Impact of changes in wine composition produced by non-*Saccharomyces* on malolactic fermentation

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Abstract

Non-*Saccharomyces* yeasts have increasingly been used in vinification recently. This is particularly true of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, which are inoculated before *S. cerevisiae*, to complete a sequential alcoholic fermentation. This paper aims to study the effects of these two non-*Saccharomyces* yeasts on malolactic fermentation (MLF) carried out by two strains of *Oenococcus oeni*, under cellar conditions. Oenological parameters, and volatile and phenolic compounds were analysed in wines. The wines were tasted, and the microorganisms identified. In general, non-*Saccharomyces* created more MLF friendly conditions, largely because of lower concentrations of SO_2 and medium chain fatty acids. The most favourable results were observed in wines inoculated with *T. delbrueckii*, that seemed to promote the development of *O. oeni* and improve MLF performance.

Keywords

Non-*Saccharomyces*, malolactic fermentation, *Oenococcus oeni*, wine

1. Introduction

Wine is the result of the alcoholic fermentation (AF) of grape must in a complex microbial environment. In the first stages of AF, a high diversity of yeast genera are involved: *Hanseniaspora*, *Pichia*, *Torulaspora*, *Metschnikowia* and *Starmerella*, among others (Beltran et al., 2002; Capozzi et al., 2015). When the ethanol concentration begins to increase, in the presence of added SO_2, a process of yeast selection begins, with *Saccharomyces cerevisiae* being the final predominant species. In this scenario, fermentation performance is highly influenced by the species fermenting the must (Capozzi et al., 2015; Comitini et al., 2011; Morata et al., 2019). To better control the process, it has long been proposed that *S. cerevisiae* be used as the most preferred yeast starter culture in must (Fleet and Heard, 1993).

During or after AF, malolactic fermentation (MLF) with lactic acid bacteria (LAB) as drivers can occur. This fermentation consists of the decarboxylation of L-malic acid to L-lactic acid. The species that dominates the process is *Oenococcus oeni* (Davis et al., 1985). MLF improves the quality of wine since this biotransformation increases pH, enhances organoleptic properties and has a positive role in microbial stabilisation (Lerm et al., 2010; Lonvaud-Funel, 1999). So, MLF is usually desirable in red wines or highly acidic white wines.

Since inoculation has become a usual cellar practice, *S. cerevisiae* has been used as the most preferred commercial starter culture. Research into non-*Saccharomyces* yeasts has increased in the last decade, and some of them have been proposed as starter cultures (Roudil et al., 2020). These non-*Saccharomyces* usually cannot finish AF, so they are inoculated with *S. cerevisiae*. The usual strategy is first to inoculate the non-*Saccharomyces* yeast and then *S. cerevisiae* (González-Royo et al., 2015; Jolly et al., 2014). In this sequential inoculation, the time that the non-*Saccharomyces* ferments by itself will determine the wine characteristics (Martín-García et al., 2020). Another good strategy is to co-inoculate both yeasts into the initial must (Azzolini et al., 2012; Belda et al., 2015; Ciani et al., 2010; Comitini et al., 2011; Jolly et al., 2014; Renault et al., 2015).

Among non-*Saccharomyces* that have been used most are *T. delbrueckii*, *M. pulcherrima* or *Lachancea thermotolerans*, which are available to oenological companies as starter cultures (Roudil et al., 2020). There are also available commercial strains of *Lachancea thermotolerans*, *P. kluyveri* and *Schizosaccharomyces pombe* (Jolly et al., 2006; Petruzzi et al., 2017). Other used non-*Saccharomyces* have been *St. bacillaris* (synonym: *Candida zemplinina*) and *H. uvarum* (anamorph *Kloeckera apiculata*) (Ciani and Maccarelli, 1998; Comitini et al., 2011; du Plessis et al., 2017; Englezos et al., 2019;

Giaramida et al., 2013; Kapsopoulou et al., 2005). Most of these species modulate the chemistry of wine by releasing aroma (Belda et al., 2017; Ramírez et al., 2016), which, among other things, decreases the ethanol concentration (Belda et al., 2017; Contreras et al., 2014), and increases glycerol and mannoprotein concentrations (Belda et al., 2016; Benito et al., 2015; González-Royo et al., 2015).

