



Impact of different temperature profiles on simultaneous yeast and bacteria fermentation

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Abstract

Purpose: The role of fermentation temperature was studied for its impact on the evolution of malolactic fermentation performed by simultaneous inoculum of yeast and bacteria in grape must. Results were discussed considering the different fermentative kinetics and the composition of obtained wines.

Methods: Two strains of bacteria belonging to the *O. oeni* and *L. plantarum* species were inoculated 24 h after the beginning of the alcoholic fermentation in 2 grape musts having different acidic and sugar profiles. Fermentations were conducted at 3 different temperature profiles (16/22 °C in 3 days, 18/24 °C in 3 days, 22/32 °C in 5 days). Evolution of microbiota was followed by flow cytometry and plate count. Chemical analysis of grape musts and wines were performed by instrumental approaches (FT-IR, enzymatic quantification of malic acid, GC-MS).

Results: *L. plantarum* resulted more efficient in malic acid consumption in the entire set of tests. These results are unexpected because, generally, *Lactobacillus* has been reported to be more sensitive to an oenological environment than *O. oeni*. In our experiments, *O. oeni* resulted inhibited by the highest fermentation temperature profile, causing incomplete malic acid degradation. Similarly, *S. cerevisiae* showed a higher sensitivity to environmental limiting factors in respect to what is generally known. Differences in the chemical composition of wines were observed in relation to the bacteria strain and the temperature profile. However, the statistical treatment of data identified temperature as the main variable able to influence the features of wines.

Conclusions: Simultaneous inoculum of yeast and bacteria in grape must is an alternative approach in the management of malolactic fermentation which showed some interesting features. However, it is necessary to consider that the dynamics of the microbial population are different to that observed in traditional winemaking and the environmental variables act against the microorganisms in a peculiar, and in certain cases unexpected, way.

Keywords: Malolactic fermentation, Simultaneous inoculum, *Oenococcus oeni*, *Lactobacillus plantarum*, Temperature, Wine

Introduction

Malolactic fermentation (MLF) is a key step in winemaking which allows the achievement of numerous objectives relevant to wine quality (Liu 2002). In the first instance, MLF leads to the degradation of malic acid, through an intracellular reaction of decarboxylation,

mediated by a specific enzyme. This process is necessary for lactic bacteria (belonging to the genera *Oenococcus* sp., *Pediococcus* sp., and *Lactobacillus* sp.) to obtain energy in wine, a harsh environment having generally lack of sugars (Battermann and Radler 1991; Wang et al. 2016). The substitution of L-malic acid with L-lactic acid gives a more pleasant palate to wine, reducing its harshness. However, the degradation of L-malic acid is not the only effect of lactic bacteria activity in wine (Liu 2002). These microorganisms are able to metabolize

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numerous components from which derive molecules having an organoleptic impact. Diacetyl and subsequently acetoin and butanediol are obtained from the catabolism of citric acid. There is a redox equilibrium between these molecules which gives aroma notes described as “lactic” or “buttery,” usually characterizing the bouquet of wines in which MLF occurred (Nielsen and Richelieu 1999, Lasik-Kurdyś et al. 2018). Other relevant classes of compounds influenced by the occurrence of lactic bacteria in wine are the higher alcohols, the glycerol, and the acetic acid due to the metabolism of sugars (Herrero et al. 1999a, 1999b). Lactic acid bacteria are also able to promote, on the one hand, the release of bound varietal aromas through their β -glucosidase and β -lyase (Smit et al. 2005, Michlmayr and Kneifel 2014) and on the other, modification of the ester profile of wines due to the esterase activity (Matthews et al. 2004). From the microbiological point of view, the occurrence of MLF stabilizes the wine due to the elimination of carbon sources, organic acids, and sugars, not fully consumed by yeast during the AF. This effect is beneficial to prevent wine spoilage due to “wild” lactic bacteria or yeast, especially belonging to the *Brettanomyces* sp. genera (Bartowsky 2009).

In view of the interest for a reliable evolution of MLF in terms of the rate of fermentation and the quality of obtained wines, in past years a few researchers have turned their attention toward the factors that are able to regulate bacterial activity in wine and to improve the strategies to facilitate the evolution of this important biologic process (Betteridge et al. 2015; Sumby et al. 2014, 2019). Wine is a harsh environment for bacteria due to the simultaneous occurrence of some limiting factors. Among these, the most known are the acidity, the presence of ethanol and sulfur dioxide, and the lack of nutritional factors such as sugars or nitrogen compounds. More recently, researchers have focused the role of phenolic molecules (tannins coming from grapes or wooden barrels) and of compounds originated from yeasts, such as fatty acids which counteract the development and the activity of lactic acid bacteria in wine (Guerzoni et al. 1995; Guzzon et al. 2009, 2013). In addition to these variables, some technological choices, made by winemakers, could promote or interfere with the evolution of MLF. Subtractive practices, such as the clarification of wine, are detrimental to bacteria because they eliminate nutrients and reduce the microbial load derived from grapes (Liu 2002). A great role is also played by the yeast strain that performs alcoholic fermentation. This must be carefully chosen by considering its compatibility with the bacteria that will be used for MLF (Costello et al. 2008). Other technological variables influencing the evolution of MLF are the methods and the duration of the pomace maceration in grape must,

the racking of wine, the oxygen availability during MLF, the presence of yeast lees during wine aging, the addition of specific nutrients for bacteria at the end of AF, or the use of chemical compounds with toxic action toward bacteria, such as lysozyme or chitosan (Azzolini et al. 2010, Elmac et al. 2014). For each of these, the limits beyond which bacterial activity can be compromised are now known. However, the incidence of these limiting factors against lactic acid bacteria is frequently regulated by agri-environmental variables, the protocol of winemaking or the technical and organizational limitations that characterize each winery. Therefore, the possibility of modulating these variables to improve the fitness of lactic bacteria during MLF is limited.

There are two other winemaking parameters that can strongly regulate bacterial activity, which are less constrained by the limits described above: the fermentation temperature and the timing of inoculation of the lactic bacteria in wine (Guerzoni et al. 1995, Garbay et al. 1995, Herrero et al. 1999a, 1999b, Guzzon et al. 2013). With regard to the fermentation temperature, its impact on alcoholic and malolactic fermentation kinetics and on the qualitative outcomes is known. Temperature fluctuations in the range that is usual in wineries, from 15 to 32 °C, can affect the fermentation rate, the extraction mechanisms from grape skins, the production of secondary metabolites with a significant organoleptic impact, and the preservation of varietal aromas liberated from grapes during fermentation (Herrero et al. 1999a, 1999b, Bordiga et al. 2016, Setford et al. 2018, Schwinn et al. 2019). The use of “cold” in cellars is a very ancient practice, traditionally obtained by building underground wineries, modulating the dimensions and materials of the vats destined for the oenological fermentations, refreshing them by cold water or, in extreme cases, by ice to lower wine temperature. In the case of MLF, the low temperatures that characterize the cellars in the months following the harvest are a critical parameter because it is proven that bacterial activity significantly slows down below 18 °C, and stops at 10 °C (Liu 2002; Guzzon et al. 2009). Moreover, the low temperatures favor the settling of bacteria and the precipitation of insoluble tartrate salts that limit the mass exchange rate between wine and bacteria. On the contrary, excessive temperatures could increase the sensitivity of bacteria to ethanol, altering cell membrane permeability.

