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Changes in the Concentration of Yeast-Derived Volatile Compounds of Red Wine during Malolactic Fermentation with Four Commercial Starter Cultures of *Oenococcus oeni*

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The effects of malolactic fermentation (MLF) on the concentration of volatile compounds released by yeasts during the production of red wine were investigated by inoculation with four commercial starters of *Oenococcus oeni*. Volatile compounds in wine at the end of MLF were extracted, analyzed by GC-MS and GC, and compared with those extracted form a noninoculated reference sample. Several esters known to play a role in the aroma profile of red wine, such as C_4-C_8 ethyl fatty acid esters and 3-methylbutyl acetate, were found to increase with MLF, and their final concentration was dependent on the bacterial starter employed for the induction of MLF. The overall increase of ethyl fatty acid esters was generally larger than the one observed for acetate esters. Ethyl lactate, 3-hydroxybutanoate, 2-phenylethanol, methionol, and γ-butyrolactone were also increased by bacterial metabolism. The impact of MLF on other volatiles or red wine, including several higher alcohols, fatty acids, and nitrogen compounds, was generally negligible.

KEYWORDS: Malolactic fermentation; esters; wine aroma; *Oenococcus oeni*

INTRODUCTION

Malolactic fermentation (MLF) is a biochemical process usually occurring in wine after the completion of alcoholic fermentation. Its main purpose is the transformation of L-malic acid into L-lactic acid, with the release of carbon dioxide. Lactic acid bacteria (LAB) genera, such as *Oenococcus*, *Pediococcus*, and *Lactobacillus*, are capable of conducting MLF in wine (*1*). As it is well adapted to the harsh conditions of wine, the species *Oenococcus oeni* is the most frequently associated with MLF (*2*).

As a result of the biochemical transformation of malic acid into lactic acid, wines that have undergone MLF generally exhibit a softened palate and increased smoothness, due to a decrease of wine acidity and increased pH. For this reason, MLF is generally viewed by winemakers as a useful tool to improve the sensory quality of red and white wines.

Wine aroma can also be modified by MLF. As bacterial growth and metabolism typically occur after alcoholic fermentation, many of the aroma modifications associated with MLF are likely to be due to either the production of odor-active compounds or the transformation of both grape- and yeast-derived volatile compounds and flavor precursors by *O. oeni*. The most frequently reported aroma modification associated with MLF consists of an increase of wine "buttery" character (*3*). A number of investigations evidenced the primary role of diacetyl (2,3-butanedione), a volatile compound largely released by LAB, in the development of this sensory character after MLF, providing extensive evidence for the biochemical pathways involved in the formation of this compound and the influence of several winemaking practices on its concentration in wine, as recently reviewed (*4*).

However, flavor modifications induced by MLF appear to be far more complex, as they often involve changes of fruity, flowery, and nutty attributes, as well as the reduction of vegetative/herbaceous aromas (*3*, *5*). Although it is clear that diacetyl alone cannot account for the occurrence of such a large array of flavor modifications, the mechanisms actually responsible for these are not completely understood. Tentative evidence is available that LAB metabolism can result in a significant increase in the concentration of several esters arising from yeast metabolism (*6*, *7*). However, other authors report that MLF coincided with a significant decrease in the concentration of esters (*8*, *9*). Maicas et al. (*6*) also reported that the concentration of some esters can be either increased or decreased by MLF, according to the type of bacterial strain used. Osborne et al. (*10*) suggested that the degradation of acetaldehyde and other aldehydes by wine LAB can contribute to the reduction of herbaceous aroma. Moreover, LAB are known to produce other potentially odor-active compounds such as higher alcohols, fatty acids, lactones, and sulfur and nitrogen compounds (*6*, *11*, *12*), some of which may either directly influence the aroma of wine or modify the contribution of other aroma compounds through enhancing or masking effects.