The chemical characteristics of wine are the consequence of the metabolism of the yeasts that dominate the AF. So, yeasts can positively, neutrally or negatively affect MLF performance (Balmaseda et al., 2018). It is difficult to classify the interactions between yeasts and *O. oeni* since these effects are highly influenced by the media -the grape matrix or the synthetic must or wine- and the strains (Alexandre et al., 2004). The studies that have been carried out showed that non-*Saccharomyces* chemical modulation affects *O. oeni* and MLF performance (Alexandre et al., 2004; du Plessis et al., 2017; Englezos et al., 2019; Martín-García et al., 2020; Ramírez et al., 2016). Of all the non-*Saccharomyces* species described as modulators of the organoleptic profile of wine, *T. delbrueckii* and *M. pulcherrima* are related with positive chemical changes in the development of MLF (Balmaseda et al., 2018; Ramírez et al., 2016). These changes are always referred to AF carried out with *S. cerevisiae* as sole starter. This is why these two species are of particular interest for stimulating the performance of MLF in harsh oenological conditions. Notably, in sequential inoculation with *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* can produce wines with less ethanol content (Belda et al., 2016; Contreras et al. 2014; Morata et al. ,2019), and higher mannoprotein concentration (González-Royo et al., 2015), and can decrease both the acetic acid and SO_2 concentration and increase the pH (Martín-García et al., 2020).

However, there is little information about how these interactions can affect wine quality after MLF under cellar conditions. For this reason, the aim of the present paper was to study the effects of non-*Saccharomyces* yeasts and *O. oeni* interactions in red and white winemaking, with particular focus on *T. delbrueckii* and *M. pulcherrima*.

2. Material and Methods

2.1. Microorganisms and inocula

Three commercial yeast strains (Lallemand Inc., Montréal, Canada) were used: *T. delbrueckii* Biodiva (Td), *M. pulcherrima* Flavia (Mp) and *S. cerevisiae* Lalvin-QA23 (Sc). Yeasts were stored at 4ºC as active dry yeasts provided by the manufacturer. For *O. oeni*, PSU-1 (ATCC BAA-331) and Viniflora CH11 (Chr. Hansen Holding.AS, Hoersholm, Denmark) were used. *O. oeni* was maintained on MRSmf

plates (Margalef-Català et al., 2017) and stored at 4°C. To obtain the inocula, a colony was picked from the plates and grown in liquid media at 27°C in a 10% CO_2 atmosphere (*O. oeni*). Then, 500 µL was inoculated in 50 mL of the same fresh liquid media.

2.2. Fermentation trials

Fermentations were carried out with white Macabeo and red Cabernet Sauvignon grape varieties (*Vitis vinifera* L.) during vintage 2018 from the AOC Tarragona (Spain) in the experimental cellar of the Rovira i Virgili University. About 100 kg of each grape variety were manually harvested. Macabeo grapes were destemmed and crushed and finally pressed using the cellar machinery and the resulting must was sulphited with 2.5 g/hL $K_2S_2O_5$ (Fisher Scientific, Madrid, Spain), transferred to a new container and cooled for 24h at 4ºC. Then, the clarified juice was transferred to the fermentation tanks (10 L). Cabernet Sauvignon grapes were destemmed and berries were randomly distributed in 9 batches of 6.5 kg. Each batch was crushed and distributed in food-grade plastic containers, used as fermenters. Then, the crushed grapes were sulphited (2.5 g/hL $K_2S_2O_5$). Fermenting white and red musts were supplemented with nutrients (0.2 g/L Nutrient Vit Nature™, Lallemand Inc., Montréal, Canada) when half the sugars were consumed. Red wines were punched down every 48 hours during the alcoholic fermentation using a stirring tool, moreover the grape skins were always submerged thanks to a flat strainer used as stopper in the fermenter.

Alcoholic fermentations were carried out with two non-*Saccharomyces* strains and by inoculating *S. cerevisiae* after 48h. In accordance with the manufacturer's instructions, each yeast was inoculated for a population of $2.5 \cdot 10^6$ cells/mL with active dry yeast. There was also a control fermentation with *S. cerevisiae* as the sole starter (Sc). All fermentations were performed in triplicate. Samples were taken every 48h to monitor the decrease in density and the evolution of the yeast population. YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac Química SLU, Castellar del Vallès, Spain) was used to calculate the total number of yeast cells present, and lysine agar medium (Oxoid LTD., Basingstoke, UK) was used to quantify the non-*Saccharomyces* yeasts (Wang et al., 2016), after incubation at 28°C for 48h. AF was considered to have finished when the sugar concentration was below 2 g/L. Macabeo fermentations were carried out at 18ºC and Cabernet Sauvignon fermentations at 22ºC.