The timing of bacteria inoculation is the second topic in the management of MLF. About 10 years ago, it was believed that bacteria had to be added to the wine at the end of AF, when sugars are exhausted, to avoid risks of alteration (Lonvaud-Funel 1999). However, a new generation of starter strains has been specifically selected for their absence of alterative activities and good compatibility with yeast, thus making it possible to anticipate the

timing of inoculation of lactic bacteria at the early stages of alcoholic fermentation (Jussier et al. 2006, Taniyasuri et al. 2006, Guzzon et al. 2013). The solution's advantages are numerous (Alexandre et al. 2004). The stress of the bacteria's adaptation to wine conditions is reduced because microorganisms develop in an environment that still contains low quantities of ethanol and sulfur dioxide. In addition, the early inoculation of lactic bacteria allows for obtaining sensorial profiles different from those wine characteristics that result from a successive inoculum of yeasts and bacteria. This innovative approach to MLF is nowadays very popular among winemakers; however, it requires a re-evaluation of the effect of wine-limiting factors against bacteria in this new scenario.

This work studies the impact of different temperature profiles, simulating what is commonly done in wineries in the early stage of winemaking, on the dynamics of alcoholic and malolactic fermentation and on the aromatic profile of obtained wines. Two different yeast-bacteria pairs were considered, exploring the behavior of obligatory hetero- and facultative hetero-fermentative strains belonging to the *O. oeni* and *L. plantarum* species. The experiments were carried out simulating the temperature profiles usually observed during winemaking: a sudden increase in the first days of fermentation, often close to 30 °C and subsequently, a constant temperature depending on the characteristics of the cellar and of the wine vessels. The combination of the two variables explored ensures a significant contribution to knowledge on the management of this winemaking process' crucial step.

Materials and methods

Microorganisms and materials involved in experimental winemaking

Winemaking experiments were carried out on two grape musts (*cv.* Chardonnay) obtained at the winery of the Edmund Mach Foundation (I). The main chemical parameters of grape musts are the following. Grape must 1 (G1): sugars 202 g/L, pH 3.12, L-malic acid 3.55 g/L, readily assimilable nitrogen 180 mg/L. Grape must 2 (G2): sugars 234 g/L, pH 3.48, L-malic acid 2.87, readily assimilable nitrogen 115 mg/L. Yeast Lalvin ICV D47 (Lallemand Inc., CA) was employed to start AF, while *Oenococcus oeni* A4 (Lallemand Inc.) and *Lactobacillus plantarum* K45H (Lallemand Inc.) were employed to drive MLF. Fermaid E (Lallemand Inc.) was used as a nitrogen supplement during AF (0.2 g/L at the beginning of AF and 0.2 g/L after 1/3 of AF).

Experimental winemaking

Winemaking from each grape must (G1 and G2) was performed by utilizing yeast and bacteria in simultaneous fermentations. Both experiments were carried out

in 1 L glass bottles ($n = 2$), we applied 3 different temperature profiles. In the first test (T1), the grape must temperature was initially set to 16 °C and subsequently increased up to 22 °C within a period of 3 days. After that, the temperature remained constant for the entire duration of the fermentation process. The second temperature protocol (T2) showed a similar dynamic to the T1, but started from 18 °C and reached 24 °C. The third protocol (T3) has an initial temperature of 20 °C and a maximum temperature of 32 °C, which was reached in 5 days. In T1 and T2, the temperature was augmented by 2 °C every 24 h; in T3, the temperature was increased by 3 °C every day. Yeast was inoculated into grape must at a concentration of 0.3 g/L. In the case of bacteria, we employed an inoculum of 0.01 g/L of freeze-dried culture for the *O. oeni* strain and 0.1 g/L for the *L. plantarum* strain. The ratio of inoculum of the two LAB strains is related to the different behavior in wine, as will be discussed in the "Discussion" section of this work. Selected microbial cultures were rehydrated according to the OIV standard (OIV 2019) and lactic bacteria were added to the fermenting grape must 24 h after the beginning of AF. AF fermentation was followed by the daily measuring of weight loss due to CO₂ production; L-malic acid was measured by enzymatic assay as reported in the next paragraph. At the end of fermentations, the vessels that contained wines were saturated by N₂, cold stored (5 °C) for 7 days, and racked to eliminate the excess of yeast lees. The wines were submitted to chemical investigations after 1 month of aging at 10 °C.

Microbiological analysis

All analyses were performed in the Edmund Mach Foundation laboratory of microbiology that operates according to the (ISO/IEC 17011 2017). The count of viable and dead yeast cells was performed by flow cytometry (FCM) (Guzzon and Larcher 2015). One milliliter of sample containing approximately 10⁵ cells, obtained by an appropriate dilution in a PBS buffer, was filtered through a 30 µm filter (CellTrics®, Partec-Sysmex GHB, D) and incubated for 10 min at 20 °C in the presence of 10 µL of a 5 mg/mL fluorescein diacetate solution (Partec-Sysmex). After incubation, samples were mixed and added to 10 µL 2 mg/mL of propidium iodide solution (Partec-Sysmex). The double-stained samples were homogenized (30" by a Vortex apparatus, IKA, S) and submitted to FCM analysis within 10 min. FCM analysis was performed by a CUBE 8 Cytometer (Partec-Sysmex), equipped with a solid blue laser emitting at 488 nm. We considered the following signals by means of four band-pass filters: a forward-angle light scatter (FSC) combined, a side-angle light scatter (SSC), and 2 fluorescence signals, the first by a 530 nm band-pass filter to

collect green fluorescence (FL1 channel), and the second by a 630 nm long-pass filter to collect red fluorescence (FL2 channel). FCM analyses were performed using logarithmic gains and specific detector settings, adjusted on a sample of unstained *Saccharomyces cerevisiae* ATTC 9763 to eliminate the background and the auto fluorescence of cells. Data were analyzed using the FCS Express 4 software (De Novo Software Inc., CA). Yeast cell population was identified and gated in the dot plot FSC/SSC; a live and dead cell differentiation was performed in the dot plot FL1/FL2, adjusted by the appropriate compensation between the two signals considering the subpopulation of yeast gated in the dot plot FSC/SSC. Lactic bacteria quantification was performed by plate count (OIV 2019) using MRS agar (Oxoid, UK) supplemented by 20% v/v of apple juice and 1% v/v of a 0.1% cicloeximide solution (Oxoid). After sample spreading, Petri plates were incubated at 25 °C for 10 days under anaerobic conditions using an Anaerogen Kit (Oxoid).