Understanding and controlling the different issues associated with the correct management of MLF is of great importance

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for winemakers. Numerous strains of *O. oeni* are now available as commercial starter cultures for the induction of MLF. To better understand the factors influencing their successful induction and completion of MLF, the compatibility between commercial *O. oeni* strains and various yeast strains, as well as their ability to conduct MLF under different winemaking conditions, has been determined (*13*−*16*). Flavor effects of individual bacterial strains are also of considerable interest for winemakers. Surprisingly, comparative studies on the influence of different commercial malolactic bacteria on the concentration of wine volatiles other than diacetyl are rather few, despite the well-documented relationship between the bacterial strain employed for the induction of MLF and the final aroma quality of wine (*3*, *4*, *17*).

The purpose of this study was to investigate the influence of MLF with four different commercial starter cultures of *O. oeni* on the concentration of yeast-derived volatile byproducts of red wine, to provide a better understanding of the contribution of MLF to the volatile composition of wine.

MATERIALS AND METHODS

Bacterial Starters. The four different commercial preparations used for this study were Lalvin 31 (referred to as MLB 1 in the text), EQ 54 (MLB 2), Lalvin O.S.U. (MLB 3), and Uvaferm Alpha (MLB 4). Dried preparations were kindly donated by Lallemand Italy (Castel d'Azzano, VR), and according to the manufacturer's literature they were all single-strain cultures, with the exception of Lalvin O.S.U., which was a mixed culture of Er1a and Eysd strains.

Wines. Aglianico grapes (100 kg) were harvested at 22.3 °Brix, destemmed, and crushed. The must was treated with potassium metabisulfite (60 mg/kg) and inoculated with 30 g/hL of D47 yeast (Lallemand Inc.), previously activated in warm water for 30 min. Fermentation was carried out in a 100 L stainless steel tank at 25−26 °C. Maceration lasted for 14 days, with the cap being punched down three times per day. Upon completion of alcoholic fermentation (residual sugars < 2 g/L), the must was racked and pressed in a pneumatic press, giving ∼65 L of wine, which was then left to settle for 2 days at 16 °C in a stainless steel tank. Wine was then racked, brought to 20 °C, and distributed among nine 5 L glass carboys. The chemical composition of the wine was as follows: ethanol, 13.2%; pH, 3.13; titratable acidity, 9.6 g/L; volatile acidity, 0.36 g/L; residual sugars, 1.72 g/L; malic acid, 3.8 g/L. Inoculation with malolactic bacteria was then carried out with one of the four commercial starter cultures, at the rate of 10 mg/L, after rehydratation of cells in warm sterile water at 30 °C for 30 min. Fermentations were carried out in duplicate, in a temperature-controlled room at 20 °C. A control wine in which MLF was inhibited by means of 100 mg/L of $K_2S_2O_5$ was stored under the same condition for the length of MLF. Weekly measurement of malic acid concentration was performed for monitoring bacterial metabolism, and MLF was deemed to be complete when malic acid was ≤0.2 g/L. At the end of MLF, wines were cold settled and racked, 60 mg/L of $K_2S_2O_5$ was added, and the wines were bottled.

Standard Chemical Analyses. Must and wine standard chemical parameters were determined according to the Official European Regulation (*18*). All samples were analyzed in triplicate. Malic acid was determined enzymatically (Roche, Mannheim, Germany).

Extraction and Analysis of Volatile Compounds. Solid-phase extraction (SPE) was employed for the extraction of volatile compounds from wines, using SDB-L styrene−divinylbenzene cartridges (Phenomenex, Torrance, CA). Wine samples (25 mL) were diluted 1:1 with water after the addition of 2-octanol as internal standard, filtered at 0.45 μm, and loaded onto SPE cartridges containing 500 mg of sorbent. Elution was carried out at ∼3 mL/min. The sorbent was rinsed with water (2 × 10 mL), and volatile compounds were eluted with dichloromethane (10 mL). The extract was dried over Na_2SO_4 and concentrated first in a Kuderna−Danish concentrator and then under a stream of pure N_2 for gas chromatographic analysis. All extractions and analyses were carried out in duplicate. A 1 μL aliquot of each concentrated extract was injected in splitless mode into a Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless injector (Hewlett-Packard, Avondale, PA), a J&W DB-Wax column (30 m length × 0.25 i.d. × 0.25 film thickness; J&W Scientific, Folsom, CA), and a flame ionization detector (FID). The temperature program used was as follows: 40 °C for 3 min, 4 °C/min to 220 °C, 20 min at maximum temperature. Carrier gas (He) velocity was 37 cm/s. Both detector and injector temperatures were maintained at 250 °C. For GC-MS, experimental mass spectra for each compound were obtained on an HP 5972 quadrupole mass spectrometer coupled with an HP5890 gas chromatograph operating as previously described for GC. Electron impact mass spectra were recorded with an ion-source energy of 70 eV. Identification of compounds was performed by comparison of their mass spectra and linear retention indices with those of pure reference compounds available in the laboratory.