After AF, the wines were transferred to a new container, cooled for 5 days and decanted. Then, samples were taken centrifuged and stored at -20ºC. In the case of Cabernet Sauvignon, the wines obtained were first pressed. Later, equal volumes of each triplicate (0.5 L) were blended, and 1.5 L was bottled and sulphited (1 g $K_2S_2O_5$/hL) in two 0.75 L bottles. These bottles were stored at 4ºC until tasting. The residual volume of the mixed wines was supplemented with L-malic acid to achieve a concentration of 2 g/L. Then, the pH was corrected to the value before L-malic acid addition. Adjusted wines were inoculated with two *O. oeni* strains, each in 1 L flasks at 20ºC to have a population of 10^7 cells/mL. There was also an uninoculated MLF (spontaneous). These fermentations were also carried out in triplicate. Samples were taken every 24h to monitor the consumption of L-malic acid and the evolution of the bacterial population. Samples were plated on MRSmf supplemented with 100 mg/L nystatin (Panreac Química SLU, Castellar del Vallès, Spain), 25 mg/L sodium azide (G BioSciences, St. Louis MO, USA) and 100 mL/L of tomato juice (Aliada, Madrid, Spain) and incubated at 27°C in a 10% CO_2 atmosphere for 7-15 days. MLF was considered to have finished when the L-malic acid was below 0.05 g/L. After MLF, samples of each triplicate were centrifuged and stored at -20ºC. Also, 1.5 L of the mixture of the triplicates was bottled, sulphited and stored as described above.

2.3. Yeast identification

Twenty-five colonies were randomly selected and isolated from the following samples: must before inoculation, must before inoculating Sc (48h) and wine at the end of AF (density below 995 g/L and residual sugars below 2 g/L). Isolates were identified, on the basis of the amplicon size of the ITS-5.8S rDNA region, to species level (Esteve-Zarzoso et al., 1999).

2.4. LAB identification and strain typing of Oenococcus oeni

At least 25 colonies were randomly selected for LAB identification from the following samples: must before inoculation, wine at the end of AF and at the end of MLF (L-malic acid below 0.05 g/L). The LAB were identified and the *O. oeni* typed as described in Franquès et al. (2018). Briefly, LAB isolates with cocci morphology were confirmed to be *O. oeni* by species-specific PCR according to Zapparoli et al. (1998). Non-*Oenococcus* isolates were identified with the 16S-ARDRA method and MseI digestion according to Rodas et al. (2003). The isolates identified as *O. oeni* were typed by the multilocus variable number tandem repeat (VNTR) method based on Claisse and Lonvaud-Funel (2014).

For LAB identification by 16S-ARDRA and for *O. oeni* typing, DNA was extracted with the High Pure PCR Template Preparation Kit (Roche, Barcelona, Spain).

2.5. Analysis of general oenological parameters

Wines after AF and after MLF were characterised. Concentration of sugars (glucose and fructose), L-malic acid, acetic acid, glycerol, D- and L-lactic acids, primary amino nitrogen (NOPA), NH_4, total and free SO_2, succinic acid and citric acid were determined by enzymatic methods using Miura One Multianalyzer (TDI, Barcelona, Spain). pH was determined using a Crison micro pH 2002 pH-meter (Hach Lange Spain, l'Hospitalet, Spain) and alcoholic content was determined by ebulliometry (Electronic ebulliometer uEBU6576, GabSystem, Moja, Spain) in accordance with the *Compendium of International Methods of Analysis of Musts and Wines* (OIV, 2009).

2.6. Analysis of volatile compounds

Wine samples (10 mL) were taken after AF and MLF. The volatile compounds were liquid/liquid extracted with 0.4 mL dichloromethane and 2.5 g $(NH_4)_2SO_4$ using 4-methyl-2-penthanol (0.8 g/L) and heptanoic acid (0.7 g/L) as internal standards, following Ortega et al. (2001). All reagents were analytical grade from Sigma-Aldrich (St. Louis MO, USA). After 90 min agitation at room temperature and centrifugation (6000 rpm, 5 min), the organic phase was extracted and 2 µL was injected in split mode (10:1, 30 mL/min) into a gas chromatograph (Agilent Technologies, Germany) with a FFAP column of 30 m × 0.25 mm × 0.25 µm. The temperature of the program started at 35ºC during 5 min, was then increased by 3ºC/min to 200ºC, and finally 8ºC/min to 220ºC. The temperatures of the injector and detector were 180ºC and 280ºC, respectively. The gas carrier was He at 3 mL/min. Aromatic volatile compounds were identified and quantified by comparison with standards.

2.7. Colour parameters and phenolic determination

Colour parameters (A420, A520 and A620) of wine samples were analysed in a 1 mm cuvette as reported by Glories (1984). CIELab coordinates: lightness (L), chroma (C), hue (h), red-greenness (a) and yellow-blueness (b) were determined in accordance with Ayala et al. (1997) and data processing was performed with MSCV software (Universidad de la Rioja, Logroño, Spain).

