

# Study of the inhibitory activity of phenolic compounds found in olive products and their degradation by *Lactobacillus plantarum* strains

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Received 21 May 2007; received in revised form 4 July 2007; accepted 8 August 2007

## Abstract

*Lactobacillus plantarum* is the main species responsible for the spontaneous fermentation of Spanish-style green olives. Olives and virgin oil provide a rich source of phenolic compounds. This study was designed to evaluate inhibitory growth activities of nine olive phenolic compounds against four *L. plantarum* strains isolated from different sources, and to explore the *L. plantarum* metabolic activities against these phenolic compounds. None of the nine compounds assayed (oleuropein, hydroxytyrosol, tyrosol, as well as vanillic, *p*-hydroxybenzoic, sinapic, syringic, protocatechuic and cinnamic acids) inhibited *L. plantarum* growth at the concentration found in olive products. Oleuropein and tyrosol concentrations higher than 100 mM were needed to inhibit *L. plantarum* growth. On the other hand, sinapic and syringic acid showed the highest inhibitory activity since concentrations ranging from 12.5 to 50 mM inhibited *L. plantarum* growth in all the strains analyzed. Among the nine compounds assayed, only oleuropein and protocatechuic acid were metabolized by *L. plantarum* strains grown in the presence of these compounds. Oleuropein was metabolized mainly to hydroxytyrosol, while protocatechuic acid was decarboxylated to catechol. Metabolism of oleuropein was carried out by inducible enzymes since a cell-free extract from a culture grown in the absence of oleuropein was unable to metabolize it. Independent of their isolation source, the four *L. plantarum* strains analysed showed similar behaviour in relation to the inhibitory activity of phenolic compounds, as well as their ability to metabolize these compounds.

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**Keywords:** *Lactobacillus plantarum*; Phenolic compounds; Antimicrobial activity; Olive wastewater

## 1. Introduction

The olive tree (*Olea europaea* L.) is one of the most important fruit trees in Mediterranean countries. Their products, olive oil and also table olives, are important components of the Mediterranean diet and are consumed worldwide. The beneficial effects of olive consumption have been attributed partly to the phenolic content of the fruit and its associated antioxidant activity. Phenolics constitute a complex mixture in both olive fruit and derived products (particularly oil) although there are notable differences in composition between them that are attributed to a series

of chemical and enzymatic alterations of some phenols during oil extraction (Ryan, Robards, & Lavee, 1999). Recently there has been increasing interest in olive products and by-products, due to their antioxidant properties.

Olive fruits may contain up to 80 mg of phenols per 100 g sample that contribute to the unique flavour of virgin olive oil. The total phenolic content and the distribution of phenolic components are affected by the cultivar, growing location, and the degree of ripeness (Ryan et al., 1999). Oleuropein is the major phenolic compound responsible for the development of bitterness in olive fruits (Romero, García, Brenes, García, & Garrido, 2002).

Table olives have different qualitative and quantitative phenolic compositions from the raw olive fruits used to prepare them. The reason for this is the diffusion of phenols

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and other water-soluble constituents from the olive fruit to the surrounding medium (water, brine or lye) and *vice versa*, the lye treatment and hydrolysis during fermentation. Commercially available table olive samples were found to contain hydroxytyrosol as the prevailing phenolic compound (Dimitrios, 2006; Pereira et al., 2006).

Phenolic compounds are important functional minor components of virgin olive oils that are responsible for the key sensory characteristics of bitterness, pungency, and astringency. The production of virgin olive oil involves mechanical pressing of mesocarp of drupes of olive trees (*O. europea* L.), washing, and decanting, centrifuging and selective filtering. The production of olive oil generates several by-products. For example, in the three-phase centrifugal mills the main by-products are (i) olive leaves, which in most cases are used for animal feed, (ii) olive press cake, which is utilized by special oil-extracting factories for the production of a lower quality olive oil and a dry olive press cake used as a fuel, and (iii) olive oil mill wastewater, which is responsible for the largest environmental problem in the oil-producing areas (Agalias et al., 2007). The presence of phenolic compounds in these residues causes difficulties for their biological treatment (Arvanitoyannis & Kassaveti, 2007). Some researchers have developed systems for the treatment of these by-products in order to recover high added-value polyphenols and the reduction of the environmental problems (Agalias et al., 2007; Brenes, Romero, & de Castro, 2004).