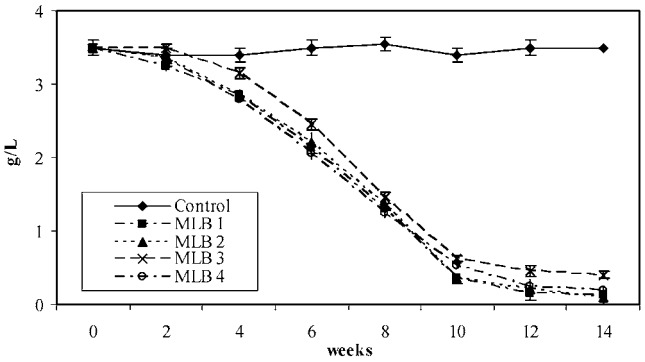

Figure 1. Evolution of malic acid during MLF.

Table 1. Analytical Parameters of the Experimental Wines at the End of MLF

	control	MLB 1	MLB 2	MLB 3	MLB 4
alcohol (%)	13.2	13.2	13.1	13.2	13
residual sugars (g/L)	1.72	1.55	1.55	1.63	1.52
pH	3.13	3.23	3.21	3.2	3.21
total acidity (g/L)	9.6	7.5	7.6	7.9	7.6
volatile acidity (g/L)	0.33	0.4	0.42	0.44	0.42
malic acid (g/L)	3.51	0.14	0.1	0.5	0.18

Statistical Analysis. Analysis of variance and LSD test were used to interpret differences in means, if any, at the 95% confidence level. Elaborations were carried out by means of Statgraphics Plus 5-PC (Manugistics, Rockville, MD).

RESULTS AND DISCUSSION

Fermentation Performances and Wine Chemistry. Figure 1 shows the evolution of malic acid after inoculation with the four different commercial strains. About 12 weeks was necessary to reach stable levels of L-malic acid. Although complete degradation was not achieved with any of the strains, final malic acid concentration was never >0.5 g/L. Metabolism of L-malic acid by *O. oeni* was generally quite slow during the first days following inoculation. MLB 3 required a longer period (∼2 weeks) before L-malic acid consumption started. In general, the fermentation rate with this strain was slightly lower than that of the other three across the whole period of MLF, and the final concentration of residual L-malic acid in experimental wines was significantly higher, albeit an overall decrease of ∼3 g/L was observed also for this strain. L-Malic acid was stable in the control sample, indicating proliferation of LAB in this treatment was inhibited by the presence of SO_2.

The analytical parameters of wines at the end of MLF are reported in **Table 1**. MLF resulted in an average increase of pH of ∼0.1 unit, whereas titratable acidity, expressed as grams per liter of tartaric acid, was decreased by ∼2 g/L. The level of

Table 2. Concentration (Micrograms per Liter)[a] of Wine Yeast-Derived Volatiles at the End of MLF

