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Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^T

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Abstract

Phenolic acids account for almost one third of the dietary phenols and are associated with organoleptic, nutritional and antioxidant properties of foods. This study was undertaken to assess the ability of *Lactobacillus plantarum* CECT 748^T to metabolize 19 food phenolic acids. Among the hydroxycinnamic acids studied, only *p*-coumaric, caffeic, ferulic and *m*-coumaric acids were metabolized by *L. plantarum*. Cultures of *L. plantarum* produced ethyl and vinyl derivatives from *p*-coumaric and caffeic acids, 4-vinyl guaiacol from ferulic acid, and 3-(3-hydroxyphenyl) propionic acid from *m*-coumaric acid. Among the hydroxybenzoic acids analysed, gallic acid and protocatechuic acid were decarboxylated to pyrogallol and catechol, respectively. Inducible enzymes seem to be involved, at least in *m*-coumaric and ferulic acid metabolism, since cell-free extracts from cultures grown in the absence of these phenolic acids were unable to metabolize them. Further work is needed for the identification of the enzymes involved, since the knowledge of the metabolism of phenolic compounds is an important issue for the food industry.

Keywords: *Lactobacillus plantarum*; Hydroxycinnamic acids; Hydroxybenzoic acids; Decarboxylase; Reductase; Inducible enzymes

1. Introduction

Vascular plants synthesize a diverse array of organic molecules, referred to as secondary metabolites. Phenolic acids are one such group of aromatic secondary plant metabolites widely spread throughout the plant kingdom. Phenolic acids have been associated with colour, sensory qualities, and nutritional and antioxidant properties of foods (Shahidi & Naczk, 2003). Phenolic acids account for almost one third of the dietary phenols, and there is an increasing awareness and interest in the antioxidant behaviour and potential health benefits associated with these simple phenolic acids. It is their role as dietary antioxidants that has received the most attention in recent literature (Lodovici, Guglielmi, Meoni, & Dolara, 2001).

The term "phenolic acids", in general, describe phenols that possess one carboxylic acid functional group. The naturally occurring phenolics acids contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic and the hydroxybenzoic structures. Hydroxybenzoic acids are components of complex structures such as hydrolysable tannins (gallotannins and ellagitannins). The hydroxycinnamic acids are more common than are hydroxybenzoic acids and mainly include *p*-coumaric, caffeic, ferulic and sinapic acids. These acids are rarely found in the free form, except in food that has undergone freezing, sterilization, or fermentation. The bound forms are glycosylated derivatives or esters of quinic acid, shikimic acid, and tartaric acid. Caffeic and quinic acids combine to form chlorogenic acid. Caffeic acid, both free and esterified, is generally the most abundant phenolic acid and represents between 75% and 100% of the total hydroxycinnamic acid content of most fruits. Ferulic acid is the most abundant phenolic acid found in cereal grains (Shahidi & Naczk, 2003).

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Lactobacillus plantarum is a lactic acid bacterial species that is most frequently encountered in the fermentation of plant materials where phenolic acids are abundant. These plant-fermentations include several food and feed products, e.g. olives, must and a variety of vegetable fermentation products. It has been reported that *L. plantarum* is able to decarboxylate the hydroxycinnamic acids, *p*-coumaric and caffeic acids (Cavin, Andioc, Etievant, & Diviès, 1993). However, controversial results were obtained about decarboxylation of ferulic acid by *L. plantarum* strains (Barthelmebs, Diviès, & Cavin, 2001; Cavin et al., 1993; Couto, Campos, Figueiredo, & Hogg, 2006; van Beek & Priest, 2000). Moreover, in this species the gene encoding a *p*-coumarate decarboxylase (PadA), having PAD activity (previously described as PDC activity), in this species has been cloned (Cavin, Barthelmebs, & Diviès, 1997a). The substrate specificity of the purified PadA enzyme was tested for ten hydroxycinnamic acids. The authors conclude that only the acids with a hydroxyl group *para* to the unsaturated side chain and with a substitution of —H or —OH *meta* to the unsaturated side chain were metabolised (Cavin et al., 1997b).

