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Main Antimicrobial Compounds in Table Olives

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The inhibitors involved in the lactic acid fermentation of table olives were investigated in aseptic olive brines of the Manzanilla and Gordal varieties. Phenolic and oleosidic compounds in these brines were identified by high-performance liquid chromatography with ultraviolet and electrospray ionization mass spectrometry detection, and several substances were also characterized by nuclear magnetic resonance. Among these compounds, the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol showed the strongest antilactic acid bacteria activity, and its presence in brines could explain the growth inhibition of these microorganisms during olive fermentation. However, it was found that the dialdehydic form of decarboxymethyl elenolic acid, identified for the first time in table olives, and an isomer of oleoside 11-methyl ester were also effective against *Lactobacillus pentosus* and can, therefore, contribute to the antimicrobial activity of olive brines. It must also be stressed that the three new inhibitors discovered in table olive brines exerted a more potent antibacterial activity than the well-studied oleuropein and hydroxytyrosol.

KEYWORDS: Table olives; antimicrobial; lactic acid bacteria; phenolic; oleosides; oleuropein; hydroxytyrosol

INTRODUCTION

Plants, herbs, spices, and certain foods have been used in folk medicine, food preservation, cosmetics, and many other applications since ancient times because of their antimicrobial activity. However, the development of the chemical synthesis during the 20th century moved most of these natural remedies out of societal uses. Nowadays, there is a great concern about these synthetic compounds along with renewed interest in natural antimicrobials (*1*, *2*).

Olives, olive oil, and olive leaf extracts are some of these foodstuffs with recognized medicinal benefits and food-preservation properties dating back to the Egyptian empire. Nevertheless, it was not until the middle of the 20th century that researchers started to look for antimicrobial compounds in these commodities, especially in table olives. It is well-known that the lactic acid fermentation step of table olive processing does not occur in many occasions (*3*), and it was earlier attributed to the presence of antimicrobial substances in olives (*4*). The traditional treatment of olives with a NaOH solution (2–3% w/v) during the processing of Spanish-style green olive produces, among other consequences, an abundant growth of lactic acid bacteria (LAB), which is not observed if the concentration of the alkali is low (<1.8%) (*5*). By contrast, olives placed directly into brines, in particular those of the Manzanilla variety, do not permit the growth of LAB (*6*). This inhibitory activity was first attributed to the presence of the bitter glucoside oleuropein in olives (*7*), which is the main phenolic compound in the flesh (*8*), but it was also demonstrated that the products of its hydrolysis (elenolic acid and oleuropein aglycon) exerted a stronger antimicrobial activity in vitro than the glucoside (*9*). These findings were confirmed in the 1980s (*10*, *11*), although these hydrolysis products were never identified in olive brines. More recently, Ruiz-Barba et al. (*12*, *13*) analyzed the phenolic compounds in olive brines and found that the main polyphenol in these solutions was hydroxytyrosol, a component of the oleuropein moiety, followed by tyrosol, verbascoside, and oleuropein. They isolated each compound by high-performance liquid chromatography (HPLC) and tested its antimicrobial activity against LAB In contrast to previous results (*9*, *10*), hydroxytyrosol showed bactericidal activity against these microorganisms, and its presence in olive brines could explain the inhibition of LAB growth. However, this polyphenol is found in brines of olives both treated (*14*) and nontreated with NaOH (*15*). Therefore, the mystery of the inhibitors of LAB growth in olive brines remained unsolved.

Apart from table olives, antimicrobial activity has been reported in olive oil mill wastewaters (*16*, *17*) and olive leaf extracts (*18*), and this bioactivity was also related to phenolic compounds, oleuropein, and hydroxytyrosol, among others. Indeed, researchers still continue studying the antimicrobial activity of these two compounds in vitro against pathogens such as bacteria and viruses (*19*–*21*). Likewise, it was recently demonstrated that the main antimicrobials in olive oil are the dialdehydic forms of decarboxymethyl elenolic acid linked to tyrosol or linked to hydroxytyrosol (*22*) and that they exert a greater bioactivity than hydroxytyrosol and many other phenolic compounds.

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Therefore, the objective of this work was to identify the inhibitors of LAB growth in table olive brines. A comprehensive knowledge of these antimicrobials is fundamental to a better understanding of the lactic acid fermentation step and could contribute to improving the quality of the product. Additionally, table olive brines could be considered as a natural source of antimicrobial compounds.

MATERIALS AND METHODS

Strains and Culture Conditions. *Lactobacillus pentosus* (ATCC 8041), *Lactobacillus plantarum* (ATCC 14917), and *Saccharomyces cerevisiae* (ATCC 9080) were obtained from the American type Culture Collection (Rockville, MD, USA). *Enterococcus faecium* (CECT 410), *Enterococcus faecaelis* (CECT 481), *Enterobacter aerogenes* (CECT 684), *Escherichia coli* (CECT 434), and *Pichia membranaefaciens* (CECT 10482) were purchased from the Spanish type Culture Collection (Burjasot, Valencia, Spain). *Lactobacillus brevis* BP and *Leuconostoc mesenteroides* LM 51 were isolated in our laboratory from table olive brines. *Lactobacillus* and *Leuconostoc* were grown in MRS broth, and *Enterococcus* in BHI (Oxoid Ltd. Basingstoke, U. K.). Enterobacteriaceae were cultured in nutrient broth containing, per liter, 5 g of "Lablemco" powder (Oxoid), 10 g of peptone (Pronadisa, Laboratorios Conda, Madrid, Spain), and 5 g of sodium chloride (Panreac, Barcelona, Spain). Yeasts were grown in YM broth (Difco, Sparks, MD). For solid media, 1.5% agar (Panreac) was added to the corresponding broth.