*Lactobacillus plantarum* is a versatile and flexible species that is encountered in a variety of niches. Its most prominent abundance is in the fermentation of plant-derived raw materials, which include several industrial and artisan food and feed products, e.g. olives, must, and a variety of vegetable fermentation products. Since it has been reported that the spontaneous fermentation of Spanish-style green olives mainly depends on *L. plantarum*, and that this species has some phenolic-degrading activities (Ciafardini, Marsilio, Lanza, & Pozzi, 1994; Vaquero, Marcobal, & Muñoz, 2004), the aims of this study were (i) to explore the antimicrobial activities of some olive phenolic compounds against *L. plantarum* strains and (ii) to analyze the *L. plantarum* metabolic activities against the same phenolic compounds, in growth culture as well as in cell-free extracts. The information obtained from this study could be used to reveal the role of *L. plantarum* phenolic compound metabolism during table olive elaboration, and help the recovery of high added-value products from olive wastes.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The phenolic compounds analyzed in this study and the concentration used, were 5 mM *trans*-cinnamic acid (*trans*-3-phenylacrylic acid) (Aldrich C8085-7), 25 mM *p*-hydroxybenzoic acid (4-hydroxybenzoic acid) (Fluka

54630), 5 mM hydroxytyrosol (Extrasynthèse 4986), 5 mM oleuropein (Extrasynthèse 0204), 15 mM protocatechuic acid (3,4-dihydroxybenzoic acid) (Sigma P5630), 5 mM sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (Fluka D7927), 15 mM syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) (Fluka 86230), 15 mM tyrosol (4-hydroxyphenethyl alcohol) (Fluka 79058) and 25 mM vanillic acid (4-hydroxy-3-methoxybenzoic acid) (Fluka 94770). The concentration used, for each phenolic compound assayed, is indicated above, and it was determined by its response in the HPLC detector used. All the phenolic compounds were dissolved in ethanol, but sinapic acid was dissolved in methanol. Catechol (Sigma C9510) was used as a standard for the identification of the protocatechuic acid degradation product.

### 2.2. Strains, media and growth conditions

Four *L. plantarum* strains, isolated from different sources, were analysed. *L. plantarum* CECT 748<sup>T</sup> (ATCC 14917, DSMZ 20174), isolated from pickled cabbage, was purchased from the Spanish Type Culture Collection (CECT). *L. plantarum* WCFS1, isolated from saliva, and *L. plantarum* LPT57/1, isolated from olives, were kindly provided by M. Kleerebezem (Wageningen Centre for Food Sciences, NIZO Food Research) and J.L. Ruiz-Barba (Instituto de la Grasa, CSIC), respectively. *L. plantarum* RM71 (previously named BIFI-71) was isolated from wine at the Instituto de Fermentaciones Industriales, CSIC (Moreno-Arribas, Polo, Jorganes, & Muñoz, 2003; Vaquero et al., 2004).

Lactobacilli strains were routinely grown in a modified basal medium (Rozès & Peres, 1998). The composition of the basal medium described for *L. plantarum* was the following: glucose (2 g/l), trisodium citrate dihydrate (0.5 g/l), D-,L-malic acid (5 g/l), casamino acids (Difco, Detroit, Mich) (1 g/l), yeast nitrogen base without amino acids (Difco) (6.7 g/l) and the pH adjusted to 5.5. This basal media was modified with the replacement of glucose by galactose in order to avoid a possible glucose carbon catabolite repression.

