

Alba MARTÍN GARCIA

**EFFECT OF THE INOCULATION STRATEGY OF NON-
Saccharomyces YEASTS ON MALOLACTIC
FERMENTATION AND *Oenococcus oeni***

MASTER THESIS

Supervised by Cristina Reguant

Master's Degree in Fermented Beverages

Facultat d'Enologia



UNIVERSITAT ROVIRA I VIRGILI

**Tarragona
14th June, 2018**

Effect of the inoculation strategy of non-*Saccharomyces* yeasts on malolactic fermentation and *Oenococcus oeni*.

Martín, A.¹, Balmaseda, A., Bordons, A. and Reguant, C.

¹alba.marting@estudiants.urv.cat

Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, Marcel·lí Domingo s/n, Tarragona 43007, Spain

Abstract

In recent years, the use of non-*Saccharomyces* yeast has increased to obtain wines with a different organoleptic profile. In general terms, these yeasts are inoculated to begin alcoholic fermentation (AF) and later *Saccharomyces cerevisiae* is inoculated to finish the process. This sequential inoculation leads to wine quality enhancement. However, non-*Saccharomyces* can affect *Oenococcus oeni* and malolactic fermentation (MLF). In the present work, there were evaluated two non-*Saccharomyces* strains, *Torulasporea delbrueckii* (Biodiva) and *Metschnikowia pulcherrima* (Flavia), through mixed and sequential fermentations with *S. cerevisiae* (QA23). Sequential fermentations were performed with different times of inoculation of *S. cerevisiae* (24h, 48h and 72h). A fermentation with *S. cerevisiae* as a single starter was also performed as a control. Next, FML was performed in all wines inoculating *O. oeni* (PSU-1). Finally, wines obtained after AF and MLF were characterized. The results obtained from mixed fermentations did not show differences compared to *S. cerevisiae* control fermentation. Nevertheless, significant differences were observed in sequential fermentations regarding, mainly, acetic acid, L-malic acid and succinic acid content. Those differences were highlighted in fermentations carried out with *T. delbrueckii*. However, none of the non-*Saccharomyces* fermentations improved MLF speed.

Keywords: *Saccharomyces cerevisiae*, non-*Saccharomyces*, *Oenococcus oeni*, malolactic fermentation

Resum

Durant els últims anys s'ha augmentat l'ús dels llevats no-*Saccharomyces* per tal d'obtenir vins amb un perfil organolèptic diferencial. De manera general, s'inoculen aquests llevats al principi de la fermentació alcohòlica (FA) per després inocular *Saccharomyces cerevisiae* i així acabar el procés. Aquesta inoculació seqüencial comporta una millora de la qualitat del vi. Tot i així, els llevats no-*Saccharomyces* poden afectar *Oenococcus oeni* i la fermentació malolàctica (FML). En aquest treball, es van estudiar dues soques de llevats no-*Saccharomyces*, *Torulaspota delbrueckii* (Biodiva) i *Metschnikowia pulcherrima* (Flavia), mitjançant fermentacions mixtes i seqüencials amb *S. cerevisiae* (QA23). Les fermentacions seqüencials es van dur a terme amb diferents temps d'inoculació per a *S. cerevisiae* (24h, 48h i 72h). També s'ha realitzat una fermentació només amb *S. cerevisiae* a mode de control. A continuació, es va realitzar la FML inoculant *O. oeni* (PSU-1). Finalment es van caracteritzar els vins després de la FA i la FML. Els resultats obtinguts a partir de fermentacions mixtes no van mostrar diferències respecte el control amb *S. cerevisiae*. En canvi, es van trobar diferències significatives entre les fermentacions seqüencials i *S. cerevisiae* respecte la concentració d'àcid acètic, àcid L-màlic i àcid succínic, principalment. Aquestes diferències eren més destacables en les fermentacions realitzades amb *T. delbrueckii*. Tot i així, cap de les fermentacions amb no-*Saccharomyces* van incrementar la velocitat de la FML.

Paraules clau: *Saccharomyces cerevisiae*, no-*Saccharomyces*, *Oenococcus oeni*, fermentació malolàctica

Contents

1	INTRODUCTION	4
1.1	Non- <i>Saccharomyces</i> yeasts in winemaking	4
1.2	Malolactic fermentation and <i>Oenococcus oeni</i>	4
1.3	Yeast - lactic acid bacteria interactions	5
1.3.1	<i>Saccharomyces cerevisiae</i> - <i>Oenococcus oeni</i> interactions	5
1.3.2	Non- <i>Saccharomyces</i> - <i>Oenococcus oeni</i> interactions	6
2	MATERIALS AND METHODS	7
2.1	Microorganisms, culture medium, solutions and reagents	7
2.2	Strains' conservation	7
2.3	Growth curves and inoculum obtainment	7
2.4	Experimental fermentations	7
2.4.1	Must characterization	8
2.4.2	Alcoholic fermentation monitoring	8
2.4.3	Wine characterization	9
2.4.4	Malolactic fermentation monitoring	9
2.4.5	Final wine characterization	9
2.5	Statistical analysis	9
3	RESULTS	10
3.1	Experimental fermentations	10
3.1.1	Alcoholic fermentation	10
3.1.2	Malolactic fermentation	11
3.1.3	Changes in wine composition	11
4	DISCUSSION	16
5	CONCLUSIONS AND PERSPECTIVES	19
6	FUNDING	20
7	ACKNOWLEDGEMENTS	20
8	REFERENCES	21
	APPENDIX I: Microorganisms, culture media, solutions and reagents	26

1. INTRODUCTION

1.1. *Non-Saccharomyces yeasts in winemaking*

The conversion of grape must to wine is a complex microbial process based in the alcoholic fermentation (AF) driven out by oenological yeasts (Beltran et al., 2002). Although *Saccharomyces cerevisiae* is recognized as the main yeast species that carries out this process, there are other yeast genera involved, such as *Hanseniaspora*, *Torulaspota* or *Metschnikowia* (Petruzzi et al., 2017). These species are, generally, found in early stages of the AF (Fleet et al., 1984), and they are known as non-*Saccharomyces* yeasts.