Chemical analysis

Chemical parameters of the grape must (sugars, pH, L-malic acid, and readily assimilable nitrogen) and wines (ethanol, pH, sugars, total acidity, acetic acid) were monitored using FT-IR (FOSS, DK). L-malic acid quantification was carried out by an enzymatic detector Miura 200 (Biogamma, I). The volatile profiles of obtained wines were determined after preparation of the samples using solid phase extraction (SPE, ENV+ cartridge), as proposed by Boido et al. (2003). Volatile compound analysis was performed with GC-MS/MS using a Varian 450 (Agilent Technologies) chromatograph coupled to a Varian 300 TQMS tandem mass spectrometer (Agilent Technologies). The sample extract was injected (1 µL) in splitless mode. The injector temperature was 250 °C. Chromatographic separation was performed using a VF-WAXms (30 m × 0.25 mm ID × 0.25 µm film thickness, Agilent Technologies) capillary column with the following oven temperature program: 40 °C for 5 min, raised to 150 °C at 5 °C min⁻¹, finally raised to 240 °C at 10 °C min⁻¹ and held for 10 min. The mass spectrometer was equipped with an electron impact ionization source (EI) (70 eV, 50 µA) and acquisition was performed in multiple reaction monitoring (MRM) mode. The transitions and collision energies were those reported by Vrhovsek et al. (2004).

Statistical analysis

Results of physico/chemical and microbiological observations were expressed as mean ± SD of the 3 or 6 replicates, as detailed in the following chapter. Statistical analysis of the data was carried out using the Statistica 7.1 software (StatSoft, Tulsa, OK). The variance among cases (LAB strains) or variables (temperature profiles) in

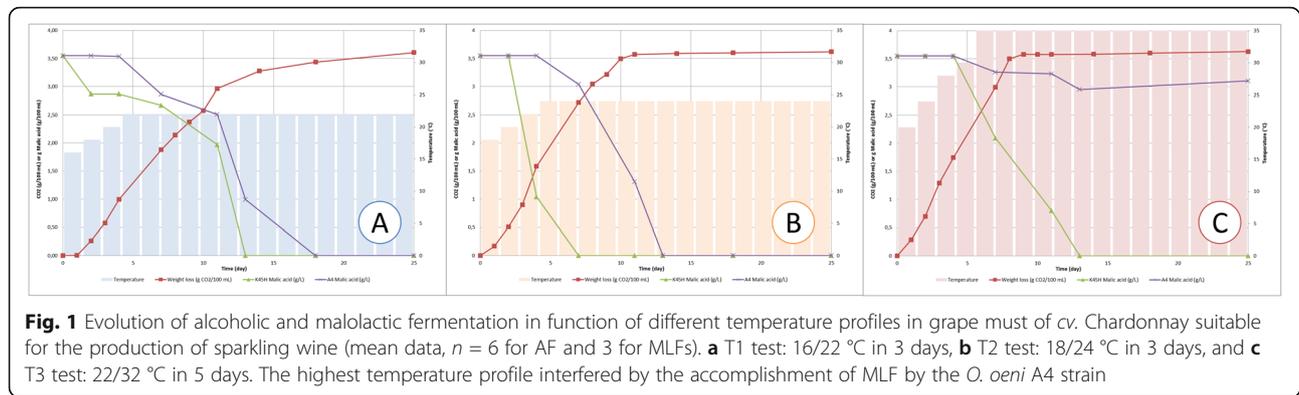
the determining of chemical parameters of wines was studied by two-way ANOVA test, $p = 0.05$. Principal component analysis (PCA) was performed to grouping the wines on the basis of the entire volatile profile.

Results

Effect of different temperature profiles on oenological fermentations

Figure 1 a, b, and c shows the weight loss curves related to the glucose + fructose consumption and the L-malic acid degradation observed in the winemaking tests performed using the two bacterial strains, in function of the different fermentation temperature profiles. G1 was obtained from early harvested grapes, simulating what is commonly done in the production of sparkling wines. The increase in temperature of the 3 experiments (T1, T2, and T3) stimulates the sugar consumption rate. Indeed, the differences observed between the evolution of AF performed in G1 with the two strains of bacteria (K45H and A4) were not significant from a statistical point of view; therefore, data are expressed as the mean of the entire set of experiments performed at the same temperature. In T1, AF required 14 days while in T2 and T3, the degradation of sugars stopped after 11 and 9 days, respectively. The initial temperature of grape must influenced the duration of lag-phase (Fig. 1) and the V_{max} of the 3 experimental trials. These are ($n = 6$): in T1, $2.7 \pm 0.3 \text{ g CO}_2 \text{ L}^{-1} \text{ day}^{-1}$, in T2, $4.0 \pm 0.3 \text{ g CO}_2 \text{ L}^{-1} \text{ day}^{-1}$, and in T3, $4.5 \pm 0.4 \text{ g CO}_2 \text{ L}^{-1} \text{ day}^{-1}$. In G1, the *Lactobacillus* strain demonstrated the best MLF performance at all the temperature profiles adopted (Fig. 1, $n = 3$). Independently, of the grape must's initial temperature, *L. plantarum* K45H showed 2 days of lag-phase to start L-malic acid consumption and MLF was accomplished in 13 days (T1 and T3) or 7 days (test T2). *O. oeni* A4 showed the same lag phase (2 days), but the degradation rate of L-malic acid was slower, accomplishing MLF in 18 days in the case of T1, 13 days in the T2 test, and it resulted unable to completely degrade L-malic acid at the highest temperature profile (T3).

G2 was derived from grapes harvested at plain ripeness, characterized by a lower acid content and higher sugar concentration with respect to G1. According to the previous tests, the presence of lactic bacteria did not interfere with AF, which trend is related to the increase of temperature (Fig. 2 a, b, and c). In G2T1 and G2T2, alcoholic fermentation required 16 and 12 days respectively, while in G2T3 the degradation of sugars stopped after 10 days. The delay with respect to G1 experiments on the conclusion of AF could be linked to the higher amount of grape must sugars. It is also relevant that a residual amount of sugars remained in the T3 experiment wines, as better discussed in the next paragraph. Temperature profiles regulated the V_{max} ($n = 6$) of AF



in experimental winemaking, reaching $2.7 \pm 0.1 \text{ g CO}_2 \text{ L}^{-1} \text{ day}^{-1}$ (T1), $3.9 \pm 0.3 \text{ g CO}_2 \text{ L}^{-1} \text{ day}^{-1}$ (T2), and $4.4 \pm 0.2 \text{ g CO}_2 \text{ L}^{-1} \text{ day}^{-1}$ (T3). The *Lactobacillus* strain confirmed its high efficiency in L-malic acid consumption. It required 5 days to consume the entire amount of L-malic acid independently of the temperature profiles (Fig. 2 a, b, and c). *O. oeni* A4 activity resulted more influenced by temperature profiles. The highest fermentation rate was obtained in T2 which concluded MLF in 9 days (Fig. 2b, $n = 3$), in T1, malic degradation required 12 days (Fig. 1a, $n = 3$) and, similarly to what was observed in the previous test, T3 (Fig. 2c, $n = 3$) inhibited the activity of this bacteria residing in L-malic acid in wine at the end of the 30-day observation period.

