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Capability of *Lactobacillus plantarum* IFPL935 To Catabolize Flavan-3-ol Compounds and Complex Phenolic Extracts

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ABSTRACT: *Lactobacillus plantarum* IFPL935 was incubated with individual monomeric flavan-3-ols and dimeric A- and B-type procyanidins to identify new metabolites and to determine the effect of compound structural features on bacterial growth and catabolism. Complex extracts rich in A-type proanthocyanidins and phenolic acids from cranberry were also tested. The results showed that *L. plantarum* IFPL935 exhibited higher resistance to nongalloylated monomeric flavan-3-ols, A-type dimeric procyanidins, and cranberry extract than to (−)-epicatechin-3-*O*-gallate and B-type dimeric procyanidins. Despite these findings, the strain was capable of rapidly degrading (−)-epicatechin-3-*O*-gallate, but not A- or B-type dimeric procyanidins. However, it was able to produce large changes in the phenolic profile of the cranberry extract mainly due to the catabolism of hydroxycinnamic and hydroxybenzoic acids. Of most relevance was the fact that *L. plantarum* IFPL935 cleaved the heterocyclic ring of monomeric flavan-3-ols, giving rise to 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, activity exhibited by only a few human intestinal bacteria.

KEYWORDS: *Lactobacillus, microbial catabolism, flavan-3-ols, B-type procyanidins, A-type procyanidins, grape seed extract, cranberry extract*

INTRODUCTION

The bioavailability and potential bioactivity of a large proportion of dietary polyphenols are largely dependent on the activity of the colonic microbiota.[1−3] Among these polyphenols are the monomeric flavan-3-ols and their oligomers and polymers, also known as proanthocyanidins, which are very abundant polyphenols in our diet, being present in many food sources such as fruits (grapes, apples, and berries), legumes, and cacao and in beverages such as wine, cider, tea, and beer.[4] Flavan-3-ols are heterogeneous flavonoid compounds, which vary according to their degree of polymerization (DP), monomeric unit composition, type of interflavan bond and position, and possible galloylation.[5] Microbial catabolism at the colon level is especially important for molecules with DP > 2, which present poor absorption in the small intestine, and also for absorbed monomeric flavan-3-ols reaching the colon through the bile.[1] Recently, it was reported that after oral administration of [^{14}C]procyanidin B2, 63% of the total radioactivity was excreted via urine, indicating that a large quantity of the parent compound was degraded by the gut microbiota.[6] One particular feature that makes flavan-3-ols interesting is that they are nonplanar molecules, presenting a saturated heterocyclic ring with two asymmetric carbons and also lacking substitution at C-4.[7] Most researchers agree that the catabolism of flavan-3-ol monomers and polymers starts with the reductive cleavage of the heretocyclic C-ring, giving rise to the intermediate metabolite 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol. From this, two characteristic metabolites are formed: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, which, after a series of decarboxylation, dehydroxylation, and oxidation reactions, result in a great variety of chemical structures corresponding to phenylpropionic, phenylacetic, and benzoic acids and simple phenols of different hydroxylation patterns.[8−12] In the case of galloylated forms, hydrolysis takes place before reductive cleavage by the action of microbial esterases.[10] Despite the health effects of microbial metabolites remaining largely unknown, anti-inflammatory and antiproliferative activities of characteristic metabolites (i.e., 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone) have been reported,[13] and findings suggest that their residence time in the organism is sufficient to produce local or systemic health effects.[14] Despite the evidence of the catabolism of flavan-3-ols by intestinal microbiota, no bacteria capable of performing the complete catabolic pathway of flavan-3-ols have been identified, a fact that has been partly attributed to their particular stereochemical features.[7,15−17]

Bacterial degradation of flavan-3-ols is hindered by the inherent antibacterial effects of these molecules. Among human bacterial populations, studies carried out with pure cultures have found that the growth of bacteria belonging to the genus *Lactobacillus* is not affected or even promoted by incubation in the presence of flavan-3-ols or flavan-3-ol-rich extracts.[18,19] Moreover, in vivo studies conducted in both humans and animals have revealed a modulatory effect in favor of an increase in *Lactobacillus* spp. and a decrease in the *Enterobacteriaceae*, *Clostridium*, and *Bacteroides* bacteria groups following administration of monomeric flavan-3-ols from green tea, red wine polyphenols, grape pomace concentrate, grape seed extract, or a

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flavan-3-ol-rich cocoa drink.[20−23] Therefore, *Lactobacillus* spp. seems to be tolerant of flavan-3-ols. Catabolism of hydrolyzable tannins and gallic acid, through certain enzymatic activities, has also been reported in some *Lactobacillus* strains.[24] However, only one recent study has presented data on the catabolism of flavan-3-ols.[25] In this study, we reported that some *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains were able to grow in the presence of a flavan-3-ol extract from grape seeds. In particular, *L. plantarum* IFPL935 showed the potential to catabolize flavan-3-ols not only by galloyl esterase, decarboxylase, and benzyl alcohol dehydrogenase activities but also by other possible enzymatic activities that resulted in the formation of a new unidentified metabolite.[25]

In the present work, we have performed incubations of *L. plantarum* IFPL935 with individual monomeric flavan-3-ols and dimeric A- and B-type procyanidins to identify this new metabolite and to determine the effect of the chemical structure, degree of polymerization, and type of interflavan linkage on the growth and catabolism of *L. plantarum* IFPL935. Complex extracts from cranberry (rich in A-type proanthocyanidins and phenolic acids) were also tested. Finally, the optimum incubation conditions (aerobic/anaerobic conditions and % glucose) for the catabolism of flavan-3-ols by *L. plantarum* IFPL935 were tested using a monomeric fraction from grape seeds, particularly useful for combining nongalloylated and galloylated monomers and dimers of flavan-3-ols.

MATERIALS AND METHODS

Phenolic Compounds and Extracts. (+)-Catechin was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). (−)-Epicatechin, (−)-epicatechin-3-*O*-gallate, and procyanidins B1, B2, and A2 were obtained from Extrasynthèse (Genay, France). A flavan-3-ol monomeric preparation obtained from a commercial grape seed extract (Vitaflavan) (GSE-M) was kindly supplied by DRT (Les Dérivés Résiniques et Terpéniques, S.A., France). Its total phenolic content was 750 mg of gallic acid equiv/g, as measured by the Folin−Ciocalteu reagent. Compounds present in this extract included gallic acid (8.00 mg/g), monomeric flavan-3-ols ((+)-catechin (136 mg/g) and (−)-epicatechin (135 mg/g)), procyanidin dimers (B1 (21.0 mg/g), B2 (42.4 mg/g), B3 (20.9 mg/g), and B4 (21.2 mg/g)) and trimers (C1 (3.37 mg/g) and T2 (2.04 mg/g)), (−)-epicatechin-3-*O*-gallate (19.1 mg/g) and procyanidin gallates (B1-3-*O*-gallate (0.13 mg/g), B2-3-*O*-gallate (3.27 mg/g), and B2-3′-*O*-gallate (2.23 mg/g)), all giving a total content of 414 mg/g.[26] A commercial cranberry extract (CE) was kindly supplied by Triarco Industries Inc. (Wayne, NJ, USA). The total phenolic content of the cranberry extract was 219 mg of gallic acid equiv/g, as measured by the Folin−Ciocalteu reagent (Merck, Darmstadt, Germany). The cranberry extract mainly contained benzoic acids (9.76 mg/g), hydroxycinnamic acids (11.1 mg/g), flavan-3-ols (2.1 mg/g), and anthocyanins (0.055 mg/g) (Sánchez-Patán et al., unpublished results). Solutions of pure compounds (0.25, 0.5, and 1 mM) and extracts (0.25, 0.5, and 1 mg/mL) were prepared in the bacterial medium and sterile-filtered before use.