For the degradation assays, the sterilized modified basal media was supplemented with the phenolic compound filter-sterilized. Inoculation (1%) was with bacteria previously grown in modified basal media supplemented with phenolic compound and incubated in darkness without shaking, at 30 °C for 10 days, under microaerophilic conditions. Incubated media with cells and without phenolic compound and incubated media without cells and with phenolic compounds were used as controls.

### 2.3. Inhibition growth assay

The inhibition growth assay was performed by the determination of the minimal inhibition concentration (MIC) values on liquid media. The inocula (1%) of the *L. plantarum* strains were prepared from broth cultures grown in

modified basal media. The phenolic compounds were dissolved and diluted to the highest concentration to be tested (100 mM), and then serial two-fold dilutions were made in a concentration range from 1.5 mM to 100 mM in 10 ml sterile test tubes containing modified basal media. The MIC was defined as the lowest concentration of the compound to inhibit bacterial growth or, similarly, the lowest concentration where absence of growth was recorded. Each tube assay was repeated at least twice.

#### 2.4. Preparation of cell-free extracts

To determine whether *L. plantarum* possess enzymes that are able to degrade the phenolic compounds assayed, cell-free extracts containing all soluble proteins were prepared. *L. plantarum* strains were grown in MRS media (Difco) under microaerobic conditions at 30 °C until a late exponential phase. 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. This suspension was disintegrated by using the French Press at 1500 psi pressure (Thermo FRENCH® Press, Thermo Electron). The cell disruption steps were carried out on ice to ensure low temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 12,000g for 20 min at 4 °C. The supernatant, containing the soluble proteins, was aseptically filtered (0.2 µm Filtropur S, Sarted). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Germany).

#### 2.5. Degradation of phenolic compounds by cell-free extracts

The enzymatic hydrolysis of phenolic compounds by cell-free extracts of *L. plantarum* strains was determined in 2 ml Eppendorf tubes, in a final volume of 1.1 ml, containing the phenolic compound. The final concentration of the phenolic compound was adjusted, taking into account the absorbance response of the compound. *L. plantarum* cell-free extracts in phosphate buffer (25 mM, pH 6.5) were incubated during 20 h at 30 °C in the presence of the phenolic compound. As control, phosphate buffer containing the phenolic compound was incubated under the same conditions.

The reaction products were extracted twice with one-third of the reaction volume of ethyl acetate (Lab-scan, Ireland). The solvent fractions were filtered through a 0.45 µm PVDF filter (Teknokroma, Spain) and analysed by HPLC.

#### 2.6. HPLC-DAD analysis

A Thermo (Thermo Electron Corporation, Waltham, Massachusetts, USA) chromatograph, equipped with a P400 SpectraSystem pump, an 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<sub>18</sub> cartridge (25 cm × 4.0 mm i.d.; 4.6 µm particle size, cartridge at room temperature) as follows: 0–55 min, 80% B linear, 1.0 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% B linear, 1.2 ml/min; 90–100 min, washing (methanol), and 100–120 min, 1.0 ml/min reequilibration of the cartridge (Bartolomé, Peña-Neira, & Gómez-Cordovés, 2000). Detection was performed by scanning from 280 to 380 nm. Samples were injected in duplicate onto the cartridge after being filtered through a 0.45 µm PVDF filter (Teknokroma, Spain).

The identification of degradation products was carried out by comparing the retention times and spectral data of each peak with those of standards from commercial suppliers.

### 3. Results and discussion

#### 3.1. Antimicrobial properties of some phenolic compounds found in olive products against *L. plantarum* strains

*L. plantarum* is a versatile and flexible species which is usually abundant in the fermentation of plant-derived raw materials where phenolic compounds are present at high concentration. In addition to these environments, *L. plantarum* is also encountered in some dairy and meat fermentation products and as a natural inhabitant of the gastrointestinal tract of humans and animals. Recently, a *L. plantarum* chromosomal region was designated a so-called “lifestyle adaptation island” and was suggested to be involved in niche adaptation (Molenaar et al., 2005). Based on this finding, and in order to find differences associated with a possible niche adaptation phenomenon, we decided to study the metabolic activities and antimicrobial effects of some olive phenolic compounds in four *L. plantarum* strains isolated from different sources.