S. cerevisiae is considered the stater yeast culture par excellence in winemaking, due to its resistance to ethanol and SO₂, and its great fermentation activity. But, because of an emerging interest in the use of non-*Saccharomyces* yeast for the AF, this industry is currently changing in order to improve product quality and complexity (Ciani and Maccarelli, 1998; Contreras et al., 2014). Thus, there is a new trend in winemaking based in the use of mixed starter cultures of non-*Saccharomyces* and *S. cerevisiae* (Padilla et al., 2016) or a sequential inoculation with *Saccharomyces cerevisiae* (Lu et al., 2017; Mendoza and Farías, 2010).

Between those improvements, some authors have reported ethanol reduction in sequential fermentations with *Metschnikowia pulcherrima* (Contreras et al., 2014; Ciani et al., 2016); increase of glycerol content in mixed fermentations with *Starmerella bacillaris* (syn. *Candida zemplinina*) (Englezos et al., 2016); and lower levels in total acidity in sequential fermentations with *Torulaspota delbrueckii* (Belda et al., 2014), among others. But, besides the enhancement of the product thanks to the use of non-*Saccharomyces* yeasts, it is noteworthy that most of these microorganisms have low or moderate fermentation power, leading to an impossibility to finish the alcoholic fermentation by themselves. As a result, it is necessary to inoculate *S. cerevisiae* to finish the AF and obtain a proper wine (Benito et al., 2015). Because of that, it is important to determine the appropriate inoculation time for *S. cerevisiae* that enables the non-*Saccharomyces* yeast to make those chemical changes and contribute to wine differentiation.

1.2. *Malolactic fermentation and Oenococcus oeni*

Malolactic fermentation (MLF) is a biological conversion consisting on L-malic acid decarboxylation to produce L-lactic acid, which improves microbiological stability (Sumbly et al., 2014), and induces some other desirable changes such as acidity decrease or the formation of volatile compounds responsible for wine aroma (Bartowsky, 2005). This process is carried out by lactic acid bacteria (LAB), being *Oenococcus oeni* the main LAB species to perform it (Versari et al., 1999).

MLF takes place during or after alcoholic fermentation, usually in red wines, but also in some high acidity white wines, from cold zones. Although it may be beneficial in some wines, in others can diminish its quality. Depending on wine's acidity after AF and the LAB present in it, MLF can produce unwanted organoleptic changes and other products like biogenic amines (Liu, 2002).

Because of the wine medium (high ethanol content, low pH, SO₂, lack of nutrients, etc.), *O. oeni*'s growth and, in consequence, MLF present some trouble (Versari et al., 1999). Nevertheless, due to *O. oeni*'s acid tolerance and flavour profile produced, out of the existing LAB, it is the preferred one to carry out the MLF (Liu, 2002).

1.3. Yeast - lactic acid bacteria interactions

As previously described, MLF is conditioned by yeasts, there exists interaction between yeast and lactic acid bacteria (Balmaseda et al., 2018). Those interactions can have different effects on MLF, they can be positive or stimulating, negative or inhibitory, or they can be neutral, with no effect over it (**Table 1**).

Because of the extended use of *S. cerevisiae*, its effect upon *Oenococcus oeni* has been widely studied. But, due to the increasing interest on non-*Saccharomyces* yeasts, it is important to determine the effect of this interactions. Fundamentally, the compounds responsible of these effects are the same as in *S. cerevisiae* fermentations, but their concentration in wine is different (Ciani and Maccarelli, 1998; Bely et al., 2008; Belda et al., 2014).

Table 1: Compounds produced by yeast with negative, positive or unknown effect on *Oenococcus oeni*. Adapted from (Balmaseda et al., 2018).

Yeast - <i>O. oeni</i> interaction		
Inhibitory effect	Stimulatory effect	Unknown effect
Ethanol	Citric acid	Other
SO ₂	Piruvic acid	
pH	Compounds derived	
Nutrient exhaustion	from yeast autolysis	
Medium Chain Fatty Acids		
Organic acids		
Antimicrobial peptides		

1.3.1. *Saccharomyces cerevisiae* - *Oenococcus oeni* interactions

Out of all the yeast-LAB interactions, the inhibition of LAB is the most studied mechanism, due to the negative impact potential on final wine quality, mostly over its acidity. This antagonistic interaction depends on multiple factors, and even the combination of them, those being, for example, ethanol, lack of nutrients, or production of toxic metabolites (Alexandre et al., 2004).

The main compound produced by yeasts causing inhibition of *O. oeni* and MLF is, of course, ethanol. But, as stated before, other compounds must be taken into account, such as SO₂ (Wells and Osborne, 2011) since it has been reported that some *S. cerevisiae* strains can produce 100mg/L or more.

As previously stated, wine's low pH and lack of nutrients are two important limiting factors for *O. oeni*'s growth, exercising an inhibition on its development and, consequently, the MLF (Guerzoni et al., 1995; Cinquanta et al., 2018). In addition, they act in synergy with other factors, like SO₂, affecting negatively the MLF (Britz and Tracey, 1990; Gockowiak and Henschke, 2003). Medium chain fatty acids (MCFA) are also known inhibitors of MLF and *O. oeni*, especially C₁₀ and C₁₂, that also act in synergy with low pH and ethanol (Capucho and San Romão, 1994; Carreté et al., 2002).

Apart from the previously mentioned factors, some *S. cerevisiae* strains can produce antibacterial proteins (Comitini et al., 2005; Mendoza et al., 2010; Branco et al., 2014), that slow the MLF. In addition, it has been reported that some cryotolerant strains have great production of succinic acid (Caridi and Corte, 1997), which acts as a competitive inhibitor of L-malic acid by the malolactic enzyme and, therefore, inhibits *O. oeni*'s growth (Lonvaud-Funel and Strasser de Saad, 1982).

However, it must be considered the positive effects of *S. cerevisiae* on *O. oeni* and MLF. The most known stimulatory effect is the performance of MLF with yeast lees. Yeast undergo an autolytic process that releases a variety of nitrogen compounds, such as aminoacids, peptides and proteins (Martínez-Rodríguez et al., 2001; Patynowski et al., 2002). This enrichment is beneficial for lactic bacteria, due to their auxotrophy for a great variety of aminoacids (Terrade and Mira de Orduña, 2009). Other macromolecules like mannoproteins or glucans are also released during autolysis and help attenuating some negative effects on bacteria's growth, since they can absorb MCFAs (Guilloux-Benatier et al., 1995).

