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Contribution of Malolactic Fermentation by *Oenococcus Oeni* and *Lactobacillus Plantarum* to the Changes in the Nonanthocyanin Polyphenolic Composition of Red Wine

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The changes in the nonanthocyanin phenolic composition during red wine malolactic fermentation carried out spontaneously and by four different starter cultures of the species *Oenococcus oeni* and *Lactobacillus plantarum* were examined to determine whether differences in nonanthocyanin polyphenolic compounds could be attributed to the lactic acid bacteria (LAB) strain that performs this important step of the wine-making process. The polyphenolic compounds were analyzed by high-performance liquid chromatography with photodiode array detection and HPLC with electrospray ionization−mass spectrometry detection. The malolactic cultures selected for this study were indigenous wine LAB strains from the A.O.C. Rioja (Spain). Results showed different malolactic behaviors in relation to wine phenolic compositions for *O. oeni* and *L. plantarum*, and also, a diversity was found within each group. The hydroxycinnamic acids and their derivatives, the flavonols and their glycosides, the flavanol monomers and oligomers, and *trans*-resveratrol and its glucoside were the main compounds modified by the different LAB. The wild LAB population exerted a greater impact in the wine content of some of these phenolic compounds than the inoculated selected monocultures of this study.

KEYWORDS: Wine; LAB; malolactic fermentation; *Oenococcus oeni*; *Lactobacillus plantarum*; nonanthocyanin polyphenolic compounds; HPLC-PAD; HPLC-(ESI)MS

INTRODUCTION

Lactic acid bacteria (LAB) are important in enology since they carry out malolactic fermentation (MLF), which has the main effect of reducing wine acidity and is almost indispensable in red wine making (*1*, *2*). Different genera of LAB including *Lactobacillus*, *Pediococcus*, and *Oenococcus* are involved in the MLF of wine. Among them, *Oenococcus oeni* is recognized as the most tolerant to wine conditions (low pH and high ethanol conditions) and is the major bacteria species found in wines during MLF (*3*, *4*). It has been demonstrated that *Lactobacillus plantarum* strains have resistance mechanisms that enable them to survive and proliferate in wine (*5*), although this species seems to be less efficient than oenococci at inducing MLF (*6*), and so far, it has not been commercialized as a MLF starter for wine making. Some significant metabolic differences have recently been reported for *O. oeni* and *L. plantarum* in modification of the amino acid and volatile composition of wines, indicating that they can make different contributions to the wine's final organoleptic properties (*6*).

Phenolic compounds are found in plant tissues, and their study in food in general and especially in wines is of great interest (*7*, *8*). These compounds are directly related to the quality of wines. They contribute to the wine's organoleptic characteristics such as its color, astringency, and bitterness (*9*). Moreover, the amount and types of phenolic compounds present in wines may play an important role in controlling oxidation in the human body. Phenolic compounds, primarily flavanols, have antioxidant properties, with mechanisms involving both free radical scavenging and metal quelation (*10*), which may be the source of putative health benefits derived from wine consumption. Wines contain a wide range of polyphenolic constituents that have reported anticancer and anti-inflammatory effects in vitro, as well as the ability to block cellular events predisposing one to atherosclerosis and coronary heart disease (*11*, *12*).

Many factors can influence the phenolic composition of wines, including grape variety (*13*), the technology applied in their manufacture (*14*), and the reactions that take place during aging in wood (*14*, *15*). It has also been reported that MLF

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affects the phenolic composition of wine, reducing the contents of anthocyanins and total polyphenols (*16*).

Most studies on the interaction of phenolic compounds and LAB in wines suggest that some phenolic compounds, depending on their structure and concentration, can affect the growth and metabolism of LAB (*17*, *18*). However, only limited data have been reported about LAB activity on phenolic compounds during wine MLF and these refer to the metabolism of hydroxycinnamic acids (ferulic and coumaric acids), by different bacteria species, resulting in the formation of volatile phenols (4-ethylguaiacol and 4-ethylphenol) (*19*). The metabolism of other phenolic compounds such as gallic acid and catechin by *L. plantarum* has also been studied in synthetic media or model solutions (*20*). There is recent evidence that caffeoyl tartaric acid (cafftaric acid) and coumaroyl tartaric acid (coutaric acid) could be changed in the corresponding free hydroxynnamic acids (caffeic acid and coumaric acid) during MLF of a wine (*14*).

The main purpose of this work was to study the effect of LAB on the nonanthocyanin polyphenolic composition during MLF carried out spontaneously and with four different starter cultures of the species *O. oeni* and *L. plantarum* in industrial red wine manufacture to evaluate whether the metabolic activity of these bacteria can alter wine phenolic composition and to determine whether differences can be attributed to the LAB strain used in this important step of the wine-making process.

MATERIALS AND METHODS

MLF and LAB. Red wine was elaborated from cv. Tempranillo red grapes from local vineyards of the northern Spanish region of La Rioja. Fermentations were carried out in 15000 L wooden tanks at 20 °C with the indigenous *Saccharomyces cerevisiae* yeast strains. The wine was not treated with commercial enzymes. After alcoholic fermentation, wine was drawn off from the yeast lees and was filtered through diatomaceous earth and a 0.45 μm filtering cartridge to eliminate the endogenous microbiota. The wine turbidity was measured, and the value obtained after filtrations was 4 NTU. The bacterial count in this starting wine was 0 CFU/mL. This wine was designated "initial wine" for the experimental design of this work.