The strains used in this study were isolated from different vegetable fermentations and one of them from human saliva, and therefore, they could have different metabolisms, reflecting selection for a specific food substrate. As shown in Table 1, phenolic compounds exert similar inhib-

Table 1  
Antimicrobial activities of phenolic compounds against *L. plantarum* strains

| Compound                      | MIC (mM)                   |       |         |      |
|-------------------------------|----------------------------|-------|---------|------|
|                               | <i>L. plantarum</i> strain |       |         |      |
|                               | CECT 748 <sup>T</sup>      | WCFS1 | LPT57/1 | RM71 |
| Cinnamic acid                 | 50                         | 50    | 50      | 50   |
| <i>p</i> -Hydroxybenzoic acid | >100                       | 100   | 100     | 100  |
| Hydroxytyrosol                | 100                        | 50    | 50      | 50   |
| Oleuropein                    | >100                       | 100   | >100    | >100 |
| Protocatechuic acid           | 100                        | 50    | 50      | 50   |
| Sinapic acid                  | 50                         | 25    | 25      | 12.5 |
| Syringic acid                 | 50                         | 25    | 25      | 50   |
| Tyrosol                       | >100                       | >100  | >100    | >100 |
| Vanillic acid                 | >100                       | 100   | 50      | 100  |

itory effects in all the four *L. plantarum* strains analyzed. Only one dilution difference in the MIC value was observed among the strains for all the compounds analyzed, but for *L. plantarum* RM71, isolated from wine, a two dilution MIC difference was observed for sinapic acid as compared to the *L. plantarum* type strain CECT 748<sup>T</sup>. Based on these results, no relevant differences were observed among strains for the inhibitory action of olive phenolics on *L. plantarum* growth.

Several authors have studied the bactericidal effect of both brines on untreated olives and have isolated phenolic compounds extracted from these olives or their brines. Nevertheless, different results have been reported for various phenolic compounds, probably because different antimicrobial assay methods were used. In some of these studies, the phenolic compound concentrations were adjusted to those in which they appeared in the brines.

Oleuropein, a bitter-tasting glucoside commonly found in leaves of the olive tree, as well as in unprocessed olives, is the major phenolic in the fresh fruit. Olives, to become edible, need to lose (at least partially), their natural bitterness. Controversy exists about the antibacterial properties of oleuropein. One of the reasons for the controversy could be the use of rich assay media to carry out the tests. In fact, it has been demonstrated that the presence of organic nitrogenous compounds (amino acids or proteins) in the assay medium can mask the antibacterial properties of certain phenolic compounds present in the green olive fermentation brines (Ruiz-Barba, Garrido-Fernández, & Jiménez-Díaz, 1991). In addition, the presence of glucose in the medium partially inhibited oleuropein  $\beta$ -glucosidase activity by *L. plantarum* (Ciafardini et al., 1994). Therefore, to avoid such enzyme inhibition, we used a modified basal medium described previously (Rozès & Peres, 1998) but containing galactose instead of glucose. In our study, *L. plantarum* strains could support elevated oleuropein concentrations, since a 100 mM oleuropein concentration corresponds to 54 g/l. The highest oleuropein concentration previously tested was 10 g/l and, at that concentration, Marsilio and Lanza (1998) reported that oleuropein (10 g/l) did not inhibit *L. plantarum* bacterial growth.