All strains were preserved at −80 °C in the same liquid media plus 20% glycerol. Before use in each experiment, microorganisms were cultured twice for 24–48 h at 37 °C and 30–32 °C for Enterobacteriaceae and the rest, respectively. They were also grown in a solid media to control the purity of the culture. If the strains were prepared to inoculate the brines, then they were previously adapted to the saline environment by adding 3% NaCl to the last broth.

Olive Samples and Aseptic Brining. Olives of the Manzanilla and Gordal varieties with a green-yellow color on the surface were obtained from four different orchards in the Seville province of Spain. They were selected to remove fruits with blemishes, cuts, and insect damage. After washing thoroughly with tap water to remove impurities, they were placed in a sodium hypochlorite solution (50 mg/L active chlorine) at 35 °C for 2 min (*23*). Then, olives were washed with sterilized water twice to remove chlorine. **S**ubsequently, 350 g of fruits were introduced into autoclaved bottles (500 mL capacity) and covered with a 5% NaCl and 0.5% acetic acid sterile solution. These manipulations were carried out in a laminar flow cabinet. Finally, the bottles were sealed and stored at room temperature for two months. They were checked for microbial growth by visual appearance and plate counts, and microorganisms were not detected in any aseptic brine. Samples of brine were taken and kept at −20 °C before analysis. Two bottles were prepared for each of the four samples of each olive variety.

Inoculation Experiments in Olive Brines. *L. pentosus* ATCC 8041 and *L. brevis* BP were separately inoculated in each of the four brines of both Manzanilla and Gordal varieties. Four mL of each aseptic brine stored for two months were inoculated with 0.1 mL of an overnight culture in MRS-3% NaCl, previously diluted in saline (0.85%) to get initial inocula between 5.5 and 6.0 log CFU/mL. Inoculated brines were then incubated at 32 °C, samples were withdrawn at 24 and 48 h, and culturable survivors were determined by plating these brines onto MRS agar plates, spreading 0.1 mL with no dilution for cloudless brines, or plating the 10^{-4} dilution (0.1% peptone water) for cloudy brines with a Spiral Plater (Don Whitley Sci. Ltd., model Wasp 2, Shirpley, U. K.). All brines were tested in duplicates.

Determination of pH. The pH was measured using a Crison model 2001 pH meter (Crison Instruments, Barcelona, Spain).

Analysis of NaCl. The concentration of sodium chloride was analyzed by titration with 0.1 N silver nitrate solution, using potassium chromate solution as indicator.

Analysis of Acetic Acid. It was measured by HPLC using a Spherisorb ODS-2 (5 μm, 250 × 4.6 mm, Waters, Inc.) column with deionized water (pH adjusted to 2.3 with phosphoric acid) as mobile phase. The flow rate was 1.2 mL/min. Samples (0.5 mL) were diluted (1:1) with deionized water, centrifuged at 11,600*g* for 5 min, and an aliquot of 20 μL was injected into the chromatograph. Acetic acid was monitored at 220 nm.

Sugar Analysis. Twenty-five grams of olive flesh were mixed with 40–50 mL of boiling water and 5 mL of sorbitol internal standard (7.5%, w/v). The mixture was triturated with an Ultraturrax T25 (Jante & Kunkel GmbH), and the homogenized paste was centrifuged at 9000*g* for 5 min. The supernatant was filtered through paper filter and was made up to 125 mL with deionized water. The solution was kept at 5 °C for 24 h to remove lipids and was subsequently filtered through a 0.45-μm pore size nylon filter.

Two milliliters of the clarified liquid or 1.5 mL of brine plus 0.5 mL of sorbitol internal standard (0.5%, w/v) were placed into contact with 1 g of the acidic resin Amberlite IR-120 and 1 g of the basic resin Amberlite IRA-93. Samples were shaken occasionally during 30 min, and 0.5–1 mL of the solution was centrifuged at 11600*g* for 5 min. An aliquot (20 μL) was injected into the chromatograph. The HPLC system consisted in a Waters 2695 Alliance with a pump and autosampler included, the detection being performed with a Waters 410 refractive index detector. A Rezex RCM-Monosaccharide Ca^{+} (8%) column (300 × 7.8 mm i.d., Phenomenex) held at 85 °C and deionized water as eluent at 0.6 mL/min were used.

