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Effects of different techniques of malolactic fermentation induction on diacetyl metabolism and biosynthesis of selected aromatic esters in cool-climate grape wines

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Abstract

We examined the effects of different malolactic bacteria fermentation techniques, including a spontaneous process – a variant with a high risk of undesirable metabolites – on the bioconversion of aromatic compounds in cool-climate grape wines.

During three wine seasons, red and white grape wines were produced by three different methods of malolactic fermentation induction: coinoculation, sequential inoculation, and spontaneous malolactic fermentation. Volatiles (diacetyl and the products of its metabolism, as well as selected ethyl fatty acids esters) were extracted by solid phase microextraction. Compounds were identified with multidimensional gas chromatograph GCxGC-ToFMS with ZOEX cryogenic (N_2) modulator. Sensory evaluation of the wines was also performed.

We found, that the fermentation-derived metabolites examined in this study were affected by the malolactic bacteria inoculation regime. Quantitatively, ethyl lactate, diethyl succinate and ethyl acetate dominated as esters with the largest increase in the concentration. The total concentration of ethyl esters was highest for the coinoculation scenario. Whereas the highest concentration of diacetyl was noted for the spontaneous processes.

A controlled malolactic fermentation, especially using the coinoculation technique, can be proposed as a safe and efficient enological practice for producing quality, cool-climate grape wines enriched with fruity, fresh and floral aromas.

Key words: malolactic fermentation, coinoculation, diacetyl, esters, aromatic compounds, grape wine

1. Introduction

Malolactic fermentation (MLF) is a secondary fermentation that takes place after alcoholic fermentation in the process of grape wine production. It is used in the majority of red wines, some white wines, and generally for wines with enhanced acidity. The process is conducted by malolactic bacteria (MLB), most often strains of *Oenococcus oeni*, and involves the decarboxylation of L-malic acid into L-lactic acid. As a result of this bioconversion, a noticeable reduction in the total acidity of the wine can be achieved. The MLB utilize not only malic acid but also the residual sugars left by yeast after the alcoholic fermentation. This reduces the potential carbon source for spoilage microflora and increases microbial stabilization of the wine [1-5].

MLF is also a process known to modify the aroma profile of the wine thought biosynthesis or bioconversion of flavor-active compounds. Some authors suggest that MLF enhances fruity notes and buttery aromas, while reduces vegetative green and grassy aromas [4, 6]. Other researchers have postulated that MLF results in a creamier palate, less fruit intensity, and more nutty, vanilla, toasty butter, and wet leather aromas [7].

A range of factors, including grape cultivar, the bacterial culture used and the conditions of vinification have been described as having an effect on the character of wines treated by MLF [8, 9]. However, little is known about the effect of different timings of malolactic bacteria inoculation on wine aroma modification. To our knowledge, only a few research groups [9-12] have compared different MLB inoculation timings and determined their effect on the differentiation of quality and quantity of the aroma compounds. However, non of these authors examined the spontaneous variant of MLF, which involves a risk of producing undesirable aroma compounds in wines.

Our research focused on selected aroma compounds (diacetyl and its metabolic products, as well as selected ethyl fatty acids esters) synthesized during malolactic fermentation in white and red grape wines produced by three different methods of inoculation: 1) coinoculation (COI), where the yeast and bacteria were inoculated at the same time, 2) sequential inoculation (SEQI), where malolactic fermentation was induced at the end of alcoholic fermentation, and 3) spontaneous malolactic fermentation (SPONT), where MLB inoculation was not performed. This is the first study to evaluate the effects of different timings of MLB inoculation – including the spontaneous process which involves a significant risk of producing undesirable compounds – on the bioconversion of aromatic compounds in red and white grape wine. The experiment was performed during three wine seasons.

2. Results and Discussion

2.1. Ethyl fatty acid esters

The synthesis and hydrolysis of esters during the malolactic fermentation of wines has been described by many research groups, but there is a disagreement concerning the influence of this secondary fermentation action on the final concentration of esters. The majority of authors indicate that there is a significant enhancement in the esters content of wines that have undergone MLF [8, 10-18], but other researchers have presented a decreasing trend [19, 20, 21]. Malolactic bacteria strain selection has also been described as an important factor that determines the final concentrations of esters [12, 13, 15, 16].

Ethyl fatty acids esters are compounds considered to be of primary importance for the aroma of wines. Of these, we analyzed: ethyl lactate, diethyl succinate, ethyl propanoate, ethyl hexanoate, ethyl octanoate, and acetate esters (ethyl acetate, isoamyl acetate and, 2-phenethyl acetate).

Ethyl lactate, one of the most characteristic aromatic compounds produced during malolactic fermentation, is synthetized in the course of the esterification of ethanol (produced by yeast during alcoholic fermentation) and lactate (produced by malolactic bacteria during secondary fermentation). When the malolactic process takes place, the concentration of ethyl lactate progressively increases. This is beneficial to the wine bouquet due to its fruity, buttery, and creamy aromas, and it also contributes to the sensations of roundness in the mouth [15, 22]. Some authors suggest that the insensitivity of ethyl lactate biosynthesis depends on the strain of *O. oeni* used [9, 14, 16, 23]. On the other hand, some other authors did not register any dependence of ethyl lactate biosynthesis on the bacteria strain [12].

In our study, the concentration of ethyl lactate after alcoholic fermentation lay in the 8.54–14.44 g/L range (Table 1), and this significantly increased as a result of malolactic fermentation. The highest concentration of ethyl lactate was registered in the coinoculation variant (at 132.57 to 173.76 mg/L). After sequential inoculation of MLB and spontaneous MLF, the concentrations of ethyl lactate were significantly lower (41.64–115.63 mg/L), though still several times higher than without MLF (Table 1).