The phenolic composition of red wines was analysed in terms of total polyphenol index (TPI), tannin concentration and anthocyanin concentration. TPI was analysed by measuring the 280 nm absorbance of a 1:100 dilution of red wine with a spectrophotometer. A 10 mm quartz cuvette was used and the absorbance value was multiplied by 100. The tannin concentration was determined using the Bate-Smith method (Ribéreau-Gayon and Stonestreet, 1966) with some modifications (Vignault et al., 2018). The total anthocyanin concentration was determined by the decolouration of wines with sodium metabisulphite (Fisher Scientific, Madrid, Spain).

2.8. Wine tasting

Sensory analyses were performed with Macabeo and Cabernet Sauvignon wines after AF and MLF after two months of bottling. Triplicates were blended for simplifying the sensory analysis. Wines were evaluated by 18 trained judges, considered as experts from the Faculty of Oenology of the Rovira i Virgili University. 20 mL of wine were presented in dark glasses to avoid subjectivity by the colour of the samples. Three series of tastings were performed for each type of wine: Sc *vs.* Td, Sc *vs.* Mp, Td *vs.* Mp. Samples were randomly numbered with 3-digit codes. Wines were served anonymously according to a Latin square of Williams design to avoid range and carry-over effect. Each wine was tasted twice in different series. The descriptive test emphasised the aroma and flavour attributes: lactic character (both white and red wines), fruitiness (both), flowery (white), reduction (both), acidity (both), bitterness (white), astringency (red), balance in mouth (both) and global impression (both). Tasters had to score in a structured scale from 0 (no detection) to 5 (the highest) the intensity of each attribute.

2.9. Statistical analyses

The statistical software XLSTAT version 2019.1.2. (Addinsoft, Paris, France) was used. The data obtained was submitted to one-way ANOVA with a subsequent analysis using the Tukey HSD (*Honestly Significant Difference*) test, with a confidence interval of 95% and significant results with a p-value $\leq$ 0.05. Principal component analyses (PCA) were also performed to determine differences between the wines.

The same XLSTAT software was used to analyse the *O. oeni* genotypic profiles obtained by VNTR with Agglomerative Hierarchical Clustering and Spearman's rank correlation. Genotypes were defined at a minimum similarity level of 95.7% as described by Cruz-Pío et al. (2017).

The results of sensory analysis were submitted to Student's t-test. They were considered significant when the associated p-value was below 0.05. The analyses were performed using PanelCheck software (V1.4.2). PanelCheck software (2006) Nofima Mat, Ås, Norway (http://www.panelcheck.com).

3. Results and discussion

3.1. Fermentation performance

Alcoholic fermentation in Macabeo wines lasted between 21 and 37 days (Table 1). When *S. cerevisiae* was the only yeast inoculated, the fermentation ended first (21 days). The delay in AF was more marked when *T. delbrueckii* was sequentially inoculated (37 days) than *M. pulcherrima* (30 days). These differences were also reflected in the rate of AF calculated by density drop per day (Table 1). This may be due to the competition between the two starters. Although *T. delbrueckii* is regarded as a good fermenter (Belda et al., 2016), the presence of *S. cerevisiae* in the media can alter its performance (Wang et al., 2016). As a result, the AF takes longer. In the case of *M. pulcherrima* sequential fermentations, the AF may have taken only a little longer because of the higher sensitivity of this particular strain to *S. cerevisiae* (Wang et al., 2016). The prevalence of the non-*Saccharomyces* population, seen by plate counts in lysine agar medium, was the same in Td and Mp wines, and was lost after 19 days. In Sc wine, we did not detect autochthonous non-*Saccharomyces* after 6 days (data not shown).

In the case of Cabernet Sauvignon fermentation, the AF lasted 14 days in all conditions. The fact that the AF lasted less in this red wine than in the white one, and mainly for the non-*Saccharomyces* wines, could be explained by the lower content of nutrients in white wine, and the higher fermentation temperature (22ºC *vs.* 18ºC for red and white AF, respectively). It is noticeable that sugar consumption did not start until *S. cerevisiae* had been inoculated in Mp wines (results not shown) after which the AF rate became the quickest (Table 1). As in Macabeo wines, the non-*Saccharomyces* population survived longer (11 days in Td and 10 days in Mp wines) than the autochthonous non-*Saccharomyces* which were viable for 4 days in Sc wines (data not shown).

As far as MLF is concerned, both the inoculated and spontaneous fermentations finished (Fig. 1). By the end of AF, in all wines except Sc Macabeo the LAB population was higher than 10^5 CFU/mL (data not shown). In this last wine, the bacterial population was around 10^2 CFU/mL and the spontaneous MLF took 15 days to reach 10^6 CFU/mL and start consuming L-malic acid. At this moment, the acid was consumed very quickly. All MLFs performed in wines previously inoculated with a non-*Saccharomyces*

were quicker than the ones performed in Sc wines (Fig. 1 and Table 1). This shows that non-*Saccharomyces* somehow diminish the harsh conditions of wine at the end of AF, and in this way these yeasts are beneficial for *O. oeni* and MLF. Mainly in Sc, spontaneous MLF took longer to completely consume the L-malic acid. No great differences were observed in the speed of L-malic acid consumption in red winemaking (Table 1), but statistically it was slower in Sc wines.