Analysis of the Phenolic and Oleosidic Compounds. A mixture of 250 μL of brine, 250 μL of internal standard (2 mM syringic acid), and 500 μL of deionized water was filtered through a 0.45-μm pore size nylon filter. An aliquot (20 μL) was injected into the chromatograph. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, a Waters column heater module, and a Waters 996 diode array detector (Waters Inc., Mildford, MA). A Spherisorb ODS-2 (5 μm, 25 × 4.6 mm i.d., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90% water (adjusted to pH 3.0 with phosphoric acid) and 10% methanol. The concentration of the latter solvent was increased to 30% over 10 min and was maintained for 20 min. Subsequently, the methanol percentage was raised to 40% over 10 min, was maintained for 5 min, and was then increased to 50%. Finally, the methanol percentage was increased to 60, 70, and 100% in 5-min periods. Initial conditions were reached in 15 min. A flow rate of 1 mL/min and a temperature of 35 °C were used in all experiments. The wavelengths selected for phenolic and oleosidic compounds were 280 and 240 nm, respectively. Tyrosol and *p*-coumaric acid were purchased from Sigma (St. Louis MO), and oleuropein was obtained from Extrasynthese (Genay, France). The rest of the substances were obtained by a semipreparative HPLC as described elsewhere (*24*). Comselogoside and caffeoyl ester of secologanoside were quantified using the response factors of *p*-coumaric and caffeic acids respectively. All oleosidic compounds were quantified using the response factor of Oleoside 11-methyl ester.

HPLC-MS Analysis. Phenolic and oleosidic extracts were analyzed by LC-MS using a quadrupole mass analyzer (ZMD4, Waters Inc.) equipped with an electrospray ionization (ESI) probe and working in the negative-ion mode. Cone voltage fragmentation was 10 V, capillary voltage was 3 kV, desolvation temperature was 200 °C, source temperature was 80 °C, and extractor voltage was 12 V. A constant flow of 1 mL/min was used for each analysis with a split ratio of approximately 5:1 (UV detector MS).

NMR Analysis. ^{1}H and ^{13}C NMR spectra at 300 and 75.4 MHz, respectively, were determined in a Bruker AC-300P (Karlsruhe, Germany). Two-dimensional NMR was used to assign the ^{13}C NMR spectra.

Isolation of Phenolic and Oleosidic Compounds. They were isolated from a Manzanilla brine of the aseptic experiments. The analytical column, mobile phases, gradient, and equipment were the same as those used for the polyphenol analysis except for the aqueous mobile phase, which was acidified with 2N HCl (522 μL/L). Fractions were collected peak-by-peak with a Waters Fraction Collector II. The pooled extract for each peak was evaporated under vacuum close to dryness, and the residue was dissolved in 1 mL of deionized water. Finally, the purity and concentration of each compound was measured by HPLC. A control run was also performed by injecting deionized water and collecting all fractions of the run.

Table 1. Growth or inhibition of lactic acid bacteria in Gordal and Manzanilla brines, respectively. Brines from olives stored aseptically for two months were inoculated with *L. pentosus* ATCC 8041 and *L. brevis* BP and were analyzed at 48 h from inoculation

	Gordal brine	Manzanilla brine
L. pentosus		
glucose consumption (mM)	18.0 (11.1)[a]	ND[b]
lactic acid formation (mM)	22.0 (17.5)	ND
log CFU/mL	8.3 (0.5)	<1.0
L. brevis		
glucose consumption (mM)	25.7 (6.6)	ND
lactic acid formation (mM)	16.7 (5.1)	ND
log CFU/mL	8.3 (0.5)	<1.0

[a] Standard deviation of four samples. [b] ND: not detected.

Table 2. Concentration of sugars (mM) in raw olives and brines of fruits aseptically prepared and stored for 2 months

	raw olives		2 months brines	
	Manzanilla	Gordal	Manzanilla	Gordal
glucose	244.7 (17.6)[a]	276.4 (15.3)	110.8 (5.5)	38.0 (1.0)
fructose	77.1 (10.5)	40.4 (1.6)	36.1 (5.0)	4.6 (0.2)
mannitol	59.9 (8.8)	83.4 (6.2)	37.7 (4.2)	25.9 (1.0)

[a] Standard deviation of four samples.

Oleoside 11-methyl ester was also isolated from a washwater of the Spanish-style green olive processing supplied by a local processor.

Antimicrobial Activity of Isolated Compounds. One hundred microliters of Gordal brine were inoculated with a diluted overnight culture of the target microorganism to get, after the addition of 50 μL of the isolated compound, a population between 5.1 and 6.2 log CFU/mL, except for yeasts where the population ranged between 3.6 and 3.7 log CFU/mL. The mixture was incubated at 32 °C for 48 h and was plated spreading both 50 μL of the stock and the 10^{-1} dilution (0.1% peptone water) with a Spiral Plater. The concentration limits for each compound were chosen by taking into account their presence in Manzanilla brines. In addition, oleuropein, hydroxytyrosol, and the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) were tested at several levels. A control test was performed with the control HPLC run extract obtained.

All experiments were done at the pH of the brines (4.0) except with *E. aerogenes* and *E. coli*, where it was increased up to pH 7.0 with a minimum added amount of 7 M NaOH.