	control	MLB 1	MLB 2	MLB 3	MLB 4
esters					
ethyl butanoate	43 a	52 b	48 ab	44 a	55 b
ethyl hexanoate	140 a	171 b	178 b	175 b	188 c
ethyl octanoate	69 a	128 b	129 b	131 b	129 b
ethyl decanoate	99 a	114 b	142 c	118 b	159 d
isoamyl acetate	264 a	322 b	332 b	317 b	347 c
hexyl acetate	3 a	4 a	4 a	4 a	5 a
2-phenylethyl acetate	68 a	81b	74 b	76 b	78 b
ethylphenyl acetate	41 a	37 a	46 a	45 a	43 a
ethyl-2-methyl butanoate	9 a	6 a	7 a	7 a	7 a
ethyl-3-methyl butanoate	11 a	9 a	8 a	8 a	10 a
ethyl-3-hydroxybutanoate	21 a	38 b	37 b	38 b	44 b
ethyl-4-hydroxybutanoate	731 a	846 c	856 c	741 a	772 b
ethyl lactate	188 a	1060 c	1211 d	946 b	1025 c
diethyl succinate	981 a	1484 c	1243 b	1217 b	1485 c
alcohols					
1-butanol	72 a	65 a	74 a	71 a	68 a
1-pentanol	55 a	52 a	65 a	55 a	62 a
1-hexanol	2923 a	3105 a	3219 b	3173 b	3203 b
1-heptanol	52 a	48 a	70 b	65 b	80 c
4-methyl-1-pentanol	48 a	51 a	54 a	53 a	54 a
3-methyl-1-pentanol	177 a	192 b	199 b	195 b	201 b
3-ethoxy-1-propanol	20 a	18 a	20 a	19 a	17 a
1-octen-3-ol	1 a	5 b	7 c	8 c	7 c
isoamyl alcohol	61694 a	61929 a	61286 a	62201 a	63516 b
2-phenylethanol	48693 a	50023 b	52569 c	50899 b	54099 d
acids					
2-methylpropanoic acid	109 a	96 a	108 a	102 a	95 a
3-methylbutanoic acid	1033 a	1002 a	1013 a	946 a	976 a
butanoic acid	126 a	123 a	149 b	139 b	144 b
hexanoic acid	1179 a	1253 b	1311 c	1218 b	1215 b
octanoic acid	712 a	759 b	834 c	743 c	747 c
decanoic acid	794 c	655 a	949 d	727 b	715 b
trans-2-hexenoic acid	65 a	75 c	78 c	69 b	69 b
benzoic acid	56 a	59 a	49 a	54 a	58 a
phenylacetic acid	176 a	192 b	163 a	162 a	169 a
lactones					
γ-butyrolactone	5 a	8 b	9 b	11 b	9 b
γ-nonalactone	22 a	24 a	29 a	27 a	24 a
sulfur and nitrogen compounds					
4-hydroxy-4-methylpentan-2-one	9 a	19 b	21 b	20 b	16 b
3-(2*H*)-dihydro-2-methylthiophenone	34 b	27 a	39 b	27 a	32 b
3-methylthio-1-propanol	174 a	170 a	190 b	205 c	198 c
N-methylbutyl acetammyde	308 a	302 a	315 a	299 a	292 a
2-phenylethyl acetammyde	184 a	179 a	200 b	178 a	160 a

[a] Means of duplicate ferments, each analyzed in duplicate. Different letters denote significant differences between bacterial starters, at $p < 0.05$.

acetic acid increased with bacterial growth. Heterofermentative LAB such as *O. oeni* are known to produce acetic acid as a byproduct of hexose metabolism; thus, an increase of volatile acidity of wine after MLF has to be invariably expected (*3*). The increase in the concentration of acetic acid observed here (between 0.07 and 0.1) is consistent with previous findings (*19*) and is unlikely to affect negatively the overall quality.

Volatile Compounds. The results of the GC analysis of the volatile fraction of wines are given in **Table 2**. A total of 40 volatiles were identified and measured, including esters, alcohols, acids, lactones, and sulfur and nitrogen compounds.

Significant changes were observed in the pool of volatile esters as a result of MLF. Quantitatively, diethyl succinate and ethyl lactate were the esters showing the largest concentration increase. Diethyl succinate arises from esterification of succinic acid, a byproduct of microbial α-ketoglutarate metabolism (*20*), hence its increase with MLF, together with other related esters such as ethyl 4-hydroxybutanoate. As for ethyl lactate, because the production of this compound by *O. oeni* is linked to the production of lactate (*6*), its accumulation during MLF is dependent on malic acid metabolism. This might explain the slightly lower concentrations observed in wines obtained with MLB 3, which was characterized by slightly reduced fermentation activity and malic acid consumption. Ethyl lactate is present in wine as a mixture of enantiomers, which distribution can vary according to the species of microrganisms contributing to the winemaking process. Particularly, it has been reported that the (*S*)-enantiomer is produced in high amounts by *O. oeni* (*21*). Although the analytical conditions in this study did not allow for quantification of the single enantiomers, the total concentration of ethyl lactate detected did not exceed the odor threshold of (*S*)-ethyl lactate (110 mg/L; *20*) or the value reported as potentially affecting the overall aroma of wine (200 mg/L; *22*).