Literature studies have reported a wide range of ethyl lactate concentrations in wines that have undergone MLF. Values significantly higher than ours were descreibed by Knoll et al. (up to 440 mg/dm^3) [16], Pozo-Bayon et al. (up to 235 mg/dm^3) [14], and Valade and Laurent

Table 1. Concentrations (mg/L) of esters in white and red grape wines produced with different MLB inoculation scenarios

		Ethyl lactate	Ethyl propanoate	Ethyl hexanoate	Ethyl octanoate	Diethyl succinate	Ethyl acetate	Isoamyl acetate	2-phenethyl acetate	Sum of esters Total / without ethyl lactate and ethyl acetate
White wine	Chardonnay									
	AF	8.54 ± 0.31^{d}	0.31 ± 0.03^{b}	0.83 ± 0.07^{a}	1.17 ± 0.09^{a}	0.68 ± 0.01^{d}	63.43 ± 1.27^{c}	0.56 ± 0.07^{b}	0.23 ± 0.01^{b}	75.75 / 3.78
	COI	134.97 ± 3.93^{a}	0.36 ± 0.05^{a}	0.86 ± 0.04^{a}	1.11 ± 0.09^{a}	2.63 ± 0.02^{a}	51.67 ± 2.03^{d}	0.42 ± 0.04^{c}	0.69 ± 0.03^{a}	192.71 / 6.07
	SEQI	115.63 ± 5.59^{b}	0.42 ± 0.04^{a}	0.79 ± 0.09^{a}	1.14 ± 0.04^{a}	1.42 ± 0.06^{b}	68.44 ± 3.16^{b}	0.63 ± 0.08^{b}	0.62 ± 0.08^{a}	189.09 / 5.02
	SPONT	72.43 ± 2.64^{c}	0.39 ± 0.05^{a}	0.81 ± 0.05^{a}	1.12 ± 0.06^{a}	0.89 ± 0.03^{c}	88.41± 2.87^{a}	0.89 ± 0.03^{a}	0.73 ± 0.05^{a}	165.67 / 4.83
	Kerling									
	AF	8.62 ± 0.68^{d}	0.14 ± 0.04^{b}	0.53 ± 0.03^{a}	0.88 ± 0.05^{a}	0.32 ± 0.05^{d}	72.66 ± 3.016^{b}	0.66 ± 0.02^{b}	0.33 ± 0.02^{b}	84.14 / 2.86
	COI	132.57 ± 4.04^{a}	0.27 ± 0.07^{a}	0.57 ± 0.05^{a}	0.93 ± 0.04^{a}	1.14 ± 0.03^{a}	58.12 ± 5.37^{d}	0.49 ± 0.04^{c}	0.49 ± 0.07^{a}	194.58 / 3.89
	SEQI	111.66 ± 5.84^{b}	0.23 ± 0.03^{a}	0.53 ± 0.04^{a}	0.89 ± 0.06^{a}	0.55 ± 0.05^{b}	63.27 ± 5.82^{c}	0.51 ± 0.03^{c}	0.52 ± 0.03^{a}	178.16 / 3.23
	SPONT	71.04 ±3.61^{c}	0.21 ± 0.04^{a}	0.55 ± 0.04^{a}	0.85 ± 0.08^{a}	0.49 ± 0.05^{c}	110.26 ± 4.93^{a}	0.94 ± 0.03^{a}	0.55 ± 0.04^{a}	184.89 / 3.59
Red wine	Pinot noir A									
	AF	14.44 ± 0.86^{d}	0.51 ± 0.03^{b}	0.74 ± 0.06^{a}	1.23 ± 0.04^{b}	0.54 ± 0.03^{d}	77.42 ± 3.28^{c}	0.33 ± 0.05^{c}	0.45 ± 0.03^{b}	95.66 / 3.8
	COI	151.25 ± 4.23^{a}	0.73 ± 0.05^{a}	0.77 ± 0.07^{a}	1.31 ± 0.03^{a}	2.07 ± 0.09^{a}	71.33 ± 6.71^{c}	0.28 ± 0.06^{c}	0.92 ± 0.03^{a}	228.66 / 6.08
	SEQI	97.32 ± 6.59^{b}	0.77 ± 0.03^{a}	0.74 ± 0.05^{a}	1.26 ± 0.02^{b}	1.32 ± 0.07^{b}	81.58 ± 4.55^{b}	0.44 ± 0.02^{b}	0.88 ± 0.04^{a}	184.31 / 5.41
	SPONT	64.97 ± 4.44^{c}	0.74 ± 0.03^{a}	0.71 ± 0.05^{a}	1.22 ± 0.05^{b}	0.76 ± 0.06^{c}	94.11 ± 5.19^{a}	0.76 ± 0.03^{a}	0.94 ± 0.06^{a}	164.21 / 5.13
	Pinot noir B									
	AF	11.43 ±0.41^{d}	0.39 ± 0.03^{b}	0.66 ± 0.08^{a}	1.08 ± 0.06^{a}	0.47 ± 0.03^{d}	69.31 ± 6.07^{c}	0.66 ± 0.09^{b}	0.37 ± 0.03^{b}	84.37 / 3.63
	COI	173.76 ± 5.72^{a}	0.62 ± 0.03^{a}	0.71 ± 0.04^{a}	1.14 ± 0.08^{a}	1.96 ± 0.07^{a}	66.93 ± 4.31^{c}	0.47 ± 0.03^{c}	0.84 ± 0.07^{a}	246.43 / 5.74
	SEQI	91.26 ± 3.66^{b}	0.60 ± 0.04^{a}	0.68 ± 0.05^{a}	1.09 ± 0.05^{a}	0.88 ± 0.05^{b}	75.21 ± 4.89^{b}	0.58 ± 0.09^{b}	0.79 ± 0.09^{a}	171.09 / 4.62
	SPONT	65.33 ± 3.43^{c}	0.61 ± 0.03^{a}	0.65 ± 0.06^{a}	1.06 ± 0.09^{a}	0.61 ± 0.07^{c}	88.13 ± 5.26^{a}	0.83 ± 0.04^{a}	0.87 ± 0.07^{a}	158.09 / 4.63
	Rondo									
	AF	9.01 ± 0.56^{d}	0.27 ± 0.03^{b}	0.64 ± 0.03^{a}	0.94 ± 0.05^{a}	0.51 ± 0.06^{d}	79.17 ± 4.11^{c}	0.31 ± 0.02^{c}	0.41 ± 0.02^{b}	91.26 / 3.08
	COI	137.41 ± 6.92^{a}	0.34 ± 0.04^{a}	0.69 ± 0.04^{a}	0.99 ± 0.04^{a}	1.88 ± 0.11^{a}	78.33 ± 4.85^{c}	0.44 ± 0.07^{b}	0.83 ± 0.05^{a}	220.91 / 5.17
	SEQI	84.78 ± 4.47^{b}	0.38 ± 0.02^{a}	0.66 ± 0.03^{a}	0.93 ± 0.07^{a}	1.12 ± 0.08^{b}	86.87 ± 3.17^{b}	0.38 ± 0.06^{b}	0.81 ± 0.09^{a}	175.93 / 4.28
	SPONT	41.64 ± 4.33^{c}	0.35 ± 0.03^{a}	0.62 ± 0.07^{a}	0.93 ± 0.08^{a}	0.74 ± 0.06^{c}	119.33 ± 4.69^{a}	0.79 ± 0.03^{a}	0.88 ± 0.05^{a}	165.28 / 4.31