Statistical Analysis. Results are expressed as mean values and standard deviations or error bars of duplicate samples. Data were subjected to statistical analysis by STATISTICA Software Package (Statistica for Windows, Tulsa, Oklahoma).

RESULTS AND DISCUSSION

Inoculation of Brines. Neither *L. pentosus* ATCC 8041 nor *L. brevis* BP was able to grow in any of the four Manzanilla brines tested (**Table 1**). In fact, survivors were not found despite initial inocula around 6 log CFU/mL. By contrast, both lactobacillus strains grew in Gordal olive brines, and consumption of glucose together with lactic acid formation took place. Overall, a higher amount of lactic acid was produced by *L. pentosus* than by *L. brevis*, which is in accordance with their different glucose fermentation pathways. A very low amount of fructose and mannitol was consumed, and the formation of acetic acid was not detected in any case (data not shown).

In short, it was confirmed that LAB grow more easily in Gordal than in Manzanilla brines (*6*). Many factors can limit the growth of LAB in olive brines such as temperature, NaCl concentration (*25*), polymers (*26*), nutrients, and others. Hence, the sugar content of the olive brines was analyzed (**Table 2**) and, surprisingly, their concentration was higher in Manzanilla than in Gordal brines. In fact, the level of sugars in raw olives was lower in Manzanilla than in Gordal fruits, which is in agreement with previous results (*27*). It seems, therefore, that the diffusion rate of these compounds was lower for the latter variety. There are no data on the diffusion rate of sugars from olives to the surrounding liquid because experiments are not run under aseptic conditions, but results on NaCl diffusion indicate that the skin of olives is the main barrier to compound exchange (*28*). Therefore, the evolution of the NaCl and acetic acid in the aseptic olive brines was also analyzed for two months, and it was confirmed that these two substances penetrate into the olive flesh at a higher rate for Manzanilla than for Gordal olives (data not shown). However, the concentration of these substances in the olive brines was almost similar for both olive varieties after two months of brining (2.8% NaCl and 0.27% acetic acid).

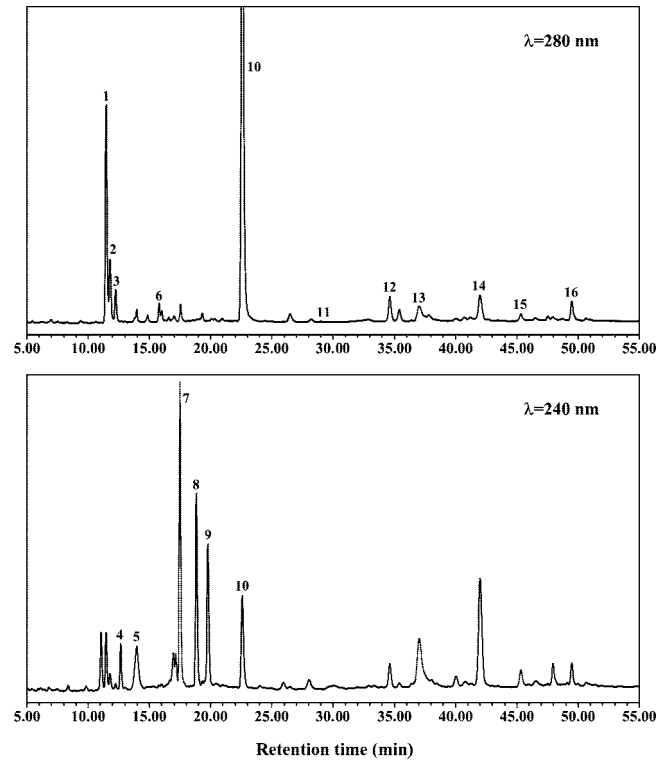


Figure 1. HPLC chromatograms of phenolic and oleosidic compounds in Manzanilla olive brine. Peaks: (1) hydroxytyrosol; (2) hydroxytyrosol 1-glucoside; (3) hydroxytyrosol 4-glucoside; (4) oleoside; (5) dialdehydic form of decarboxymethyl elenolic acid (EDA); (6) tyrosol; (7) secoxyloganin; (8) secologanoside; (9) oleoside 11-methyl ester; (10) internal standard (syringic acid); (11) *p*-coumaric acid; (12) verbascoside; (13) dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA); (14) oleuropein; (15) caffeoyl ester of secologanoside; and (16) comselogoside.

In consequence, sugars, salt, or acetic acid were not the direct factors that limited the growth of lactobacilli in the Manzanilla olive brines, and the presence of antimicrobial substances in these solutions again emerged as a possible cause (*4, 29*).