Two *O. oeni* strains, Oe-18 and Oe-159, and two *L. plantarum* strains, Lp-39 and Lp-51, isolated from red wines of the Rioja Appellation of Origin, which never before had been used as wine starters, were selected for MLF. The cultures were maintained in 20% sterile skim milk (Difco, Madrid, Spain) at −80 °C. Prior to wine making, the *L. plantarum* cultures were grown in MRS broth (Scharlau Chemie S.A., Barcelona, Spain) and *O. oeni* cultures were grown in MLO broth (Scharlau Chemie S.A.) at 25 °C with continuous shaking to obtain the appropriate biomass (4 10^7 cfu/mL) and were adapted to wine conditions performing the following steps: 250 mL of a sterile solution of 0.4 g/L of MLF activator Opti'malo (Lallemand Inc., Toulouse, France) was mixed with 250 mL of filtered wine, and bacterial samples were incubated in this medium (50% wine) for 2−7 days until the L-malic acid concentration decreased 90%. The resulting 500 mL samples were added to 2 L of the filtered wine and kept for 5−10 days at 20 °C until L-malic acid concentration decreased 60%. These "pieds de cuve" were added to stainless steel tanks and filled with 25 L of filtered wine. Experiments were carried out in duplicate, and control samples were prepared with 1 L of wine lees obtained after alcoholic fermentation. These lees were submitted to the same process of habituation and dilution in filtered wine as described for *L. plantarum* and *O. oeni* strains. Therefore, 10 tanks, containing 25 L of wine in each one, underwent MLF: Two control samples were inoculated with wine lees, four were inoculated with *L. plantarum* strains, either Lp-39 or Lp-51, and four were inoculated with *O. oeni* strains, either Oe-18 or Oe-159. MLF was followed by measuring wine the L-malic acid content using the L-malic acid Enzymatic BioAnalysis (Boehringer-Mannheim/R-Biopharm, Darmstadt, Germany). When MLF had finished (L-malic acid concentration < 0.02 g/L), wines were sulfited and samples were taken for polyphenolic compound analysis. Ten milliliter wine samples were taken at time intervals during the MLF and analyzed for bacterial counts and identification. LAB were enumerated by appropriate serial dilutions of wine samples and growth onto fresh plates of MRS agar (Scharlau Chimie S. A.) with 200 μg of nystatin/mL (Acofarma, S. Coop, Terrassa, Spain). Samples were incubated at 30 °C under strict anaerobic conditions (GasPak, Oxoid Ltd., Basingstoke, England) for at least 5 days, and viable counts were obtained as the number of cfu/mL. Five colonies from each wine sample were selected for reisolation and identification. Strains were identified by their morphology, Gram staining, and species−species polymerase chain reaction analysis for *O. oeni* (*21*) and *L. plantarum* (*22*). Clonal characterization of strains was carried out by pulsed field gel electrophoresis (PFGE).

All MLF experiments were carried out in duplicate in independent 25 L stainless steel tanks; therefore, a total of 11 wines were analyzed (10 wines after MLF plus one sample of the initial wine without MLF). Results reported here are the average values of two independent experiments.

Extraction of Phenolic Compounds. Samples of 50 mL of wine were extracted three times with 3 × 30 mL of diethyl ether and 3 × 30 mL of ethyl acetate. The organic fractions were combined and evaporated to dryness in vacuum at 30 °C. The residue was dissolved in 2 mL of MeOH/H_2O (1:1), then filtered (0.45 μm), and analyzed by high-performance liquid chromatography (HPLC) with photodiode array detection (PAD) and mass spectrometry (MS).

HPLC-PAD Analysis. The chromatographic system was equipped with an autoinjector, a quaternary pump, a photodiode array detector 2001 (Waters, Milford, MA), and a column Nova-Pak C_{18} (300 mm × 3.9 mm, 4 μm). The analytical conditions were based on those described by Dueñas et al. (*23*) with some modifications. Two mobile phases were employed for elution: A, water/acetic acid (98:2 v/v), and B, water/acetonitrile/acetic acid (78:20:2 v/v/v). The gradient profile was 0−55 min, 100%-20% A; 55−70 min, 20%-10% A; 70−80 min, 10%-5% A; and 80−100 min, 100% B. The flow rate was 1 mL/min from the beginning to 55 min and 1.2 mL/min from this point to the end. The column was reequilibrated between injections with 10 mL of acetonitrile and 25 mL of the initial mobile phase. Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1 s. A volume of 25 μL was injected. The samples were analyzed in duplicate.

HPLC-MS Analysis. Mass spectra were obtained using a Hewlet Packard 1100MSD (Palo Alto, CA) chromatograph equipped with an atmospheric pressure ionization source, using an electrospray ionization (ESI) interface and the conditions reported by Dueñas et al. (*23*). The solvent gradient and column used were the same as for HPLC-PAD but with a flow rate of 0.7 mL/min. ESI conditions were as follows: negative mode, nitrogen was used as the nebulizing pressure at 40 psi; drying gas, 10 L/min at 340 °C; voltage at capillary entrance, 4000 V; and variable fragmentation voltage, 100 (m/z < 200), 200 (m/z 200−1000), and 250 V (m/z 1000−2500). Mass spectra were recorded from m/z 100 to m/z 2500.

