted confirm the capability of *L. plantarum* IFPL935 to initiate flavan-3-ols catabolism when added to a complex colonic microbiota, which may have an impact on the bioavailability of these dietary polyphenols. Some of the microbial-derived metabolites may play a key role in the protective effects that have been linked to a polyphenol-rich diet.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +34910017900. Fax: +34910017905. E-mail: carmen.martinez@csic.es.

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Notes

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