Analysis of Phenolic and Oleosidic Compounds. They were studied in the Manzanilla and Gordal brines because of previous reports on the antimicrobial activity of phenolic compounds in brines, in particular oleuropein and hydroxytyrosol (*7, 13*). **Figure 1** shows two HPLC chromatograms of these substances obtained at 280 and 240 nm. Among the phenolic compounds, hydroxytyrosol and tyrosol were found in all brines; these results were predictable because they have been detected in olive brines by many researchers (*14, 30, 31*). In contrast, the two phenolic

Table 3. Concentration of phenolic and oleosidic compounds (mM) in brines obtained from olives stored aseptically for two months

compound	Gordal brine	Manzanilla brine
hydroxytyrosol	1.68 (0.18)[a]	5.78 (1.42)
hydroxytyrosol 1-glucoside	0.31 (0.13)	3.59 (2.25)
hydroxytyrosol 4-glucoside	0.23 (0.05)	2.41 (1.34)
oleoside	0.02 (0.01)	0.27 (0.10)
EDA[b]	0.03 (0.01)	1.05 (0.66)
tyrosol	0.27 (0.06)	0.61 (0.16)
secoxyloganin	0.51 (0.29)	1.15 (0.65)
secologanoside	0.07 (0.01)	1.18 (0.42)
oleoside 11-methyl ester	0.18 (0.08)	1.33 (0.45)
p-coumaric acid	0.08 (0.01)	ND[c]
verbascoside	ND	0.01 (0.01)
HyEDA[d]	ND	1.33 (0.94)
oleuropein	ND	1.54 (1.00)
caffeoyl ester of secologanoside	0.01 (0.01)	0.02 (0.01)
comselogoside	0.01 (0.01)	0.05 (0.01)

[a] Standard deviation of four samples. [b] Dialdehydic form of decarboxymethyl elenolic acid. [c] Not detected. [d] Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol.

compounds recently identified in olive fruits, caffeoyl ester of secologanoside (*32*) and comselogoside (*33*), have never been described in table olive brines and were found in all aseptic brines. These substances were identified because of their characteristic UV and mass spectra.

Oleuropein and *p*-coumaric acid were found only in Manzanilla and Gordal brines, repectively (**Table 3**). Oleuropein is the main phenolic compound in the flesh of many varieties (*8*) and it has been found in Gordal olives (*34*), although in a lower amount than Manzanilla olives. The lack of oleuropein in Gordal brines could also be attributed to the diffusion of this substance from the olives to the surrounding brines and its rapid hydrolysis, as has been described for other olive varieties (*14, 30*).

The identification of hydroxytyrosol glucosides was achieved by analyzing ESI-MS and UV spectra. Three isomers of hydroxytyrosol glucoside have been reported in olives (*35*), and our laboratory has found hydroxytyrosol 4-glucoside in table olive brines (*36*). Thus, the compound eluting at 12.5 min was assigned to hydroxytyrosol 4-glucoside (**Figure 1**) by comparison with its spectroscopic characteristics and those of the standard. A peak between those of the hydroxytyrosol and hydroxytyrosol 4-glucoside was also detected with a molecular weight of 316 uma and an UV absorbance maximum at 276.6 nm. Because the UV absorption maximum of both *o*-diphenols hydroxytyrosol and hydroxytyrosol 4-glucoside was at 280.2 nm, we tentatively named the peak eluting after hydroxytyrosol as hydroxytyrosol 1-glucoside.

The dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) is one of the main phenolic compounds in olive oil (*37, 38*), and it has also been found in leaves (*39*), flesh (*40*) and olive oil mill wastewaters (*41*). This compound has been detected in brines of Italian table olives (*31*), and its presence in the Manzanilla and Gordal brines was confirmed on the basis of its ESI-MS spectrum. It is assumed by many researchers that HyEDA is formed via enzymatic degradation of oleuropein during the crushing of olives (*37*), although Australian researchers have also proposed HyEDA as an intermediate in the route of oleuropein biosythesis (*39*).

Oleosidic compounds were monitored at 240 nm (**Figure 1**) because all of them showed an absorption maximum close to this value (**Figure 2**). There were two peaks with absorption maximum at 238.8 nm and the ESI-MS spectra with a deprotonated moiety $[M - H]^-$ at *m*/*z* 403. Another two peaks had absorption maxima at 232.9 nm and a deprotonated moiety

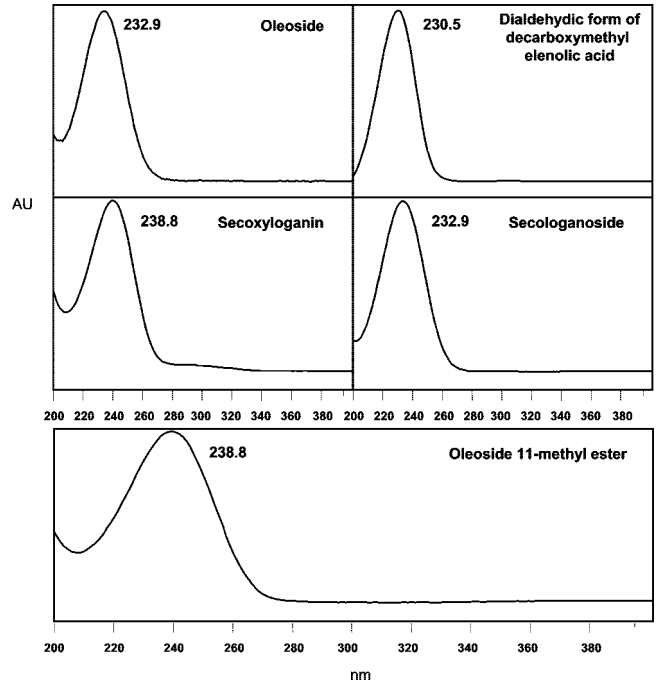


Figure 2. UV spectra of oleosidic compounds present in table olive brines.