Hydroxytyrosol, tyrosol, and luteolin are the prevailing phenols in all samples of table olives (Pereira et al., 2006). The quantification of the identified phenolics revealed that hydroxytyrosol was the compound present in the highest amount, varying from 60.7 to 85.9% of total phenolics in table olives. This compound results from the hydrolysis of oleuropein. Oleuropein could be hydrolyzed in the acidic medium of directly brined olives or by action of oleuropeinolytic *L. plantarum* strains, to glucose plus aglycone and conversion of the latter, in turn, to more simple, non-bitter compounds such as elenoic acid and hydroxytyrosol. Marsilio and Lanza (1998) reported that hydroxytyrosol (2 g/l) did not inhibit *L. plantarum* bacterial growth. The *L. plantarum* strains analyzed in this study, were inhibited by 7.7 or 15.4 g/l hydroxytyrosol, representing a 50 or 100 mM concentration, respectively. However, previously,

Ruiz-Barba, Brenes-Balbuena, Jiménez-Díaz, García-García, and Garrido-Fernández (1993) reported that hydroxytyrosol, at the maximum concentration found in olive brines (approx. 7.5 mM or 1.15 g/l), showed a strong bactericidal effect against *L. plantarum*. In fact, similarly to oleuropein, controversial results were obtained, possibly because different antimicrobial assays were used.

Ruiz-Barba et al. (1993) have shown that tyrosol has no antimicrobial effect when was used at 1.5 mM concentration. In our study, higher tyrosol concentrations were tested. All the *L. plantarum* strains were not inhibited, even by the highest concentration assayed, 100 mM, corresponding to 13.8 g/l. Vanillic acid, when assayed even at the maximal concentrations found in brines (0.01 mM), showed inhibition against *L. plantarum* (Ruiz-Barba et al., 1993). In the antimicrobial assay used in this study, *L. plantarum* strains were inhibited by 50 or 100 mM vanillic acid concentrations. In a different lactobacillus species, *L. hilgardii*, Campos, Couto, and Hogg (2003) reported that vanillic acid, or *p*-hydroxybenzoic acid, did not show an inhibitory effect at the highest concentration assayed (500 mg/l). In our study, *p*-hydroxybenzoic acid, at 100 mM concentration, inhibited *L. plantarum* growth. Sinapic and syringic acids showed the highest inhibitory activity against the *L. plantarum* strains analyzed.

Surprisingly, none of the phenolic compounds assayed seemed to inhibit *L. plantarum* growth at the concentrations found in olive food product. However, caution should be applied in comparing the results observed in this study, that were conducted in culture medium, to those in real olive systems, which are more complex. In fact, Ruiz-Barba et al. (1993) concluded that phenolic compounds showed a combined effect in the inhibition; they demonstrated the additive antimicrobial effect of some olive phenolics, whereas, when assayed as single fractions, they had no bacterial effect against *L. plantarum*. In addition, the presence of non-phenolic compounds, such as sugars, pectin, salts, acids, lipids and polyalcohols, also abundant in olive-related products, could significantly affect the inhibitory action of the phenolic compounds.

### 3.2. Degradation of some phenolic compounds found in olive products by *L. plantarum* strains

The spontaneous lactic acid fermentation of Spanish-style olives is due mainly to *L. plantarum*. Lactic acid bacteria are strongly recommended for preserving ripe olives, since they produce adequate acidity, resulting from the metabolism of the sugar eluted from olives in brine. With the exception of oleuropein, it is not known whether some phenolic compounds present in olive food products can be modified by the *L. plantarum* metabolism. Recently, Kachouri and Hamdi (2004) reported the enhancement of polyphenols in olive oil by contact with olive mill wastewater fermented by *L. plantarum*. Simple polyphenol content was increased in olive oil when *L. plantarum* was added to OMW, especially for oleuropein, *p*-hydroxyphenylacetic,

vanillic and ferulic acids and tyrosol. Since this approach was done in olive mill wastewater, a complex polyphenolic mixture, we decided to elucidate the abilities of several *L. plantarum* strains to metabolize some of the simple, low molecular weight, and commercially available, phenolic compounds found in olive food products.

As explained above, we analyzed four *L. plantarum* strains isolated from different sources, in order to observe possible differences in the metabolism of these compounds related to the presence or absence of these compounds in their isolation habitat. However, all the strains analysed showed similar behaviour, and no differences were observed among the different strains analyzed.