1.3.2. Non-*Saccharomyces* - *Oenococcus oeni* interactions

When it comes to non-*Saccharomyces* yeast, similarly as *S. cerevisiae*, there are interactions between the yeasts and *O. oeni* that can be inhibitory, stimulatory or neutral, but it has not been as studied as *S. cerevisiae* interactions (Balmaseda et al., 2018).

One of the main reasons to use non-*Saccharomyces* yeasts, as previously described, is the reduction of wine ethanol content (Contreras et al., 2014), which facilitates *O. oeni*'s survival and, in consequence, the MLF performance. As for glycerol, some LAB can assimilate and degrade it to an acrolein precursor, but *O. oeni* has not been described as one of them. Therefore, a modification of the glycerol concentration in wine has not been reported to affect the MLF or the bacteria.

Among the organic acids present in wine, L-malic acid is, logically, the most important in MLF. Different authors agree to observe a reduction of L-malic acid when the AF is performed with non-*Saccharomyces* yeasts. Belda et al. (2014) observed that fermentations with *T. delbrueckii* reduced from 10% (co-inoculation with *S. cerevisiae*) to 15% (*T. delbrueckii* as a sole starter) the L-malic acid content. This can affect negatively *O. oeni*, since it is its principal substrate during the MLF and one of the few energy sources left in wine after the AF. Regarding to the citric acid, another important organic acid related to *O. oeni*, it has been reported a light increase in mixed fermentations with *S. bacillaris* (Giaramida et al., 2013); whereas other authors showed no changes in citric acid concentration in sequential fermentations with *Wickerhamomyces anomalus* and (Izquierdo Cañas et al., 2014).

As previously introduced, some *S. cerevisiae* strains can produce significant concentrations of succinic acid (Caridi and Corte, 1997), comparably to citric acid, some authors observed an increase of succinic acid in mixed fermentations with non-*Saccharomyces* (Contreras et al., 2014), while others reported a decrease of its concentration (Magyar et al., 2014).

To summarize, it needs to be considered the quantitative and qualitative differences between the compounds that can be found at the end of an AF with *S. cerevisiae* single-culture and the one with sequential or mixed inoculation of non-*Saccharomyces* yeast and *S. cerevisiae*. And, also, how this differences can affect the MLF and *O. oeni*. Therefore, the aim of this study was to determine the effect of non-*Saccharomyces* yeasts on *O. oeni* and the MLF, with different times of inoculation for *S. cerevisiae*.

2. MATERIALS AND METHODS

2.1. Microorganisms, culture medium, solutions and reagents

Every microorganism, culture medium, solution and reagent used in the present study is described in **Appendix I**.

2.2. Strains' conservation

In the present study, three yeasts species (*S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima*) and a strain of *O. oeni* were used (**Appendix I**). The microorganisms were maintained on YPD and MRS_{mf} plates, respectively, and stored at 4 °C. Those plates were renewed monthly through a subculture in new ones.

2.3. Growth curves and inoculum obtainment

First, each strain growth was characterized. To establish the growth curve, yeasts were incubated at 28 °C; and bacteria was incubated at 27 °C in a 10% CO₂ atmosphere. Their growth was followed by measuring the optical density at 600 nm (Spectro Genesis 10UV, ThermoScientific), and plating the appropriate dilution to determine the viable cells of the culture.

Regarding the inoculum, before obtaining it, it was prepared a pre-inoculum. From the YPD and MRS_{mf} plates, a colony was picked and grown in 6 mL of liquid medium (YPD for yeasts, MRS_{mf} for LAB). Then, yeasts were grown at 28 °C and *O. oeni* at 27 °C in a 10% CO₂ atmosphere and, after 24h and 3 days, respectively, 400 µl of the pre-inoculum were passed to 40 mL of fresh medium.

2.4. Experimental fermentations

Experimental fermentations were performed in 500 mL flasks containing 400 mL of sterile must, with a sugar concentration of 200 ± 10 g/L.

The must was inoculated with the non-*Saccharomyces* yeasts separately (**Appendix I**) to a population of 10⁶ CFU/mL, following the experimental design shown in **Figure 1**. After 24h, 48h and 72h of the inoculation of those yeasts, *S. cerevisiae* was inoculated also in a population of 10⁶ CFU/mL. Also, a fermentation with *S. cerevisiae* as sole starter was performed as control. All fermentations were carried out by triplicate.

The AF was considered finished when the sugar concentration was below 1.00 g/L. Then, in order to eliminate all yeasts, the resulting wine was centrifuged at 8500 rpm for 5 minutes and filtered (MF-Millipore™ 0.45 µm MCE Membrane; Ref.: HAWP04700).

Once the wine was filtered and there were no remaining viable yeasts, it was inoculated with *O. oeni* with a population of 2x10⁷ CFU/mL. The MLF was considered finished when the L-malic acid concentration was 0.00 g/L. These malolactic fermentations were also carried out by triplicate.

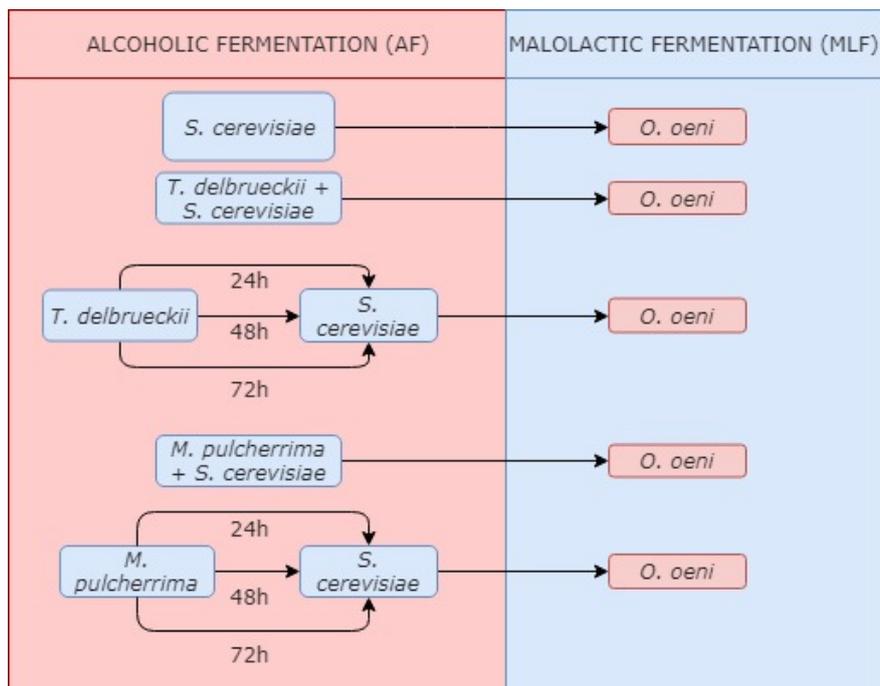


Figure 1: Diagram of the experimental fermentations. Each one was carried out by triplicate.