Evolution of microbial population

The monitoring of the yeast population during AF by flow cytometry (Table 1) ensured information on cell density and its physiological state, differentiating between live, dead, and damaged cells depending on the permeability of the cell membrane and on cytoplasmic esterase activity (Guzzon and Larcher 2015). Yeast population growth dynamics appeared mostly influenced by the different temperatures and contributed toward

explaining the data in Figs 1 and 2. In G1, no differences were observed in the yeast's dynamics in terms of the different strains of bacteria involved in the MLF; therefore, data were aggregated on the basis of the 3 temperature profiles tested (T1, T2, and T3 $n = 6$). Two days after bacteria inoculum (day 4, Table 1), experiment live cells in T1 reached $6.9 \pm 4.4 \text{ log cell/mL}$ and the subpopulation represented by dead cells was negligible. In addition, no subpopulations of cells having a damaged membrane (Fig. 3a) were observed. At the end of experiments (day 18), live cells represented more than 98% of the yeast population. In the T2 test, yeast growth was more rapid, reaching $7.6 \pm 5.4 \text{ log cell/mL}$ in 7 days (Table 1), but dead cells accounted for about 50% of the population on the 18th day of fermentation. Winemaking performed at T3 showed a concentration of yeast similar to that of T2 but cell decay was faster after 11 days of AF (Table 1). The differences observed in the ratio between live and dead cells at different temperature could be correlated at the different AF rate and, consequently, at long permanence of T2 and T3 yeast's population in starvation conditions (post AF). The determination of lactic bacteria concentration during MLF was carried out by plate count; therefore, only information on cells capable of growing on MRS medium

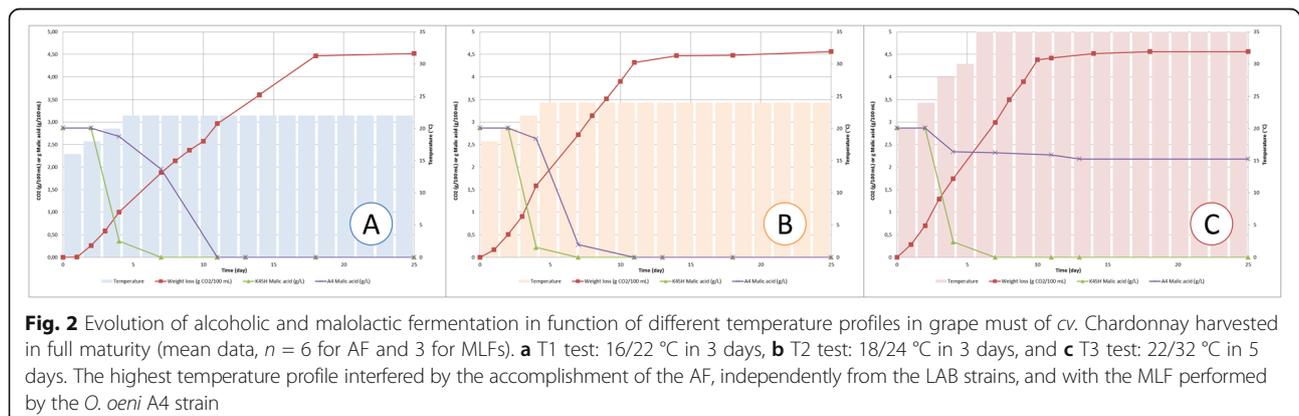


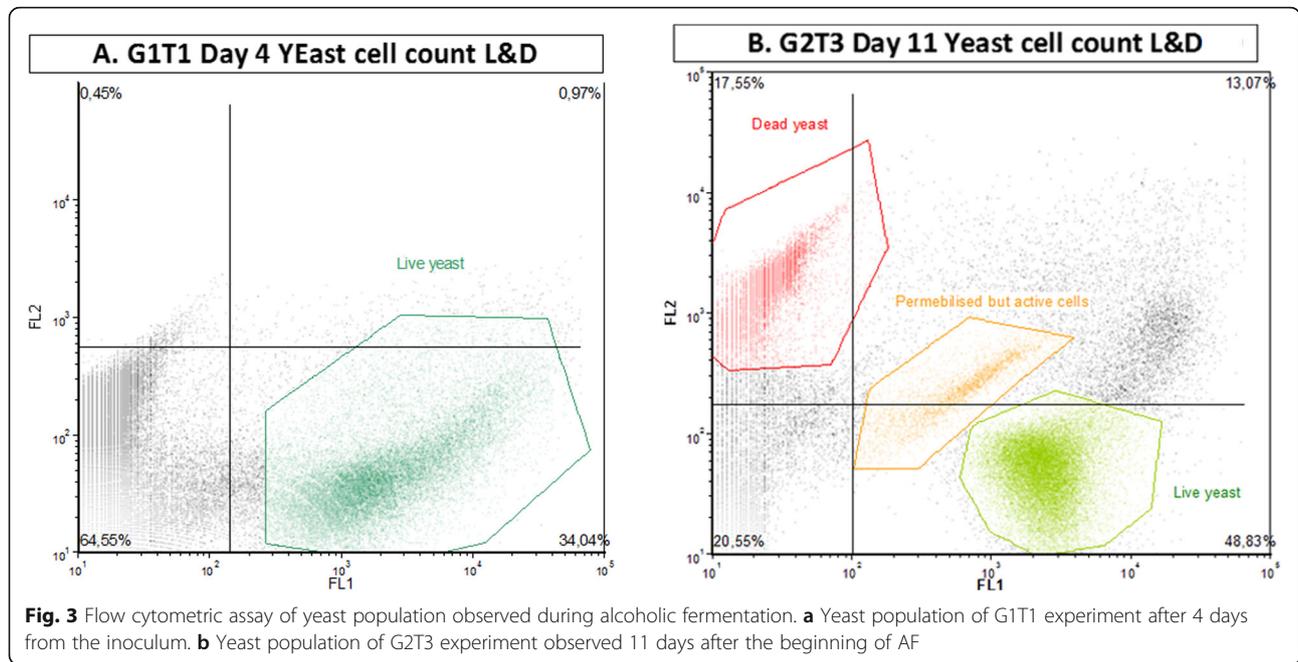
Table 1 Result of yeast (flow cytometric determination) and bacteria count (plate count onto MRS + TJ) performed during the 3 experimental winemaking. T1 test: 16/22 °C in 3 days, B. T2 test: 18/24 °C in 3 days, and C. T3 test: 22/32 °C in 5 days. (Mean, $n = 6$ in the case of yeast and $n = 3$ in the case of LAB)