As far as we know, there is no information about the ability of *L. plantarum* to metabolize hydroxybenzoic acids, or other phenolic acids frequently found in foods. Therefore, in this paper, we studied the degradation of 19 phenolic acids by *L. plantarum* and report the identification of the degradation compounds obtained.

2. Material and methods

2.1. Chemicals

The 19 phenolic acids analyzed in this study were seven hydroxycinnamic acids, nine hydroxybenzoic acids, and three other food phenolic acids, such as phloretic acid (Aldrich H524006), chlorogenic acid (Sigma C3878) and ellagic acid (Sigma E2250). The hydroxycinnamic acids were: *p*-coumaric acid (Sigma C-9008), *o*-coumaric acid (Fluka 28170), *m*-coumaric acid (Aldrich H23007), cinnamic acid (Aldrich C8, 085-7), caffeic acid (Sigma C0625), ferulic acid (Sigma F3500) and sinapic acid (Sigma D7927). The hydroxybenzoic acids assayed were: syringic acid (Fluka 86230), gallic acid (Fluka 48630), salicylic acid (Merck 631), benzoic acid (Merck 6391513), gentisic acid (Aldrich 149357), veratric acid (Fluka 94872), *p*-hydroxybenzoic acid (Fluka 54630), protocatechuic acid (Sigma P5630), and vanillic acid (Fluka 94770).

The phenolic acid derivatives, 4-ethyl phenol (Fluka 04700), 4-ethyl catechol (Lancaster A12048), 4-ethyl guaiacol (Aldrich W 24,360-4-K), 4-vinyl phenol (Lancaster L10902), 4-vinyl guaiacol (Lancaster A13194), pyrogallol (Merck 612), catechol (Sigma C9510) and 3-(3-hydroxyphenyl) propionic acid (Lancaster L01279), were used as standards for the identification of the degradation compounds.

2.2. Bacterial strain and growth conditions

L. plantarum CECT 748^T (ATCC 14917, DSMZ 20174), isolated from pickled cabbage, was purchased from the Spanish Type Culture Collection. This strain was selected as it represents the type strain of this species.

The bacterium was cultivated in a modified basal medium described previously for *L. plantarum* (Rozès & Peres, 1998). The basal medium has the following composition: glucose, 2.0 g/l; trisodium citrate dihydrate (SO 0200, Scharlau), 0.5 g/l; D-, L-malic acid (AC 1420, Scharlau), 5.0 g/l; casamino acids (223050, BD), 1.0 g/l; yeast nitrogen base without amino acids (239210, BD), 6.7 g/l; pH was adjusted to 5.5. The basal medium was modified by the replacement of glucose by galactose (216310, Difco). This defined medium was used to avoid the presence of phenolic compounds included in non-defined media. For the degradation assays, the sterilized modified basal medium was supplemented at 1 mM final concentration with the phenolic compound filter-sterilized. The *L. plantarum* inoculated media were incubated at 30 °C, in darkness, under microaerophilic conditions, without shaking, for 10 days. A long incubation period was used to find the dead-end products of phenolic acid degradation. Incubated media with cells and without phenolic compound and incubated media without cells and with phenolic compounds were used as controls. From the supernantants, the phenolic products were extracted twice with one third of the reaction volume of ethyl acetate (Lab-Scan, Ireland).

2.3. Degradation of phenolic acids by cell-free extract

In order to prepare cell-free extracts, *L. plantarum* CECT 748^T strain was grown in MRS media (Difco, France) under microaerobic conditions at 30 °C until a late exponential phase was reached. The cells were harvested by centrifugation and washed three times with phosphate buffer (50 mM, pH 6.5), and subsequently resuspended in the same buffer for cell rupture. Bacterial cells were disintegrated twice by using the French Press at 1500 psi pressure (Thermo Electron). The disintegrated cell suspension was centrifuged at 12000*g* for 20 min at 4 °C in order to sediment cell debris. The supernatant containing the soluble proteins was filtered aseptically using sterile filters of 0.2 μm pore size (Sarstedt, Germany).