Identification and Quantification of the Compounds. Chromatographic peaks were identified by comparing the retention times, UV spectra, and data of UV spectral parameters (*24*, *25*) with those of standards and confirmed by HPLC-MS(ESI). The standards, gallic, protocatechuic, vanillic, syringic, ellagic, *trans*-*p*-coumaric, *trans*-caffeic, and *trans*-ferulic acids, *trans*-resveratrol, methylgallate, ethylgallate, tyrosol, dihydroquercetin, quercetin 3-*O*-glucoside, myricetin 3-*O*-galactoside, myricetin, quercetin, (+)-catechin, and (−)-epicatechin, were from Extrasynthese (France). The trytophol was from Aldrich (Germany). *cis*-Resveratrol was obtained from the standard of *trans*-resveratrol after exposure to UV light (340 nm) for 1 h. Other compounds with the same shape and maxima wavelength of UV spectra as that of hydroxycinnamic, proanthocyanidins, *cis*- and *trans*-resveratrol, and dihydroquercetin for which no standards were available, were identified by HPLC-PAD as derivatives of these compounds, based on the study of data of UV spectral parameters and confirmed by HPLC-MS (ESI).

Quantification was done using the external standard method with commercial standards. The calibration curves were made by injecting different volumes from the stock solutions (0.25 μg/mL for phenolic

Table 1. Implantation of *O. oeni* Strains in the Inoculated Wines Determined by PFGE[a]

inoculate strain	wine tank	MLF stage	appearance (%)[a]
Oe-18	1	initial[b]	100
Oe-18	2	initial[b]	100
Oe-18	1	full[c]	80
Oe-18	2	full[c]	100
Oe-139	3	initial[b]	100
Oe-139	4	initial[b]	80
Oe-139	3	full[c]	100
Oe-139	4	full[c]	100

[a] Appearance = number of isolates that presented the inoculated strain PFGE pattern × 100/total number of isolates per sample. [b] Initial: MLF stage when the L-malic acid concentration is 90% of its initial concentration. [c] Full: MLF stage when the L-malic acid concentration is less than 40% of its initial concentration.

acids and 0.10 μg/mL for flavonoids) over the range of concentrations observed for each compound, using a linear regression to relate the area sum vs concentration, under the same conditions as for the samples analyzed. The hydroxycinnamic acid derivatives were quantified using the calibration curves of the corresponding free acid. Quercetin 3-*O*-glucuronide was quantified by the curve of the quercetin 3-*O*-glucoside. Resveratrol glucosides were quantified with the calibration curve of *trans*-resveratrol. Procyanidins and prodelphinidin were quantified as (+)-catechin. The dihydroquercetin derivative was quantified using the curve of dihydroquercetin.

RESULTS AND DISCUSSION

MLF. The wines with spontaneous MLF, which carried out MLF with wine lees, finished their fermentation in 9 days. Inoculated wines required between 18 and 33 days to complete MLF. The volume of added lees contained a biomass (10^5 cfu/mL) of indigenous LAB 10-fold higher than that of the selected cultures (10^4 cfu/mL), and this accounted for the shorter MLF of control wines. Before MLF, the initial wine had an alcohol degree of 12.4%, 1.3 g/L of malic acid, 0.4 g acetic acid/L of volatile acidity, 5.8 g tartaric acid/L total acidity, and a pH of 3.4. After MLF, pH values increased (0.01−0.09 units) as did volatile acidity (0.05−0.32 g/L), whereas, as expected, the total acidity decreased (0.46−0.91 g/L). No major differences were observed in these three parameters among the different wines, and all of the wines were within the normal range for good quality wines. In all of the cases, residual malic acid was below the detection limit of the method, attesting completion of MLF. Microbiological identification of isolates at initial (90% of L-malic acid initial concentration) and full MLF (less than 40% of L-malic acid initial concentration) revealed that the inoculated strains were responsible for MLF. In the case of *L. plantarum* strains, 79% of the studied isolates were *L. plantarum* species at initial and full MLF. In the case of *O. oeni* strains, PFGE was necessary to differentiate among indigenous strains and the inoculated *O. oeni* strains Oe-18 and Oe-159, and **Table 1** shows the resulting implantation percentages of the inoculated *O. oeni* strains. The wine that carried out MLF with the indigenous microbiota (wine lees) showed a mixed population fully constituted by the indigenous *O. oeni* strains (100%).

Phenolic Compounds Identified in the Wines. The HPLC chromatogram of polyphenolic compounds shown in **Figure 1** corresponds to the spontaneous MLF wine (MLFs). Over 39 phenolic compounds were identified in all samples taken, including nonflavonoid polyphenolic compounds, hydroxybenzoic and hydroxycinnamic acids and derivatives, alcohols and stilbenes, and the flavonoids, flavonols, flavanols, and dihydroflavonols. **Table 2** presents the wavelength of maximum UV absorption and the molecular ions of identified compounds from HPLC-MS obtained for all of the wines analyzed. Peak numbers correspond to that of the chromatogram. In some batches, several of the above-mentioned phenolic compounds were detected at very low levels.