Bacterial Growth. *L. plantarum* IFPL935 was previously isolated from raw goat's milk cheese[27] and belongs to the bacterial culture collection of the Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, Spain. It was routinely cultured at 37 °C in de Man, Rogosa, and Sharpe (MRS) broth (Pronadisa, Madrid, Spain). For experiments of growth and catabolism of *L. plantarum* IFPL935 in the presence of polyphenols, a chemically defined medium (ZMB1)[28] was used. Unless otherwise stated, the glucose content in the medium was 0.4% (w/v).

Bacterial growth under aerobic conditions was carried out in triplicate wells of sterile 96-well microplates with a lid (Sarstedt Inc., Newton, NC, USA), containing 300 μL of ZMB1 medium with or without different concentrations of phenolic compounds and extracts. Wells were inoculated (1%; about 10^7 colony-forming units/mL) with a fresh culture of the strain incubated in MRS broth at 37 °C for 16 h. Appropriate controls were used by incubating noninoculated ZMB1 supplemented with phenolic compounds/extracts (blank) and ZMB1 inoculated with the strain (growth control). Incubations were carried out at 37 °C for 48 h under stationary conditions. Growth was monitored at 60 min intervals (preceded by 15 s of shaking at variable speed) by assessing the optical density at 600 nm (OD_{600}) using an automated microplate reader (Varioskan Flash, Thermo Electron Corp., Vantaa, Finland). The bacterial growth data collected were fitted to determine maximum specific growth rates (μ_{max}) and lag phase (lag) by using the Microsoft Excel add-in DMfit v. 2.1 (Baranyi and Roberts, 1994; available at http://www.ifr.ac.uk/safety/DMfit/default.html).

Bacterial growth under anaerobic conditions was carried out using anaerobic jars (Gas-Pack, Anaerogen; Oxoid). Growth was monitored by recording the OD_{600} variations after 7.5, 24, and 48 h of incubation at 37 °C.

Cultures used to follow catabolism of flavan-3-ol compounds and phenolic extracts by *L. plantarum* IFPL935 were performed in ZMB1 by scaling volumes up to 10 mL into 15 mL sterile Falcon tubes (VWR, Germany). During incubation (37 °C, 48 h), samples were taken at regular intervals and centrifuged (10000*g*, 10 min). Supernatants were filtered through 0.45 μm pore-size filters and kept at −20 °C until UPLC analysis. Experiments were carried out in duplicate.

Analysis of Phenolic Compounds and Microbial Metabolites by UPLC-DAD-ESI-TQ MS. A UPLC system coupled to an Acquity PDA eλ photodiode array detector and an Acquity TQD tandem-quadrupole mass spectrometer (UPLC-PAD-ESI-TQ MS) (Waters, Milford, MA, USA) was used. A previously developed UPLC-ESI-TQ MS method for the analysis of food flavan-3-ols and their metabolites was applied.[29] Separation was performed on a Waters BEH C18 column (2.1 × 100 mm; 1.7 μm) at 40 °C. A gradient composed of solvent A (water/acetic acid (98:2, v/v)) and solvent B (acetonitrile/acetic acid (98:2, v/v)) was applied at a flow rate of 0.5 mL/min as follows: 0−1.5 min, 0.1% B; 1.5−11.17 min, 0.1−16.3% B; 11.17−11.5 min, 16.3−18.4% B; 11.5−14 min, 18.4% B; 14−14.1 min, 18.4−99.9% B; 14.1−15.5 min, 99.9% B; 15.5−15.6 min, 0.1% B; 15.6−18 min, 0.1% B. The DAD was operated in the 250−420 nm wavelength range at a 20 point/s rate and 1.2 nm resolution. The ESI parameters were 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. The MRM transitions used for detection of flavan-3-ols were (+)-catechin and (−)-epicatechin (289 → 245), (−)-epicatechin-3-*O*-gallate (441 → 289), procyanidins B1 and B2 (577 → 289), procyanidin A2 (575 → 449), and B1-, B2-, and B2′-3-*O*-gallates (729 → 577). For detection of different structures of phenolic metabolites, the MRM transitions used were as follows:[29] for phenylpropionic acids, 3-(3,4-dihydroxyphenyl)propionic acid (181 → 137), 3-(3- and 3-(4-hydroxyphenyl)propionic acids (165 → 121), and phenylpropionic acid (149 → 105); for phenylacetic acids, 3,4-dihydroxyphenylacetic acid (167 → 123), 3- and 4-hydroxyphenylacetic acids (151 → 107), and phenylacetic acid (135 → 91); and for benzoic acids and others, gallic acid (169 → 125), benzoic acid (121 → 77), syringic acid (197 → 182), protocatechuic acid (153 → 109), vanillic acid (167 → 152), *p*-coumaric acid (163 → 119), caffeic acid (179 → 135), ferulic acid (193 → 134), and catechol/pyrocatechol (109 → 81).

In the absence of commercial standards, procyanidin B1-, B2-, and B2′-3-*O*-gallates were quantified using the epicatechin-3-*O*-gallate curve. The metabolite 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (i.e., diphenylpropan-2-ol) was quantified using the calibration curve of 3-(3,4-dihydroxyphenyl)propionic acid. Data acquisition and processing were carried out using MassLynx 4.1 software. Before injection, frozen samples were thawed at room temperature and then centrifuged (14926*g*, 20 °C, 10 min) and filtered through a 0.22 μm filter. The filtrate was diluted (1:1, v/v) with a mixture of water/acetonitrile (60:40, v/v), and 2 μL of the diluted sample was injected onto the column.