To determine whether uninduced *L. plantarum* cells possessed enzymes able to metabolize phenolic acids, the cell-free extract was incubated in the presence of each phenolic acid at 1 mM final concentration. *L. plantarum* cell-free extract in phosphate buffer (25 mM, pH 6.5) containing approximately 1 mg of total protein, was incubated during 20 h at 30 °C in the presence of each phenolic acid. As control, phosphate buffer containing the phenolic acid was incubated under the same conditions. The reaction products were extracted twice with ethyl acetate (Lab-Scan, Ireland).

2.4. HPLC–DAD analysis

A Thermo (Thermo Electron Corporation, Waltham, Massachussetts, USA) chromatographic system equipped with a P400 SpectraSystem pump, and AS3000 autosampler, and a UV6000LP photodiode array detector was used. A gradient of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2, v/v/v) was applied to a reversed-phase Nova-pack C_{18} cartridge (25 cm × 4.0 mm i.d.; 4.6 μm particle size) at room temperature. The elution programme was as follows: 0–55 min, 80% B linear, 1.1 ml/min; 55–57 min, 90% B linear, 1.2 ml/min; 57–70 min, 90% B isocratic, 1.2 ml/min; 70–80 min, 95% B linear, 1.2 ml/min; 80–90 min, 100% linear, 1.2 ml/min; 100–120 min, washing at 1.0 ml/min. Detection was performed by scanning from 220 to 380 nm (Bartolomé, Peña-Neira, & Gómez-Cordovés, 2000). Samples were injected in duplicate onto the cartridge after being filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filter (Teknokroma, Spain).

The identification of degradation intermediates was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers or by LC–DAD/ESI–MS.

2.5. High-performance liquid chromatography–diode array detector–electrospray mass spectrometry (HPLC–DAD/ESI–MS)

A Hewlett-Packard series 1100 (Palo Alto, CA) chromatographic system equipped with a diode array detector (DAD) and a quadrupole mass spectrometer (Hewlett-Packard series 1100 MSD) with an electrospray interface was used. Separation was performed on a reversed-phase Waters Nova-Pak C18 column at room temperature. The elution programme described above was applied. DAD detection was performed from 220 to 380 nm, with 0.7 ml/min. The ESI parameters were as follows: drying gas (N2) flow and temperature, 10 l/min at 340 °C; nebulizer pressure, 40 psi; capillary voltage, 4000 V. The ESI was operated in negative mode, scanning from 100 to 3000 *m/z* using the following fragmentator voltage gradient: 100 V from 0 to 200 *m/z* and 200 V from 200 to 3000 *m/z*.

3. Results and discussion

3.1. Hydroxycinnamic acids degradation by L. plantarum

There is a great variety of hydroxycinnamic acids in foods. The biosynthesis of this diversity of hydroxycinnamic acids has been studied. In plants, phenylalanine ammonia lyase (PAL) catalyzes the release of ammonia from phenyl-alanine and leads to the formation of a carbon–carbon double bond, yielding *trans*-cinnamic acid. In some plants and grasses, tyrosine is converted into 4-hydroxycinnamic via the action of tyrosine ammonia lyase (TAL). Introduction of a hydroxyl group into the *para* position of the phenyl ring of cinnamic acid proceeds via catalysis by monooxygenase, utilizing cytochrome P_{450} as the oxygen binding site. The *p*-coumaric acid formed may be hydroxylated further in positions 3 and 5 by hydroxylases and possibly methylated via *O*-methyl transferase with *S*-adenosylmethionine as methyl donor; this leads to the formation of caffeic, ferulic and sinapic acids. These compounds posses a phenyl ring and a C3 side chain and serve as precursors for the synthesis of lignins and many other compounds.

Studies were limited to seven commercially available hydroxycinnamic acids. In order to find whether *L. plantarum* has the ability to degrade these acids, two procedures were carried out. First, *L. plantarum* cultures were grown for 10 days in the presence of each hydroxycinnamic acid at 1 mM final concentration. So, if *L. plantarum* cells are able to metabolise the hydroxycinnamic acid, the dead-end degradation products could be detected in the culture media. In addition, cell-free extracts containing all the soluble proteins were incubated at 37 °C during 20 h in the presence of 1 mM of each commercial hydroxycinnamic acid. Since the soluble proteins were present in phosphate buffer (50 mM, pH 6.5), control samples were prepared in this buffer and incubated under the same conditions. By using this second approach, information about induction of the involved enzymes could be obtained since, in the extracts, the only proteins present were synthesized in the absence of the corresponding hydroxycinnamic acid.