a, b, c, d – denotes statistically significant differences ($p<0.05$) between the different inoculation scenarios

(up to 190 mg/dm^3) [24]. Our results are similar to those of Lloret et al. who found ethyl lactate in wines after MLF at concentrations ranging from 90 to150 mg/L [25]. On the other hand, Fleet [26] and Maicas et al. [13] described standard concentrations for ethyl lactate in red wines of up to 50 mg/dm^3. The aroma threshold for ethyl lactate has been determined to be 110 mg/L [25]. In line with this, ethyl lactate was discernible in our experiment only in wines in which the yeast and MLB had been coinoculated.

Diethyl succinate is another volatile compound that contributes to wine aroma. Succinic acid (a by-product of microbial α-ketoglutarate metabolism) is esterified to diethyl succinate, which brings fruity melon notes. This compound occurs naturally in apples, grapes, and cocoa. Its odor threshold has been set on 1.2 mg/L [27]. When only alcoholic fermentation was performed in our experiments, the concentration of this compound was found to be under the threshold value (up to 0.68 mg/L). In the wines that underwent MLF, the concentration of diethyl succinate was in the 0.49–2.63 mg/L range (Table 1). The highest concentration of diethyl succinate was always observed in the case of coinoculated wines. Similar observations have been described by Knoll et al. [9], who noted that sequential inoculations resulted in lower concentrations of ethyl lactate and diethyl succinate than in the case of coinoculation.

With regard to other esters, we observed no effect of the malolactic process on the biosynthesis of **ethyl hexanoate** (fruity, strawberry, green apple aromas) or **ethyl octanoate** (fruity, sweet, banana, pear aromas). On the other hand, all wines that underwent malolactic fermentation showed a significantly enhanced concentration of **ethyl propanoate** (pineapple aroma) (Table 1). Different observations have been described by Knoll et al. [9]. In their study, a decrease in the concentrations of ethyl hexanoate and ethyl octanoate was seen while, in agreement with our study, there was an increase in ethyl propanoate concentration after MLF. Summarizing the fluctuations in ethyl fatty acids esters, ethyl lactate and diethyl succinate quantitatively dominated and were the esters showing the greatest increase in concentration. Similarly to our results, a significant increase in the concentration of ethyl lactate, ethyl propanoate, and diethyl succinate was also observed after MLF during vinification of Riesling wine [9, 16, 28], Aglianico wine [15], Tempranillo and Merlot wine [12, 14].

A second important group of wine esters is the acetate esters group, from of we have selected the most common three: ethyl acetate, isoamyl acetate and 2-phenethyl acetate.

Ethyl acetate is synthetized from ethanol and acetic acid, which are key metabolites in the vinification process. When its concentration does not exceed 100 mg/L, a desirable and fruity aroma enriches the wine. Its presence in higher concentrations leads to solvent, nail, varnish, and chemical aromas [4, 13, 29]. I**soamyl acetate** introduces pleasant fruity notes (mostly banana) to wine aroma profiles. This ester is formed from isoamyl alcohol and acetic acid, intermediate metabolites of alcoholic and malolactic fermentation. We observed that the highest concentrations of both ethyl acetate (88.13–119.33 mg/L) and isoamyl acetate (0.76–0.94 mg/L) were always noted with spontaneous MLF (Table 1). This may be due to the notably higher concentrations of volatile acidity (as acetic acid) for this variant of vinification, as described in our previous research [30]. The different inoculation scenarios significantly affected the final concentration of ethyl acetate and isoamyl acetate in the white and red wines, but the concentrations did not exceed 86.87 and 0.66 mg/L respectively (Table 1). Maicas et al. [13] found additionally that the production of these compounds was dependent on the MLB strain. In their study, the concentrations after MLF varied from 36.94 to 216.04 mg/L of ethyl acetate and from 0.25 to 0.68 mg/L of isoamyl acetate.

2-phenethyl acetate is a volatile metabolite which brings to wine floral, honey, and raspberry aromas. In all the examined wines, malolactic bioconversion significantly increased (by almost a factor of two) its concentration over that of the control process (only alcoholic fermentation). However, no effect of inoculation scenario on the biosynthesis of 2-phenethyl acetate was noted. Knoll et al. [9] found that wines with sequential MLF had lower concentrations of acetate esters and ethyl esters than coinoculated wines. Esters concentrations were also affected by the bacteria strain used.