Table 1. Effect of Phenolic Compounds on the Growth of *L. plantarum* IFPL935[a]

	phenolic concentration	max OD_{600}	growth rate (μ_{max}; h^{-1})	lag time (h)
control		1.05 ± 0.04	0.14 ± 0.01	5.65 ± 0.31
(+)-catechin	0.25 mM	1.01 ± 0.06	0.13 ± 0.01	6.02 ± 0.38
	0.50 mM	0.94 ± 0.06	0.12 ± 0.01	5.92 ± 0.24
	1.0 mM	0.81 ± 0.16	0.11 ± 0.01	5.89 ± 0.27
(−)-epicatechin	0.25 mM	0.99 ± 0.04	0.13 ± 0.01	5.90 ± 0.21
	0.50 mM	0.94 ± 0.08	0.11 ± 0.01	5.91 ± 0.27
	1.0 mM	0.78 ± 0.16	*0.10 ± 0.01	6.33 ± 0.65
(−)-epicatechin-3-*O*-gallate	0.25 mM	1.00 ± 0.01	0.11 ± 0.01	6.81 ± 0.16
	0.50 mM	0.92 ± 0.06	*0.09 ± 0.01	7.44 ± 0.86
	1.0 mM	*0.75 ± 0.07	*0.03 ± 0.01	*7.74 ± 1.43
procyanidin B1	0.25 mM	0.96 ± 0.06	0.13 ± 0.01	6.04 ± 0.03
	0.50 mM	*0.67 ± 0.14	*0.07 ± 0.02	6.03 ± 1.44
	1.0 mM	*0.56 ± 0.14	*0.08 ± 0.02	6.83 ± 1.02
procyanidin B2	0.25 mM	0.97 ± 0.04	0.12 ± 0.01	5.97 ± 0.10
	0.50 mM	0.89 ± 0.01	0.11 ± 0.01	6.08 ± 0.01
	1.0 mM	0.68 ± 0.02	*0.09 ± 0.01	6.74 ± 0.42
procyanidin A2	0.25 mM	1.02 ± 0.01	0.14 ± 0.03	5.83 ± 0.06
	0.50 mM	0.97 ± 0.06	0.13 ± 0.02	6.05 ± 0.92
	1.0 mM	0.86 ± 0.02	*0.10 ± 0.01	6.45 ± 0.35
cranberry extract	0.25 mg/mL	1.12 ± 0.03	0.14 ± 0.03	−
	0.50 mg/mL	1.12 ± 0.04	0.14 ± 0.03	−
	1.0 mg/mL	1.16 ± 0.02	0.14 ± 0.04	−

[a]An asterisk to the left of a mean value indicates significant difference ($p < 0.05$) from the mean value corresponding to the control. −, not determined.

Statistical Analysis. One-way analysis of variance (ANOVA) was applied to data of growth response and/or residual phenolic concentration for testing the effect of different factors (i.e., initial concentration of phenolic compounds, anaerobic/aerobic conditions, and glucose content), and the Dunnett test was used to compare mean values. The Statistica program version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

RESULTS AND DISCUSSION

Effect of Flavan-3-ol Compounds and Phenolic Extracts on the Growth of *L. plantarum* IFPL935. Among several lactic acid bacteria and bifidobacteria tested, *L. plantarum* IFPL935 was proven to be resistant and able to grow in the presence of a complex phenolic extract from grape seeds or fractions enriched in either monomeric or oligomeric flavan-3-ols.[25] In the present work, to assess the specific capability of this strain to degrade individual phenolic compounds, incubations were performed in the presence of monomeric flavan-3-ols ((+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate) and B-type (B1 and B2) and A-type (A2) procyanidins at different concentrations (0.25, 0.50, and 1.0 mM) (Table 1). *L. plantarum* IFPL935 was capable of growing in the presence of the different phenolic compounds but the growth was slightly affected in a dose-dependent manner. The results showed a progressive decrease in maximal OD_{600} and μ_{max} together with an increase in lag time, from a concentration of 0.25 to 1.0 mM, although statistically significant differences were found for only some cases (Table 1). The decrease in the growth rate was most evident in the presence of (−)-epicatechin-3-*O*-gallate and B-type procyanidins, although the compounds did not completely inhibit the *L. plantarum* IFPL935 growth. Galloylated flavan-3-ols are reported to have higher antimicrobial activity than their homologues lacking this moiety.[30] This effect has been attributed to the fact that the galloyl moiety increases the hydrophobicity of the flavan-3-ol molecule, resulting in a higher affinity for phospholipid cell membranes.[31] The sensitivity of *L. plantarum* IFPL935 to B-type procyanidins could explain the growth delay observed when the strain was incubated in the presence of a flavan-3-ol oligomeric fraction from grape seed extract (GSE-O) in comparison to a monomeric fraction (GSE-M).[25]

In contrast to B-type procyanidins, maximum growth rates were observed in the presence of procyanidin A2. In view of these results, *L. plantarum* IFPL935 was also grown in the presence of a cranberry extract mainly composed of A-type procyanidins and phenolic acids. The cranberry extract did not produce any inhibition on the growth of *L. plantarum* IFPL935 at any of the tested concentrations (0.25, 0.50, and 1.0 mg/L) (Table 1).

Identification of Metabolites after Incubation of Phenolic Compounds and Extracts with *L. plantarum* IFPL935. As *L. plantarum* IFPL935 was found to tolerate the presence of individual phenolic compounds (<1.0 mM for standards and <1.0 mg/mL for extracts), incubations were carried out to assess whether the strain was able to catabolize these compounds. For these experiments, the phenolic concentration was fixed at 0.5 mM for pure compounds and at 0.50 mg/L for extracts. As an example, Figure 1 illustrates the

