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

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Abstract

The species *Lactobacillus plantarum* is the main responsible of 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 know 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) inhibits *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 contrary, sinapic and syringic acid showed the highest inhibitory activity since concentrations ranging from 12.5 to 50 mM inhibit *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 presence of this compounds. Oleuropein was metabolized mainly to hydroxytyrosol, and protocatechuic acid was decarboxylated to catechol. Metabolism of oleuropein was carried out by inducible enzymes since cell-free extract from a culture grown in absence of oleuropein was unable to metabolize it. Independent of their isolation source, the four *L. plantarum* strains analysed showed a similar behaviour in relation to the inhibitory activity of phenolic compounds, as well as their ability to metabolize these compounds.

Keyworks: *Lactobacillus plantarum*; Phenolic compounds; Antimicrobial activity; olive wastewater

1. Introduction

The olive tree (*Olea europea* L.) is one of the most important fruit trees in the Mediterranean countries. Their products, olive oil and also table olives, are important components of the Mediterranean diet and are largely consumed in the world. 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 (notably oil) although there are some 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 is an increasing interest in olive products and byproducts, due to their antioxidant properties

Olive fruits may contain up to 80 mg of phenols per 100 g sample that are responsible for 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, Robards, & Lavee, 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 a different qualitative and quantitative phenolic composition than the raw olive fruits from which they are prepared. The reason is the diffusion of phenols 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. Commercial 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 (*Olea europea* L.), washing, and decanting, centrifuging and selective filtering. The production of olive oil generates several byproducts. As example, in the three-phase centrifugal mills the main byproducts are (i) olive leaves, which in most cases are used by 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 byproducts in order to recovery of high added value-contained 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, like olives, must, and a variety of vegetable fermentations. Since it has been reported that the spontaneous fermentation of Spanish-style green olives mainly depends on *L. plantarum*, and that this species possess some phenolic degrading activities (Ciafardini, Marsilio, Lanza, & Pozzi, 1994; Vaquero, Marcobal, & Muñoz, 2004), the aims of this study were (i) to know 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 known the role of *L. plantarum* phenolic compound metabolism during table olive elaboration as well as for the recovery of high added-value from olive wastes.

2. Material 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 compounds assayed is indicated above, and it was determined based on 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 standard for the identification of the protocatechuic acid degradation product.

2.2. Strains, media, and growth conditions

Four *Lactobacillus plantarum* strains isolated from different sources were analysed. *L. plantarum* CECT 748^T (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. Ruíz-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, Marcobal, & Muñoz, 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) (1g/l), yeast nitrogen base without amino acids (Difco) (6.7 g/l) and the pH adjusted to 5.5. This basal media was modified by 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%) with bacteria previously grown in modified basal media supplemented with phenolic compound was incubated in darkness without shaking, at 30 ºC 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

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148 The inhibition growth assay was performed by the determination of the minimal
149 inhibition concentration (MIC) values on liquid media. The inocula (1%) of the *L.*
150 *plantarum* strains were prepared from broth cultures grown in modified basal media.
151 The phenolic compounds were dissolved and diluted to the highest concentration to be
152 tested (100 mM), and then serial two-fold dilutions were made in a concentration range
153 from 1.5 mM to 100 mM in 10 ml sterile test tubes containing modified basal media.
154 The MIC was defined as the lowest concentration of the compound to inhibit bacterial
155 growth or, similarly, the lowest concentration where absence of growth was recorded.
156 Each tube was repeated at least twice.

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158 *2.4. Preparation of cell-free extracts*

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160 To determine if *L. plantarum* possess enzymes able to degrade the phenolic
161 compounds assayed, cell-free extracts containing all soluble proteins were prepared. *L.*
162 *plantarum* strains were grown in MRS media (Difco) under microaerobic conditions at
163 30 ºC until a late exponential phase. The cells were harvested by centrifugation and
164 washed three times with phosphate buffer (50 mM, pH 6.5), and subsequently
165 resuspended in the same buffer for cell rupture. This suspension was disintegrated by
166 using the French Press at 1500 psi pressure (Thermo FRENCH® Press, Thermo
167 Electron). The cell disruption steps were carried out on ice to ensure low temperature
168 conditions required for most enzymes. The disintegrated cell suspension was
169 centrifuged at 12000 x g for 20 min at 4 ºC. The supernatant containing the soluble
170 proteins was aseptically filtered (0.2 μm Filtropur S, Sartedt). Protein concentration was
171 determined using the Bio-Rad protein assay (Bio-Rad, Germany).