Among the seven hydroxycinnamic acids assayed, only *p*-coumaric and caffeic acids were metabolized by cell cultures or by cell-extracts of *L. plantarum* CECT 748^T. Fig. 1a shows the HPLC chromatograms obtained with *p*-coumaric acid. Compared to the control, in cell-free extracts, a proportion of *p*-coumaric acid was decarboxylated, and vinyl phenol was obtained (Fig. 1a (3)). However, supernatants obtained from cell cultures showed the presence of vinyl- and ethyl phenol, resulting from the decarboxylation, and decarboxylation plus reduction of *p*-coumaric acid (Fig. 1a (2). Previously it was reported that, in a *L. plantarum* LPNC8 strain, no *p*-coumaric acid degradation was detected in the uninduced cell extracts (Barthelmebs, Divies, & Cavin, 2000). However, no information was provided about the reaction time used.

A similar situation was observed in the caffeic acid sample (Fig. 1b). Cell-free extracts were able to fully decarboxylate the caffeic acid present in the reaction (Fig. 1b (3)) whereas, in the supernatants from the cultures, the products of the decarboxylation (4-vinyl catechol) as well as the decarboxylation plus reduction (4-ethyl catechol) of caffeic acid were identified (Fig. 1b (2)). The caffeic acid derivative, 4-vinyl catechol was identified by LC–DAD/ESI–MS. The degradation peak, at retention time 37 min, was identified as 4-vinyl catechol by its molecular ion, $[M\text{-}H^-]$ *m/z* 135 (data not shown).

From these results we could conclude that uninduced cell-free extracts contained decarboxylases able to

