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# Influence of lactic acid bacteria strains on ester concentrations in red wines: Specific impact on branched hydroxylated compounds

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ABSTRACT

This research investigated the influence of lactic acid bacteria (LAB) strains on ester levels in Bordeaux red wines. These wines were made in five Bordeaux areas in two vintages, using three yeast strains. Malolactic fermentation (MLF) was carried out using industrial starters or indigenous strains, each in triplicate. Ester concentrations were determined by liquid-liquid-extraction- or HS-SPME-GC/MS at various stages in the winemaking process. The levels of most compounds were slightly impacted by LAB, depending on grape variety. Nevertheless, branched hydroxylated esters, such as ethyl 2-hydroxy-3-methylbutanoate and ethyl 2-hydroxy-4-methylpentanoate were the only compounds to be strongly influenced by the bacteria strain, regardless of matrix composition or the yeasts used for alcoholic fermentation. Moreover, the effect observed after MLF persisted over time, for at least 12 months. These esters are apparently important markers of LAB esterase activity. To our knowledge, this was the first time they had been identified in this role.



## 1. Introduction

Red wine is not only the result of the fermentation of sugars by yeasts, but is almost always followed by malolactic fermentation (MLF), conducted by lactic acid bacteria (LAB), which may occur spontaneously or be induced by inoculation with commercial starters (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Early works by Ribéreau-Gayon and Peynaud (1964) revealed the usefulness of this second fermentation, which usually ensures the stability of wines, as well as improving their aromas and flavors. The main result of MLF is to transform L-malic acid into L-lactic acid, accompanied by a release of carbon dioxide. Of all enological LAB species, *Oenococcus oeni* is preferred for MLF, as it is resistant to the harsh environmental conditions, decomposes the malic acid first, followed by the sugars, and forms little volatile acidity. This decarboxylation naturally reduces the total acidity and is accompanied by a slight increase in pH, which contributes to softening the flavor on the palate and enhancing its smoothness. MLF also promotes the microbial stability of wines by substrate depletion. These secondary bacterial metabolisms associated to bacterial development are responsible for chemical modifications affecting the olfactory and gustatory perception of wine (Bartowsky, Francis, Bellon, & Henschke, 2002; Henick-Kling, 1993; Matthews et al., 2004).

The most frequently-reported aromatic compound associated with MLF is diacetyl (butane-2,3-dione), mainly released by LAB (Bertrand, Zmirou-Bonnamour, & Lonvaud-Funel, 1984; de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel, & Bertrand, 1999) and associated with an increase in buttery character (Bartowsky & Henschke, 2004). Ethyl lactate is another marker of bacterial activity (Boido et al., 1999), but its impact on fruity aroma is quite limited, contrary to other esters, which are considered some of the most important fruity compounds in wines (Ebeler, 2001; Ferreira, López, & Cacho, 2000).

From a qualitative point of view, all red wines contain the same set of ester compounds. However, their respective proportions vary considerably from one wine to another (Antalick, Perello, & de Revel, 2014). Generally, these molecules are present at concentrations well below their perception thresholds, so it would be logical

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to assume that they do not modulate wine aroma. Since 2009, new data has revealed that these compounds play a central role in the fruity expression of red wines, via synergistic phenomena (Lytra, Tempere, Le Floch, de Revel, & Barbe, 2013; Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2009). Thus, small variations in the concentrations of one or more esters may have a significant effect on the perception of fruity aroma. In particular, previous research demonstrated the impact of ethyl esters, acetates, and branched ethyl esters on the fruity character of red wines (Falcão, Lytra, Darriet, & Barbe, 2012; Ferreira et al., 2016).

Since the late 1960's, studies have highlighted the capacity of LAB strains (*Lactobacillus*, *Pediococcus*, *Leuconostoc*) to increase concentrations of some esters in wine during MLF (Pilone, Kunkee, & Webb, 1966). Screening the enzyme activity of several wine LAB strains revealed that some of them were also able to hydrolyze esters (Davis, Wibowo, Fleet, & Lee, 1988). In that regard, several studies exploring the modulation of wine aromas revealed that ester concentration increased or decreased after MLF (Antalick, Perello, & de Revel, 2012; Delaquis et al., 2000; Zeeman, Snyman, & van Wyck, 1980). These results suggested that the esterase activity of wine LAB, like that found in the cheese industry, was capable of synthesizing and/or hydrolyzing these compounds. This hypothesis was recently validated by Sumby, Jiranek, and Grbin (2013), highlighting the role of the synthesis and hydrolysis of two enzymes, EstA2 and EstB28, involved in the ester biosynthesis pathway in *O. oeni*. LAB ester metabolisms are apparently strongly influenced by several enological parameters. Maicas, Gil, Pardo, and Ferrer (1999) reported that the concentrations of some esters either increased or decreased during MLF, according to the type of bacterial strain used. Delaquis et al. (2000) reported that the aromatic composition of wines was influenced by both yeast and LAB strains, as well as winemaking conditions. Finally, Knoll et al. (2011) demonstrated the influence of ethanol and pH on MLF and ester profiles.