2.4.1. Must characterization

Fermentation must was prepared using rectified concentrated must (RCM) and sterile MilliQ purified water in order to get a must with a sugar concentration of 200 ± 10 g/L. In addition, six samples of 1 mL each were taken to measure different chemical compounds with the multianalyzer Miura One (TDI SL, Gavà, Spain) (ISE S.r.l., Ref.: 13310001200).

2.4.2. Alcoholic fermentation monitoring

Samples were taken every 24h to monitor sugar (glucose + fructose) consumption and yeast population evolution. Sugar samples consisted on 1 mL of the experimental fermentation. They were centrifuged at 8500 rpm for 5 minutes, diluted if necessary, and measured with the multianalyzer Miura One.

Regarding the yeast population dynamics, samples of 100 μ l were taken and diluted in sterile saline solution. The appropriate dilution was plated on YPD and lysine agar medium plates, and incubated at 28 °C for 48h.

The use of the lysine agar medium is due to the inability of *Saccharomyces cerevisiae* to grow in an environment with lysine as its only source of nitrogen. For that reason, the YPD medium provides total yeast count and lysine agar medium provides non-*Saccharomyces* cell count.

AF was considered finished when the sugar concentration is below 1 g/L. At this point, the wine was filtered (MF-Millipore™ 0.45 μ l MCE Membrane; Ref.: HAWP04700) in order to eliminate all yeasts. Then, 100 mL of the filtered wine were destined to perform malolactic fermentation, 6 mL were kept frozen for further chemical analysis and 250 mL were taken to measure ethanol content and pH.

2.4.3. Wine characterization

Measurement of ethanol content was performed by using an ebulliometer (Electronic ebulliometer uEBU6576, GabSystem). 100 mL of wine were destined to this purpose.

Of the remaining 150 mL of wine, pH was determined and different chemical compounds were analyzed. Those compounds were as it follows: glucose + fructose, L-malic acid, L-lactic acid, acetic acid, glycerol, citric acid, sulfite (total and free) and succinic acid. They were determined using a multianalyzer Miura One.

2.4.4. Malolactic fermentation monitoring

Samples were taken every 24h to monitor L-malic consumption and bacterial population evolution. Analogously to AF monitoring (**Section 2.4.2**), L-malic acid samples consisted on 1 mL of the fermenting wine, centrifuged at 8500 rpm for 5 minutes and measured with the Miura One multianalyzer. For bacterial population dynamics, samples of 100 μ l were taken and diluted in sterile saline solution. The appropriate dilution was plated on MRS_{mf} and incubated at 27 °C, with a 10% CO₂ atmosphere, for 7 days.

2.4.5. Final wine characterization

Once MLF was finished, the final wine was characterized following the same parameters explained in **Section 2.4.3**.

2.5. Statistical analysis

For the statistical treatments and analysis of the results, the statistics software *XLSTAT* version 2018.4.51298 was used.

Each fermentation was performed in three assays and its mean value and standard deviation were calculated. The obtained data was submitted to one-way ANOVA with a subsequent analysis using the Tukey test, considering a confidence interval of 95% and significant results when p-value ≤ 0.05 . A major component analysis (PCA) was also performed to determine differences between the wines obtained.

3. RESULTS

3.1. Experimental fermentations

3.1.1. Alcoholic fermentation

Alcoholic fermentations were carried out with two non-*Saccharomyces* yeasts (*T. delbrueckii* and *M. pulcherrima*) and inoculating *S. cerevisiae* with the following time regimes: co-inoculation (Td-Sc and Mp-Sc) and after 24h (Td.24h and Mp.24h), 48h (Td.48h and Mp.48h) and 72h (Td.72h and Mp.72h). There was also a control fermentation conducted with *S. cerevisiae* as a sole starter (Sc).

Figure 2.A shows the AF kinetics of *T. delbrueckii* (Td) fermentations, while the kinetics for *M. pulcherrima* (Mp) fermentations are showed in **Figure 2.B**. In both figures, there is the AF kinetics of the control fermentation with *S. cerevisiae* (Sc). In addition, **Table 3** shows the average AF speed for each fermentation. In both cases, the co-inoculated fermentations were the fastest (Td-Sc $26.25 \pm 0.00 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ and Mp-Sc ($27.00 \pm 0.00 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$), along with the control of *S. cerevisiae* ($31.33 \pm 0.00 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) as a sole starter. Nevertheless, there was a tendency to slow down the AF the longer it took to inoculate *S. cerevisiae*. On one hand, Td fermentations took more time to finish the AF, being Td.48h the slowest ($10.60 \pm 0.00 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and the one that took more time (25 days). Mp fermentations had a slow beginning. In fact, Mp fermentations did not start to consume significantly sugar until Sc was inoculated, and their kinetics showed similar behavior to ones of Sc fermentations.

Regarding yeast population, *S. cerevisiae* and non-*Saccharomyces* increased the initial concentration of 10^6 CFU/mL to $10^7 - 10^8 \text{ CFU/mL}$ after 2-3 days of inoculation. *M. pulcherrima* remained viable during all the AF, although its population decreased to less than 10^5 CFU/mL in the fermentation stages. As for *T. delbrueckii*, Td-Sc and Td.72h were the only fermentations in which *T. delbrueckii* remained viable until the end of AF (data not shown).