Identified were the following compounds: the hydroxybenzoic acids gallic (peak 1), protocatechuic (peak 3), vanillic (peak 13), syringic (peak 17), and ellagic (peak 33); the hydroxycinnamic acids in free form, as *trans*-caffeic (peak 14), *trans*- and *cis*-*p*-coumaric (peaks 23 and 24), and *trans*-ferulic (peak 27); flavanols, (+)-catechin (peak 12) and (−)-epicatechin (peak 20); flavonols, myricetin and quercetin (peaks 36 and 39, respectively); dihydroflavonol as dihydroquercetin (peak 29); stilbenes, *trans*- (peak 37) and *cis*-reveratrol (peak 38); and alcohols, tyrosol (peak 10) and tryptofol (peak 32). All of these compounds were identified by comparison of retention times and UV spectra with those of standards and confirmed by HPLC/MS (ESI) analysis (**Table 2**).

Moreover, other compounds for which no standards were available were also identified (**Table 2**). In addition to the free hydroxycinnamic acids, some esterified hydroxycinnamic acids have been identified. Peaks 2 and 6 showed a UV spectrum similar to that of *cis*- and *trans*-caffeic acids, presenting a

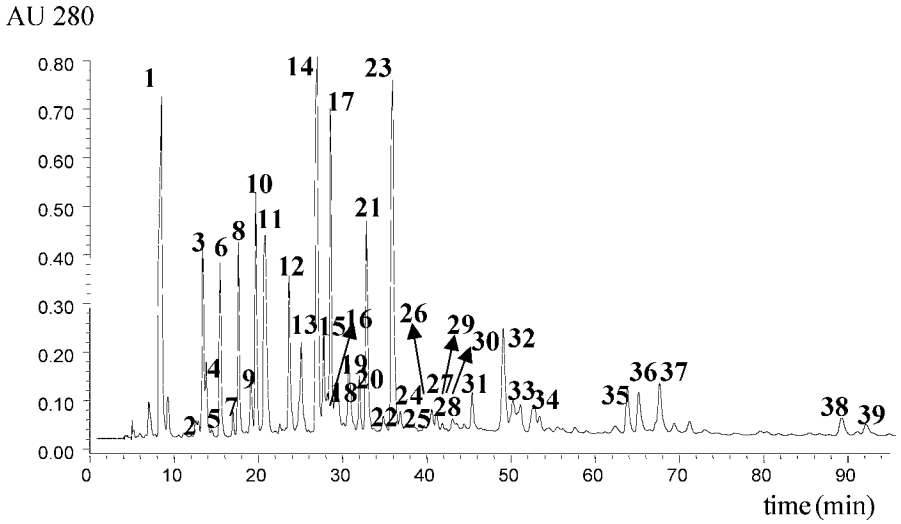

Figure 1. Chromatographic profile of the phenolic compounds determined in the spontaneous MLF wine (MLFs). The retention times of compounds not detected in this wine are also indicated in the chromatogram. For peak identification, see **Table 1**.

Table 2. Phenolic Compounds Identified by HPLC-PAD-MS in the Analyzed Wines[a]

peak	compounds	λ max (nm)	[M − H]⁻	fragments
	hydroxybenzoic acids and derivatives			
1	gallic	271.7	169.1	
3	protocatechuic	259.9/294.2	153.0	
8	methyl gallate	272.9	182.9	
13	vanillic	261.1/293.0	167.1	
17	syringic	274.6	197.1	
21	ethyl gallate	272.9	197.1	
33	ellagic	256.2/367	301.1	
	hydroxycinnamic acids and derivatives			
2	*cis*-cafftaric	310.9	311.1	149.0/179.1
6	*trans*-cafftaric	325.9	311.1	149.0/179.1
9	*cis*-coutaric	310.8	295.0	149.0/163.1
11	*trans*-coutaric	313.2	295.0	149.0/163.1
14	*trans*-caffeic	322.7	179.2	
15	*trans*-*p*-coumaric hexose	312.0	325.1	162.0/163.1
19	*trans*-*p*-coumaric hexose	312.0	325.1	162.0/163.1
23	*trans*-*p*-coumaric	314.3	163.1	
24	*cis*-*p*-coumaric	295.4	163.1	
27	*trans*-ferulic acid	322.7	193.1	
	stilbenes			
31	*trans*-resveratrol glucoside	319.2	389.1	227.1
35	*cis*-resveratrol glucoside	286.7	389.1	227.1
37	*trans*-resveratrol	306.1	227.1	
38	*cis*-resveratrol	284.7	227.1	
	alcohols			
10	tyrosol	276.4	137.1	
32	tryptophol	280.0	160.1	
	flavanols			
4	procyanidin trimer	278.8	865.1	289.1
5	prodelphinidin dimer	276.4	593.0	
7	procyanidin dimer	278.8	577.1	289.1
12	(+)-catechin	278.8	289.1	
16	procyanidin trimer	278.8	865.1	577.1/289.1
20	(–)-epicatechin	278.8	289.1	
22	procyanidin trimer	278.8	865.1	577.1/289.1
25	procyanidin dimer	278.8	577.1	289.1
26	procyanidin dimer	278.8	577.1	289.1
30	procyanidin dimer	278.8	577.1	289.1
	flavonols			
18	dihydroquercetin derivative	283.1	465.1	303.0
28	myricetin 3-*O*-galactoside	261.1/354.9	479.1	317.1
29	dihydroquercetin	289.1	303.1	
34	quercetin 3-*O*-glucuronide	256.3/353.7	477.1	176.1/301.0
36	myricetin	252.8/372.1	317.0	
39	quercetin	255.2/369.0	301.0	