2.2. Diacetyl and its metabolic products

Diacetyl (2,3-butanedione) is produced by yeast during alcoholic fermentation by a pathway linked to the amino acid metabolism. At the end of alcoholic fermentation, diacetyl is reduced by diacetyl reductase to acetoin (3-hydroxy-2-butanone) and to 2,3-butanediol. At this stage of the winemaking process, the concentration of diacetyl thus has no olfactive effect. Significantly higher amounts of diacetyl can be produced during malolactic fermentation as an intermediate product in citric acid metabolism [4, 8, 13, 29, 31].

In the course of the MLB carbohydrate metabolism pathway, pyruvate is reduced to lactate. However, when the concentration of residual sugars is too low, citric acid begins to be utilized as a carbon source and additional pyruvate is synthetized. This pyruvate is than a

precursor in the process of diacetyl production. The utilization of citric acid starts simultaneously with malic acid degradation, but it is a very slow process. It is particularly observed in states of sugar deficiency [31]. Subsequently, diacetyl because of its low chemical stability, can be easy transformed into acetoin and 2,3-butanediol [4, 7, 32].

Malolactic fermentation is thus highly recommended to allow diacetyl to enter into the wine aroma profile. During the secondary fermentation diacetyl, acetoin, and 2,3-butanediol appear in different concentrations, which has a direct effect on wine aroma. Each of these compounds has a different odor detection threshold. Acetoin and 2,3-butanediol have significantly higher threshold values of perceptibility than diacetyl, at an average of 150 and 600 mg/L, respectively [7, 33]. The aroma detection threshold value for diacetyl depends on the type and style of wine. In general, for good quality young red wines, it ranges from about 0.2 to about 1.84 mg/L, and for aged red wines from 1.25 to 3.39 mg/L [33].

Table 2. Concentrations (mg/L) of diacetyl, acetoin and 2,3-butanediol in white and red grape wines produced with different MLB inoculation scenarios

		2,3-butanedione (diacetyl)	3-hydroxy-2-butanone (acetoin)	2,3-butanediol
White wines	Chardonnay 2009			
	AF	1.13 ± 0.22^{d}	0.86 ± 0.06^{d}	254.32 ± 11.34^{d}
	COI	2.19 ± 0.15^{c}	3.11 ± 0.39^{c}	331.56 ± 15.33^{c}
	SEQI	3.42 ± 0.19^{b}	5.95 ± 0.26^{b}	479.93 ± 9.14^{b}
	SPONT	7.44 ± 0.08^{a}	8.79 ± 0.42^{a}	712.42 ± 23.65^{a}
	Kerling 2010			
	AF	0.94 ± 0.24^{d}	0.73 ± 0.04^{d}	165.36 ± 12.75^{d}
	COI	3.02 ± 0.16^{c}	4.31 ± 0.22^{c}	349.62 ± 16.83^{c}
	SEQI	4.09 ± 0.08^{b}	6.18 ± 0.42^{b}	513.88 ± 8.31^{b}
	SPONT	8.33 ± 0.22^{a}	11.05 ± 0.98^{a}	783.63 ± 10.63^{a}
Red wines	Pinot noir 2009			
	AF	1.71 ± 0.15^{d}	1.62 ± 0.17^{d}	288.97 ± 12.06^{d}
	COI	3.80 ± 0.19^{c}	5.11 ± 0.36^{c}	361.35 ± 9.72^{c}
	SEQI	5.24 ± 0.16^{b}	6.94 ± 0.45^{b}	647.55 ± 17.83^{b}
	SPONT	9.22 ± 0.23^{a}	12.39 ± 0.41^{a}	806.37 ± 14.97^{a}
	Pinot noir 2012			
	AF	1.31 ± 0.14^{d}	1.04 ± 0.12^{c}	267.42 ± 8.53^{d}
	COI	4.06 ± 0.09^{c}	6.48 ± 0.48^{b}	493.52 ± 11.84^{c}
	SEQI	5.91 ± 0.11^{b}	7.17 ± 0.56^{b}	631.67 ± 17.59^{b}
	SPONT	8.72 ± 0.15^{a}	12.52 ± 0.46^{a}	718.13 ± 13.77^{a}
	Rondo 2012			
	AF	1.72 ± 0.05^{d}	1.53 ± 0.14^{d}	264.94 ± 6.48^{d}
	COI	3.81 ± 0.09^{c}	4.97 ± 0.32^{c}	355.13 ± 15.83^{c}
	SEQI	5.50 ± 0.13^{b}	7.03 ± 0.26^{b}	584.42 ± 18.28^{b}
	SPONT	8.80 ± 0.07^{a}	11.43 ± 0.31^{a}	652.36± 15.75^{a}

a, b, c, d – denotes statistically significant differences ($p<0.05$) between the different inoculation scenarios

The lowest concentration of diacetyl noted in our study occurred in the case of alcoholic fermentation without MLF. The concentration of diacetyl synthesized by the yeasts then

ranged from 0.94 to 1.72 mg/L (Table 2) being too low to have an impact on the wine aroma (Figure 1). A significantly higher concentration of diacetyl was noted with malolactic fermentation. In the coinoculation variant, this ranged from 2.19 to 4.06 mg/L, and in the sequential inoculation it varied from 3.42 to 5.91 mg/L. For these wines, light, pleasant buttery and nutty aromas were perceptible (Figure 1). The highest concentration of diacetyl was observed for the spontaneous malolactic fermentation (7.44 to 9.22 mg/L); this was characterized as an intensive and unacceptable buttery aroma.

According to Lerm et al. [34], the accumulation of diacetyl and acetoin depends on the dynamics of malolactic fermentation. The higher, the MLF rate, the lower the concentration of diacetyl and acetoin. In our study, the dynamics of MLF were as follows: COI < SEQI < SPONT [30]. This explains why less diacetyl was observed in the case of coinoculation, and why higher concentration were seen for the SEQI and SPONT variants, in which the dynamics of the MLF process were significantly lower.

The presence of oxygen during MLF can also affect the diacetyl content of wine. This is directly associated with the oxidation of α-acetolactate to diacetyl [29, 34]. In our study, microoxygenation was performed to support the initiation of spontaneous MLF (Table 3) [30]. The additional amount of oxygen present during the vinification process could thus also have led to the significantly higher concentration of diacetyl in this variant (Table 2).