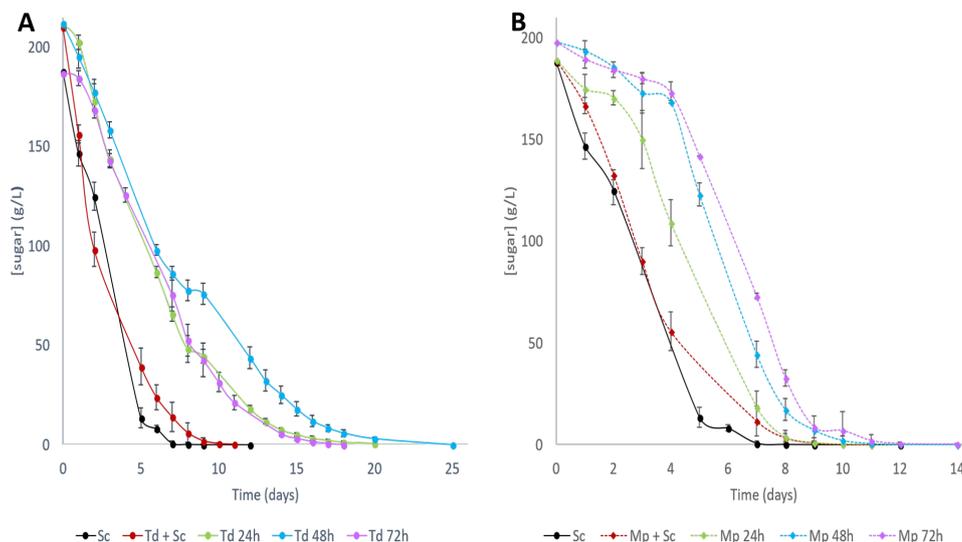


Figure 2: Evolution of alcoholic fermentation. Monitoring of sugar consumption by yeasts. A) *T. delbrueckii* fermentations and control; B) *M. pulcherrima* fermentations and control.

3.1.2. Malolactic fermentation

When the AF was finished and yeasts were removed completely, *O. oeni* PSU-1 was inoculated to perform the MLF. Different kinetics of L-malic consumption were observed during MLF (**Figure 3**). *O. oeni* showed better performance of MLF in *S. cerevisiae* fermented wines ($0,48 \pm 0,01 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$), and, contrary to AF, co-inoculated *T. delbrueckii* wines took longer to finish MLF. **Table 3** shows the average of MLF speed for each fermentation. In general, in *T. delbrueckii* wines, *O. oeni* had a better performance when it took longer to inoculate *S. cerevisiae*. In contrast, *M. pulcherrima* fermented wines, it was the opposite.

As for *O. oeni*'s population, the initial concentration of 2×10^7 CFU/mL remained constant in control wines, while it increased up to 4×10^7 CFU/mL, and remained constant, in non-*Saccharomyces* wines.

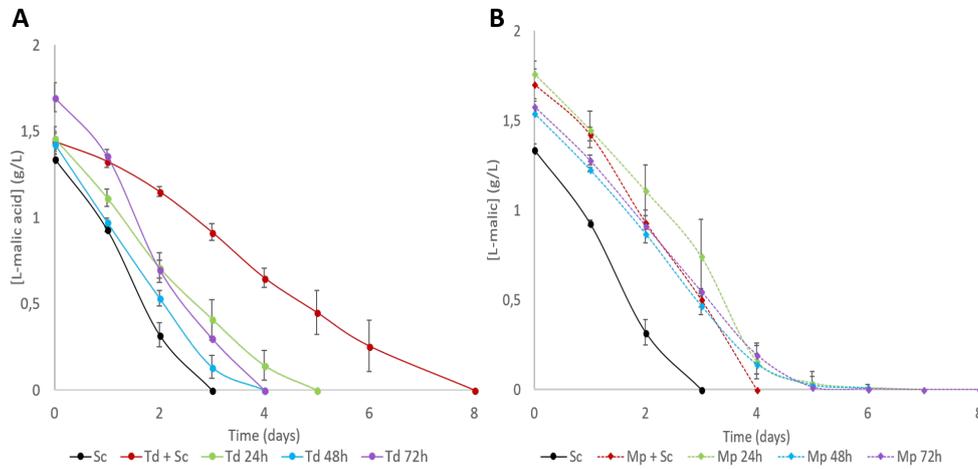


Figure 3: Evolution of malolactic fermentation. Monitoring of L-malic acid consumption by *O. oeni*. A) *T. delbrueckii* fermentations and control; B) *M. pulcherrima* fermentations and control.

3.1.3. Changes in wine composition

Differences in wine composition were found at the end of AF and MLF depending on the inoculation time of *S. cerevisiae*. **Table 2** shows the characterization of the initial must, and **Table 3** shows the wines characterization after both fermentations.

Table 2: Characterization of initial must.

	Sugar (g/L)	L-malic acid (g/L)	Citric acid (g/L)	α -NH ₂ (mg/L)	Ammonium (mg/L)	pH
Initial must	$198,11 \pm 10,72$	$2,14 \pm 0,08$	$0,25 \pm 0,05$	$153,90 \pm 11,21$	$70,22 \pm 4,44$	$3,94 \pm 0,01$

Ethanol content of *T. delbrueckii* mixed ($11,3 \pm 0,1 \text{ \%}(v/v)$) and sequential fermentations was significantly higher than in *S. cerevisiae* control wines, highlighting Td 24h ($13,5 \pm 0,2 \text{ \%}(v/v)$) and Td 48h ($13,7 \pm 0,2 \text{ \%}(v/v)$). As for *M. pulcherrima* wines, there was no significant difference from the control wines. Although pH after AF of control and co-inoculate wines was similar, there was a tendency of a higher pH in Td.24h, T.48h and Td.72h wines, closer to the initial must pH ($3,85 \pm 0,05$; $3,93 \pm 0,04$; $3,81 \pm 0,01$, respectively). The same occurred with pH after MLF.

Table 3: Characterization of wines after AF and MLF.