3.2. Microbial population's analysis

Prior to inoculation, grape musts had a high initial yeast concentration of $1.1 \cdot 10^5$ CFU/mL (YPD) and $5.3 \cdot 10^4$ CFU/mL (Lys) for Macabeo and $3.2 \cdot 10^5$ CFU/mL (YPD) and $1.1 \cdot 10^5$ CFU/mL (Lys) for Cabernet Sauvignon. The implantation of the active dry yeast strains used to inoculate the grape musts was 90% or higher in all cases (data not shown). The inoculations were successful in both white and red wines, and the imposition of non-*Saccharomyces* at 48h (before *S. cerevisiae* was inoculated), and the imposition of *S. cerevisiae* at the end of AF was confirmed. In fact, by the end of AF the non-*Saccharomyces* population had been completely replaced by *S. cerevisiae*.

During MLF, *S. cerevisiae* was still found in populations between 10^3-10^5 CFU/mL (data not shown). After AF, wines were racked, cooled and decanted, but not filtered, so this would explain the presence of viable yeasts. During the time that MLF was carried out, the remaining viable yeasts began to die, losing their ability to grow on YPD plates.

Small concentrations of LAB were detected in must ($1.8 \cdot 10^2$ CFU/mL in Macabeo and less than 10^2 CFU/mL in Cabernet Sauvignon). All wines underwent a successful MLF, including those not inoculated with *O. oeni* (spontaneous MLF). In all cases, the population of the inoculated MLF remained constant at 10^7-10^8 CFU/mL until the end of the fermentation.

LAB isolates were identified from musts, wines after AF and wines after MLF. Of 576 isolates, 575 were identified as *O. oeni*. Firstly, 545 isolates were identified by species-specific PCR as *O. oeni* (Zapparoli et al., 1998). Then, the 31 isolates that resulted in no amplification by species-specific PCR were analysed by 16S rRNA ARDRA (Rodas et al., 2003) and 30 were confirmed as *O. oeni*. The one isolate that was not *O. oeni* was found in Cabernet Sauvignon must and was identified as *Pediococcus pentosaceus*. As expected, the predominant LAB found during the fermentation process were *O. oeni*.

The 575 *O. oeni* isolates were typified by VNTRs of 5 polymorphic alleles (Claisse and Lonvaud-Funel, 2014). The VNTR analysis revealed 13 different genotypes (Table 2), two of which – IN1 and IN2 –

corresponded to the inoculated *O. oeni* strains PSU-1 and Viniflora-CH11, respectively. Each genotype can be regarded as a different strain. The presence of strain diversity in MLF is a common phenomenon in wine (Lorentzen and Lucas, 2019). In the VNTR profiles obtained, the number of repeats of the alleles varied from 37 to 9 for TR1, 3 to 2 for TR2, 6 to 1 for TR3, 4 to 2 for TR4 and 4 to 1 for TR5.

The imposition of the commercial *O. oeni* strains was dependent on the grape variety of the wines (Table 2). Macabeo wines inoculated with *O. oeni* showed, to one extent or another, the presence of each inoculated strain at the end of MLF. Instead, all the Cabernet Sauvignon wines showed the highest imposition percentage for the IN1 genotype (corresponding to the PSU-1 VNTR profile), even in the wines inoculated with the CH11 strain (genotype IN2) and in spontaneous MLF. In fact, the IN1 genotype was already detected in Cabernet Sauvignon at the end of AF before *O. oeni* inoculation, meaning that the IN1 strain took over the other strains (inoculated or autochthonous) during MLF. The presence of the IN1 genotype in all Cabernet Sauvignon wines, including spontaneous MLF, indicates that this strain may have adapted to cellar conditions. The type of wine and winemaking practices can modulate the dynamics and prevalence of *O. oeni* strains. As described by several authors, commercial strains previously used in a cellar for several vintages can be detected in wines not inoculated with these strains (El Khoury et al., 2017; Franquès et al., 2017; González-Arenzana et al., 2014; Reguant and Bordons, 2003).

The highest number of different genotypes was detected in Td wines (Table 3). This suggests that the changes in wine composition produced by this yeast would enhance *O. oeni* strain diversity. Altogether, the prevalence of *O. oeni* strains during MLF depends on the type of winemaking (white or red) but also on the yeast species used.