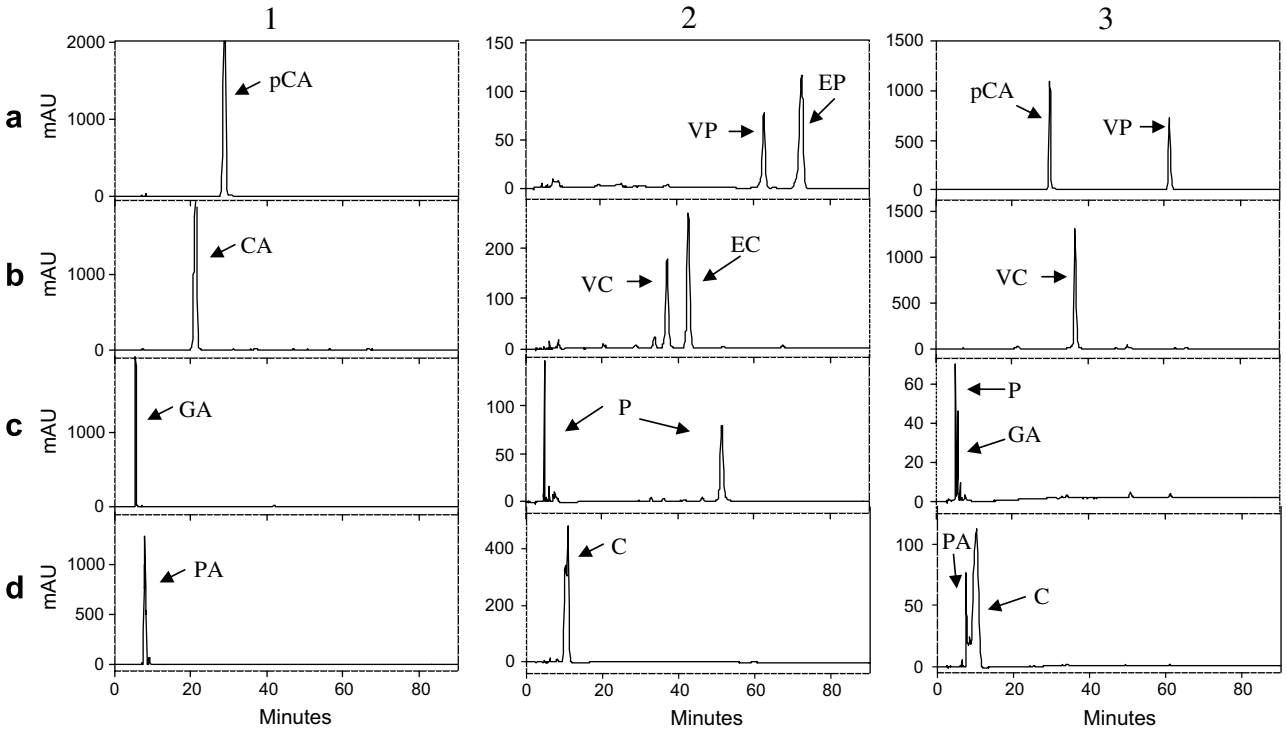

Fig. 1. HPLC chromatograms of the degradation of *p*-coumaric, caffeic, gallic and protocatechuic acids by *Lactobacillus plantarum*. Chromatograms of supernatants from *L. plantarum* CECT 748^T grown for 10 days in the presence of *p*-coumaric (a), caffeic (b), gallic (c) and protocatechuic acid (d) (2) or from cell-free extracts after a 20 h incubation in the presence of the same phenolic acids (3) are shown. The HPLC chromatograms from the control samples are also indicated (1). The chromatograms were recorded at 280 nm. pCA, *p*-coumaric acid; CA, caffeic acid; GA, gallic acid; PA, protocatechuic acid; VP, vinyl phenol; EP, ethyl phenol; VC, vinyl catechol, EC, ethyl catechol; P, pyrogallol; C, catechol.

decarboxylate *p*-coumaric and caffeic acids. In fact, a *p*-coumaric acid decarboxylase (PadA), able to metabolize *p*-coumaric and caffeic acid, was purified, and its corresponding gene was cloned and heterologously expressed (Cavin et al., 1997a). However, culture induction is needed to synthesize the reductase involved in the conversion of the vinyl derivatives to the corresponding ethyl derivatives. It has previously been reported that *L. plantarum* has a phenolic acid reductase activity (named PAR), induced by *p*-coumaric and ferulic acid in the presence of glucose (Barthelmebs et al., 2000).

Unlike *p*-coumaric and caffeic acids, ferulic and *m*-coumaric acids were found to be metabolized only by *L. plantarum* cell cultures (Fig. 2a); however, in both acids, no degradation was observed by cell-free extracts (data not shown). These results indicated that the enzymes involved in their metabolism need to be synthesized after their induction by the presence of the corresponding phenolic acid.

As shown in Fig. 2a (1) ferulic acid was decarboxylated to vinyl guaiacol, as determined by comparing its retention time and spectral data with the commercial standard (Fig. 2b (1)). Controversial results were obtained in relation to the decarboxylation of ferulic acid. Cavin et al. (1997b) reported that only *p*-coumaric and caffeic acids were metabolised by the *L. plantarum* purified *p*-coumaric acid decarboxylase (PdaA), and they concluded that the absence of detectable ferulic acid decarboxylase activity of the purified PdaA confirmed the existence of another phenolic acid decarboxylase, which was able to decarboxylate ferulic acid and was induced by ferulic acid only. However, later, the same authors reported that purified PadA appeared to decarboxylate ferulic acid *in vitro* (Bart-