Microorganism	Temperature profile	Cell concentration (log units/mL)				
		Day 4	Day 7	Day 11	Day 13	Day 18
Grape must 1 (G1)						
Yeast (live cells)	T1	6.9 ± 4.4	7.1 ± 5.3	6.8 ± 5.4	6.2 ± 5.2	5.4 ± 4.2
Yeast (dead cells)	T1	nd	nd	2.2 ± 2.1	2.7 ± 2.2	3.8 ± 2.3
Yeast (live cells)	T2	7.2 ± 5.2	7.6 ± 5.4	7.2 ± 5.2	6.8 ± 5.4	4.8 ± 4.5
Yeast (dead cells)	T2	nd	nd	2.4 ± 1.2	2.8 ± 2.3	3.6 ± 2.4
Yeast (live cells)	T3	7.3 ± 5.2	7.1 ± 5.2	6.9 ± 5.2	5.8 ± 5.3	4.4 ± 4.2
Yeast (dead cells)	T3	nr	2.8 ± 1.4	3.2 ± 2.2	4.5 ± 3.1	5.8 ± 0.4
A4	T1	6.0 ± 3.2	6.1 ± 4.5	6.5 ± 4.1	6.5 ± 4.7	6.3 ± 4.1
A4	T2	6.4 ± 4.1	6.5 ± 4.9	6.8 ± 5.2	6.9 ± 5.3	6.8 ± 5.1
A4	T3	5.6 ± 3.3	5.5 ± 4.2	4.9 ± 4.5	4.9 ± 3.9	nd
K45 H	T1	6.1 ± 3.7	6.3 ± 4.3	6.6 ± 3.9	6.6 ± 4.2	6.2 ± 4.1
K45 H	T2	6.4 ± 3.2	6.4 ± 4.7	6.8 ± 4.1	6.5 ± 4.2	6.6 ± 3.9
K45 H	T3	5.9 ± 3.3	6.3 ± 3.9	6.4 ± 4.2	6.5 ± 4.3	6.3 ± 4.4
Grape must 2 (G2)						
Yeast (live cells)	T1	7.0 ± 5.2	7.2 ± 5.2	7.1 ± 5.7	6.8 ± 5.3	5.8 ± 4.4
Yeast (dead cells)	T1	nd	nd	2.5 ± 2.2	2.7 ± 2.1	3.7 ± 2.9
Yeast (live cells)	T2	7.4 ± 4.9	7.8 ± 6.2	7.7 ± 6.3	6.9 ± 5.9	6.2 ± 5.3
Yeast (dead cells)	T2	nd	3.1 ± 2.2	3.3 ± 2.4	3.6 ± 2.9	3.9 ± 3.1
Yeast (live cells)	T3	7.1 ± 5.3	7.2 ± 5.7	6.0 ± 5.1	5.2 ± 4.2	4.9 ± 4.5
Yeast (dead cells)	T3	nd	2.9 ± 2.4	5.3 ± 2.9	5.4 ± 3.3	5.8 ± 4.6
A4	T1	5.5 ± 4.1	5.4 ± 4.3	5.8 ± 4.2	5.7 ± 4.4	5.6 ± 4.3
A4	T2	6.5 ± 5.2	6.6 ± 5.1	6.9 ± 5.2	7.0 ± 6.1	6.9 ± 5.8
A4	T3	5.5 ± 4.2	5.5 ± 4.3	nd	nd	nd
K45 H	T1	6.5 ± 5.2	6.9 ± 5.3	6.9 ± 5.2	6.6 ± 5.1	6.7 ± 5.4
K45 H	T2	6.6 ± 5.3	6.6 ± 6.1	7.0 ± 6.4	6.7 ± 5.3	6.8 ± 5.1
K45 H	T3	6.0 ± 5.3	6.1 ± 5.3	6.6 ± 5.2	6.1 ± 5.9	6.2 ± 5.2

were available (Table 1). In G1 experiments, *L. plantarum* K45H was characterized by the highest cellular density in the first days after inoculum in grape must, while the *O. oeni* A4 strain reached its maximum concentration later. The differences observed in terms of bacterial density are not relevant from a technological point of view and microbial load were generally sufficient to ensure an efficient MLF (Lonvaud-Funel 1999; Liu 2002). In tests performed at T2, the *L. plantarum* K45H strain reached 6.8 ± 4.8 log CFU/mL ($n = 3$) after 11 days, while the *O. oeni* A4 strains grew to 6.9 ± 4.9 log CFU/mL ($n = 3$) after 13 days of AF. T1 and T3 experiments showed poorer performance in terms of maximum bacterial concentration for the two bacterial strains, not passing 6.6 log CFU/mL in the case of the *Lactobacillus* strain (T1, G1, Table 2) and 6.5 log CFU/mL in the case of the *O. oeni* strain (T1, G1, Table 2). It

is worth noting that in the T3 experiments *O. oeni* A4 decreases below 5 log units 11 days after the inoculum. This sudden mortality explains the incomplete L-malic degradation observed in this test.

Yeast populations in experiments G2 made at T1 and T2 grew quickly, after a lag-phase of 24 h, up to a cell density of 7 log units. The T1 test showed a live yeast concentration of 7.2 ± 5.9 log cell/mL ($n = 3$) on the 7th day (Table 1), with a negligible presence of dead cells; live cells represented more than 90% of the yeast population for the test's entire duration. T2 data are similar to the previous ones, with a maximum cell load of 7.8 ± 6.2 ($n = 3$) log cell/mL at day 7. On the contrary, in the T3 experiment, after the yeast population's initial growth, a rapid decline in the cellular vitality was observed (Fig. 3) which reduced the population of *S. cerevisiae* below the critical value of 6 log cell/mL 11 days after the beginning



of the test (Table 1). This dynamic resulted in an arrest in sugar degradation and in their residue in wine above 30 g/L, as we will discuss in the next paragraph. The evolution of the bacterial population of the G2 experiments are similar to G1, with the difference that the lower acidity of the second grape must favored bacterial growth (Table 2). *L. plantarum* K45H showed a concentration of 7.0 ± 6.4 log CFU/mL after 7 days, a similar

value of microbial load was reached by the *O. oeni* A4 strain in 11 days. However, in the T1 and T2 tests, the bacterial population's evolution showed a progressive adaptation to these specific environmental conditions with a relevant cell growth with respect to the initial inoculum, maintaining cellular load up to 6 log CFU for the entire duration of MLF. The highest temperature profile (T3, Table 2) appeared detrimental because

Table 2 Chemical parameters of wines obtained by simultaneous fermentation of *S. cerevisiae* and *L. plantarum* or *O. oeni* at different temperature profiles. T1 test: 16/22 °C in 3 days, B. T2 test: 18/24 °C in 3 days, and C. T3 test: 22/32 °C in 5 days. (Mean, n = 3). *Differences are statistically significant both in terms of bacteria strain and temperature profile effect (two-way ANOVA test, p = 0.05).°Differences are statistically significant in terms of temperature effect (two-way ANOVA test, p = 0.05)

Bacteria strain	Grape must/temperature profile	Ethanol (% vol)	pH	Tot. acidity (as tartaric acid) g/L	Acetic acid	Glucose and fructose	Tartaric acid	Malic acid	Lactic acid	Glycerol
Grape must 1 (G1)										
A4	T1	11.4*	3.25*	3.6°	0.15°	< 1.0	1.58*	< 0.35°	2.15*	4.7*
A4	T2	11.3*	3.24*	3.9°	0.22°	< 1.0	1.59*	< 0.35°	2.20*	5.2*
A4	T3	10.9*	3.07*	5.4°	< 0.10°	3.0*	1.69*	2.98°	< 0.50*	5.5*
KA5 H	T1	11.4*	3.19*	4.3°	0.12°	< 1.0	1.64*	< 0.35°	3.00*	4.2*
KA5 H	T2	11.3*	3.15*	4.4°	0.17°	< 1.0	1.77*	< 0.35°	2.94*	4.9*
KA5 H	T3	11.2*	3.17*	4.3°	0.10°	< 1.0	1.70*	< 0.35°	2.47*	5.3*
Grape must 2 (G2)										
A4	T1	13.8*	3.57*	3.0°	0.26*	2.7*	1.07*	< 0.35°	1.67*	6.3*
A4	T2	13.8*	3.60*	3.4°	0.30*	2.7*	1.00*	< 0.35°	1.81*	7.4*
A4	T3	11.4*	3.49*	4.5°	0.60*	35.6*	1.02*	2.68°	< 0.50*	7.4*
KA5 H	T1	13.7*	3.54*	3.2°	0.23*	2.1*	1.12*	< 0.35°	1.63*	6.1*
KA5 H	T2	13.8*	3.55*	3.6°	0.27*	2.9*	1.14*	< 0.35°	1.98*	7.2*
KA5 H	T3	11.7*	3.51*	3.8°	0.32*	30.1*	1.57*	< 0.35°	1.84*	7.3*

bacterial concentration remained below 6.6 log CFU/mL in the case of K45H, and below 5 log CFU/mL for *O. oeni* A4, which was not detectable 11 days after the beginning of the experiments.