at *m*/*z* 389. Oleoside 11-methyl ester has already been reported in olive brines (*15*), and it was also generated when oleuropein was hydrolyzed with 0.15 N NaOH for 1 h (data not shown). Thus, this compound was assigned to the peak eluting at 19.8 min. Furthermore, the treatment of oleuropein with 2N NaOH for 1 h gave rise to oleoside (a demethyl form of oleoside 11-methyl ester), which was confirmed by LC-MS, and this compound was assigned to peak eluting at 12.8 min. Additionally, peaks at retention times of 17.5 and 19 min were tentatively identified as secoxyloganin and secologanoside, respectively. Secoxyloganin exhibited a similar absorption maximum (238.8 nm) and molecular mass (404 uma) as that of oleoside 11-methyl ester, and the same situation occurred for secologanoside and oleoside. Indeed, these two couples are isomers. Oleoside 11-methyl ester and oleoside are probably formed from the oleuropein hydrolysis, and secoxyloganin and secologanoside are probably formed from the oleuroside hydrolysis. The latter compound is an isomer of oleuropein, which has been found in olive leaves and pulp (*39, 40*).

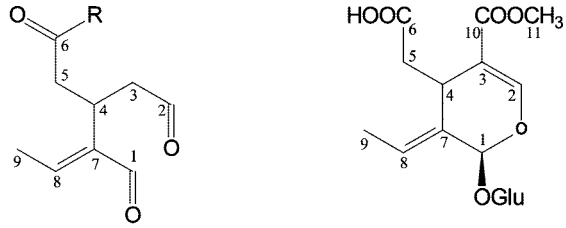
The compound that eluted at retention time of 14 min (peak 5) was only detected in the 240 nm chromatograms of the Manzanilla brines. This compound showed an absorption maximum at 230.5 nm and an intense pseudomolecular peak at *m*/*z* 183 in the ESI-MS spectrum. The ^{13}C NMR study (**Table 4**) revealed that resonances of carbons agreed with those reported by Montedoro et al. (*37*) for HyEDA, except for carbons 5 and 6 (**Figure 3**). Furthermore, the acid hydrolysis (2N HCL) of HyEDA at 100 °C for 5 min gave rise to two peaks, one corresponding to hydroxytyrosol and the other to peak 5 (data not shown). Taking all these data together, peak 5 was identified as the dialdehydic form of decarboxymethyl elenolic acid (EDA), never found before in table olives, although it has been tentatively detected in olive oils (*41*)

Once the identification of most of the phenolic and oleosidic compounds was achieved, the concentration of these substances in the olive brines of the Manzanilla and Gordal varieties was quantified (**Table 3**). Overall, most compounds were in higher concentration in Manzanilla than in Gordal brines, except for

Table 4. ^{13}C NMR data (ppm) for the dialdehydic form of decarboxymethyl elenolic acid (EDA) and isomers of Oleoside 11-methyl ester.

carbon	EDA	oleoside 11-methyl ester "a"[a]	oleoside 11-methyl ester "b"[a]
1	195.3	95.8	95.9
2	200.4	155.2	155.1
3	46.1	109.7	109.9
4	26.8	31.8	32.0
5	36.4	42.4	43.2
6	172.2	178.6	179.5
7	143.0	129.4	129.5
8	154.8	125.6	125.5
9	15.4	13.8	13.8
10		170.3	170.4
11		52.8	52.8

[a] Isomer "a" was isolated from a washwater of the Spanish-style green olive processing, and isomer "b" was isolated from a Manzanilla brine that was not treated with NaOH.



Dialdehydic form of decarboxymethyl elenolic acid (R=OH)
Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (R=hydroxytyrosol)

Oleoside 11-methyl ester

Figure 3. Structures of the main antimicrobial compounds in table olives.

p-coumaric acid. Additionally, verbascoside, HyEDA, and oleuropein were not detected in the Gordal brines, and the concentration of EDA in these brines was very low in comparison with that observed in the Manzanilla brines. Hydroxytyrosol and its glucosides were the most concentrated phenolic compounds in the latter brines, and the oleosidic substances showed a rather similar concentration.

Identification of LAB Growth Inhibitors. As commented above, none of the four Manzanilla brines allowed *lactobacillus* growth after inoculation. One of these brines, with the lower concentration in phenolic and oleosidic compounds, was chosen to compare each single compound with that of the Gordal brine used as growth medium (**Figure 4**). The spiked amount of each compound in the Gordal brine can also be observed to reach a concentration similar to the Manzanilla brine, except for secoxyloganin, which was studied at a concentration higher than that found in the Manzanilla brine. Verbascoside, *p*-coumaric acid, comselogoside, and caffeoyl ester of secologanoside were not tested because of their very low concentrations in all olive brines.