[a] The numbers of compounds correspond to those of the peak chromatogram.

negative molecular ion $[M - H]^-$ at *m*/*z* 311.1 and two fragment ions, $[M - H]^-$ at *m*/*z* 179.1, which corresponded to the caffeic acid, and $[M - H]^-$ at *m*/*z* 149.0 from the tartaric acid. These compounds were identified as *cis*- and *trans*-caffeoyl tartaric esters or *cis*- and *trans*-caftaric acids.

Peaks 9 and 11 with UV spectra similar to *cis*- and *trans*-*p*-coumaric acids, respectively, presented a $[M - H]^-$ at *m*/*z* 295.0 and two fragments ions, $[M - H]^-$ at *m*/*z* 163.1 corresponding to the *p*-coumaric acid and $[M - H]^-$ at *m*/*z* 149.0 corresponding to the tartaric acid. Peaks 9 and 11 were identified as *trans*- and *cis*-*p*-coumaroyltartaric acid or *trans*- and *cis*-coutaric acids.

Peaks 15 and 19 have UV spectra similar to *trans*-*p*-coumaric acids and presented a $[M - H]^-$ at *m*/*z* 325.1 and two fragments ions, $[M - H]^-$ at *m*/*z* 163.1 corresponding to the *p*-coumaric acid and $[M - H]^-$ at *m*/*z* 162.1, which corresponds to a hexose. These compounds were identified as *trans*-*p*-coumaric hexoses.

Peak 31 had a UV spectrum similar to *trans*-resveratrol, which presented a molecular ion $[M - H]^-$ at *m*/*z* 389.1 corresponding to resveratrol glucoside and a fragment $[M - H]^-$ at *m*/*z* 227.1, which corresponded to free resveratrol. *cis*-Resveratrol (peak 38) was identified in the same way (**Table 2**).

Peaks 7, 22, 25, 26, and 30 were identified as procyanidin dimers (**Table 2**) because of their maximal UV spectrum (278.1) and the analysis of HPLC-MS(ESI) in which they presented a molecular ion $[M - H]^-$ at *m*/*z* 577.1, corresponding to a dimer of procyanidin. Peaks 4, 16, and 22, with maximal UV spectrum of 278.1 nm, presented a molecular ion $[M - H]^-$ at *m*/*z* 865.1, corresponding to a trimer of procyanidin. Peak 5 had a maximal UV spectrum of 276.4, corresponding to prodelphinidin and a molecular ion $[M - H]^-$ at *m*/*z* 593.1, and was identified as a prodelphinidin dimer (gallocatechin-catechin).

The flavonols identified as myricetin 3-*O*-galactoside (peak 28) and quercetin 3-*O*-glucuronide (peak 34) have a λ_{max} corresponding to derivatives of myricetin and quercetin, respectively. Peak 28 presents a molecular ion $[M - H]^-$ at *m*/*z* 479.1 from myricetin 3-*O*-galactoside and two fragments $[M - H]^-$ at *m*/*z* 317.1 from myricetin and $[M - H]^-$ at *m*/*z* 162.0 from galactoside. Peak 34 presents a molecular ion $[M - H]^-$ at *m*/*z* 477.1 and two fragments $[M - H]^-$ at *m*/*z* 301.1 from quercetin and $[M - H]^-$ at *m*/*z* 176.0 from glucuronide acid.

Peak 18 shows a UV spectrum with a maximum at 291.1 similar to that of a dihydroflavonols and presented a molecular ion $[M - H]^-$ at *m*/*z* 427.1 and a fragment $[M - H]^-$ at *m*/*z* 303.1 corresponding to the dihydroquercetin. This peak was identified as a dihydroquercetin derivative.

Changes in Phenolic Compounds during MLF. Mean levels of the phenolic compounds in the wines studied are reported in **Table 3**. In the initial wine, after alcoholic fermentation, the most abundant compounds correspond to gallic, protocatechuic, syringic, *trans*-coutaric, and *trans*-cafftaric acids, the flavanols (+)-catechin and (−)-epicatechin, the flavonols quercetin glucuronide, myricetin, and quercetin, and the phenolic alcohol tyrosol. In the wines obtained after MLF, the same compounds as those identified in the initial wine were found. However, MLF also gave rise to some new phenolic compounds not detected in the initial wine, such as the hydroxycinnamic acid, *trans*-ferulic, and four flavanols, three of them identified as procyanidin dimers (peaks 4, 25, and 26) and a prodelphinidin dimer (peak 5) (**Table 3**). Moreover, other compounds were more abundant in the wines after MLF than in the initial wine, among them, the flavanols catechin and epicatechin, and the alcohols tyrosol and tryptophol, two compounds generally produced as a consequence of fermentation processes (*26*).