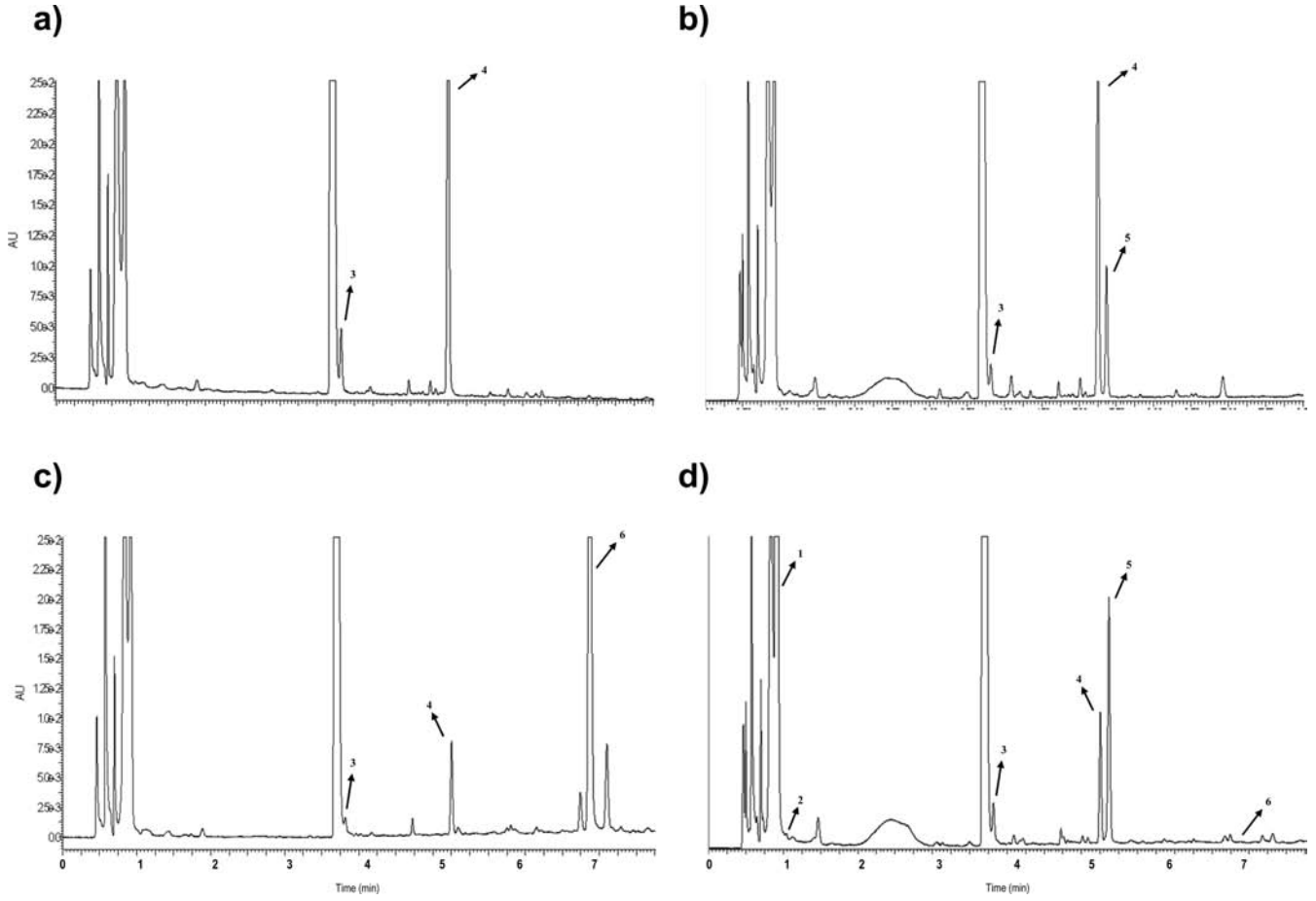

Figure 1. Chromatograms (A_{280}) of 48 h incubations of 0.5 mM (−)-epicatechin (a, b) and (−)-epicatechin gallate (c, d) in the absence (a, c) and in the presence (b, d) of *L. plantarum* IFPL935. Peaks: 1, gallic acid; 2, pyrogallol; 3, (+)-catechin; 4, (−)-epicatechin; 5, unknown; 6, (−)-epicatechin-3-*O*-gallate.

UPLC chromatograms of the 48 h incubations of (−)-epicatechin and (−)-epicatechin-3-*O*-gallate in the absence and presence of *L. plantarum* IFPL935. Peaks with identical response in the absence and presence of the bacteria (e.g., 3.68 min) corresponded to compounds present in the culture medium. In the case of (−)-epicatechin, incubations with *L. plantarum* IFPL935 (Figure 1b) resulted in its partial depletion together with the appearance of a new peak (peak 5). This compound was also detected in the incubations of *L. plantarum* IFPL935 with (+)-catechin (data not shown) and (−)-epicatechin-3-*O*-gallate (Figure 1d). The MS/MS spectrum of this unknown peak presenting a $[M - H]^-$ at m/z 291 revealed that it could correspond to 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (from here onward, diphenylpropan-2-ol) (Figure 2).[12] This compound would arise from the reductive cleavage of the heterocyclic C-ring of flavan-3-ols.[9,10,32] To the best of our knowledge, this intermediate degradation has not been described before in relation to *L. plantarum.* In addition, the activity seems to be strain-dependent because either some *L. plantarum* strains are very sensitive to flavan-3-ol compounds[25] or, as in the case of *L. plantarum* RM71, growth was stimulated by catechin but the strain did not catabolize it during fermentation.[33] Further microbial transformations of diphenylpropan-2-ol would lead to 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.[9,10,34] Thus, the mass signal of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (207 → 163, 40 V, and 18 V for MRM transition, cone energy, and collision energy, respectively)[35] was screened, but no response was found. In the case of (−)-epicatechin-3-*O*-gallate, gallic acid and pyrogallol (1,2,3-benzenetriol) were also found in the incubations of *L. plantarum* IFPL935 (Figure 1d). Gallic acid is known to originate from the cleavage of the ester link by microbial galloyl esterases and can be further decarboxylated into pyrogallol (1,2,3-benzenetriol) by decarboxylase enzymes.[10,36] Further catabolism of pyrogallol can lead to the formation of catechol by means of benzyl alcohol dehydrogenases,[24] but this metabolite was not detected in

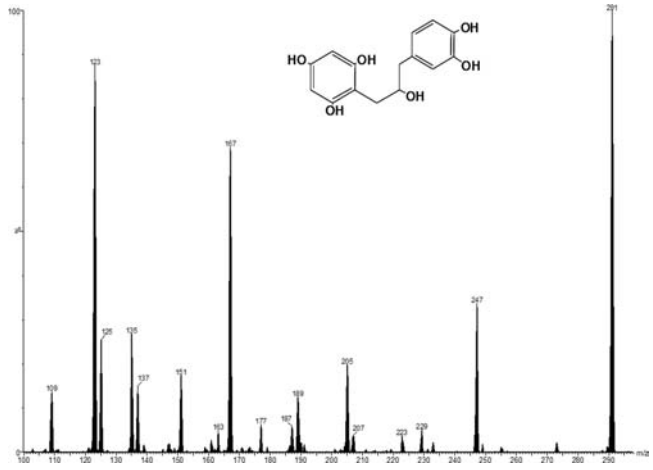

Figure 2. MS/MS spectrum of diphenylpropan-2-ol.

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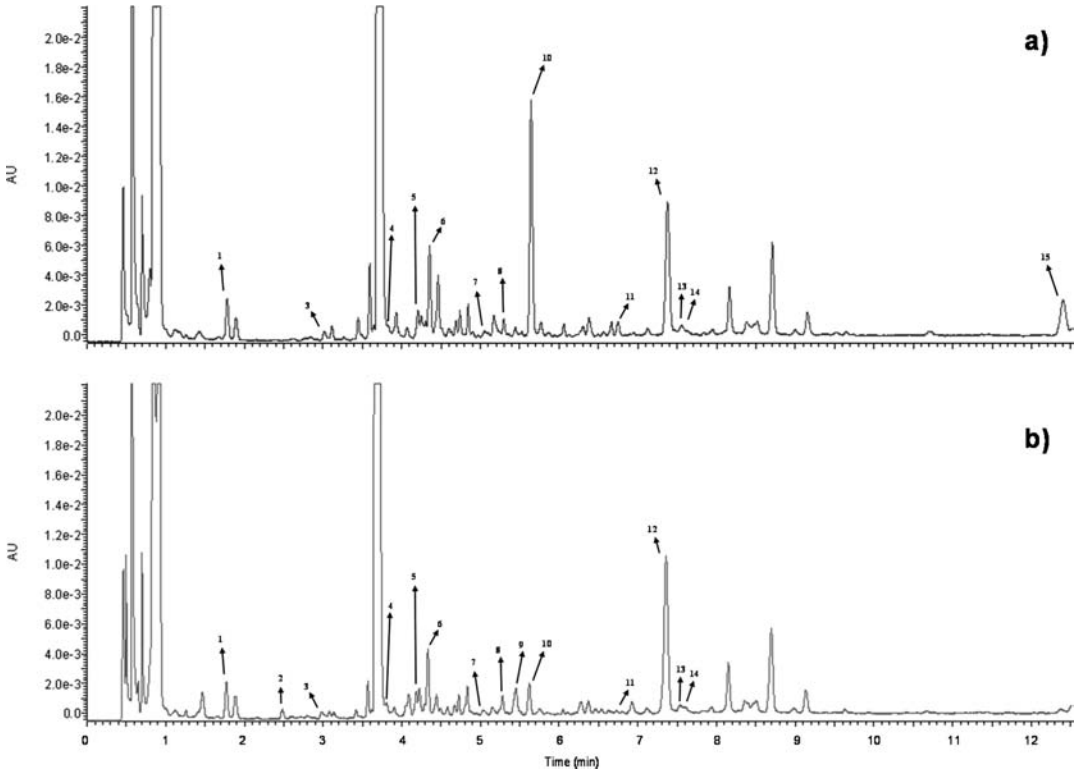