The products of diacetyl degradation, acetoin (3-hydroxy-2-butanone) and 2,3-butanediol, were also evaluated. In all the wines, the lowest levels of both compounds were noted when only alcoholic fermentation was performed; this concentration ranged from 0.73 to 1.62 mg/L of acetoin, and from 165 to 288 mg/L of 2,3-butanediol (Table 2). Similarly, as in the case of diacetyl, MLF significantly increased the concentration of both metabolites. The highest concentrations were noted for the spontaneous process, in which the biosynthesis yield was as high as 8.79–12.52 mg/L of acetoin and 652–806 mg/L of 2,3-butanediol. Coinoculation resulted in significantly lower values of both metabolites than sequential inoculation. According to Francis and Newton [33] and Bartowsky and Henschke [7], acetoin levels remained under the sensory threshold, but 2,3-butanediol reached a concentration of sensory significance for wine (over 600 mg/dm^3), though only in the spontaneous variants. This was reflected in the sensory evaluation of the produced wines (Figure 1). Generally, 2,3-butanediol is not expected to affect the sensory qualities of wine appreciably [35] but we did note a bitter taste in these variants. Some authors have also described very low or undetectable levels of diacetyl in wines that had undergone the MLF [36]. They suggest, that

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this may be a result of the enzymatic reduction of diacetyl to 2,3-butanediol. Acetoin, the other intermediate metabolite of diacetyl involved in the same metabolic pathway, is also reduced to 2,3-butanediol. This may explain the high levels of 2,3-butanediol found in our wines.

2.3. Sensory evaluation

The sensory evaluation indicated that the timing and method of MLB inoculation significantly affected the taste and aroma of the wines. In general, malolactic fermentation diversified the wine aroma profile (Figure 1). The coinoculated wines were noted to be higher in fruity, fresh, and floral sensations than the wines which had used sequential MLF. The spontaneous process was perceived as producing wines with more buttery and bitter notes. Pleasant balanced buttery and nutty aromas were also found in the coinoculated wines. The sequential and spontaneous regimes had no nutty aromas but instead strong buttery aromas were noted.

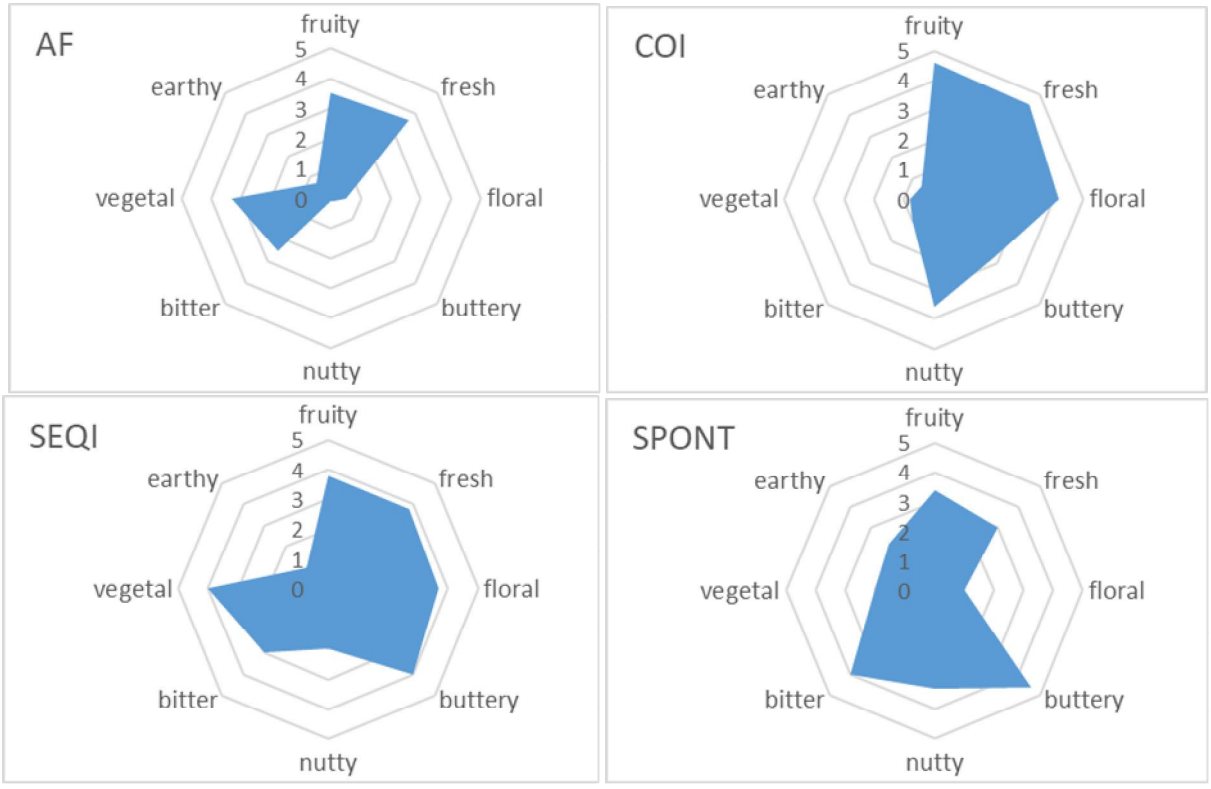

Figure 1. Descriptive sensory evaluation of the red an white wines produced with different inoculation regimes. Scale 0 – 5: 0-lack, 1-very light, 2- light, 3- noticeably, 4- intensive, 5-very intensive sensibility (value 5 is the most desirable for fruity, fresh and floral aromas, but undesirable and not acceptable for buttery, nutty, bitter, vegetal, earthy aromas)

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3. Materials and methods

3.1. Microorganisms

Saccharomyces cerevisiae yeast (Lalvin EC-118, Lallemand, USA) and *Oenococcus oeni* bacteria (Lalvin VP41, Lallemand, USA) were used in the experiments. Before use, the preparates were rehydrated according to the producer's instructions.