In the case of spontaneous MLF (**Table 3**), the most marked changes observed were for the hydroxycinnamic acids and their derivatives. Caffeoyl- and *p*-coumaroyl tartaric esters (i.e., cafftaric and coutaric acids) are the most abundant cinnamate esters in grape juices (*27*, *28*). These phenolic acids can be released as free acids mainly by certain cinnamoyl esterase activities from commercial enzyme preparations (*29*, *30*). It has been reported that during MLF, LAB were able to hydrolyze hydroxycinnamic esters, *trans*-cafftaric and *trans*-coutaric acids, increasing the corresponding free forms (*14*). These preliminary findings were obtained from only one wine-making process carried out under industrial conditions, in which commercial pectinases are usually added. Because these commercial enzyme preparations may have potential cinnamoyl esterase side activities and with the intention of determining whether LAB could also be responsible for this transformation, we planned the present study in which the action of commercial enzymes was prevented, the development of MLF was controlled, and the

Table 3. Mean ± Standard Deviation Values of the Concentration (mg/L) of Nonanthocyanin Polyphenolic Compounds in Wines[a]

peak	compounds	initial wine	MLFs	Oe-18	Oe-159	Lp-51	Lp-39
			hydroxybenzoic acids and derivatives				
1	gallic	41.65 ± 0.20	51.65 ± 0.80	53.76 ± 0.45	44.51 ± 0.44	42.54 ± 2.14	46.02 ± 3.21
3	protocatechuic	15.54 ± 0.54	12.31 ± 0.65	13.32 ± 0.25	7.96 ± 0.40	8.84 ± 0.78	11.16 ± 1.24
8	methyl gallate	6.33 ± 1.20	8.11 ± 0.74	7.99 ± 0.85	6.73 ± 0.10	7.17 ± 0.98	7.71 ± 0.14
13	vanillic	8.51 ± 2.02	9.42 ± 1.24	11.00 ± 0.78	8.17 ± 0.10	7.99 ± 0.44	9.09 ± 0.54
17	syringic	13.04 ± 1.20	11.82 ± 0.08	11.94 ± 0.98	12.54 ± 0.14	11.36 ± 0.25	11.86 ± 0.45
21	ethyl gallate	9.51 ± 0.89	11.89 ± 2.15	12.35 ± 1.27	9.91 ± 0.01	9.26 ± 1.22	10.11 ± 0.47
33	ellagic	2.03 ± 0.42	2.02 ± 0.25	2.29 ± 0.02	1.94 ± 0.58	0.54 ± 0.10	0.61 ± 0.64
			hydroxycinnamic acids and derivatives				
2	*cis*-cafftaric	2.11 ± 1.21	0.18 ± 0.21	0.73 ± 0.24	1.49 ± 0.54	1.16 ± 1.47	0.77 ± 2.54
6	*trans*-cafftaric	14.98 ± 2.14	6.85 ± 0.54	16.04 ± 1.58	12.01 ± 2.17	7.68 ± 0.17	12.32 ± 2.24
9	*cis*-coutaric	4.33 ± 0.21	2.17 ± 0.15	5.37 ± 0.54	4.97 ± 0.40	3.91 ± 0.25	4.41 ± 0.87
11	*trans*-coutaric	13.75 ± 1.21	8.68 ± 1.87	17.98 ± 0.24	13.84 ± 0.21	8.98 ± 0.65	12.77 ± 0.24
14	*trans*-caffeic	2.68 ± 1.54	26.24 ± 1.54	4.54 ± 0.14	2.96 ± 0.54	9.89 ± 0.54	4.79 ± 0.25
15	*trans*-*p*-coumaric hexose	1.23 ± 0.04	1.84 ± 0.10	1.49 ± 0.24	1.39 ± 0.03	2.23 ± 0.12	7.16 ± 0.57
19	*trans*-*p*-coumaric hexose	1.51 ± 0.24	2.63 ± 0.21	1.69 ± 0.57	1.79 ± 2.14	2.06 ± 0.15	1.95 ± 0.24
23	*trans*-*p*-coumaric	2.06 ± 0.24	16.01 ± 1.01	3.45 ± 0.32	2.34 ± 2.14	7.16 ± 0.57	3.83 ± 0.25
24	*cis*-*p*-coumaric	1.91 ± 0.58	0.52 ± 0.04	2.13 ± 1.20	0.91 ± 2.14	0.93 ± 0.05	1.45 ± 0.21
27	*trans*-ferulic acid	ND	0.84 ± 0.05	0.55 ± 0.04	0.38 ± 2.14	0.42 ± 0.01	0.40 ± 0.04
			stilbenes				
31	*trans*-resveratrol glucoside	1.64 ± 0.01	2.85 ± 0.09	3.93 ± 0.54	1.88 ± 0.32	0.67 ± 0.24	1.53 ± 0.34
35	*cis*-resveratrol glucoside	0.87 ± 0.09	0.98 ± 0.07	1.05 ± 0.04	0.88 ± 0.02	0.85 ± 0.01	0.72 ± 0.03
37	*trans*-resveratrol	0.63 ± 0.21	5.25 ± 0.32	3.59 ± 0.31	1.91 ± 0.78	1.59 ± 0.03	2.63 ± 0.02
38	*cis*-resveratrol	0.26 ± 0.11	0.59 ± 0.22	0.49 ± 0.34	0.37 ± 0.32	0.33 ± 0.04	0.50 ± 0.03
			alcohols				
10	tyrosol	31.25 ± 1.12	49.35 ± 0.98	48.70 ± 0.79	38.59 ± 2.13	41.43 ± 3.12	41.75 ± 1.22
32	tryptophol	0.11 ± 0.09	11.88 ± 0.11	9.38 ± 0.32	7.98 ± 0.16	1.22 ± 0.22	2.54 ± 0.05
			flavanols				
4	procyanidin trimer	ND	3.75 ± 0.98	3.39 ± 0.98	0.10 ± 0.98	2.8 ± 0.98	3.9 ± 0.98
5	prodelphinidin dimer	ND	0.49 ± 0.98	0.57 ± 0.98	0.49 ± 0.98	0.22 ± 0.98	0.22 ± 0.98
7	procyanidin dimer	ND	2.58 ± 0.98	ND	ND	ND	ND
12	(+)-catechin	11.50 ± 0.12	23.50 ± 0.22	24.13 ± 0.44	18.04 ± 1.45	14.48 ± 0.32	17.43 ± 1.98
16	procyanidin trimer	ND	0.52 ± 0.03	0.77 ± 0.06	ND	ND	0.33 ± 0.08
20	(−)-epicatechin	3.69 ± 0.08	7.53 ± 0.12	7.48 ± 0.32	5.96 ± 0.94	4.75 ± 0.22	5.09 ± 0.95
22	procyanidin trimer	1.55 ± 0.06	3.04 ± 0.02	2.96 ± 0.22	2.82 ± 0.54	1.02 ± 0.14	1.78 ± 0.38
25	procyanidin dimer	ND	0.71 ± 0.02	0.39 ± 0.45	0.25 ± 0.08	0.24 ± 0.09	0.28 ± 0.45
26	procyanidin dimer	ND	0.47 ± 0.01	0.50 ± 0.09	0.36 ± 0.08	0.19 ± 0.08	ND
30	procyanidin dimer	0.13 ± 0.98	0.20 ± 0.01	0.22 ± 0.04	ND	0.19 ± 0.06	ND
			flavonols				
18	dihydroquercetin derivative	1.93 ± 0.21	1.63 ± 0.18	1.56 ± 0.21	1.96 ± 0.11	1.71 ± 0.06	1.46 ± 0.08
28	myricetin 3-*O*-galactoside	0.88 ± 0.14	0.95 ± 0.33	1.17 ± 0.02	1.31 ± 0.03	1.16 ± 0.02	0.96 ± 0.18
29	dihydroquercetin	1.72 ± 0.09	1.45 ± 0.07	1.47 ± 0.02	1.20 ± 0.09	1.45 ± 0.01	ND
34	quercetin 3-*O*-glucuronide	2.47 ± 0.06	3.24 ± 0.03	3.05 ± 0.66	2.51 ± 0.05	5.18 ± 0.11	3.37 ± 0.04
36	myricetin	3.57 ± 0.11	9.65 ± 0.03	8.55 ± 0.32	6.34 ± 0.07	3.58 ± 0.13	4.23 ± 0.48
39	quercetin	2.42 ± 0.32	4.72 ± 0.22	4.02 ± 0.09	3.55 ± 0.02	0.98 ± 0.34	1.64 ± 0.43