Figure 3. Chromatograms (A_{280}) of 48 h incubations of a cranberry extract (0.5 mg/L) in the absence (a) and in the presence (b) of *L. plantarum* IFPL935. Peaks: 1, protocatechuic acid; 2, catechol/pyrocatechol; 3, 3-*O*-methylgallic acid; 4, (+)-catechin; 5, caffeic acid; 6, vanillic acid; 7, syringic acid; 8, (−)-epicatechin; 9, 4-hydroxyphenylpropionic acid; 10, *p*-coumaric acid; 11, ferulic acid; 12, benzoic acid; 13, salicylic acid; 14, procyanidin A2; 15, *trans*-cinnamic acid.

our incubations, probably because of insufficient incubation time and/or precursor concentration.

None of the procyanidin dimers tested (B1, B2, and A2) were degraded when incubated with *L. plantarum* IFPL935 (data not shown), indicating that the strain is tolerant of these compounds but is not able to catabolize them under the culture conditions used in this study.

Previously, we showed that *L. plantarum* IFPL935 clearly modified the phenolic profile of complex phenolic mixtures such as grape seed extracts (GSEs) containing flavan-3-ols.[25] The results described above allow us to confirm that the appearance of gallic acid, pyrogallol, and catechol reported after the incubation of *L. plantarum* IFPL935 with GSEs was due to the bacterial catabolism of the galloylated flavan-3-ols present in the extract ((−)-epicatechin-3-*O*-gallate and procyanidin gallates). Additionally, the appearance of the unknown compound (peak 5), also reported in the GSE incubations (now identified as diphenylpropan-2-ol), can be attributed to the bacterial catabolism of monomeric flavan-3-ols ((+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate). Therefore, *L. plantarum* IFPL935 is able to produce a reductive cleavage of the C-ring of monomeric flavan-3-ols and their gallates, giving rise to diphenylpropan-2-ol, from solutions containing both pure compounds and complex phenolic extracts, but not from A- or B-type procyanidins or extracts containing these compounds. To the best of our knowledge, besides *L. plantarum* IFPL935, the strains *Eubacterium* SDG-2[15] and *Eggerthella lenta* rK3,[37] both isolated from human feces, are the only bacteria capable of producing the metabolite diphenylpropan-2-ol from monomeric flavan-3-ols. None of these bacteria were able to further the catabolism of

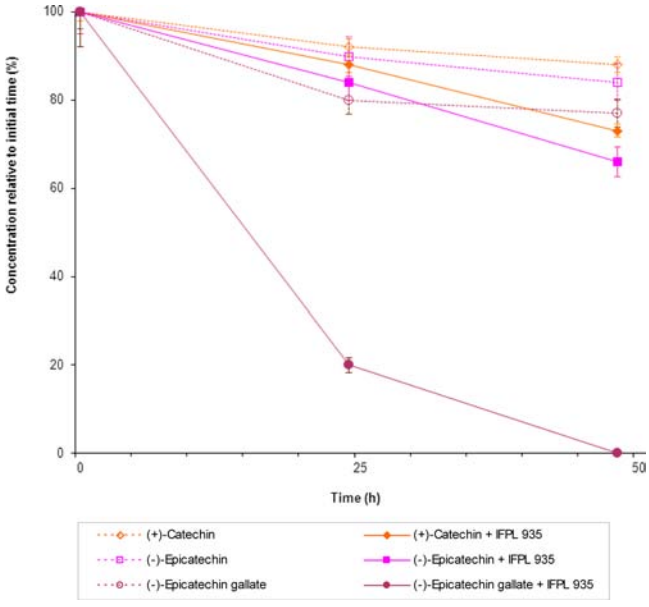

Figure 4. Concentration relative to initial time (%) of (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate (0.5 mM) after 48 h of incubation in the absence and in the presence of *L. plantarum* IFPL935.

diphenylpropan-2-ol to 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. However, in contrast to *Eubacterium* SDG-2, which was incapable of producing the same metabolite from the galloylated esters of monomeric flavan-3-ols (*R* or *S* form),[15] *L. plantarum* IFPL935 was capable of producing it from (−)-epigallocatechin-3-*O*-gallate.