3.3. General oenological parameters of wines

The composition of the two musts Macabeo (M) and Cabernet-Sauvignon (CS) was the same in sugars (152 g/L glucose and fructose), acetic acid (0.06 g/L) and NH_4 (30 mg/L), and similar in L- malic acid (1.5 g/L in M, 1.4 in CS) and citric acid (0.33 g/L in M, 0.5 in CS). The main differences were observed in pH, which was lower in Macabeo must (3.42) than in CS (3.75), and primary amino nitrogen (NOPA), which was higher in CS must (50.92 mg/L) than in M must (35.2). It should be noted that the YAN (Yeast Assimilable Nitrogen) for the two grape musts was lower than the accepted limit concentration (140 mg N/L) for finishing AF. Therefore, nitrogen was added in the middle of AF.

The composition of wines showed some differences when different yeast species were used. The main compounds analysed in both Macabeo and Cabernet Sauvignon wines are shown in Table 3. Other compounds, such as sugars, D-lactic acid, nitrogen compounds and succinic acid, were also quantified (Suppl. Table 1), but there were no relevant differences in their concentrations.

3.3.1. Macabeo wines

The production of acetic acid at the end of AF in Td wines was significantly lower than in Sc and Mp wines. The concentrations were statistically invariable after MLF in all but Mp wines, in which the concentrations increased. During MLF, LAB can metabolise citric acid, which increases the volatile acidity (Augagneur et al., 2007). At the end of AF, citric acid was similar in Sc and Mp wines whereas in Td wines, curiously, the concentration was almost undetectable. During MLF, it was consumed both in Sc and Mp wines by *O. oeni*. However, the increase in acetic acid was more noticeable in Mp wines. Although statistically significant, the increases detected in acetic acid concentration were low and the maximum increase was 0.09 g/L in Mp-P wine. Td wines showed the lowest concentrations of acetic acid because there was no citric acid at the end of AF that could be metabolised by *O. oeni* during MLF.

No differences were found in ethanol and glycerol analyses. In this study we observed no decrease in alcohol content associated with non-*Saccharomyces*, as has been described by other authors (Contreras et al., 2014; Quirós et al., 2014). The behaviour of Td Biodiva was similar to that found in a previous study in which this strain did not decrease ethanol content (Martín-García et al., 2020). However, in the same study Mp Flavia did significantly decrease it. The ability to reduce ethanol may be dependent on the must and winemaking conditions. The use of non-*Saccharomyces* tended to decrease the pH after AF, and this decrease was significant in Mp wine. After MLF, pH increased as expected due to the decarboxylation of L-malic acid although the pH in Td wines increased less. The lower pH in final Td wines may be because some organic acid compounds were not included in the analysis performed.

Total SO_2 decreased in Td wines after AF as determined in a previous study using the same Td strain (Martín-García et al., 2020). Anyway, the content of total SO_2, always less than 10 mg/L, was much lower than 35 mg/L, the limit of toleration for some of *O. oeni* strains, such as CH11 (Lerm et al., 2010).

3.3.2. Cabernet Sauvignon wines

Citric acid was present in similar concentrations at the end of AF and was consumed in all cases during MLF. Consequently, the acetic acid concentration increased to similar amounts in all wines due to *O. oeni* metabolism. The decarboxylation of L-malic acid increased pH, which reached a value close to 4. The high pH of these wines could have enhanced the strain diversity observed. In fact, Td-C wine had the highest pH (4.23) and showed the highest number of different strains at the end of MLF. Total SO_2 at the end of AF was significantly lower in Td wines, and anyway less than 10 mg/L, as for Macabeo wines. This could also have contributed to the greater diversity of *O. oeni* strains observed in the MLF of these wines. In Cabernet Sauvignon vinification, *M. pulcherrima* reduced the ethanol content by 0.5% (vol/vol). This result confirms that the ability of non-*Saccharomyces* to reduce ethanol depends on the type of must and winemaking conditions, since the behaviour of Mp was different in Macabeo wines. The reduction of ethanol could be due to the presence of higher levels of nitrogen and temperature during the fermentation process in Cabernet Sauvignon, compared to Macabeo must.

3.4. Volatile compounds

The volatile composition of wines showed that non-*Saccharomyces* had a considerable influence on the organoleptic profile of wines after AF. Both Macabeo and Cabernet Sauvignon wines presented clearly different profiles (Figure 2A and 2C). Interestingly, Td wines were characterised by higher concentrations of 1-butanol, ethyl butanoate, diethyl succinate and 2-methylpropanoic acid in white and red vinifications (Suppl. Figure 1, Suppl. Table 2). Ethyl esters, such as ethyl butanoate and diethyl succinate are compounds considered to be of primary importance for the fruity aroma of wine. Related compounds with aromas have been previously found also in Td wines by other authors (Azzolini et al., 2012; Ramírez et al., 2016; Renault et al., 2015). Fusel alcohol acetates were the only volatiles related to Sc in both wines and 2-methylpropanoic acid in Mp (Table 4, Suppl. Table 2).