Influence of temperature profiles on the chemical composition of wines

Table 2 lists the main chemical parameters of wines obtained by the simultaneous fermentation of yeast and bacteria at different temperature profiles. Wines resulting from G1 and fermented by *L. plantarum* K45H did not show differences in their composition in terms of the main oenological features. Alcoholic and malolactic fermentations were accomplished with the complete consumption of sugars (< 0.1 g/L) and L-malic acid (< 0.35 g/L). Volatile acidity reached a mean concentration ($n = 9$) of 0.13 ± 0.03 g/L, a value that can be considered negligible in winemaking. The wines obtained by *O. oeni* A4 at T1 and T2 (Table 3) showed a similar composition to the former, with the exception of a lower conversion yield of malic acid to lactic acid. Its concentration was about 0.8 g/L lower than in wines where MLF was performed with *L. plantarum*. This difference is also reflected in the pH, which is slightly higher, and could be related to a partial consumption of malic acid by yeasts (Su et al. 2014). Indeed, T3 evidenced a residual content of sugars (Table 2) and the absence of consumption of the malic acid which resided in wines (mean 2.98 ± 0.12 g/L, $n = 3$). Volatile acidity (mean 0.13 ± 0.04 g/L, $n = 9$) and ethanol content (mean 11.2 ± 0.6 % v/v, $n = 9$) did not differ significantly from those of wines made by *L. plantarum* K45H. Wine obtained from G2 by the two bacterial strains at T1 and T2 have a similar chemical composition (Table 3). The stress observed in the microbial population (Table 1) in G2T3 resulted in an alteration in the chemical profile of obtained wines. In the case of the experiment performed by *L. plantarum* K45H, 30.1 ± 0.3 g/L ($n = 3$) of sugars resided in wines, causing a loss in ethanol accumulation of about 2% (Table 3). In the case of trials performed by *O. oeni* A4, the lack of sugar consumption (35.4 ± 0.6 g/L, $n = 3$) is accompanied by MLF failure (2.61 ± 0.02 g/L of L-malic acid, $n = 3$) and by the increase in volatile acidity (0.60 ± 0.07 g/L, $n = 3$). The statistical analysis of data (two-way ANOVA test, Table 2) underlines that both variables considered (temperature profile and LAB strain) influence the variability of the majority of the enological parameters. Regarding the variations in the acidic profile, in particular in acetic and malic acid concentration, the temperature is the only statistically significant variable. Some hypothesis about the effect of the high temperature on the metabolism of the two microbial populations will be discussed in the next chapter.

The volatile profiles of the wines were determined by GC-MS, taking 71 molecules into consideration, of which 51 resulted quantifiable in at least one sample (Table 3). Gas chromatographic analysis detected 38 free volatile compounds and revealed differences in the set of data in terms of the degree of ripeness of grapes (G1 or G2). However, the technological variables considered (temperature and strain of LAB) resulted relevant in driving the evolution of the wines' volatile profile. According to the features of *cv.* Chardonnay, which contains less concentration of volatile precursors, esters (ethyl lactate and other ethylic esters), higher alcohols (1-hexanol and 2-phenyl ethanol), and fatty acids (hexanoic acid, octanoic acid, and decanoic acid) dominated the aromatic profile of the wines. Temperature proved to be the most significant variable (two-way ANOVA test, Table 3), influencing the content of 47% of the volatile compounds, while the differences between the two strains were found to be relevant only in the 18% of cases. Finally, the cooperative interaction of both variables was observed significant in 4 compounds (7% of the aromatic profile, Table 3). In details, the test performed by *O. oeni* A4, the differences among T1 and T2 data are included in the variability observed in the different replicates. To the contrary, the highest fermentation temperature (T3) reduces the content of esters and fatty acids, thus increasing the presence of higher alcohols (Table 3). By contrast, *L. plantarum* K45H proportionally accumulated esters at fermentation temperature, with a similar trend observed for 2-phenyl ethanol. In the tests with *O. oeni* the increase in temperature reduced the concentration of fatty acids, independently of the length of the carboxylic chain. The analysis of bound volatile compounds allowed the quantification of 13 molecules with relevant differences between the wines obtained from the two grape musts although, in general, the quantities of these compounds are very low. Benzyl alcohol is the most represented compound from among bonded molecules in G1. We observed its increases in T3 with respect to the other two fermentation temperature profiles and this trend appeared independent of the LAB strain involved in MLF. In G2, GC-MS quantified a small amount of terpins, whose accumulation is probably favored by the advanced ripeness of the grapes (Gambetta et al. 2016). These molecules did not vary as a function of the fermentation temperature or the LAB strain. Similar results were observed for 2-phenyl ethanol and benzyl alcohol, as the most represented bounded volatile compounds in G2: the different variables of the experiments did not alter their concentration.

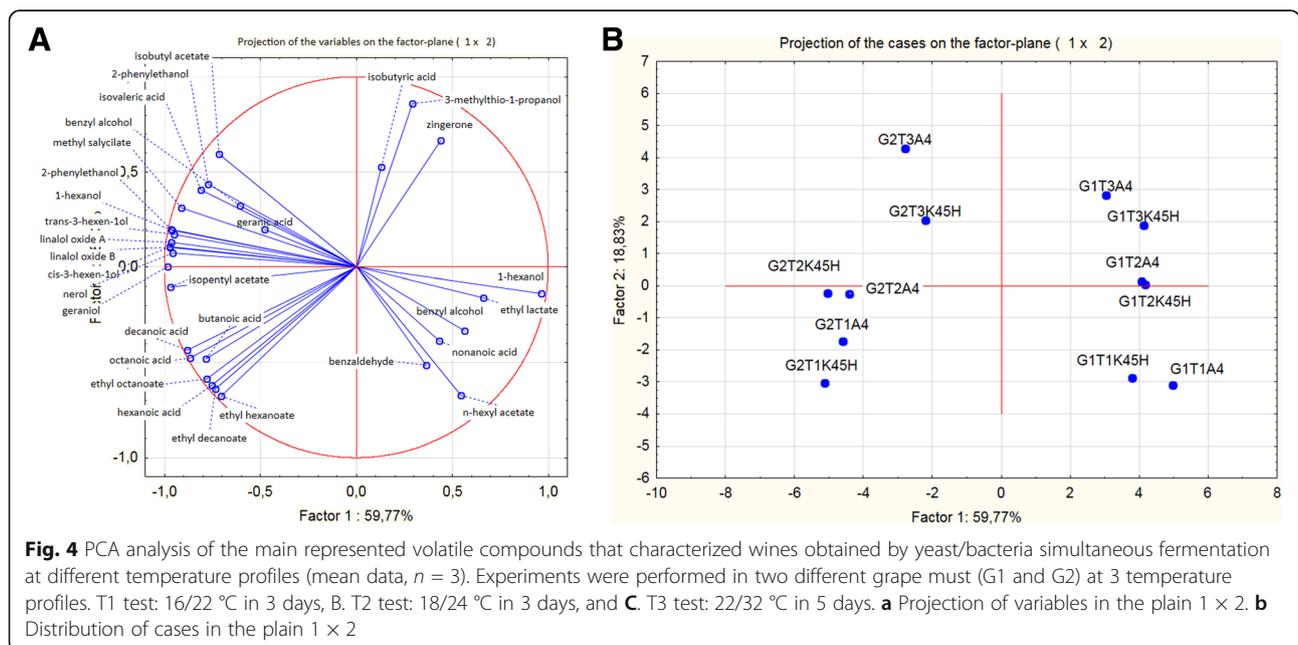
A PCA analysis was performed (Fig. 4) considering the 20 volatile compounds having the highest concentration among free volatiles in order to verify the