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173 *2.5. Degradation of phenolic compounds by cell-free extracts*

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175 The enzymatic hydrolysis of phenolic compounds by cell-free extracts of *L.*
176 *plantarum* strains was determined in 2-ml Eppendorf tubes in a final volume of 1.1 ml
177 containing the phenolic compound. The final concentration of the phenolic compound
178 was adjusted taking into account the absorbance response of the compound. *L.*
179 *plantarum* cell-free extracts in phosphate buffer (25 mM, pH 6.5) were incubated during
180 20 h at 30 ºC in presence of the phenolic compound. As control, phosphate buffer
181 containing the phenolic compound was incubated in the same conditions.
182 The reaction products were extracted twice with one third of the reaction volume
183 of ethyl acetate (Lab-scan, Ireland). The solvent fractions were filtered through a 0.45
184 μm PVDF filter (Teknokroma, Spain) and anlaysed by HPLC.

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186 *2.6. HPLC-DAD analysis*

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188 A Thermo (Thermo Electron Corporation, Waltham, Massachusetts, USA)
189 chromatograph equipped with a P400 SpectraSystem pump, an AS3000 autosampler,
190 and a UV6000LP photodiode array detector were used. A gradient of solvent A
191 (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 78:20:2,
192 v/v/v) was applied to a reversed-phase Nova-pack C_{18} cartridge (25 cm x 4.0 mm i.d.;
193 4.6 μm particle size, cartridge at room temperature) as follows: 0-55 min, 80% B linear,
194 1.0 ml/min; 55-57 min, 90% B linear, 1.2 ml/min; 57-70 min, 90% B isocratic, 1.2
195 ml/min; 70-80 min, 95% B linear, 1.2 ml/min; 80-90 min, 100% B linear, 1.2 ml/min;
196 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 to a possible niche adaptation phenomenon, we decided to study the metabolic activities and antimicrobial effect 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 the human saliva, and therefore, they could posses a different metabolism, reflecting a selection for a specific food substrate. As showed in

Table 1, phenolic compounds exert a similar inhibitory effect 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 dilutions MIC difference was observed for sinapic acid as compared to the *L. plantarum* type strain CECT 748^{T}. Based on these results, no relevant differences were observed among strains on 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 appears 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, & Jimenez-Diaz, 1991). In addition, the presence of glucose in the medium partially inhibited oleuropein β-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 supports 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 & Lanza (1998) reported that oleuropein (10 g/l) did not inhibit *L. plantarum* bacterial growth.

Hydroxytyrosol, tyrosol, and luteolin were 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, in more simple, nonbitter compounds like elenoic acid and hydroxytyrosol. Marsilio & 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, & 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 are obtained, possibly due to that 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 concentration. In a different lactobacilli species, *L. hilgardii*, Campos, Couto, & Hogg (2003) reported that vanillic acid, as well as *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 seems to inhibit *L. plantarum* growth at the concentrations found in olive food product. However, caution should be paid in applying 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, polyalcohols, etc, also abundant in olive-related products could significantly affect the inhibitory effect 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 exception of oleuropein, it is not known whether some