The Gordal brine without spiked compounds was inoculated and, as expected, *L. pentosus* ATCC 8041 grew (**Figure 5**); viable counts were higher than 10^8 CFU/mL after 48 h of inoculation. A similar growth was observed when this brine was spiked with hydroxytyrosol and its glucosides, oleoside, tyrosol, secoxyloganin, secologanoside, and oleuropein. Surprisingly, neither hydroxytyrosol nor oleuropein were active against this strain. Indeed, oleuropein was even tested at a concentration as high as 8 mM and did not show any inhibitory activity (data not shown). It is well-known that the inhibition of LAB growth

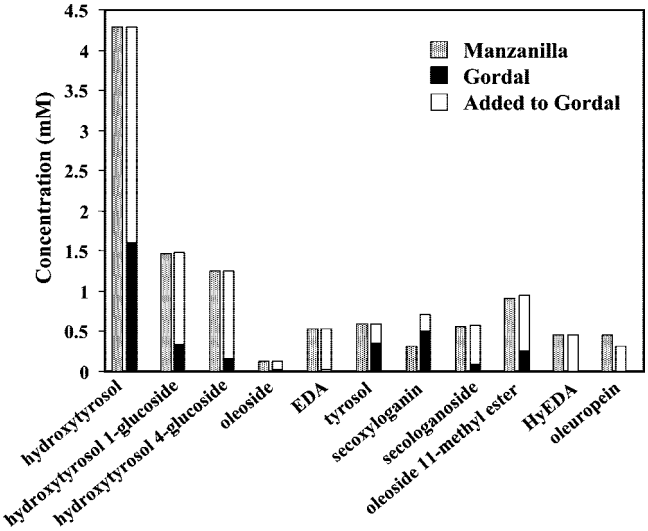


Figure 4. Concentration (mM) of phenolic and oleosidic compounds in Manzanilla and Gordal brines used to test the antimicrobial activity of isolated compounds, which were added to the Gordal brine to reach the concentration of the Manzanilla brine.

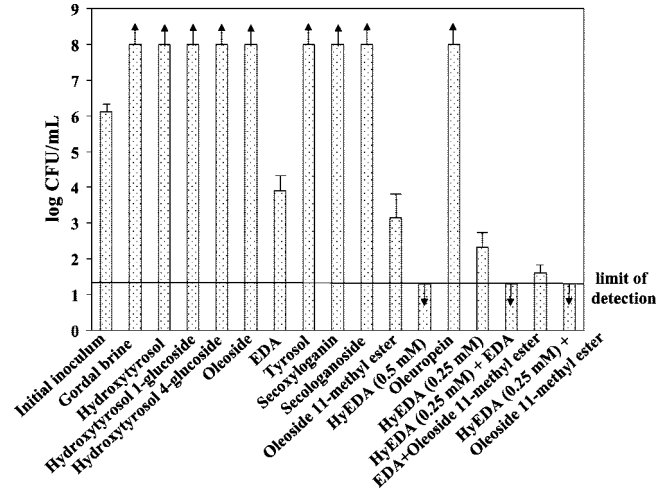


Figure 5. Effect of isolated phenolic and oleosidic compounds on the viability of *L. pentosus* ATCC 8041 in the Gordal brine spiked at the concentration showed in **Figure 4**. Bars indicate the standard errors of the mean. Arrows ↓ mean below the detection limit (1.3 Log CFU/mL). Arrows ↑ mean above 8 log CFU/mL.

depends on the inhibitor concentration along with many other factors (*13*, *20*), but oleuropein and hydroxytyrosol were totally ineffective against *L. pentosus*, and their presence in Manzanilla olive brines cannot explain the inhibition of lactic acid fermentation.

By contrast, both the dialdehydic form of decarboxymethyl elenolic acid (EDA) and oleoside 11-methyl ester inhibited the growth of *L. pentosus* and decreased the viable counts of the initial inoculum more than 2 log CFU/mL. To our knowledge, the antimicrobial activity of EDA has never been tested before, although this bioactivity could be predictable on the basis of its dialdehydic structure (**Figure 3**), which is similar to that of the antiseptic glutaraldehyde (*42*). Likewise, it was surprising to find that oleoside 11-methyl ester at a concentration of 1 mM inhibited the growth of *L. pentosus* because this compound is found in a very high concentration in the washwaters of the Spanish-style green olive processing, and LAB can grow in this medium (*43*). To clarify these contradictory results, oleoside 11-methyl ester was isolated from a Manzanilla washwater,