	AF speed (g·L ⁻¹ ·day ⁻¹)		Sugar (g/L)		Ethanol content (% (v/v))		MLF speed (g·L ⁻¹ ·day ⁻¹)		L-malic acid (g/L)		pH	
	AF	MLF	AF	MLF	AF	MLF	AF	MLF	AF	MLF	AF	MLF
Sc	31.33 ± 0.00 ^a	0.11 ± 0.09	0.11 ± 0.09	0.11 ± 0.09	10.7 ± 0.2 ^d	0.48 ± 0.01 ^{ab}	1.36 ± 0.03 ^d	nd	3.45 ± 0.03 ^e	3.86 ± 0.02 ^{bc}		
Td + Sc	26.25 ± 0.00 ^b	0.23 ± 0.21	0.23 ± 0.21	0.23 ± 0.21	11.3 ± 0.1 ^{bc}	0.23 ± 0.04 ^{ab}	1.46 ± 0.06 ^{cd}	nd	3.56 ± 0.09 ^{de}	3.74 ± 0.00 ^{de}		
Td 24h	13.25 ± 0.79 ^e	0.63 ± 0.23	0.58 ± 0.17	0.58 ± 0.17	13.5 ± 0.2 ^a	0.29 ± 0.01 ^{ab}	1.46 ± 0.07 ^{cd}	nd	3.85 ± 0.05 ^{ab}	4.20 ± 0.02 ^a		
Td 48h	10.60 ± 0.00 ^f	nd	nd	nd	13.7 ± 0.2 ^a	0.36 ± 0.01 ^{ab}	1.43 ± 0.06 ^{cd}	nd	3.93 ± 0.04 ^a	4.23 ± 0.00 ^a		
Td 72h	12.47 ± 0.00 ^e	0.10 ± 0.09	0.10 ± 0.09	0.10 ± 0.09	11.5 ± 0.1 ^b	0.42 ± 0.02 ^{ab}	1.70 ± 0.08 ^{ab}	nd	3.81 ± 0.01 ^{abc}	4.03 ± 0.02 ^b		
Mp + Sc	27.00 ± 0.00 ^b	0.16 ± 0.05	0.14 ± 0.02	0.14 ± 0.02	10.8 ± 0.1 ^{cd}	0.43 ± 0.02 ^a	1.70 ± 0.08 ^{ab}	nd	3.49 ± 0.04 ^{de}	3.93 ± 0.01 ^{bc}		
Mp 24h	23.63 ± 0.00 ^c	0.23 ± 0.20	0.19 ± 0.32	0.19 ± 0.32	10.7 ± 0.3 ^d	0.29 ± 0.08 ^a	1.76 ± 0.07 ^a	nd	3.46 ± 0.00 ^{de}	3.97 ± 0.09 ^{bc}		
Mp 48h	19.80 ± 0.00 ^d	0.13 ± 0.09	0.12 ± 0.06	0.12 ± 0.06	11.1 ± 0.1 ^{cd}	0.31 ± 0.01 ^{ab}	1.54 ± 0.02 ^c	nd	3.73 ± 0.07 ^{bc}	3.88 ± 0.13 ^{bc}		
Mp 72h	18.00 ± 0.00 ^d	0.25 ± 0.20	0.23 ± 0.18	0.23 ± 0.18	11.0 ± 0.1 ^{cd}	0.32 ± 0.04 ^b	1.58 ± 0.02 ^{bc}	nd	3.65 ± 0.03 ^{cd}	3.81 ± 0.08 ^c		
	L-lactic acid (g/L)		Citric acid (g/L)		Acetic acid (g/L)		Glycerol (g/L)		Succinic acid (mg/L)			
	AF	MLF	AF	MLF	AF	MLF	AF	MLF	AF	MLF		
Sc	nd	0.99 ± 0.01	0.22 ± 0.02	0.01 ± 0.01 ^{cd}	0.23 ± 0.04 ^{bc}	0.41 ± 0.03 ^{ab}	5.06 ± 0.20 ^d	5.80 ± 0.44	331.94 ± 2.98 ^a			
Td + Sc	nd	0.93 ± 0.02	0.23 ± 0.03	0.17 ± 0.03 ^a	0.18 ± 0.02 ^{cd}	0.28 ± 0.02 ^{cd}	5.56 ± 0.38 ^{cd}	6.17 ± 0.19	323.82 ± 3.66 ^{ab}			
Td 24h	nd	0.94 ± 0.02	0.28 ± 0.01	0.09 ± 0.01 ^b	0.12 ± 0.02 ^d	0.22 ± 0.01 ^d	6.28 ± 0.09 ^{cd}	6.52 ± 0.02	311.53 ± 3.96 ^{bc}			
Td 48h	nd	1.04 ± 0.01	0.24 ± 0.01	nd ^d	0.12 ± 0.03 ^d	0.25 ± 0.03 ^d	5.74 ± 0.27 ^{cd}	5.92 ± 0.07	309.66 ± 1.81 ^c			
Td 72h	nd	0.92 ± 0.01	0.22 ± 0.02	nd ^d	0.10 ± 0.01 ^d	0.29 ± 0.01 ^{bcd}	5.87 ± 0.27 ^{cd}	4.59 ± 0.01	314.20 ± 1.15 ^{bc}			
Mp + Sc	nd	0.91 ± 0.01	0.22 ± 0.02	0.01 ± 0.01 ^{cd}	0.35 ± 0.02 ^a	0.49 ± 0.02 ^a	6.19 ± 0.21 ^{cd}	4.97 ± 0.01	320.28 ± 1.28 ^{bc}			
Mp 24h	nd	0.94 ± 0.02	0.21 ± 0.03	nd ^d	0.31 ± 0.04 ^{bc}	0.35 ± 0.06 ^{abc}	6.45 ± 0.14 ^{bc}	5.29 ± 0.18	320.29 ± 7.01 ^{bc}			
Mp 48h	nd	1.02 ± 0.00	0.23 ± 0.01	0.05 ± 0.04 ^{bc}	0.29 ± 0.03 ^{ab}	0.35 ± 0.01 ^{abc}	7.27 ± 0.21 ^b	7.05 ± 0.16	315.53 ± 2.60 ^{bc}			
Mp 72h	nd	0.97 ± 0.08	0.29 ± 0.04	0.03 ± 0.01 ^{cd}	0.19 ± 0.05 ^{cd}	0.30 ± 0.05 ^{bcd}	8.01 ± 0.25 ^a	7.50 ± 0.65	309.42 ± 1.69 ^c			
	α-NH ₂ (mg/L)		Ammonium (mg/L)		Total sulfite (mg/L)		Free sulfite (mg/L)					
	AF	MLF	AF	MLF	AF	MLF	AF	MLF				
Sc	32.54 ± 1.24 ^b	32.23 ± 2.48 ^b	3.00 ± 0.71	0.00 ± 0.00	45.50 ± 0.71 ^{ab}	13.67 ± 2.31 ^{ab}	4.00 ± 1.00 ^a	1.67 ± 0.58 ^{cd}				
Td + Sc	36.89 ± 2.34 ^b	41.79 ± 0.09 ^b	0.67 ± 0.57	3.00 ± 0.00	39.50 ± 0.71 ^{abc}	2.00 ± 1.41 ^d	2.00 ± 1.73 ^{ab}	0.33 ± 0.58 ^d				
Td 24h	65.73 ± 5.54 ^a	61.57 ± 2.93 ^a	2.50 ± 0.71	3.00 ± 1.00	26.67 ± 3.05 ^{cde}	4.67 ± 3.79 ^{bcd}	3.33 ± 0.58 ^{ab}	1.00 ± 1.00 ^{cd}				
Td 48h	73.06 ± 5.38 ^a	66.37 ± 2.71 ^a	2.50 ± 0.71	5.50 ± 0.71	16.00 ± 2.82 ^e	5.00 ± 1.41 ^{cd}	1.67 ± 0.58 ^{ab}	0.50 ± 0.71 ^{cd}				
Td 72h	63.80 ± 3.52 ^a	60.47 ± 0.74 ^a	1.00 ± 0.00	3.50 ± 0.71	18.00 ± 0.00 ^{de}	7.00 ± 0.00 ^{bcd}	1.50 ± 0.71 ^b	6.50 ± 0.71 ^{bc}				
Mp + Sc	26.98 ± 4.02 ^b	28.90 ± 1.24 ^b	3.50 ± 1.41	2.00 ± 0.00	45.67 ± 0.00 ^a	18.00 ± 2.83 ^a	3.67 ± 0.57 ^a	8.00 ± 0.00 ^{ab}				
Mp 24h	20.26 ± 2.00 ^b	20.00 ± 3.70 ^b	2.00 ± 1.00	2.00 ± 1.00	28.00 ± 4.24 ^{abc}	15.33 ± 3.79 ^a	2.33 ± 0.58 ^{ab}	9.00 ± 1.00 ^a				
Mp 48h	39.07 ± 8.30 ^b	38.77 ± 3.03 ^b	4.00 ± 0.00	3.50 ± 0.71	33.00 ± 2.00 ^{abc}	20.67 ± 3.79 ^a	2.33 ± 0.58 ^{ab}	1.67 ± 0.58 ^{cd}				
Mp 72h	28.32 ± 5.33 ^b	19.97 ± 3.87 ^b	7.00 ± 0.00	3.33 ± 1.53	29.67 ± 2.89 ^{bcd}	13.33 ± 1.53 ^{abc}	2.33 ± 0.58 ^{ab}	2.00 ± 1.00 ^{cd}				