One of the difficulties in finding a consensus is that the previous work on this topic focused mainly on a few cases of bacterial strains or wines, whereas many enological parameters may affect the influence of LAB strains on the ester composition of red wines. Thus, it was essential to conduct a comprehensive study. To investigate the influence of LAB strains on ester levels, MLF was triggered using two different commercial *O. oeni* starters and compared with spontaneous MLF. To elucidate the influence of the yeast strain on LAB metabolism, alcoholic fermentation (AF) was triggered by inoculation with three different commercial *Saccharomyces cerevisiae* starters. To evaluate the impact of the matrix on ester metabolism by LAB, experiments were conducted during two vintages, using two cultivars, Merlot and Cabernet Sauvignon. Finally, to confirm the influence of LAB strains on some ester levels, particularly in micro-vinification, some of the wines tested were made on an industrial scale.

## 2. Material and methods

### 2.1. Winemaking

Two different experimentations were conducted in the Bordeaux region during the 2011 and 2012 vintages. Micro-vinifications were carried out with Cabernet Sauvignon grapes (named WEC 2011 and WEC 2012). Vinifications in four wineries were conducted with Cabernet Sauvignon or Merlot grapes at industrial scale (MRGX 2011, MDC 2011, PCLN 2012, and STEM 2012) (Table 1). In all six experiments, AF was initiated by inoculation with rehydrated dried yeast, according to the manufacturer's recommendations (*S. cerevisiae* yeasts strains: Actiflore cerevisiae, 522D; Zymaflore FX10, Biolaffort, Floirac, France; and Excellence XR, Lamothe-Abiet, Canéjan, France). AF was performed in 2 h L stainless steel tanks in triplicate under micro-vinification conditions. In wineries, AF was completed in stainless steel tanks in bigger volume (Table 1). Implantation in each tank under all experimental conditions was checked at the middle of AF (density close to 1.040). Yeast starter culture implantation was monitored by PCR at SARCO laboratory (Biolaffort, Floirac, France) (data not shown). It confirms that, for each wine, AF was carried out by the yeast strain implanted. MLF was triggered using starters (*O. oeni* bacterial strains: Lactoenos 450 PreAc and Lactoenos B28 PreAc, Biolaffort, Floirac, France) or indigenous strains (spontaneous flora), in triplicate for all experimental conditions (Table 1) at the end of AF. In wines inoculated with bacteria, starters were rehydrated with bacterial nutrient (Energizer®, Biolaffort, Floirac, France), according to the manufacturer's instructions, and added to wines at the recommended dose. Malic acid concentrations were measured once a week throughout MLF under the various conditions, to monitor the bacterial metabolism. Implantation control of commercial bacterial starter cultures (data not shown) was performed by the Microflora® laboratory (ISVV, Bordeaux University, France), based on a method developed by Claisse and Lonvaud-Funel (2012). This analysis also confirmed that the indigenous strains (IND1 and IND2) responsible for MLF in wineries, MRGX 2011 and MDC 2011, were different from each other and from the commercial strains used in this study (data not shown). At the end of MLF (<0.1 g/L malic acid), 5 g/h L $SO_2$ were added. Wines made under winery and micro-vinification conditions were sampled for oenological and volatile compound analyses at the end of AF (<0.2 g/L glucose/fructose) and after completion of MLF (malic acid $\leq$0.1 g/L). Samples were collected for volatile compound analysis in 0.75 L glass bottles, stored at 10 °C for 1 week, decanted, and frozen at −18 °C prior to analysis. The remaining wine was stored in a 30 L stainless-steel barrel for aging. $SO_2$ content was measured and adjusted if necessary. Samples were collected for chemical analyses after 3, 6, and 12 months' aging under the same conditions as those applied after AF and MLF.

### 2.2. Standard chemical analysis

The standard chemical parameters of wine (total acidity, sugar, malic acid, yeast assimilable nitrogen, $SO_2$, pH, and alcohol) were analyzed by SARCO laboratory (Biolaffort, Floirac, France), using the official methods or those recommended by the International Organization of Viticulture and Wine (OIV).

### 2.3. Volatile compound analyses

#### 2.3.1. Chemicals

Compounds used as internal standards, including octan-3-ol (99%), were obtained from Sigma-Aldrich (Steinheim, Germany); deuterated compounds, including ethyl butyrate-4,4,4-$d_3$ (>99%), ethyl hexanoate-$d_{11}$ (>98%), ethyl octanoate-$d_{15}$ (>98%), and ethyl *trans*-cinnamate-$d_5$ (phenyl-$d_5$) (>99%), were obtained from Cluzeau (Sainte-Foy-la-Grande, France). Dichloromethane (>99%) and sodium chloride (norma pure) were from VWR Chemicals (Fontenay-sous-Bois, France), anhydrous sodium sulfate (99%) was supplied by Scharlau Chemie (Sentmenat, Spain), and ethanol ($\geq$99.9%) was obtained from Merck (Damstadt, Germany). R-ethyl 2-hydroxy-3-methylbutanoate (>98%), S-ethyl 2-hydroxy-3-methylbutanoate (>98%), R-ethyl 2-hydroxy-4-methylpentanoate (>98.7%), and S-ethyl 2-hydroxy-4-methylpentanoate (>98.7%) were synthesized by Hangzhou Imaginechem Co., (Hangzhou, China).