Ethyl fatty acid esters (4−10 carbons) were significantly affected by MLF, with increases in the ranges of 2−28% (C4), 22−34% (C6), 85−89% (C8), and 20−31% (C10). Although these compounds are generally considered to be of primary importance for the aroma characteristic of white wines, recent investigations highlighted their significant contribution to the flavor of red wines (*23*, *24*). As for acetate esters, the concentrations of the powerful odorant isoamyl acetate, characterized by banana notes, increased following MLF, consistent with previous findings (*6*, *25*). 2-Phenylethyl acetate also increased after MLF, although the final concentration of this

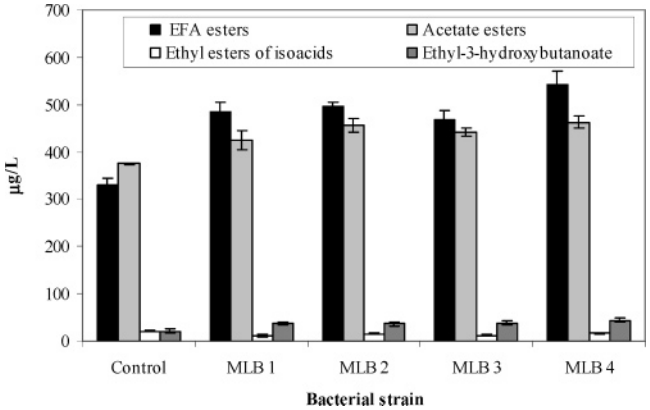

Figure 2. Effect of MLF and bacterial strain on ester composition of red wine.

compound was well below its odor threshold (250 μg/L; *26*). Another compound, the concentration of which significantly increased during MLF, with all four bacterial strains, was ethyl 3-hydroxybutanoate. In a recent study on the nature of the odorants involved in the flavor of red wines from different grape varieties, this compound was indicated as a possible contributor to the strawberry character of Pinot Noir wines, its odor being described as strawberry or burnt marshmallow (*24*). Finally, MLF did not cause any change in the concentration of the two branched esters ethyl 2-methylbutanoate and ethyl 3-methylbutanoate, both known to play an important role in the flavor of red wines (*24*). As a whole, for all four bacterial strains tested, MLF resulted in a significant change of the overall ester profile of wine, with ethyl fatty acid esters becoming quantitatively the most representative class of esters after MLF (**Figure 2**). Although no remarkably large difference in the amount of esters synthesized by the four different strains was observed, significantly higher concentrations were observed for MLB 4, indicating that the bacterial starter employed for MLF can influence the final concentration of esters in wine.

Early observations by Davis et al. (*27*) indicate that LAB of enological interest possess esterase activities which may degrade esters during MLF, although the stability of these enzymes under winemaking conditions was not demonstrated. Consistent with these results, some authors reported a decrease in the concentration of esters following either *O. oeni* inoculated (*8*) or spontaneous MLF (*9*), associated with a reduction in the fruity attributes of wine. Reduction of wine fruity attributes has been also reported by Savaugeot and Vivier (*28*), although in this case quantitative data regarding esters were not given. In contrast with these findings, other authors report that MLF can increase the fruity aromas of wine (*3*, *5*, *29*). Our results indicate that MLF can result in a significant increase in the concentration of individual esters potentially involved in the perceived aroma of wine, such as ethyl esters and acetates, confirming the observations of Maicas et al. (*5*) and Delaquis et al. (*6*). The production of esters by *O. oeni* could play a role in the increase of wine fruitiness reported in some cases as a consequence of MLF. In any case, the great inconsistency of experimental results regarding the influence of MLF on the ester composition and the related sensory attributes of wine suggests the need for further detailed studies on the factors influencing ester metabolism in *O. oeni*.