3.2. Grape variety

During three wine seasons (2009, 2010, and 2012), two white varieties, *Chardonnay* and *Kerling,* and two red varieties, *Pinot noir* and *Rondo,* were used to produce the experimental wines. The grapes were obtained from Mierzecin Vineyard in Poland. Typically for grapes from cool-climate countries the musts were characterised with enhanced total acidity (9.38-12.14 g/L, as tartaric acid) and low pH (3.19-3.64) [30], what significantly singularize them from others studied and described in the literature [9-12, 37, 38]. Detailed chemical characterisation of the grape musts is presented in our previous study [30].

3.3. Parameters of the vinification process

The wines were produced on a laboratory scale in 15-liter glass containers. Four different variants of the vinification process were performed: 1) alcoholic fermentation only, as a control (AF); 2) coinoculation (COI), where the yeast and bacteria were inoculated at the same time; 3) sequential inoculation (SEQI), where malolactic fermentation was induced at the end of alcoholic fermentation; and 4) spontaneous malolactic fermentation (SPONT), where we did not perform MLB inoculation. The consecutive steps of the vinification process are presented in Table 3 and follow those of our previous study [30].

The process was started with the inoculation of yeast in the first day for all variants. The timing of malolactic bacteria inoculation was dependent on the variant: this was done on the first day (together with yeasts) in coinoculation, and on the seventh day of winemaking for sequential inoculation. To avoid spontaneous MLF in the AF variant, an additional sulfitation process was performed after one month of vinification (10 g/hL of $K_2S_2O_5$). In the spontaneous scenario, MLF was induced by microoxygenation, supplementation with bacteria nutrients, and lower sulfitation. No malolactic bacteria starter culture was added in this case.

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Table 3. Schedule of white and red wine production [30]

	AF	COI	SEQI	SPONT
First Day (temp. 20-22°C)				
White wine:				
Crushing and destemming , pressing	+	+	+	+
Sulfitation (g $K_2S_2O_5$/hL)	5	5	5	3
Yeast inoculation (30 g/hL)	+	+	+	+
Bacteria inoculation (1 g/hL)*	-	+	-	-
Red wine:				
Crushing and destemming,	+	+	+	+
Sulfitation (g $K_2S_2O_5$/hL)	5	5	5	3
Yeast inoculation (30g/hL)	+	+	+	+
Bacteria inoculation (1 g/hL)*	-	+	-	-
After 7 days (temp. 20-22°C)				
White wine:				
Racking	+	+	+	+
Bacteria inoculation (1 g/hL)	-	-	+	-
Micro-oxygenation	-	-	-	+
Nutrient supplementation for bacteria**	-	-	-	+
Red wine:				
pressing	+	+	+	+
Bacteria inoculation (1 g/hL)	-	-	+	-
Microoxygenation	-	-	-	+
Nutrient supplementation for bacteria **	-	-	-	+
After 1-st month (temp. 15-17°C)				
Racking	+	+	+	+
Sulfitation (g $K_2S_2O_5$/hL)	10***	-	-	-
After 3 months (temp. 7-10°C)				
Racking	+	+	+	+
Sulfitation (g $K_2S_2O_5$/hL)	3	3	3	3
After 6 months (temp.7-10°C)				
Racking	+	+	+	+
Bottling	+	+	+	+

*after 24h of sulfitation; ** Optimalo Plus (Lallemand, USA) 20g/hL; *** for inhibiting MLF in the AF

3.4. Analysis of volatile compounds

The volatiles were extracted by solid phase microextraction using a 2cm carboxene/pdms/dvb fiber (Supelco) with a CTC Combipal autosampler (Agilent Technologies). For each analysis, a 10 mL sample of wine was placed into 20 mL vials, spiked with an internal standard ([$^2H_8$]-naphthalene), sealed with PTFE/silicon septa caps, and incubated for 2 minutes at 50 °C prior to extraction. Compounds were extracted from the headspace at 50 °C for 35 minutes. Compounds wee identified using multidimensional gas GCxGC-ToFMS chromatography with a ZOEX cryogenic (N_2) modulator (Pegasus IV, LECO, St. Joseph, MI). The GC was equipped with a DB-5 column (30 m x 0.25 mm x 0.25 µm) and has a Supelcowax 10 (1 m x 0.1 mm x 0.1 µm) as a second column with a helium flow rate of 0.8 mL/min. For the two-dimensional analysis, the modulation time was optimized and set at 3 seconds, and the mass spectra were collected at a rate of 150 scans/s. The transfer line was heated to 280 °C and the

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ion source was heated to 220 °C, respectively. The injector temperature was set to 280 °C for the Carboxen/PDMS/DVB fiber. During the injection, the fiber was maintained for 5 min in splitless mode and than for 1 minute in the split mode (20:1). Identification of volatiles was performed by comparison of retention indices and mass spectra of eluting compounds to those of the NIST 05 library match. The calculation was done using Chroma TOF software (version 4.23) upgraded with additional post data processing software Statistical Compare (LECO, St. Joseph, MI) for calculation of Fisher ratio. Semiquantification of the volatile compounds was performed using the internal standard; they thus do not represent the absolute amount of the compound present in the wine samples, but were instead calculated and used to observe the differences between the wine samples. Each measurement was repeated three times.

3.5. Sensory evaluation

Sensory analysis was performed in order to evaluate the differences between the wines obtained with different inoculation scenarios. The sensory panel members included 120 persons between 24 and 55 years old. They evaluated the wine samples using a 0–5 point scale (0 = very low discernible aroma; 5 = very intensive discernible aroma).

3.6. Statistical analysis

All data are presented as the mean value of at least three repetitions ± standard deviations. Statistical data analysis was performed using analysis of variance (ANOVA) in Statistica V.7 (Statsoft Inc., USA). Turkey's test was used for significantly different samples ($p < 0.05$).