Oleuropein is the main phenolic glucoside of olive fruit. As explained above, oleuropein degradation by *L. plantarum* strains has previously been demonstrated. Marsilio, Lanza, and Pozzi (1996) reported the results of a gas-chromatographic study of the oleuropein derivatives released by incubation with *L. plantarum*. The results indicated that *L. plantarum* strains initially hydrolyze the oleuropein by means of  $\beta$ -glucosidase action, with formation of an aglycone (the first observable intermediate compound), and in a second step, this derivative, by means of esterase action, gives rise to hydroxytyrosol (identified) and elenoic acid (not identified). These results are in agreement with the description of a  $\beta$ -glucosidase activity present in *L. plantarum* strains (Sestelo, Poza, & Villa, 2004). In our study, most of the oleuropein was degraded by *L. plantarum* strains (Fig. 1a). No marked differences were observed among the *L. plantarum* strains analyzed (data not shown). As reported by Marsilio et al. (1996), oleuropein rearranges

to other aglycone structures before transforming into stable final compounds such as hydroxytyrosol. We only observed aglycone structures and hydroxytyrosol in the chromatograms after 10 days of incubation. The aglycones were identified by their oleuropein-like spectra (Fig. 1b, 1). The identity of the hydroxytyrosol was determined by comparing the retention time and spectral data with the commercial sample (Fig. 1b, 2) and, as reported by Marsilio et al. (1996), no elenoic acid was detected. Hydroxytyrosol is a strong antioxidant whose antioxidant efficiency in water is comparable to that of ascorbic acid, whereas in lipidic medium it is four times higher (Briante, La Cara, Tonziello, Febbraio, & Nucci, 2001). However, hydroxytyrosol is not commercially available in high amounts as a food additive. Several methods have been proposed for the production of hydroxytyrosol by means of chemical (Tuck, Tan, & Hayball, 2000) or enzymatic synthesis (Espin, Soler-Rivas, Cantos, Tomás-Barberán, & Wichers, 2001). This work confirms previous results, since hydroxytyrosol is produced by *L. plantarum* from oleuropein, the main phenolic of olive fruit. By-products from processing materials of biological origin, such as wastewaters from olive oil mills, may therefore become important sources of high added-value compounds, such as hydroxytyrosol or other antioxidants phenols.

Phenyl alcohols, such as hydroxytyrosol and tyrosol, are identified in olive products. When *L. plantarum* strains were grown in the presence of these phenyl alcohols, none of them was degraded (data not shown).

Acids which often appear in lists of olive products are vanillic acid, *p*-coumaric acid, ferulic acid, caffeic acid,