Table 3 Profile of free and bounded volatile compounds, determined by GC-MS, of wines obtained by simultaneous fermentation of *S. cerevisiae* and *L. plantarum* or *O. oeni* at different temperature profiles. T1 test: 16/22 °C in 3 days, B. T2 test: 18/24 °C in 3 days, and C. T3 test: 22/32 °C in 5 days. °Differences are statistically significant both in terms of bacteria strain and temperature profile effect (two-way ANOVA test, $p = 0.05$). *Differences are statistically significant in terms of temperature effect (two-way ANOVA test, $p = 0.05$). *Differences are statistically significant in terms of strain effect (two-way ANOVA test, $p = 0.05$) (Continued)

LAB	A4	A4	A4	K45H	K45H	K45H	A4	A4	A4	K45H	K45H	K45H
Bound compound (mg/L)												
1-hexanol ^{°*}	0.025	0.025	0.038	0.031	0.030	0.031	0.056	0.051	0.054	0.052	0.052	0.052
trans-3-hexen-1ol	0.000	0.000	0.001	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.002
cis-3-hexen-1ol ^{°*}	0.004	0.004	0.006	0.004	0.005	0.004	0.018	0.016	0.018	0.018	0.016	0.017
2-phenylethanol ^{°*}	0.123	0.131	0.183	0.136	0.144	0.176	0.392	0.366	0.383	0.326	0.363	0.343
Benzyl alcohol [†]	1.219	1.382	1.798	1.447	1.518	2.052	2.166	1.763	1.695	1.779	1.944	1.942
Linalol oxide A [†]	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.004	0.004	0.004	0.004	0.004
Linalol oxide B [†]	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.002	0.002	0.002	0.002	0.001
Nerol	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.002	0.002	0.002	0.002	0.002
Geraniol	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.003	0.003	0.003	0.003	0.003
Geranic acid	0.000	0.000	0.001	0.000	0.005	0.000	0.004	0.000	0.004	0.003	0.004	0.004
Benzaldehyde	0.013	0.015	0.012	0.012	0.016	0.012	0.017	0.013	0.010	0.014	0.012	0.013
Methyl salicylate	0.002	0.002	0.002	0.002	0.002	0.002	0.004	0.005	0.006	0.005	0.006	0.006
Zingerone	0.001	0.002	0.003	0.001	0.004	0.003	0.001	0.002	0.003	0.000	0.001	0.002

capacity of the technological variables and the composition of grape musts to differentiate the volatile profile of the wines obtained. This choice is designed to simplify data analysis considering that the PCA analysis on the entire set of data does not differ (data not shown). Figure 4 a portrays the spatial distribution of the variables on the plane defined by factors 1

and 2, explaining 78.21% of total variability, while Fig. 4 b shows the spatial distribution of the wine samples. The volatile compound profile distinguishes wines belonging to the two different grape musts, particularly due to the higher concentration of bounded compounds that characterized G2. Among the two technological variables considered in this



work, the temperature profile appears to be more capable of distinguishing between the different samples than the bacterial strains. In the G1 experiments, wine made from the lowest fermentation temperature (G1T1, Fig. 4) resulted clearly separated to that obtained at T2 and T3, independently of the LAB strain. To the contrary, in G2 based tests, the wine obtained at T3 is segregated from the others. The reasons for the distribution of cases in the PCA analysis could be found in the microbial population evolution described above and in the resulting different volatile compound profile, as discussed in the next part of the work.

Discussion

The addition of bacteria to grape must during AF to obtain quick MLF is a practice that has gained increasing interest among winemakers. The reasons of this trend have already been discussed in the first chapter of the work. To summarize, the improved microbiological control that the simultaneous fermentation ensured, is correlated to the unique organoleptic profile of wines derived from this winemaking approach (Jussier et al. 2006, Guzzon et al. 2013). The simultaneous presence of two high-concentration microbe populations, yeast and bacteria, in the same environment does not frequently occur in winemaking. Usually, the bio-transformation of grape must in wine drives the evolution of microflora due to the progressive disappearance of essential substrates (sugars and nitrogen compounds) and the accumulation of toxic agents, such as ethanol and SO₂. This environmental evolution selects the oenological microflora (Barata et al. 2012). LAB is able to develop after glucose and fructose depletion, when the more vigorous *S. cerevisiae* disappears (Liu 2002, Alexandre et al. 2004, Lonvaud-Funel 1999). The interaction between yeast and bacteria during winemaking has been already studied, identifying molecules released by yeast that are capable of repressing LAB activity (Alexandre et al. 2004). In addition, Costello et al. (2003) have underlined the importance of a careful choice of the yeast-bacterial pair to avoid inhibition of MLF. In this work, the yeast strain Lalvin ICV D47 was chosen because no negative interactions with the two lactic bacteria strains involved in the experiments are known. However, observing the results of this work, it is hypothesizable that the response of microorganisms to the environmental stress, including non-ideal fermentation temperatures, is influenced by the competition between different microbial populations that can develop together.

In the first grape must (G1), which has a low sugar concentration and consequently, low potential alcohol content, *S. cerevisiae* does not apparently experience bacterial competition resulting in the regular evolution of AFs (Fig. 1). This behavior is consistent with those of

previous works concerning the interaction between yeast and bacteria during oenological fermentations (Alexandre et al. 2004; Jussier et al. 2006; Guzzon et al. 2013; Capozzi et al. 2019) which opened the doors to the practice of simultaneous inoculum in grape must of these two microbial populations. However, it is hypothesizable, observing the result of experiments performed at T3, that the co-presence of yeast and LAB in grape must at the beginning of AF makes microorganisms more sensitive to environmental stress factors, and in particular at the high temperature of fermentation. In G1 experiments, an incomplete AF was observed in T3 experiment, residing in wine with 3 g/L of sugars. This result is quite singular considering the low selective pressure that G1 exerts over microorganisms (Alcohol content around 11%, Table 3) and that the maximum temperature reached (32 °C), although unusual for the strain Lalvin ICVD47, within the survival range of the *S. cerevisiae*, and commonly employed in the reactivation of ADY (Rodríguez-Porrata et al. 2008).