Conclusion

The fermentation-derived metabolites examined in this study were affected by the malolactic bacteria inoculation regime. The total concentration of the analyzed ethyl esters was highest for the coinoculation case. Quantitatively, ethyl lactate, diethyl succinate and ethyl acetate dominated as the esters that showed the greatest increase in concentration. Excess of diacetyl, perceive as a serious danger of MLF which can have an adverse effect on the quality of wine, was noted only for spontaneous processes. Whereas, coinoculation was a treatment with the unbeatably balanced nutty notes.

The present investigations, highlighted that controlled malolactic fermentation, and specially the coinoculation technique, can be proposed as a safe and efficient enological practice for producing quality grape wines. Our results, noted in the three following wine

seasons, clearly indicate that simultaneous inoculation of yeast and bacteria offer not only dynamic deacidification of low pH grape wines [30], but also modified qualitatively and quantitatively the profile of volatile compounds enriching the cool-climate and low aromatic wines in fruity, fresh and floral aromas.

Human / animal rights

This article does not contain any studies with human or animal subjects.

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Author Contributions

Małgorzata Lasik-Kurdyś (leader of the project) conceived, designed and performed the experiments; analyzed the data, wrote the manuscript. Małgorzata Majcher analyzed the volatiles in the wine samples. Jacek Nowak designed the experiment. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Davis, C.R.; Wibowo, D.; Eschenbruch, R.; Lee, T.H.; Fleet, G.H. Practical implications of malolactic fermentation: A review. Am J Enol Vitic **1985**, 36(4), 290-301.

[2] Davis, C.R.; Wibowo, D.J.; Lee, T.H.; Fleet, G.H. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. Appl Environ Microbiol **1986**, 51(3), 539–545.

[3] Wibowo, D.; Eschenbruch, R.; Davis, C.R.; Fleet, G.H.; Lee, T.H. Occurrence and growth of lactic acid bacteria in wine: A review. Am J Enol Vitic **1985**, 36, 302-313.

[4] Henick-Kling, T. Malolactic fermentation. In: Wine microbiology and biotechnology, ed. Fleet, G.H.; Harwood Academic Publishers, Switzerland **1993;** pp. 289-327; ISBN 0-415-27850-3.

[5] Versari, A.; Parpinello, G.P.; Cattaneo, M. *Leuconostoc oenos* and malolactic fermentation in wine: a review. J Ind Microbiol Biotechnol **1999**, 23, 447–455. https://doi.org/10.1038/sj.jim.2900733

[6] Liu, S.Q. A review: Malolactic fermentation in wine – beyond deacidification. J Appl Microbiol **2002**, 92(4), 589-601. https://doi.org/10.1046/j.1365-2672.2002.01589.x

[7] Bartowsky, E.J.; Henschke, P.A. The 'buttery' attribute of wine – diacetyl – desirability, spoilage and beyond. Int J Food Microbiol **2004**, 96(3), 235-252. https://doi.org/10.1016/j.ijfoodmicro.2004.05.013

[8] Antalick, G.; Perello, M.C.; de Revel, G. Characterization of fruity aroma modifications in red wines during malolactic fermentation. J Agric Food Chem **2012**, 60(50), 12371-12383. DOI: 10.1021/jf303238n

[9] Knoll, C.; Fritsch, S.; Schnell, S.; Grossmann, M.; Krieger-Weber, S.; du Toit, M.; Rauhut, D. Impact of different malolactic fermentation inoculation scenarios on *Riesling* wine aroma. World J Microbiol Biotechnol **2012**, 28(3), 1143–1153. DOI: 10.1007/s11274-011-0917-x

[10] Tristezza, M.; di Feo, L.; Tufariello, M.; Grieco, F.; Capozzi, V.; Spano, G.; Mita, G.; Grieco, F. Simultaneous inoculation of yeasts and lactic acid bacteria: effects on fermentation dynamics and chemical composition of Negroamaro wine. Food Sci Technol **2016**, 66, 406–412. https://doi.org/10.1016/j.lwt.2015.10.064

[11] Abrahamse, C.E.; Bartowsky, E.J. Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition. World J Microbiol Biotechnol **2012**, 28(1), 255–265. https://doi.org/10.1007/s11274-011-0814-3

[12] Izquierdo Canas, P.M.; Perez Martin, F.; Garcia Romero, E.; Sesena Prieto, S.; Palop Herreros, M.L. Influence of inoculation time of an autochthonous selected malolactic bacterium on volatile and sensory profile of Tempranillo and Merlot wines. Int J Food Microbiol **2012**, 156(3), 245–254. https://doi.org/10.1016/j.ijfoodmicro.2012.03.033

[13] Maicas, S.; Gil, J.V.; Pardo, I.; Ferrer, S. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Rech Int **1999**, 32(7), 491-496. DOI: 10.1016/S0963-9969(99)00122-2

[14] Pozo-Bayon, M.A.; Alegria, E.G.; Polo, M.C.; Tenorio, C.; Martin-Alvarez, P.J.; Calvo de la Banda, M.T.; Ruiz-Larrea, F.; Moreno-Arribas, M.V. Wine volatile and amino acid composition after malolactic fermentation: Effect of *Oenococcus oeni* and *Lactobacillus plantarum* Starter Cultures. J Agric Food Chem **2005**, 53, 8729-8735. DOI: 10.1021/jf050739y

[15] Ugliano, M.; Moio, L. Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. J Agric Food Chem **2005**, 53, 10134-10139. DOI: 10.1021/jf0514672

[16] Knoll, C.; Fritsch, S.; Schnell, S.; Grossmann, M.; Rauhut, D.; du Toit, M. Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines. Food Sci Technol **2011**, 44(10), 2077-2086. https://doi.org/10.1016/j.lwt.2011.05.009