297 phenolic compounds present in olive food products can be modified by the *L. plantarum*
298 metabolism. Recently, Kachouri & Hamdi (2004) reported the enhancement of
299 polyphenols in olive oil by contact with fermented olive mill wastewater by *L.*
300 *plantarum*. Simple polyphenols content was increased in olive oil when *L. plantarum*
301 was added to OMW, especially for oleuropein, *p*-hydroxyphenylacetic, vanillic and
302 ferulic acids and tyrosol. Since this approach was done in olive mill wastewater, a
303 complex polyphenolic mixture, we decided to known the ability of several *L. plantarum*
304 strains to metabolize some of the simple, low molecular weight, and commercially
305 available, phenolic compounds found in olive food products.
306 As explained above, we analyzed four *L. plantarum* strains isolated from
307 different sources in order to observe possible differences in the metabolism of these
308 compounds related to the presence or absence of these compounds in their isolation
309 habitat. However, all the strains analysed showed a similar behaviour, and no
310 differences were observed among the different strains analyzed.
311 Oleuropein is the main phenolic glucoside of olive fruit. As explained above,
312 oleuropein degradation by *L. plantarum* strains have been demonstrated previously.
313 Marsilio, Lanza & Pozzi (1996) reported the results of a gas-chromatographic study of
314 the oleuropein derivatives released by incubation with *L. plantarum*. The results
315 indicated that *L. plantarum* strains initially hydrolyze the oleuropein by means of β-
316 glucosidase action with formation of an aglycone (the first observable intermediate
317 compound), and in a second step, this derivative, by means of esterase action, gives rise
318 to hydroxytyrosol (identified) and elenoic acid (not identified). These results are in
319 agreement with the description of a β-glucosidase activity present in *L. plantarum*
320 strains (Sestelo, Poza, & Villa, 2004). In our study, most of the oleuropein was
321 degraded by *L. plantarum* strains (Fig. 1A). No remarkable 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 like hydroxytyrosol. We only observed aglycone structures and hydroxytyrosol in the chromatograms after 10 days 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 which 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 amount as 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 from olive fruit. By-products from processing materials of biological origin, such as wastewaters from olive oil mills, may then 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 presence of these phenyl alcohols, it was observed that 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, 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 was already studied in *L. plantarum*, we decided to study the ability of *L. plantarum* strains to metabolize seven different phenolic acids frequent in olive products. From these acids, only protocatechuic acid was metabolized by *L. plantarum* strains growing on 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 & Coggins (1971) reported that *L. plantarum* show 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 if 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 to degrade 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 known, protocatechuate decarboxylase activity has been only reported in *Klebsiella aerogenes* (Grant & Patel,

371 1969) and in *Clostridium hydroxybenzoicum* (He & Wiegel, 1996), and no genetical and
372 biochemical enzyme characterization have been described so far.
373 As described above, by using *L. plantarum* cell cultures, degradation of
374 oleuropein and protocatechuic acid was observed; however, by using cell-free extracts,
375 only protocatechuic acid was degraded (data not shown). Therefore, it could be assumed
376 that degradation of oleuropein is carried out by inducible enzymes. Previously, Whiting
377 & Coggins (1971) reported that the *L. plantarum* enzymes involved in the reduction of
378 quinate and shikimate are induced. The induced nature of the enzymes involved in the
379 *L. plantarum* metabolism of phenolic acids has also been reported more recently (Cavin
380 et al. 1997; Barthelmebs, Divies, & Cavin, 2000). Phenolic acid decarboxylase activity
381 was only detected for bacteria grown in presence of the enzyme substrates indicating
382 that this activity was inducible. Moreover, it has been described that *L. plantarum* also
383 possess a second inducible acid phenol decarboxylase enzyme, which also displays
384 inducible acid phenol reductase activity (Barthelmebs et al. 2000).
385
386 In summary, the results of this work have shown that none of the nine phenolic
387 compounds analyzed and present in olive food products inhibit *L. plantarum* growth at
388 the concentration found in olive food products. In addition, the present study showed
389 that for the compounds analyzed, only oleuropein and protocatechuic acid were
390 metabolized by *L. plantarum* cultures containing the phenolic compound. This
391 metabolism seems to be carried out partially by inducible enzymes since a cell-free
392 extract from a culture grown in absence of oleuropein was unable to metabolize it. In
393 spite of the genomic variability reported among *L. plantarum* strains, we found a similar
394 behaviour in relation to phenolic metabolism in the four *L. plantarum* strains isolated
395 from different sources. In addition, the information obtained in this work will be useful

for the management of the olive-mill wastewater treatment and disposal, since they are a critical environmental problem for the Mediterranean countries.

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Figure captions

Fig. 1. (A) HPLC analysis of *L. plantarum* oleuropein metabolism. Modified basal media containing 5 mM oleuropein was inoculated with *L. plantarum* WCFS1 strain and incubated for 10 days at 30 ºC (2); a non-inoculated control medium was incubated in 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.

Fig. 2. (A) HPLC analysis of *L. plantarum* protocatechuic acid metabolism. Modified basal media containing 15 mM protocatechuic acid was inoculated with *L. plantarum* CECT 748^T strain and incubated for 10 days at 30 ºC (2); a non-inoculated control medium was incubated in 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).