spiked into the Gordal brine up to a final 1 mM concentration, and tested for activity. In this case, the growth of *L. pentosus* was observed and, therefore, this oleoside 11-methyl ester was ineffective against this microorganism. Then, it was confirmed that both oleoside 11-methyl esters isolated from washwater or Manzanilla brines had similar UV and ESI-mass spectra. Additionally, both compounds exhibited identical ^{1}H NMR spectra and very similar ^{13}C NMR spectra (**Table 4**), which were consistent with the proposed structure (**Figure 3**) (*44*). The only significant differences were observed in the chemical shifts of C5 and C6. Thus, C5 appeared at δ 42.4 for isomer "a" and at 43.2 for isomer "b". In addition, C6 appeared at δ 178.6 for isomer "a" and at 179.5 for isomer "b". These differences might be tentatively suggested to be a consequence of a different configuration of C4. Because C4 is a chiral carbon, isomers "a" and "b" may be diastereomers. Further studies are needed to confirm this hypothesis as well as to determine the absolute configurations of both isomers. Hence, the diastereomer "b" found in the Manzanilla brines from olives not treated with NaOH possesses antimicrobial activity, but diasteromer "a" isolated from the washwaters of the Spanish-style green olive processing does not. It is not surprising that the stereospecific structure determines the antimicrobial activity, as it has been reported for catechins (*45*).

The most potent antimicrobial compound in the Manzanilla brines was, however, the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA), and viable counts of *L. pentosus* were under the limit of detection in the Gordal brine spiked up to 0.5 mM of HyEDA. This compound has a dialdehydic structure similar to that of EDA (**Figure 3**) and glutaraldehyde but was more effective than EDA against *L. pentosus*; both were tested at the same concentration (**Figure 5**). Therefore, the dialdehydic structure is important not only for the antimicrobial activity but for the rest of the moiety too. In fact, studies carried out with compounds isolated from olive oil indicated that the presence of tyrosol in the moiety instead of hydroxytyrosol increased the bioactivity to a large extent (*22*).

Considering all the results together, it seems that a concentration as low as 0.5 mM of HyEDA can itself explain the inhibition of lactobacillus growth in the Manzanilla brine. It must be stressed that this was the lowest concentration found in all Manzanilla brines, but it could reach up to 2.7 mM. However, an additive effect was also observed by the three antimicrobial compounds identified (**Figure 5**), and depending on their concentration, a synergistic effect of all inhibitors must not be ruled out to explain LAB growth inhibition in olive brines.

Finally, the antimicrobial activity of oleuropein, hydroxytyrosol, and HyEDA against several microorganisms found in table olives was studied (**Figure 6**). It must be noted that the concentration of HyEDA was around 10 times lower than that of the other two substances. As expected from a previous work (*22*), all compounds were ineffective against yeasts, which is also predictable because they are the main microorganisms involved in the fermentation of olives that are not treated with NaOH (*46*, *47*).

Furthermore, oleuropein was ineffective against most microorganisms, and hydroxytyrosol allowed for the growth of the *L. plantarum* and *L. pentosus* strains used. Nevertheless, hydroxytyrosol at a 5.2 mM concentration inhibited the growth of *L. mesenteroides* and *E. faecium* and significantly reduced the viable counts of the initial inoculum of *E. faecalis*, *E. aerogenes*, and *E. coli*. It could be deduced that hydroxytyrosol exerted a stronger antibacterial activity against Gram-negative than Gram-positive bacteria, although this hypothesis must be confirmed.

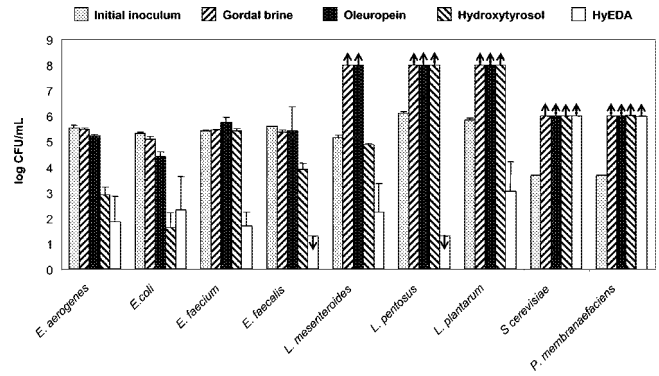


Figure 6. Effect of oleuropein (5.8 mM), hydroxytyrosol (5.2 mM), and HyEDA (0.5 mM) on the viability of several microorganisms inoculated in a Gordal brine. Bars indicate the standard errors of the mean. Arrows ↓ mean below the detection limit (1.3 Log CFU/mL). Arrows ↑ mean above 8 or 6 Log CFU/mL for bacteria and yeast, respectively.

It was again confirmed that HyEDA exerted the highest activity against all bacteria tested. A reduction of 2.5–3.5 log CFU/mL was observed for most bacteria, and *E. faecalis* and *L. pentosus* counts were even below the detection limit. It is important to keep in mind that HyEDA was tested at a concentration as low as 0.5 mM.

In summary, it has been demonstrated in this study that the main antimicrobial compounds in table olive brines are the dialdehydic form of decarboxymethyl elenolic acid (EDA), EDA linked to hydroxytyrosol (HyEDA) and an isomer of oleoside 11-methyl ester, and the traditional inhibitors (oleuropein, its aglycon, hydroxytyrosol, and elenolic acid) (*7*, *9*–*13*) were ineffective or not found in olive brines. In view of these findings, table olive brines could be a good source of natural antimicrobials.

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