In our opinion, the increase in yeast sensitivity to wine limiting factors due to the simultaneous presence of LAB becomes more evident in the experiments carried out in G2. In these conditions, *S. cerevisiae* was unable to conclude AF at the T3 temperature profile, leaving more than 30 g/L of sugar in the wine (Table 3). The ethanol potential of G2, around 13.5% (Table 3), should not represent a limit for selected yeast strains (Carrasco et al. 2001). Therefore, the causes of incomplete AF could be researched in the yeast sensitivity to high temperature of fermentation that, in our hypothesis, could be due to the interaction between yeast and LAB. Flow cytometric analysis would seem to confirm this hypothesis (Fig. 3). Under the T3 conditions, the yeast population suffered a rapid decline. This should not be surprising since the interaction, negative for cells, between ethanol and high temperatures in determining alterations of the cellular membrane is known (Woo et al. 2014). However, attention must be given to the fact that the two stress factors are detrimental for yeast even at such low values. The hypothesis suggested by the authors regarding the increase in the sensitivity of yeasts to stress factors in the presence of LAB could have relevant oenological implications and, therefore, must be confirmed with further tests.

The experimental plan envisaged the use of two strains of LAB with different characteristics. *L. plantarum* K45H is a homofermentative bacteria with poor tolerance to acidity. It is suitable for use in wine in the presence of sugar residues because an eventual glucose/fructose intake cannot cause the accumulation of acetic acid in wine (Guerzoni et al. 1995). *O. oeni* A4 is a heterofermentative microorganism with a proven resistance to difficult MLF conditions due to

the presence of limiting environmental factors (Lonvaud-Funel 1999; Guzzon et al. 2009). In experiments performed in G1, *L. plantarum* K45H resulted more efficient in the consumption of malic acid independently of pH and temperature. *O. oeni* A4 sustained reliable behavior at the first two temperature profiles (T1 and T2) although the conclusion of the MLFs was generally delayed with respect to the K45H strain, probably because the strain of *O. oeni* accumulated a lower amount of cells (Table 1). Tests carried out with the A4 strain at T3 did not register cell growth below 6 log units (Table 1) and consequently, the degradation of malic acid did not occur (Table 2). In the test performed in G2, both bacteria showed an increased sensitivity to fermentation temperature, resulting in the inability to conclude MLF in the tests carried out at T3. As for the yeasts, these results are unexpected because the characteristics of the grape must were not so selective as to inhibit LAB activity (Liu 2002; Guzzon et al. 2009). Again, it can be assumed that the coexistence of two microbial populations reduces their resistance to sub-optimal environmental conditions and, among these, to high fermentation temperature. Between the two species of lactic bacteria, *O. oeni* appeared the least suitable for simultaneous fermentation at high temperature. In G2T3 tests, the stopping of AF in the presence of an *O. oeni* population led to an increase in volatile acidity (Table 3) that is probably caused by the heterofermentation of sugars by bacteria. It is interesting to note that in the same conditions the activity of *L. plantarum*, a homofermentative bacteria, induces a slight increase in the concentration of lactic acid, without the accumulation of acetic acid (Table 2).

The GC-MS analysis of wine volatile profiles underlined differences referable both to the features of the grape musts and the technological variables adopted in experimental winemaking. An extensive discussion about the changes in the profile of volatile compounds of wines linkable to the ripeness of grapes is outside the topic of this work. However, it is plausible that the high number of volatile compounds found in G2 samples, which explains their positioning in the quadrant designed by the negative values of the 1st and 2nd factors of PCA (Fig. 4 a and b), was correlated at the accumulation kinetics of volatiles during grape ripening. The different technological variables tested (temperature and LAB strains) do not seem to have minimized these differences, since the wine samples obtained from G1 and G2 are clearly differentiated in the PCA analysis. This result could respond to doubts, frequently advanced by winemakers, regarding the use of selected cultures of yeasts and bacteria and the risks that these may standardize the features of wines, reducing their

typicality. Just a delay of about 10 days in the harvest time of grapes, coming from the same vineyard, was able to segregate the obtained wines in a more significant way than some technological variables, such as the yeast or bacteria strain and the fermentation temperature. However, in both grape musts, the strain of bacteria and the fermentation temperature caused differences in the volatile profile of wines, evident in the PCA analysis. The fermentation temperature was the most significant factor in the determining the features of wines. This observation is confirmed by the statistical treatment of data of Table 2 and 3 (two-way ANOVA test) and by the positioning of cases (wine samples) in the plane described from the first two variables of the PCA (Fig. 4). Considering that, for a large part of the compounds, the concentration in wine is inversely correlated to the 1st variable, it is evident that the increase in fermentation temperature causes a loss of volatile molecules, both for G1 and for G2 samples. This effect is maximized in the G2T3 sample, but shows a similar trend in G1 samples. Among the two strains of LABs, wines made by A4 had a lower content of volatile molecules, especially at the extreme T1 and T3 temperature profiles, while at T2, the profile of wines made by the different LAB strains almost overlap. These results fit well with the microbiological observation that underlined the stress exerted by high temperatures on LAB activity. The sensitivity of *S. cerevisiae* to high-fermentation temperature was recently confirmed by Pattanakittivorakul et al. (2019) who characterized some species of yeast in order to improve bioethanol production. Schwinn et al. (2019) observed differences in volatile accumulation by *S. cerevisiae* when increasing the temperature from 14 to 19 °C. Similarly, experiments performed in cider by Herero et al. (Herero et al. 1999a, 1999b, Herrero et al. 2006) indicated that the LAB metabolism's optimum activity is round 22–25 °C, with a reduction in acetic acid accumulation and the best efficiency in malic acid consumption. Temperatures above 30 °C affect the performances of bacteria, and in particular of *O. oeni*, with a reduction in malic acid degradation and an increase in fructose assimilation, potentially dangerous due to the accumulation of acetic acid (Pimentel et al. 1994; Woo et al. 2014). Regarding the comparison between *Lactobacillus* sp. and *Oenococcus* sp., back in 1994, Guerzoni et al. had already observed that the first genera showed the best resistance at the simultaneous action of different limiting factors at sub-lethal level, the same situation that is observable in G2.

In conclusion, this work enhances knowledge on the simultaneous fermentation of yeast and bacteria in wine confirming the potential interest of this practice in winemaking. However, the results obtained suggest that the behavior of microorganisms, both in terms of resistance

to limiting factors and in the production of valuable metabolites, is affected by the interaction of different and highly concentrated microbial populations that coexist in the same environment. Therefore, a careful choice of the yeast and bacteria strain and the control of fermentation temperature are mandatory to prevent wine deterioration due to the loss of valuable volatile fractions and the accumulation of unpleasant compounds, such as acetic acid.

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Authors' contributions

Guzzon Raffaele and Larcher Roberto have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data. Tomas Roman has been involved in drafting the manuscript or revising it critically for important intellectual content. The authors read and approved the final manuscript.

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Ethics approval and consent to participate

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Competing interests

The authors declare that they have no conflict of interest. All experiments were performed in total autonomy and without any pressure or interference from external companies or financiers

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