The use of non-*Saccharomyces* yeasts reduced the concentration of medium chain fatty acids (MCFA). This reduction was significant in all Td and Mp wines after AF with respect to Sc wines although the differences were more relevant in Macabeo than in Cabernet Sauvignon wines (Table 4). The release of MCFA by wine yeast can inhibit *O. oeni* growth and malolactic activity and is considered to cause yeast-bacteria antagonism (Edwards and Beelman, 1987; Lonvaud-Funel et al., 1988). Capucho and San Romao (1994) reported the inhibitory effect on MLF of decanoic and dodecanoic acids at concentrations above 12.5 and 2.5 mg/L, respectively. The negative impact of MCFA can act synergistically with either low pH

and ethanol, inhibiting ATPase activity which is associated to MLF (Carreté et al., 2002). The lower concentrations of MCFA observed in Td and Mp wines in comparison to Sc wines could be due to the action of yeast ghosts generated towards the end of AF of sequential fermentations. In the second half of AF non-*Saccharomyces* viable populations dramatically decreased and dead cells may have adsorbed toxic compounds such as MCFA. In fact, the capacity of yeast ghosts of removing the inhibitory effect of some MCFA has been suggested by several authors (Edwards and Beelman, 1987; Lafon-Lafourcade et al., 1984). The decrease of MCFA was more noticeable in Td Macabeo wines where hexanoic and decanoic acids completely disappeared after AF and octanoic acid was reduced to more than 50% with respect to Sc wines (Suppl. Table 2). These evidences let us to hypothesise that the lowest MCFA concentrations in Td Macabeo wines would have been one of the reasons of a faster MLF than in the rest of the wines.

Ethyl lactate was higher in Td and Mp than in Sc Macabeo wines (Table 4). This would be due to the metabolic activity of autochthonous LAB, found in higher populations in these wines before *O. oeni* inoculation. Presumably due to the same reason, the development of autochthonous LAB, ethyl lactate was also high in all Cabernet Sauvignon wines at the end of AF. In fact, the increase of ethyl lactate in wine is associated to MLF metabolism (Liu, 2002).

No changes were observed in fusel alcohol concentration in the different conditions (Table 4) and also remained constant throughout the vinification process (Suppl. Table 2).

In Macabeo and Cabernet Sauvignon, independently of whether non-*Saccharomyces* were used, wines clustered in general on the basis of MLF strategy (Figure 2).

The Macabeo wines that clustered in terms of MLF strategy were all similar with the exception of Mp-C wines, which presented a high concentration of ethyl esters, which differentiated them from Sc-C and Td-C wines (Figure 2, Suppl. Figure 1). The combination of strains used in Mp-C was clearly the one producing a profile of volatile compounds more different from the rest. It should also be mentioned that the Mp-S wines clustered together with the wines inoculated with *O. oeni* PSU-1 (Figure 2B). This was mainly due to the similar production of propionic and pentanoic acids (Suppl. Table 2). Despite the fact that in all wines with spontaneous MLF the main *O. oeni* genotype detected at the end of the fermentation was AB1 (Table 3), the different profile of volatile compounds in Mp-S wines might be due to the metabolism of diverse strains developing along MLF. Spontaneous fermentations, lacking the pressure of

the massive inoculation of one strain, may allow a wider diversity of strains succession which can result in more unpredictable metabolic changes.

In contrast to white winemaking, in which spontaneous MLF presented the lowest concentrations of volatile compounds, in Cabernet Sauvignon this was the MLF strategy that resulted the most aromatic (Figure 2D). Despite the strain detected in higher proportion in all Cabernet Sauvignon wines at the end of MLF belonged to IN1 genotype (Table 3), these wines showed differences in the aromatic profile and clustered according to the MLF inoculation strategy (Figure 2D). Even if the inoculated strain was not detected at the end of MLF, as in the case of CH11, it may have been present in the early stages of the fermentation contributing to define the aromatic profile.

In summary, the volatile composition of wines was modulated by non-*Saccharomyces* yeasts to produce different wines. However, the aromatic composition was homogenised after MLF, which was dependent on the *O. oeni* strain inoculated.