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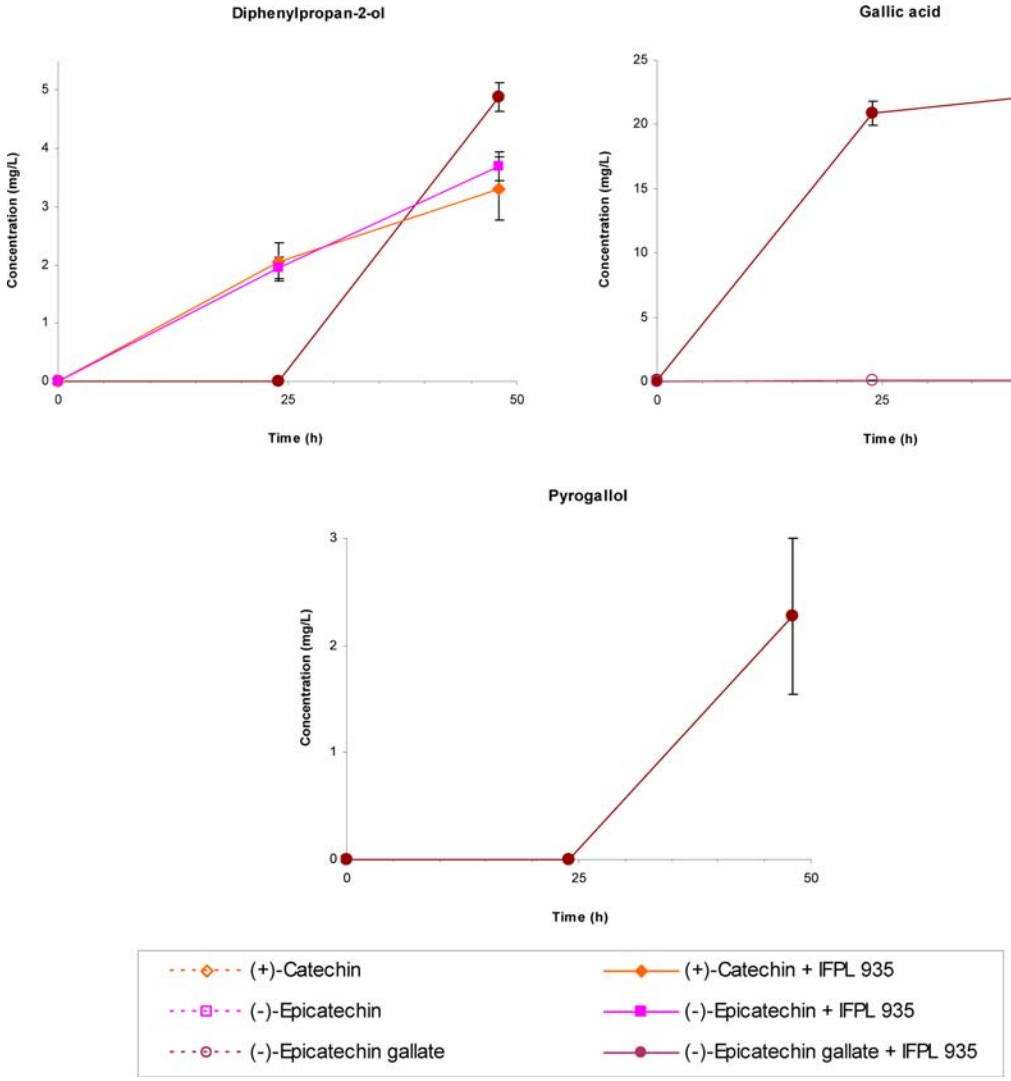

Figure 5. Formation of diphenylpropan-2-ol, gallic acid, and pyrogallol after 48 h of incubation of (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate (0.5 mM) in the absence and presence of *L. plantarum* IFPL935.

To expand the spectrum of phenolic substrates catabolized by *L. plantarum* IFPL935, we have also tested its capability to degrade a cranberry extract (*Vaccinium macrocarpon*). Figure 3 compares the chromatograms of the 48 h incubations of the cranberry extract in the absence and presence of *L. plantarum* IFPL935. Although the concentration of procyanidin A2 was not affected by the catabolic activity of *L. plantarum* IFPL935 (Figure 3), in accordance with the previous experiments with individual compounds, the catabolic activity of the bacteria clearly modified the phenolic profile of the extract. A decrease in the response was observed for hydroxycinnamic acids, including *p*-coumaric, *trans*-cinnamic, ferulic, and caffeic acids (the latter to a lesser extent), as well as for hydroxybenzoic acids, including vanillic and protocatechuic acids (the latter to a lesser extent). Concurrently with the disappearance of these compounds, the formation of 4-hydroxyphenylpropionic acid and catechol, together with a significant increase in the response of benzoic acid, was observed. None of these metabolites were found in the incubations of *L. plantarum* IFPL935 without the extract (growth control) (chromatogram not shown). On the other hand, diphenylpropan-2-ol (unknown peak 5) could not be detected after incubation with *L. plantarum* IFPL935, probably because of the small content of (+)-catechin and (−)-epicatechin in the cranberry extract. Other compounds (i.e., 3-*O*-methylgallic, syringic, and salicylic acids), which were present at relatively low concentration in the cranberry extract, were not found to be affected by incubation with the bacteria.

The formation of 3-hydroxyphenylpropionic and 4-hydroxy-3-methoxyphenylpropionic acids by *L. plantarum* from the C−C double-bond reduction of *m*-coumaric and ferulic acids, respectively, has been described. [24] Also in this regard, Gonthier et al.[38] found that caffeic acid esters could be converted into 3-hydroxyphenylpropionic and benzoic acids by human fecal microbiota. Therefore, the formation of 4-hydroxyphenylpropionic and benzoic acids probably arises from the catabolism of *p*-coumaric acid, which was the most abundant hydroxycinnamic acid in the cranberry extract and the compound showing the largest decrease in response after incubation with *L. plantarum* IFPL935 (Figure 3). Enzymes involved in the formation of benzoic acid from 4-hydroxyphenylpropionic acid may include *β*-oxidases and dehydroxylases. Benzoic acid could also arise from the degradation of vanillic and protocatechuic acids, which also contributes to explaining its high increase after the strain incubation. The formation of 4-ethylcatechol has been reported to occur after the catabolism of caffeic acid or its esters by human bacteria[39] or by the action of reductases from

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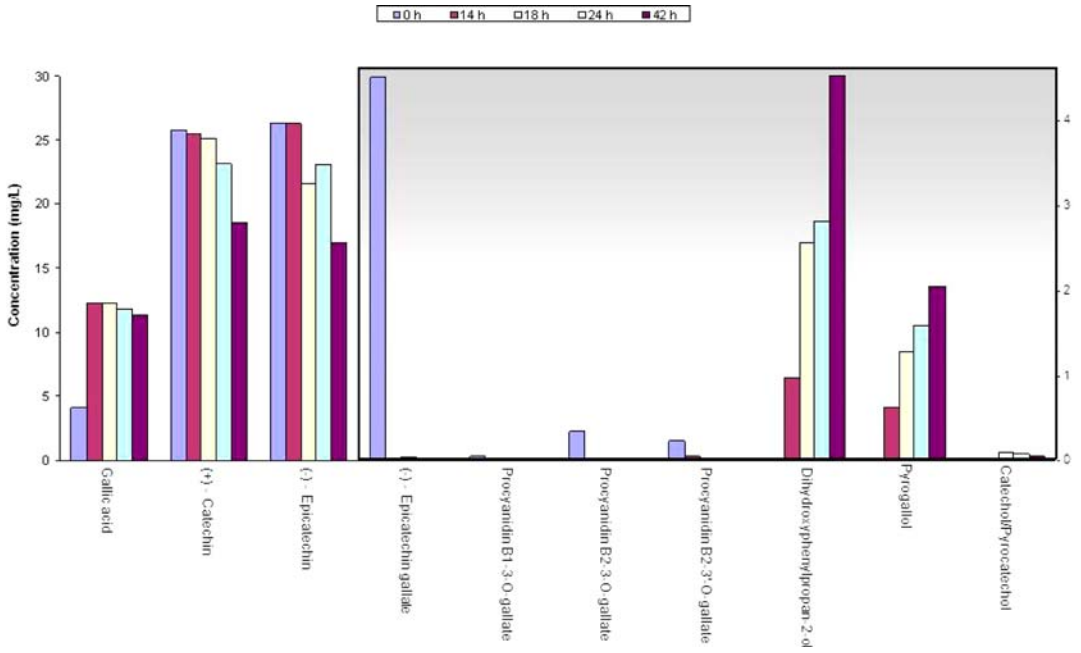

Figure 6. Catabolism of the grape seed monomeric extract (GSE-M) (0.5 mg/mL) by *L. plantarum* IFPL935.