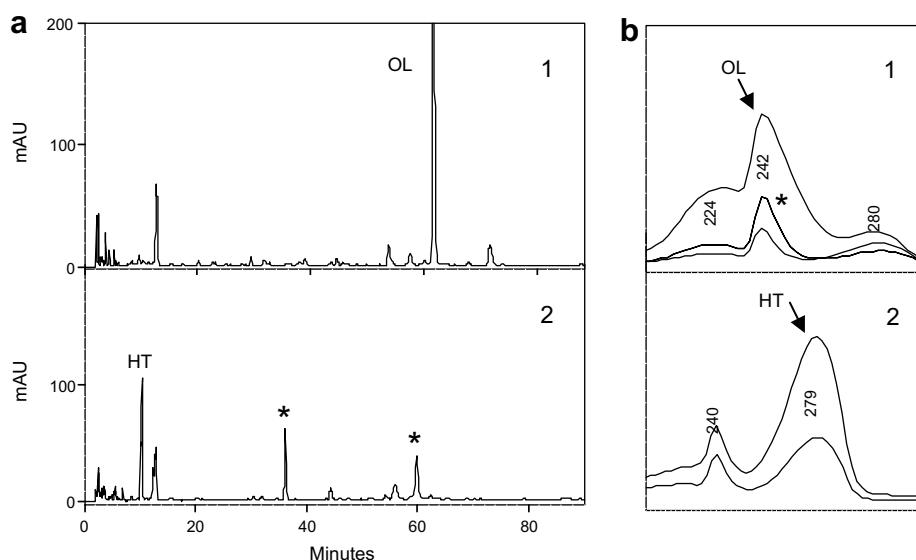


Fig. 1. (a) HPLC analysis of *L. plantarum* oleuropein metabolism. Modified basal medium containing 5 mM oleuropein was inoculated with *L. plantarum* WCFS1 strain and incubated for 10 days at 30 °C (2); a non-inoculated control medium was incubated under the same conditions (1). Detection was performed at 280 nm. OL, oleuropein; HT, hydroxytyrosol. (b) Comparison between spectra of the phenolic compounds identified from the *L. plantarum* culture and the standards. (1) Oleuropein standard (OL) and oleuropein (RT 59.9 min) and oleuropein-like compound (RT 36.3 min) found in the *L. plantarum* growth media that are indicated by asterisks, and (2) hydroxytyrosol standard (HT) and the hydroxytyrosol produced by *L. plantarum* growth. The spectra corresponding to the standard compounds are indicated by arrows.

cinnamic acid (not a phenol), protocatechuic acid, *p*-hydroxybenzoic acid, sinapic acid and syringic acid among others (Dimitrios, 2006). Previously, three cinnamic acids (*p*-coumaric, caffeic and ferulic acids) have been reported to be metabolised by *L. plantarum* strains (Cavin, Andioc, Etievant, & Divies, 1993). These phenolics acids were decarboxylated. When decarboxylation was observed, volatile phenols, such as 4-ethyl phenol and 4-ethyl guaiacol, were detected, indicating the possibility of reduction of the side chain before or after decarboxylation.

Since *p*-coumaric, caffeic and ferulic acid metabolism have already been studied in *L. plantarum*, we decided to study the ability of *L. plantarum* strains to metabolize seven different phenolic acids frequently present in olive products. Among these acids, only protocatechuic acid was metabolized by *L. plantarum* strains growing in its presence. Protocatechuic acid was completely degraded to catechol (Fig. 2a). The identity of catechol was determined by comparing retention times and spectral data with commercial catechol (Fig. 2b, 2). As early as 1971, Whiting and Coggins (1971) reported that *L. plantarum* showed an oxidative route of metabolism of quinate and shikimate, and described that the oxidative route gives catechol as end-product, and there was no indication of its further metabolism under anaerobic conditions. They observed that cells grown in a medium containing protocatechuate completely metabolised it to catechol. Since a phenolic acid decarboxylase, able to decarboxylate *p*-coumaric, caffeic and ferulic acids, has been purified from *L. plantarum*, it will be interesting to test whether this decarboxylase is also able to decarboxylate protocatechuic acid, since this compound has not been tested previously (Cavin et al., 1997). The non-oxidative decarboxylation of protocatechuic acid to produce catechol is an unusual bacterial pathway for

degrading phenolic compounds since, in the main aromatic compound-degrader bacteria, the pathways for recycling aromatic compounds converge into catechol or protocatechuate, which are ring-cleaved by dioxygenases. As far as we know, protocatechuate decarboxylase activity has been reported only in *Klebsiella aerogenes* (Grant & Patel, 1969) and in *Clostridium hydroxybenzoicum* (He & Wiegel, 1996), and no genetical and biochemical enzyme characterization data have been described so far.

As described above, by using *L. plantarum* cell cultures, degradation of oleuropein and protocatechuic acid was observed; however, by using cell-free extracts, only protocatechuic acid was degraded (data not shown). Therefore, it could be assumed that degradation of oleuropein is carried out by inducible enzymes. Previously, Whiting and Coggins (1971) reported that the *L. plantarum* enzymes involved in the reduction of quinate and shikimate are induced. The induced nature of the enzymes involved in the *L. plantarum* metabolism of phenolic acids has also been reported more recently (Barthelmebs, Divies, & Cavin, 2000; Cavin et al., 1997). Phenolic acid decarboxylase activity was only detected for bacteria grown in the presence of the enzyme substrates, indicating that this activity was inducible. Moreover, it has been described that *L. plantarum* also has a second inducible acid phenol decarboxylase enzyme, which also displays inducible acid phenol reductase activity (Barthelmebs et al., 2000).