[a] ND, not detected.

establishment of malolactic inocula was studied. As can be seen in **Table 3**, the levels of both esters, that is, *trans*-cafftaric acid and *trans*-coutaric acid, declined dramatically with a concomitant increase in the corresponding free phenolic acids in some of the studied wines. Thus, in the spontaneous MLF wine (MLFs), the concentrations of *trans*-cafftaric and *trans*-coutaric acids dropped sharply, 54 and 37%, respectively, with respect to the initial wine, resulting in a rise in the corresponding free forms, *trans*-caffeic and *trans*-coumaric. The same behavior was found in the wine inoculated with *L. plantarum* Lp-51, in which the losses of *trans*-cafftaric acid and *trans*-coutaric acid were 7.30 and 4.77 mg/L, whereas the increase in the concentration of *trans*-caffeic acid and *trans*-*p*-coumaric acid was 7.18 and 5.10 mg/L (**Table 3**). However, the other LAB studied had little or no effect on caffeoyl and *p*-cumaroyltartaric acid esters, nor did they produce its subsequent hydrolysis products. Our results are consistent with the observations of Hernández et al. (*14*), indicating that *trans*-cafftaric and *trans*-coutaric acids are substrates of LAB, which can exhibit cinnamoyl esterase activities during MLF, increasing the concentration of the hydroxycinnamic acids. An additional source of caffeic and *p*-coumaric acids may come from the hydrolysis of cinnamoylglucoside anthocyanins (*12*) as well as from other hydroxycinnamic derivatives by LAB enzymatic activity. Phenolic acid esterase enzymes have been previously reported for the bacteria *Streptomyces* spp. (*31*), *Bacillus* spp. (*32*), certain gut bacteria, including species belonging to the genera *Bifidobacterium* and *Lactobacillus* (*33*), and the fungi *Penicillium* spp. (*34*) and *Aspergillus* spp. (*35*), among others. Furthermore, our results seem to indicate that among wine LAB, this activity could be strain-dependent and could also depend on the isomeric form of the above-mentioned esters, since only the *trans*-isomers were involved in the reaction. Free phenolic acids can be metabolized by different wine microorganisms, including *S. cerevisiae* (*29*), some LAB species, mainly *Lactobacillus brevis*, *L. plantarum*, and *Pediococcus* spp. (*19*), and *Brettanomyces*/*Dekkera* (*36*) to form 4-vinyl derivatives, which can be reduced to 4-ethyl derivatives in wine. These derivatives can have a significant influence on wine aroma since they are regarded as sources of phenolic off-flavors in wine, due to their characteristic aroma