3.5. Colour

The colour parameters of both Cabernet Sauvignon and Macabeo wines were analysed, but no changes were observed in the latter. Due to the considerable chemical changes in MLF, mainly driven by the increase in pH, the colour parameters were affected in the red wine (Suppl. Figure 2). As expected, wines after AF had higher values of h*, C* and a*. Interestingly, colour parameters, after both AF and MLF, are grouped in terms of yeast inoculation strategy. Wines inoculated with a non-*Saccharomyces* belong to a cluster different from those inoculated with *S. cerevisiae* as sole starter (Suppl. Figure 2).

3.6. Polyphenolic compound content

The overall content of polyphenols in red Cabernet Sauvignon wines did not change during the vinification process. The total polyphenolic index (TPI) remained around 40 in all wines (data not shown). This low value was associated with a less effective colour extraction due to the method used to punch down in the small volume fermenters. Regarding to the anthocyanin concentration, Td wines after AF presented higher anthocyanin amount than Sc wines (Suppl. Figure 3). These differences were maintained after MLF disregarding the *O. oeni* strain used. Also, all Mp wines after MLF showed higher concentrations of anthocyanin than Sc wines. These results are in accordance with previous works describing incremented amounts of anthocyanin in wines inoculated with *T. delbrueckii* and *M.*

pulcherrima when compared to wines inoculated only with *S, cerevisiae* (Escribano-Viana et al., 2019; Minnaar et al., 2015). No changes were observed in tannin concentrations.

3.7. Wine tasting

Cabernet Sauvignon sensory analysis did not result in concluding remarks. Wines could be clearly distinguished before and after MLF but there was not a clear clustering based on the inoculation strategy (data not shown). Macabeo wines were classified by tasters into three main clusters (Suppl. Figure 4): (i) wines after AF (red circle), (ii) wines after inoculated MLF (green circle) and (iii) wines after spontaneous MLF (blue circle). Wines after AF were the most acidic and oxidised. Wines with spontaneous MLF had the most intense lactic character. Just one inoculated MLF wine is not included in the second cluster: Td-P. Interestingly, this was the wine which tasters preferred and described with the most moderate marks.

The wine tasting revealed that the most important changes in the chemical composition of the wines were not perceived by tasters. In this regard, the wine which was the most different in terms of volatile composition, Mp-C, was grouped in the inoculated wine cluster. The tasting data on acidity correlated with the pH values of the most acidic wines after AF (Table 2).

Sensory analysis was more variable in Macabeo wines, probably because fermentation was slower. Moreover, most of the chemical changes brought about by the different starter cultures were not noticed in the sensory evaluation of the resulting wines.

4. Conclusion

This paper reports novel research into the evaluation of the effect of two non-*Saccharomyces* on MLF in white and red winemaking under cellar conditions. The changes in wine composition was dependent on the type of winemaking and on the yeast strains used. Regarding the colour in red winemaking, wines inoculated with non-*Saccharomyces* showed higher concentrations of anthocyanin at the end of MLF than those inoculated only with *S. cerevisiae*. The aromatic profile of the wines was very dependent on the MLF strategy, highlighting the impact of *O. oeni* metabolic traits on the organoleptic characteristics. The inoculation of non-*Saccharomyces* yeasts caused longer AF in Macabeo wines. The MLF was faster in most of the wines inoculated with non-*Saccharomyces*, and the differences were more evident in Macabeo wines. The use of *M. pulcherrima* and *T. delbrueckii* resulted in lower SO_2 and MCFA

concentrations at the end of the AF, offering more MLF friendly conditions than *S. cerevisiae* alone. The use of *T. delbrueckii* resulted in the fastest MLF in Macabeo wines and in the maximum *O. oeni* strain diversity in Cabernet Sauvignon wines. Altogether, *T. delbrueckii* metabolic fingerprint in wine seems to promote the development of *O. oeni* and improve MLF performance. Future research should attempt to provide greater insight into the impact of different *T. delbrueckii* strains on MLF in different types of wine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1. Evolution of malolactic fermentation after AF by monitoring the L-malic acid consumption. Left: Macabeo wines fermented with *S. cerevisiae* (A1), *M. pulcherrima* (A2) and *T. delbrueckii* (A3). Right: Cabernet Sauvignon wines fermented with *S. cerevisiae* (B1), *M. pulcherrima* (B2) and *T. delbrueckii* (B3).

Figure 2. Principal component analysis (PCA) biplots of varimax rotated PCA for wine volatile composition in which observations are plotted. (A) Macabeo wines after AF. (B) Macabeo wines after MLF. (C) Cabernet Sauvignon wines after AF. (D) Cabernet Sauvignon wines after MLF. Sc, Td and Mp correspond to *S. cerevisiae*, *T. delbrueckii- S. cerevisiae* and *M. pulcherrima-S. cerevisiae* fermented wines, respectively. P, C and S refers to the MLF strategy were *O. oeni* PSU-1, *O. oeni* CH11 or non-*O. oeni* was inoculated.