In summary, the results of this work have shown that none of the nine phenolic compounds analyzed and present in olive food products inhibit *L. plantarum* growth at the concentrations found in olive food products. In addition, the present study showed that, for the compounds analyzed, only oleuropein and protocatechuic acid were metabolized by *L. plantarum* cultures containing the

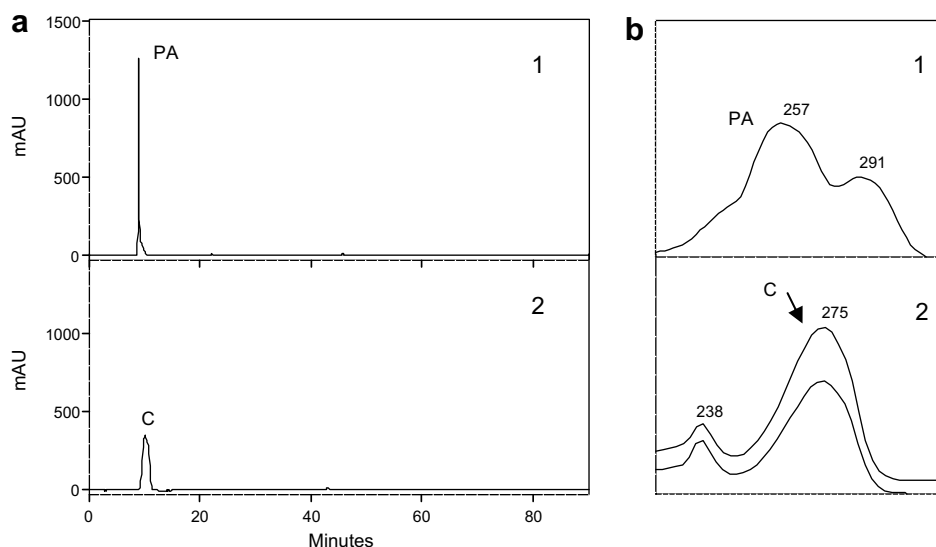


Fig. 2. (a) HPLC analysis of *L. plantarum* protocatechuic acid metabolism. Modified basal medium containing 15 mM protocatechuic acid was inoculated with *L. plantarum* CECT 748<sup>T</sup> strain and incubated for 10 days at 30 °C (2); a non-inoculated control medium was incubated under the same conditions (1). Detection was performed at 280 nm. PA, protocatechuic acid; C, catechol. (b) Spectra of protocatechuic acid (PA) (1) and comparison between spectra of the catechol identified and the catechol standard (C) (indicated by an arrow).

phenolic compound. This metabolism seems to be carried out partially by inducible enzymes since a cell-free extract from a culture grown in the absence of oleuropein was unable to metabolize it. In spite of the genomic variability reported among *L. plantarum* strains, we found similar behaviour, in relation to phenolic metabolism, in the four *L. plantarum* strains isolated from different sources. In addition, the information obtained in this work will be useful for the management of olive mill wastewater treatment and disposal, since they constitute a critical environmental problem for the Mediterranean countries.

## Acknowledgements

This work was supported by grants AGL2005-00470 (CICYT), FUN-C-FOOD Consolider 25506 (MEC), RM03-002 (INIA), and S-0505/AGR/000153 (CAM). We thank C. Gómez-Cordovés for her help with the HPLC analysis. The technical assistance of M.V. Santamaría is greatly appreciated. J.M. Landete and J. A. Curiel are recipients of a postdoctoral and predoctoral fellowship, respectively, from the MEC. H. Rodríguez is a recipient of a predoctoral fellowship from the I3P-CSIC.

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