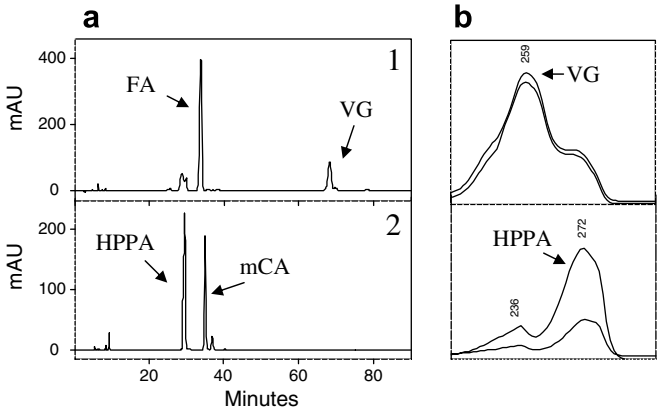

Fig. 2. HPLC chromatograms showing the degradation of the hydroxycinnamic acids, *m*-coumaric and ferulic acids, by *L. plantarum* cultures. (a) Chromatograms of supernatants from *L. plantarum* CECT 748^T grown for 10 days in the presence of ferulic acid (FA) (1) or *m*-coumaric acid (mCA) (2). Chromatograms were recorded at 280 nm. (b) Comparison between spectra of the compounds identified and the standards: vinyl guaiacol (VG) and 3-(3-hydroxyphenyl) propionic acid (HPPA).

helmebs et al., 2000) and therefore, they suggest that the PDC activity present in *L. plantarum* should be renamed PAD due to its decarboxylase activity on *p*-coumaric, ferulic and caffeic acids (Barthelmebs et al., 2001).

So far, the observed decarboxylation of *p*-coumaric, ferulic and caffeic acids appear to be due to the activity of the PadA enzyme. However, purified PadA enzyme was unable to decarboxylate *m*-coumaric acid (Cavin et al., 1997b). We have observed *m*-coumaric acid degradation (Fig. 2b (2)), with the production of a degradation intermediate showing a retention time of 28.8 min (Fig. 2b (2) and UV absorbance maxima at 236/272 nm (Fig. 2b (2), as determined by using a diode array detector. In order to identify the compound obtained, LC–DAD/ESI–MS was applied to the sample. The compound eluted at a retention time of 28.8 min, was identified by its molecular ion, [M-H$^-$] *m*/*z* 165, as 3-(3-hydroxyphenyl) propionic acid (HPPA) (data not shown). Later, HPPA was additionally identified by comparison with the commercial compound. Microbial degradation of *m*-coumaric acid has been only scarcely reported. As early as 1968, it was reported that cells of the wood-destroying fungus, *Sporobolomyces roseus,* were able to convert *m*-coumaric acid into *m*-hydroxybenzoic acid, but the latter compound, which accumulated in the medium, was not further metabolized (Moore, Subba Rao, & Towers, 1968). Later, it was reported that the bacterium *Clostridium glycolicum* transformed *m*-coumaric acid to HPPA by reducing the double bond of the side chain (Chamkha, Labat, Patel, & Garcia, 2001). Therefore, it seems that *C. glycolicum* and *L. plantarum* share a similar mechanism for the degradation of *m*-coumaric acid.

3.2. Hydroxybenzoic acids degradation by L. plantarum

In plants, benzoic acid derivatives are produced via the loss of a two-carbon moiety from cinnamic acids. Salicylic acid is a benzoic acid derivative that acts as a signal substance. After infection or UV irradiation, many plants increase their salicylic acid content, which may induce the biosynthesis of defence substances. Aspirin, the acetyl ester of salicylic acid, was first isolated from the bark of the willow tree. As with hydroxycinnamic acids, hydroxylation and possibly methylation of hydroxybenzoic acid leads to the formation of dihydroxybenzoic acid (protocatechuic acid), vanillic acid, syringic acid and gallic acid. Hydroxybenzoic acids are commonly present in the bound form in foods and are often the components of complex structures such as lignins and hydrolysable tannins.

Among the hydroxybenzoic acids assayed, only gallic and protocatechuic acids were metabolized by both cell cultures and cell-free extracts from *L. plantarum* CECT 748^T. Fig. 1c shows the HPLC chromatograms obtained with gallic acid. As compared to the control, in the cell-free extracts, a proportion of gallic acid was decarboxylated, and pyrogallol was obtained (Fig. 1c (3)). However, in the supernatants obtained from cell cultures only pyrogallol was detected (Fig. 1c (2)). These results are in agreement with a previous study reporting gallate decarboxylase activity in *L. plantarum* (Osawa, Kuroiso, Goto, & Shimizu, 2000).