Table 2. Effect of Anaerobic/Aerobic Conditions on the Catabolism of a Monomeric-Enriched Grape Seed Extract (GSE-M) (0.5 mg/mL) during Growth of *L. plantarum* IFPL935 in ZMB1 Containing 0.4% Glucose[a]

	residual concentration (mg/L) after incubation for			
	7.5 h		24 h	
phenolic compound	anaerobic	aerobic	anaerobic	aerobic
(+)-catechin	40.0 ± 2.1	38.3 ± 1.8	30.3 ± 1.3	33.4 ± 2.0
(−)-epicatechin	40.2 ± 1.7	40.3 ± 1.6	29.8 ± 2.5	33.4 ± 1.9
epicatechin-3-*O*-gallate	1.53 ± 0.14	1.31 ± 0.15	nd	nd
procyanidin B1-3-*O*-gallate	0.048 ± 0.011	0.060 ± 0.011	nd	nd
procyanidin B2-3-*O*-gallate	0.113 ± 0.008	*0.055 ± 0.006	nd	nd
procyanidin B2-3′-*O*-gallate	0.303 ± 0.011	*0.257 ± 0.014	0.023 ± 0.002	0.027 ± 0.001
gallic acid	19.6 ± 1.3	20.0 ± 0.6	13.7 ± 0.4	*15.7 ± 0.6
diphenylpropan-2-ol	nd	nd	5.34 ± 0.57	*3.45 ± 0.21
pyrogallol	nd	nd	7.66 ± 0.93	6.58 ± 0.71
catechol/pyrocatechol	nd	nd	0.313 ± 0.014	*0.425 ± 0.014

[a]An asterisk to the left of a mean value indicates significant difference ($p < 0.05$) from the mean value corresponding to anaerobic conditions. nd, not detected.

L. plantarum.[24] Consequently, the decrease in caffeic acid found after the *L. plantarum* IFPL935 incubation may partly explain the formation of catechol, the product resulting from the posterior degradation of ethylcatechol. Protocatechuic acid is another source of catechol because *L. plantarum* is also able to convert protocatechuic acid into catechol by the action of decarboxylases.[24] However, to the best of our knowledge, β-oxidases and dehydroxylases have not been reported before in *L. plantarum*.

Time Course of the Phenolic Catabolism by *L. plantarum* IFPL935. The time course of the catabolism by *L. plantarum* IFPL935 of (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate (0.5 mM), as well as that of a monomeric fraction from grape seed extract (GSE-M, 0.5 mg/mL), was studied. A slight chemical degradation of the compounds (12, 16, and 23%, respectively, for (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate) was observed after 48 h of incubation in the absence of the strain (Figure 4). Among the three monomeric forms, (−)-epicatechin-3-*O*-gallate was completely degraded by *L. plantarum* IFPL935 after 48 h of incubation, whereas only 27 and 34% of the initial amount of (+)-catechin and (−)-epicatechin were respectively catabolized (Figure 4). For the catechin-converting strain *E. lenta* rK3, the opposite has been described, (+)-catechin exhibiting a significantly faster degradation than (−)-epicatechin.[37] Parallel to the disappearance of these compounds, formation of diphenylpropan-2-ol was detected at 24 h for (+)-catechin and (−)-epicatechin (Figure 5). However, for (−)-epicatechin-3-*O*-gallate, the metabolite diphenylpropan-2-ol was detected after 48 h of fermentation (Figure 5), coinciding with the disappearance of this compound (Figure 4) due to the breakdown of the ester linkage and the subsequent release of gallic acid (Figure 5) and reaching higher concentration levels than for nongalloylated monomeric forms. Pyrogallol, the subsequent decarboxylation product of gallic acid, was found only at 48 h (Figure 5).

Figure 6 depicts the concentration of phenolic compounds after 24 h of incubation of *L. plantarum* IFPL935 with GSE-M. As observed for the individual incubations, (−)-epicatechin-3-*O*-gallate,

Table 3. Growth Response and Residual Phenolic Concentration of *L. plantarum* IFPL935 Liquid Cultures in the Presence of Different Concentrations of GSE-M and Glucose, after 24 h of Incubation[a]

	2.0 mg/mL GSE-M				3.0 mg/mL GSE-M			
	0.0% glucose	0.1% glucose	0.2% glucose	0.3% glucose	0.0% glucose	0.1% glucose	0.2% glucose	0.3% glucose
Growth Response								
max OD_{600}	0.37 ± 0.14	*0.61 ± 0.11	*0.75 ± 0.01	–	0.39 ± 0.20	*0.56 ± 0.08	*0.78 ± 0.04	–
growth rate (μ_{max}, h^{-1})	nd	*0.04 ± 0.01	*0.07 ± 0.01	–	nd	*0.01 ± 0.004	*0.0 ± 0.01	–
Residual Concentration of Phenolic Compounds (mg/L)								
(+)-catechin	40.9 ± 0.4	*34.2 ± 0.4	*30.6 ± 0.4	*27.8 ± 0.3	40.1 ± 0.3	39.2 ± 0.3	*37.5 ± 0.3	*38.5 ± 0.3
(−)-epicatechin	42.5 ± 0.3	*34.6 ± 0.6	*28.2 ± 0.3	*25.8 ± 0.3	44.0 ± 0.1	42.2 ± 0.3	40.7 ± 0.3	*39.2 ± 0.1
epicatechin-3-*O*-gallate	17.2 ± 0.1	*0.168 ± 0.003	*0.076 ± 0.001	*0.057 ± 0.001	24.3 ± 0.3	*0.411 ± 0.001	*0.111 ± 0.001	*0.067 ± 0.001
procyanidin B1-3-*O*-gallate	0.072 ± 0.001	*nd	*nd	*nd	0.174 ± 0.001	*0.021 ± 0.001	*nd	*nd
procyanidin B2-3-*O*-gallate	1.93 ± 0.02	*0.018 ± 0.001	*0.025 ± 0.001	*0.016 ± 0.001	1.84 ± 0.007	*0.101 ± 0.001	*0.025 ± 0.001	*0.016 ± 0.001
procyanidin B2-3′-*O*-gallate	1.23 ± 0.02	*0.112 ± 0.001	*0.071 ± 0.001	*0.066 ± 0.001	1.48 ± 0.02	*0.27 ± 0.001	*0.133 ± 0.002	*0.110 ± 0.007
gallic acid	13.6 ± 0.2	*21.8 ± 0.1	*19.4 ± 0.2	*19.8 ± 0.2	23.3 ± 0.2	22.5 ± 0.2	*35.1 ± 0.2	*34.4 ± 0.3
diphenylpropan-2-ol	nd	*6.29 ± 0.50	*16.9 ± 0.4	*19.5 ± 0.7	nd	*4.84 ± 0.50	*7.32 ± 0.42	*12.2 ± 0.4
pyrogallol	0.009 ± 0.001	*0.620 ± 0.035	*1.77 ± 0.028	*2.59 ± 0.042	0.023 ± 0.002	*0.475 ± 0.004	*2.95 ± 0.212	*5.27 ± 0.028
catechol/pyrocatechol	nd	*0.050 ± 0.004	*0.067 ± 0.003	*0.035 ± 0.003	nd	*0.038 ± 0.003	*0.349 ± 0.006	*0.556 ± 0.055