and their low detection threshold (*19*). In wines, the amounts of these compounds are generally low and usually limited by the concentrations of their precursors (*29*). Thus, on the basis of these observations, the present results suggest that LAB could contribute to the differences in the vinylphenol levels found in wines.

Table 3 also shows differences in other compounds when comparing spontaneous MLF wine with the inoculated samples. Some compounds, such as *trans*-resveratrol (4.62 mg/L), tyrosol (18 mg/L), (+)-catechin (12 mg/L), (−)-epicatechin (3.84 mg/L), myricetin (6.1 mg/L), and quercetin (2.3 mg/L) were observed to increase. Samples corresponding to wines inoculated with the four LAB strains presented changes in various polyphenolic compounds, showing differences between strains and with respect to the MLFs sample. Hydroxybenzoic compounds seem to be the least affected by the different MLF conditions, and wines inoculated with *O. oeni* Oe-18 and *L. plantarum* Lp-39 were especially very similar to MLFs.

In the case of resveratrol, when comparing the concentration of the *cis*- and *trans*-isomers, their glucosides, and the sum of the *cis*- and *trans*-forms, the wines were observed to present higher values of the *trans*-isomers than of the *cis*-ones and these differences were more evident in the wines after MLF. In fact, the sum of the *trans*-isomers in the wines after MLF ranged from 2.26 to 6.21 mg/L, whereas the sum of the *cis* ranged from 1.18 to 1.57 mg/L. Moreover, after MLF, differences related to inoculation were found. The *trans*-isomers were higher after both natural (MLFs) and inoculated Oe-18 fermentation (**Table 3**). Conversely, the content of the *cis*-forms remained almost constant or increased only slightly in these wines. It should be pointed out that their contents in the MLFs and the Oe-18 wines were more than twice that in the initial wine. *trans*-Resveratrol is one of the most investigated phenolics in red wine because of its potential beneficial effects on human health, which include anticancer and antioxidant activities, inhibition of platelet aggregation, and inhibition of tissue factor expression in vascular cells (*10*, *37*). According to the present results, it may be suggested that malolactic bacteria could be among the factors that contribute to the antioxidant activity of wine.

After gallic acid, the largest concentration of the 39 phenolic compounds examined in the wines was recorded for (+)-catechin (**Table 3**). Independently of the MLF experiment, (+)-catechin occurred at higher concentrations than the other compounds. Moreover, similarly to *trans*-resveratrol, we also found differences between the mean levels of (+)-catechin in the MLF wines. The higher concentration was detected in the MLFs and the Oe-18 wines (23.50 and 24.13 mg/L, respectively). The levels of (−)-epicatechin in these two samples were also very similar (7.53 and 7.78 mg/L, respectively) and were higher than those recorded in the other wines. The proanthocyanidin contents (dimers and trimers) showed a general increase after MLF (**Table 3**), and the highest concentration of these compounds was recorded in the wine undergoing spontaneous MLF. The reaction between flavanols, such as catechin, epicatechin and proanthocyanidins, and anthocyanins, plays an important role in the color of red wines (*38*, *39*) because of the formation of complex (copigment−pigment), which explains some of the color modification, since these complexes affect the stability of anthocyanins.

The total flavonol content in the initial wine (before MLF) was 12.99 mg/L, whereas in the wines analyzed after MLF this varied from 11.66 to 21.64 mg/L. We found an increase in the aglycones myricetin and quercetin during spontaneous MLF as well as in the wine inoculated with *O. oeni* Oe-18. The content of myricetin and quercetin in these wines was twice that recorded in the initial wine (**Table 3**). These wines are characterized by a high percentage of free flavonols (i.e., myricetin and quercetin): 45 and 22% in the MLF wine and 43 and 20% in the Oe-18 wine, for myricetin and quercetin, respectively. Flavonols, mainly myricetin and quercetin, are the best copigments to complex with anthocyanins to modify wine color (*39*). These compounds are known to exhibit antioxidant activities (*40*). According to Williamson and Manach (*41*), the bioavailability of flavonol glycosides and flavonol aglycons in humans is different. From the results obtained here, we can deduce that the quantity of free flavonols in red wines could depend on the LAB strain that performs MLF.

It is interesting to note that polyphenolic compounds with free radical scavenging activity, which are present in grapes and wines, can be transformed by bacteria, and differences can arise after MLF, as shown in the wines of this study. These differences will render different antioxidant activities and organoleptic characteristics, which could be of value to both winemakers and consumers.

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