[17] Izquierdo Canas, P.M.; Garcia Romero, E.; Perez Martin, F.; Sesena Prieto, S.; Heras Manso, J.M.; Palop Herreros, M.L. Bahaviour during malolactic fermentation of three strains of Oenococcus oeni used as direct inoculation and acclimatization cultures. S Afr J Enol Vitic **2013**, 34, 1-9. http://dx.doi.org/10.21548/34-1-1023

[18] Izquierdo Canas, P.M.; Mena Morales, A.; Garcia Romero, E. Malolactic fermentation before and during wine aging in barrels. Food Sci Technol **2016**, 66, 468-474. https://doi.org/10.1016/j.lwt.2015.11.003

[19] Sauvaugeot, F.; Vivier, P. Effects of malolactic fermentation on sensory properties of four Burgundy wines. Am J Enol Vitic **1997**, 48, 187-192

[20] Gambaro, A.; Boido, E.; Zlotejablko, A.; Medina, K.; Lloret, A.; Dellacassa, E.; Carrau, F. Effect of malolactic fermentation on the aroma properties of Tannat wine. Aust J Grape Wine Res **2001**, 7(1), 27-32. https://doi.org/10.1111/j.1755-0238.2001.tb00190.x

[21] Du Plessis, H.W.; Steger, C.L.; Du Toit, M.; Lambrechts, M.G. The occurrence of malolactic fermentation in brandy base wine and its influence on brandy quality. J Appl Microbiol **2002**, 92(5), 1005-10013. https://doi.org/10.1046/j.1365-2672.2002.01616.x

[22] Sumby, K.M.; Grbin, P.R.; Jiranek, V. Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chem **2010**, 121, 1-16. https://doi.org/10.1016/j.foodchem.2009.12.004

[23] Ruiz-Perez, P.; Izquierdo Canas, P.M.; Sesena Prieto, S.; Garcia Romero, E.; Palop Herreros, M.I.I. Malolactic fermentation and secondary metabolite production by *Oenococcus oeni* strains in low pH wines. J Food Sci **2012**. 77(10), 579-585. https://doi.org/10.1111/j.1750-3841.2012.02895.x

[24] Valade, M.; Laurent, M. La maitrise de la fermentation malolactique en champagne. Biologgia Oggi **1992**, 6, 35-41

[25] Lloret, A.; Boido, E.; Lorenzo, D.; Medina, K.; Carrau, F.; Dellacassa, E. Aroma variation in Tannat wines: Effect of malolactic fermentation on ethyl lactate level and its enantiomeric distribution. Ital J Food Sci **2002**, 14(2), 175-180

[26] Fleet, G.H. The microorganisms of winemaking – isolation, enumeration and identification. In: Wine microbiology and biotechnology, ed. Fleet, G.H.; Harwood Academic Publishers, Switzerland **1993;** pp. 55-77; ISBN 0-415-27850-3.

[27] Peinado, R.A.; Moreno, J.; Bueno, J.E.; Moreno, J.A.; Mauricio, J.C. Comparative study of aromatic compounds in two young white wines subjected to pre-fermentative cryomaceration. Food Chem **2004**, 84, 585-590. https://doi.org/10.1016/S0308-8146(03)00282-6

[28] Herjavec, S.; Tupajic, P.; Majdak, A. Influence of malolactic fermentation on the quality of Riesling wine. Agric Conspec Sci **2001**, 66(1), 59-64.

[29] Nielsen, J.C.; Richelieu, M. Control of Flavor Development in Wine during and after Malolactic Fermentation by *Oenococcus oeni*. Appl Environm Microbiol **1999**, 65, 740-745

[30] Lasik-Kurdyś, M.; Gumienna, M.; Nowak, J. Influence of malolactic bacteria inoculation scenarios on the efficiency of the vinification process and the quality of grape wine from the Central European region. Eur Food Res Technol **2017**, 243(12), 2163-2173. https://doi.org/10.1007/s00217-017-2919-x

[31] Ribereau Gayon, J.; Dubourdieu, D.; Doneche, B.; Lonvaud, A. Lactic acid bacteria. In: Handbook of enology: the microbiology of wine and vinifications, vol 1, 2nd Edition; ed. Ribereau-Gayon, J.; Dubourdieu, D.; Doneche, B.; Lonvaud, A.; Whiley, London **2006**; pp. 115-138; ISBN 0-470-01034-7.

[32] Costello, P. The chemistry of malolactic fermentation. In: Malolactic fermentation in wine – understanding the science and the practice, ed. by Morenzoni, R., Lallemand, Montreal, **2006** pp. 4.1-4.11

[33] Francis, I.L.; Newton, J.L. Determining wine aroma from compositional date. Aust J Grape Wine Res **2005**, 11(2), 114-126. https://doi.org/10.1111/j.1755-0238.2005.tb00283.x

[34] Lerm, E.; Engelbrecht, L.; du Toit, M. Malolactic fermentation: The ABC's of MLF. S Afr J Enol Vitic **2010**, 31, 186-212.

[35] Romano, P.; Brandolini, V.; Ansaloni, C.; Menziani, E. The production of 2,3-butanediol as a differentiating character in wine yeasts. World J Microbiol Biotechnol **1998**, 14, 649-653. https://doi.org/10.1023/A:1008804801778

[36] Martineau, B.; Henick-Kling, T. Performance and diacetyl production of commercial strains of malolactic bacteria in wine. J Appl Bacteriol **1995**, 78(5), 526-536. DOI: 10.1111/j.1365-2672.1995.tb03095.x

[37] Massera, A.; Soria, A.; Catania, C.; Krieger, S.; Combina, M. Simultaneous inoculation of Malbec (*Vitis vinifera*) musts with yeast and bacteria: effects on fermentation performance, sensory and sanitary attributes of wines. Food Technol Biotechnol **2009**, 47, 192–201.

[38] Munoz, V.; Beccaria, B.; Abreo, E. Simultaneous and successive inoculations of yeasts and lactic acid bacteria on the fermentation of an unsulfited *Tannat* grape must. Braz J Microbiol **2014**, 45, 59–66. http://dx.doi.org/10.1590/S1517-83822014000100009