A relatively large number of higher alcohols were detected in the volatile fraction of the experimental wines. Generally, under our experimental conditions, MLF does not have major effects on the concentration of these compounds, although small increases were observed for 1-hexanol, 1-heptanol, 3-methyl-1-pentanol, and 1-octen-3-ol. Important wine odorants such as isoamyl alcohol and 2-phenylethanol, characterized by herbaceous and floral/rose notes, respectively, were significantly increased by bacterial metabolism for MLB 4.

Volatile short-chain fatty acids are natural components of many alcoholic beverages, being produced by yeasts during alcoholic fermentation. When present at high concentrations, these compounds can negatively affect the aroma quality of wine, due to their odors reminiscent of cheese and rancid cheese (*30*). Many LAB isolated from cheese are known to possess lipases that can hydrolyze lipids, giving rise to the formation of volatile fatty acids (*31*). However, lipolitic systems in wine LAB are not well-known, and neither are other metabolic pathways potentially leading to the production of volatile fatty acids (*32*). With the observation of lipase activities in wine LAB (*27*), it has been proposed that these microrganisms have the ability to degrade lipids and produce short-chain fatty acids. Nevertheless, none of the four commercial strains of *O. oeni* tested in the present study caused increases in the concentration of short-chain fatty acids, although some minor but statistically significant increases were noted for hexanoic, octanoic, and decanoic acids by MLB 2. In general, our findings indicate that the four commercial strains tested in this study are unlikely to cause the occurrence of any flavor change or off-flavor associated with the production of volatile fatty acids, supporting the findings of Rodriguez et al. (*33*) regarding the stability of wine cheesy aroma attributes during MLF with *O. oeni*.

The concentration of 3-(methylthio)-1-propanol (methionol) increased following MLF with MLB 1, 2, and 3. The levels of methionol in wine have been reported to vary largely according to the grape variety, its occurrence being related to yeast-driven transamination of methionine followed by decarboxylation and reduction (*34*). Production of variable amounts of this compound by different strains of LAB has been also reported (*5*, *11*). Methionol has a cooked cabbage odor and may be responsible for off-flavor when present in high concentrations (*30*). However, even after the completion of MLF, the concentrations of methionol seen in this study are far below its odor threshold of in wine (500 μg/L; *26*). Nevertheless, it has been shown that, in white wine, the oxidation of methionol may result in increased concentrations of methional, a more powerful aroma compound (odor threshold = 0.5 μg/L; *34*) involved in the typical flavor of oxidation-spoiled wines (*35*). In red wine, however, the oxidation of methionol did not seem to determine increases in the concentration of methional (*36*).

Among lactones, γ-butyrolactone, a well-known byproduct of α-ketoglutarate metabolism in *O. oeni* (*20*), was found to increase with MLF, although the low levels detected for this compound compared to other studies (*6*) could be due to the low affinity of the resin used for volatiles extraction. Conversely, γ-nonalactone, which occurred at concentrations close to its odor threshold (25 μg/L; *37*) was stable during MLF. This compound has been recently reported among the volatiles released by acid-catalyzed hydrolysis of odorless precursors of red grapes (*38*).

In summary, our findings indicate that MLF can significantly influence the volatile composition of red wine by increasing the concentration of several wine volatiles known to play an important role in wine aroma characteristics. Particularly, the release by *O. oeni* of several esters is of great interest, considering the positive contribution of these compounds to the aroma attributes of wine. Other major volatile compounds, such as higher alcohols, fatty acids, lactones, and sulfur compounds, were scarcely affected by MLF. Further experiments are needed to elucidate the mechanisms involved in the biosynthesis of

esters and other volatiles by *O. oeni* as well as the impact of different bacterial starters and winemaking practices on their evolution during MLF. Sensory investigations are also needed to establish a definitive correlation between the production of volatile compounds by *O. oeni* and the flavor modifications associated with MLF.

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