Figure 1

Figure 1 (Landete, Curiel, Rodríguez, de las Rivas & Muñoz)

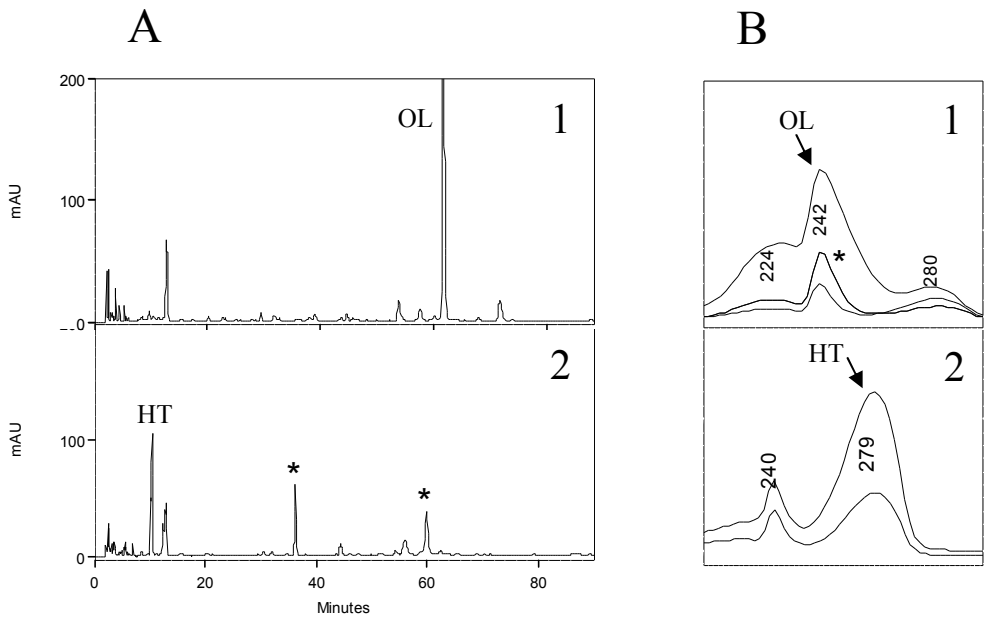

Figure 2

Figure 2 (Landete, Curiel, Rodríguez, de las Rivas & Muñoz)

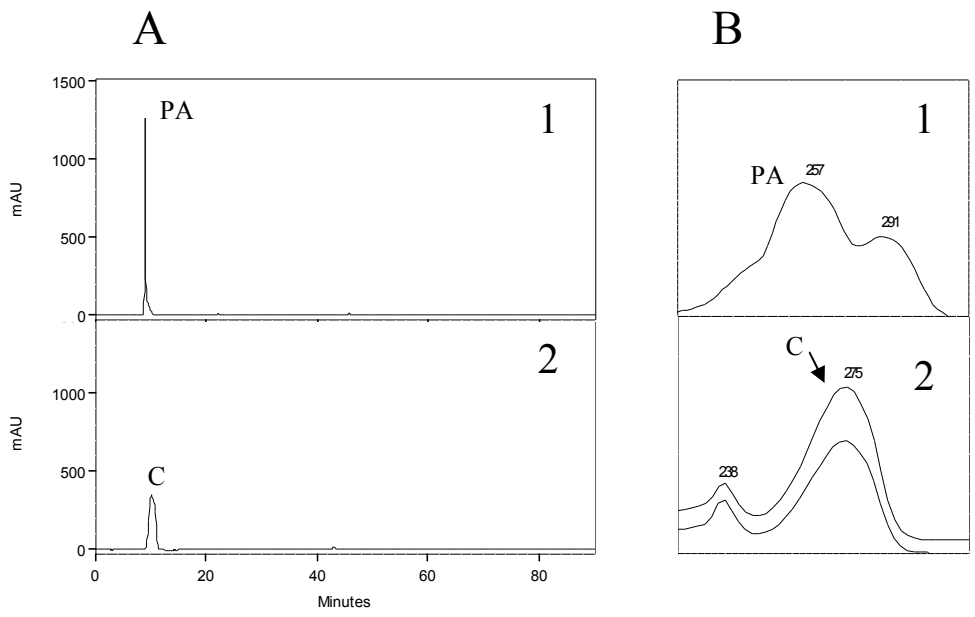

Table 1 (Landete, Curiel, Rodríguez, de las Rivas & Muñoz)

Table 1

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

	MIC (mM)			
	L. plantarum strain			
Compound	CECT 748^T	WCFS1	LPT57/1	RM71
Cinnamic acid	50	50	50	50
p-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