[a] An asterisk to the left of a mean value indicates significant difference ($p < 0.05$) from the mean value corresponding to the parallel experiment in the absence of glucose (0%). −, not determined; nd, not detected.

initially present in the extract, was rapidly (<24 h) and completely degraded by the strain. Procyanidin gallates (B1-3-*O*-gallate, B2-3-*O*-gallate, and B2-3′-*O*-gallate), present at a lower concentration in GSE-M, followed the same trend, confirming that *L. plantarum* IFPL935 was also able to hydrolyze the ester bond of these compounds, although it was not capable of further catabolizing the free dimeric structure. (+)-Catechin and (−)-epicatechin were catabolized to a lesser extent: 28 and 35% of the initial concentration after 42 h of incubation (Figure 6). The nondegradation of (−)-epicatechin during the first 14 h of incubation could be due to the release of this compound from (−)-epicatechin-3-*O*-gallate by the galloyl esterase activity. In fact, the concentration of gallic acid clearly increased after 14 h of incubation, as a result of the release of the galloyl moiety from galloylated monomers and dimers. However, as the incubation time progressed, the net concentration of gallic acid slightly decreased due to further degradation into pyrogaloll and catechol, which were detected at very low concentration (<0.1 mg/L). As a characteristic metabolite of flavan-3-ols, a continuous formation of diphenylpropan-2-ol was observed, reaching a concentration of 4.51 mg/L after 42 h of incubation with *L. plantarum* IFPL935 (Figure 6).

Effect of Anaerobiosis on the Catabolism of Flavan-3-ols by *L. plantarum* IFPL935. Because *L. plantarum* IFPL935 is a facultative anaerobic micro-organism, further experiments were conducted to determine whether anaerobic conditions could stimulate the catabolism of flavan-3-ols by this strain. Table 2 reports the residual concentrations of phenolic compounds after 7.5 and 24 h of incubation of *L. plantarum* IFPL935 liquid cultures in the presence of 500 mg/L GSE-M. At 7.5 h of incubation, no statistically significant differences were observed in the concentration of (+)-catechin and (−)-epicatechin from the GSE-M extract as their catabolism requires a longer incubation time (Figure 4). For some galloylated forms, which are usually degraded at a higher rate (Figure 4), statistically significant differences in concentration were observed between anaerobic and aerobic conditions, but with no specific trend or translation into the formation of gallic acid. At 24 h, a decrease in the concentration of all precursor compounds was observed, together with the formation of the characteristic metabolites. For the metabolite diphenylpropan-2-ol, anaerobic conditions led to a statistically significantly higher concentration than aerobic conditions (35% more). However, the opposite was found for gallic acid and catechol, which were found at a significantly higher concentration under the aerobic incubations (12 and 20% more, respectively), probably because of the higher specificity of the galloyl esterase activity of the bacteria under this condition. Because the largest difference in metabolite concentration between the two incubation conditions was observed for diphenylpropan-2-ol, these results suggest that the reductive heterocyclic ring cleavage of the flavan-3-ols by *L. plantarum* IFPL935 is favored under anaerobic conditions.

Effect of Glucose Content on the Catabolism of Flavan-3-ols by *L. plantarum* IFPL935. The effect of limiting the glucose content at a concentration <0.4% (i.e., 0, 0.1, 0.2, and 0.3%) on the growth and catabolism of flavan-3-ols by *L. plantarum* IFPL935 was evaluated using GSE-M as phenolic substrate at two different concentrations (2 and 3 mg/mL). The composition of this extract, combining nongalloylated and galloylated monomers and dimers of flavan-3-ols, proved useful for setting up this experiment. Table 3 reports kinetic data and the

concentration of phenolic compounds initially present in the GSE-M extract (gallic acid, (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, and procyanidin gallates), as well as that of microbial metabolites (diphenylpropan-2-ol, pyrogallol, and catechol) formed after 24 h of incubation with *L. plantarum* IFPL935. The strain growth rate was negligible in the absence of glucose, progressively increasing from 0.1 to 0.3% glucose at both GSE-M concentrations (2 and 3 mg/mL), indicating that *L. plantarum* IFPL935 is not able to use phenolic compounds as the unique source of carbon and that metabolites were formed throughout the strain growth in the presence of monomeric flavan-3-ols. In this regard, the concentrations of (+)-catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, and procyanidin gallates (B1-3-*O*-gallate, B2-3-*O*-gallate, and B2-3′-*O*-gallate), after 24 h of incubation, were significantly higher in the absence than in the presence of glucose, at both GSE-M tested concentrations. As expected from the lower degradation rates of precursor flavan-3-ols, the concentrations of diphenylpropan-2-ol, pyrogallol, and catechol were negligible when incubations were carried out using GSE-M as the unique source of carbon, progressively increasing in a glucose concentration-dependent manner. The concentration of gallic acid, initially present in the GSE-M extract but also formed by the action of *L. plantarum* IFPL935, was also significantly higher in the incubations in the presence of glucose. All of these observations indicated that the extent of the catabolism of GSE-M by *L. plantarum* IFPL935 was favored by the growth of the strain in the presence of glucose. (−)-Epicatechin-3-*O*-gallate as precursor compound and diphenylpropan-2-ol as microbial metabolite both required a minimum of 0.1% glucose to be either degraded or formed, respectively.

In conclusion, the results showed that (a) *L. plantarum* IFPL935 had a higher resistance to nongalloylated monomeric flavan-3-ols, A-type dimeric procyanidins, and complex extracts enriched in these compounds (i.e., cranberry extract) than to galloylated monomers (i.e., (−)-epicatechin-3-*O*-gallate) and B-type dimeric procyanidins; (b) despite this structure-dependent effect, *L. plantarum* IFPL935 was capable of rapidly degrading (−)-epicatechin-3-*O*-gallate and dimer gallates, releasing gallic acid, which subsequently undergoes further catabolism to pyrogallol and catechol; (c) besides this activity, *L. plantarum* IFPL935 was capable of cleaving the heterocyclic ring of monomeric flavan-3-ols, including (−)-epicatechin-3-*O*-gallate, giving rise to 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol (i.e., diphenylpropan-2-ol), which is the first metabolite in the microbial catabolic pathway of flavan-3-ols; (d) the catabolism was largely favored by the growth of the strain in the presence of glucose, whereas anaerobic conditions seem to induce a slightly higher production of the metabolite diphenylpropan-2-ol; (e) no degradation was observed in the case of A-type or B-type dimers, suggesting that resistance is not linked to catabolism under the conditions used in this study; and (f) however, *L. plantarum* IFPL935 was capable of modifying the phenolic profile of the cranberry extract, mainly due to catabolism of hydroxycinnamic and hydroxybenzoic acids.

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Notes

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