Column values followed by different superscript letters are significantly different at $p \leq 0.05$, according to a Tukey post-hoc comparison test.

Knowing that the initial must had a L-malic acid concentration of 2.14 ± 0.08 g/L, both *S. cerevisiae* and non-*Saccharomyces* yeasts consumed L-malic acid during AF. In fact, this consumption was up to a 30% in control wines, 35% in Td, and 20% in Mp. In sequential fermentations the L-malic acid consumption was higher when the non-*Saccharomyces* yeasts were more time left alone.

L-lactic acid concentrations were similar at the end of MLF, indicating no statistically significant differences between wines with different inoculation time of *S. cerevisiae*. As for citric acid, the same happened at the end of AF. However, at the end of MLF, in co-inoculated Td-Sc (0.17 ± 0.03 g/L) and Td.24h (0.09 ± 0.01 g/L) wines, some significant citric acid was not consumed. Regarding acetic acid, Mp wines were similar to control, even though the co-inoculated Mp-Sc (0.35 ± 0.02 g/L) had a higher value than single-culture *S. cerevisiae* (0.23 ± 0.04 g/L) wine. As for Td wines, they had significantly lower acetic acid production. As a general rule, the longer it took to inoculate *S. cerevisiae*, there was less production of acetic acid, reducing it up to a 60% (Td.72h 0.10 ± 0.01 g/L).

S. cerevisiae single culture (5.06 ± 0.20 g/L) and *T. delbrueckii* wines had similar glycerol production, while *M. pulcherrima* wines produced more when left more time alone before inoculating *S. cerevisiae* (Mp.48h 7.27 ± 0.21 g/L; Mp.72h 8.01 ± 0.25 g/L). Concerning succinic acid, control wines had the highest production (331.94 ± 2.98 mg/L), and in non-*Saccharomyces* fermented wines there was a tendency to decrease that production by 10% when increasing the time of inoculation of *S. cerevisiae*.

Initial ammonium concentration was of 70.22 ± 4.44 mg/L. Its consumption by yeast was similar in all fermentations, showing no statistically significant differences. Concerning α -NH₂, its initial concentration was of 153.90 ± 11.21 mg/L. Nonetheless, during AF, there was a higher consumption of α -NH₂ by *S. cerevisiae* (32.54 ± 1.24 mg/L), *M. pulcherrima* mixed and sequential fermentations and *T. delbrueckii* mixed fermentations compared to Td.24h (65.73 ± 5.54 mg/L), Td.48h (73.06 ± 5.38 mg/L) and Td.72h (63.80 ± 3.52 mg/L) sequential fermentations.

M. pulcherrima mixed fermentations produced the highest concentration of SO₂ (45.67 ± 0.00 mg/L of total SO₂ and 3.67 ± 0.57 mg/L of free SO₂), similar to control, with $45,50 \pm 0,71$ mg/L of total SO₂ and 4.00 ± 1.00 mg/L of free SO₂ after AF. Regarding to *T. delbrueckii* wines, it was observed that the production of SO₂ decreased with time of Sc inoculation, being Td.48h the wines with lower content of SO₂ with 16.00 ± 2.82 mg/L.