Protocatechuic acid was completely decarboxylated to catechol by cultures of *L. plantarum* grown in the presence of this hydroxybenzoic acid (Fig. 1d (2)). However, and similarly to gallic acid, cell-free extracts produced catechol but non-decarboxylated protocatechuic acid was also detected (Fig. 1d (3)). Whiting and Coggins (1971) reported that *L. plantarum* cells, grown in a medium containing protocatechuic acid, completely metabolised it to catechol, and there was no indication of a further metabolism of catechol (Whiting & Coggins, 1971). Both results seem to indicate that catechol is a dead-end product of protocatechuate degradation in *L. plantarum* cultures. No information is available about the *L. plantarum* enzyme involved in the protocatechuic acid decarboxylation. As far as we know, enzymes possessing protocatechuic acid decarboxylase activity have only been reported in *Clostridium hydrobenzoicum* (He & Wiegel, 1996).

Recently, hydroxybenzoic acid derivatives (including gallic acid and protocatechuic acid) have been proposed as minor polyphenols that could serve as characteristic indices for discrimination of varietal red wines (Kallithraka, Mamalos, & Makris, 2007). This choice was based on the consideration that these components are, in general, chemically and microbiologically stable; thus, they could be viewed as indices for a reliable differentiation. However, after the results obtained in this study, caution should be applied in relation to this proposal, since *L. plantarum* is frequently associated with malolactic fermentation in wines and, as reported in this work, this is able to metabolize some of the hydroxybenzoic acids chosen for the discrimination of varietal red wines.

3.3. Degradation of other phenolic acids by L. plantarum

As mentioned above, the metabolism of three food phenolic acids, phloretic, chlorogenic and ellagic acid, was also studied. Ellagic and chlorogenic acids were not detected by the chromatographic method used in this study. Phloretic acid was not metabolized by cell cultures or by the cell-free extracts (data not shown). As explained above, PadA from *L. plantarum* was purified, and its corresponding gene was cloned and heterologously expressed (Cavin et al., 1997a). The substrate specificity of the purified enzyme was tested for several phenolic acids, and phloretic acid was not decarboxylated by this enzyme. Later, a *L. plantarum* mutant strain deficient in PDC activity, *L. plantarum* LPD1, was constructed (Barthelmebs et al., 2000). In a LPD1 mutant, in cells induced with *p*-coumaric acid, this acid was metabolized but vinyl phenol was not the product of the reaction. Instead, phloretic acid or ethyl phenol appeared to be produced, based on the UV spectrum. Phloretic acid was not further degraded, as in the results of this work.

Table 1
Metabolism of phenolic acids by *L. plantarum* CECT 748^T

Phenolic acid	Compound produced	Enzyme involved
p-Coumaric acid	4-Vinyl phenol/4-ethyl phenol	PadA decarboxylase/Reductase
Caffeic acid	4-Vinyl catechol/4-ethyl catechol	PadA decarboxylase/Reductase
Ferulic acid	4-Vinyl guaiacol/4-ethyl guaiacol	PadA decarboxylase/Reductase
m-Coumaric acid	3-(3-Hydroxyphenyl) propionic acid)	Reductase
Gallic acid	Pyrogallol	Decarboxylase
Protocatechuic acid	Catechol	Decarboxylase

To improve our understanding of phenolic acid degradation by *L. plantarum*, further work on the identification of the involved enzymes is required. Table 1 summarizes the results obtained in this work. These results indicate that *L. plantarum* is able to degrade some hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and *m*-coumaric acid) and some hydroxybenzoic acids (gallic and protocatechuic acid). The reactions involved in their metabolism are decarboxylation and reduction of the phenolic acid. A phenolic acid decarboxylase (PadA) has been previously characterized in *L. plantarum*. This enzyme, only decarboxylates *p*-coumaric, caffeic and ferulic acid, among the hydroxycinnamic acids assayed, and does not decarboxylate *m*-coumaric acid. However, no information is available about the decarboxylation of hydroxybenzoic acids by this enzyme. Therefore, additional information is needed in relation to the substrate specificity of this decarboxylase; at least any additional enzymes, e.g. reductases (o reductases) involved in the formation of ethyl derivatives from their corresponding vinyls, and in the reduction of *m*-coumaric acid to HPPA, need to be identified. For the food industry, the knowledge of the enzymes involved in the metabolism of compounds playing an important role in food quality is of great interest.

Acknowledgments

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