The results obtained were subjected to a Principal Component Analysis (PCA) to group the wines produced based on their similitudes and differences after AF (**Figure 4**) and MLF (**Figure 5**). The first PCA clustered wines after AF into three groups according to the yeast strain used. Even so, the second PCA clustered the wines into two groups: one for *M. pulcherrima* sequential fermentation wines; and another for *T. delbrueckii* sequential fermentation wines.

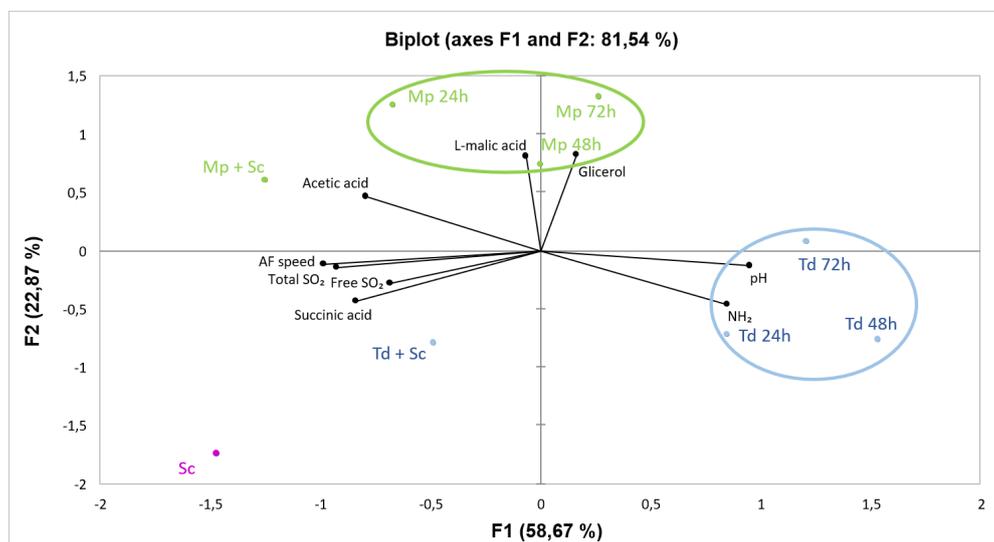


Figure 4: Principal components analysis (PCA) biplot of wines obtained at the end of alcoholic fermentation.

PCA in **Figure 4** explains a 81.54% of the wines variance. The first principal component (F1) explains a 57,94% of the samples variance, whereas F2 explains a 21,60%. On one hand, F1 variables that have a positive correlation are pH, glycerol and NH_2 ; while having a negative correlation with AF speed, L-malic acid, acetic acid, succinic acid and SO_2 (both total and free). On the other hand, F2 variables with a positive correlation are AF speed, L-malic acid, pH, acetic acid and glycerol; and the ones with negative correlation are succinic acid, NH_2 and SO_2 (both total and free). Variables with more importance in F1 are pH, NH_2 , AF speed, total SO_2 , acetic acid and succinic acid; and variables with more importance in F2 are glycerol and L-malic acid. This PCA clusters samples into two groups: one for *T. delbrueckii* sequential fermentations, and another for *M. pulcherrima* sequential fermentations, leaving mixed fermentations and control (*S. cerevisiae*) out of any cluster. Regarding *M. pulcherrima* sequential fermentations, they all show high content in L-malic acid, acetic acid, glycerol, SO_2 and succinic acid, along with more AF speed. They also have in common less NH_2 content and pH than *T. delbrueckii* sequential fermentations. As for them, it is the opposite in every parameter. Finally, Mp-Sc is not in the cluster with *M. pulcherrima* because, even though their content in some of the parameters is similar, Mp-Sc has greater production of acetic acid in comparison with Mp sequential fermentations. *S. cerevisiae* control fermentation and both mixed fermentations have similar AF speed, pH and SO_2 ; and, *S. cerevisiae* and Td-Sc have similar L-malic acid and NH_2 content. Succinic acid concentration is closer between the non-*Saccharomyces* mixed fermentations.

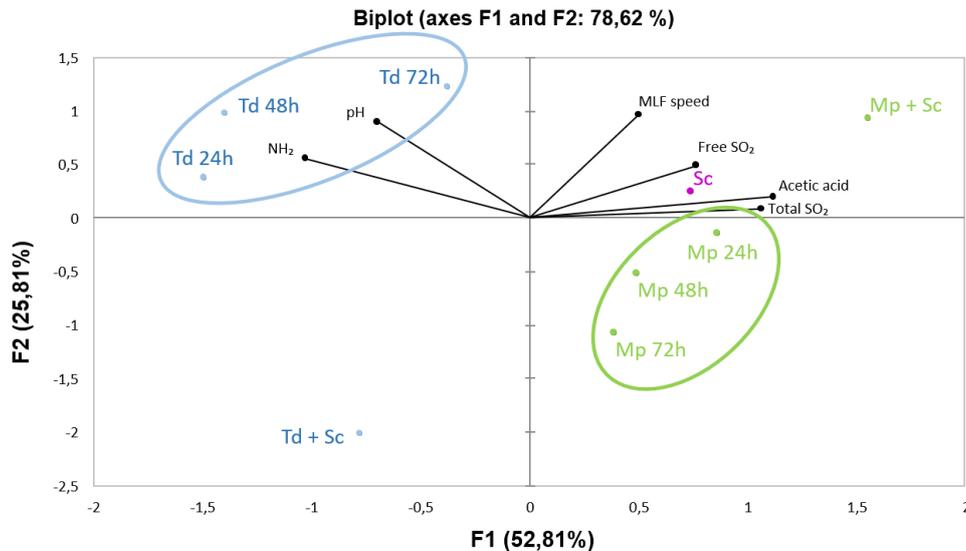


Figure 5: Principal components analysis (PCA) biplot of wines obtained at the end of malolactic fermentation.

PCA in **Figure 5** explains a 78,62% of the wines variance. F1 explains a 52,81% of the samples variance, while F2 explains a 25,81%. Variables in F1 that show a positive correlation are FML speed, acetic acid and SO_2 (both total and free); and variables with a negative correlation are pH and NH_2 . All variables in F2 show a positive correlation. Variables with more importance in F1 are acetic acid, NH_2 , SO_2 and pH; while variables with more importance in F2 are MLF speed and pH. PCA clusters *T. delbrueckii* sequential fermentations and describes them with higher pH and NH_2 but lower MLF speed, SO_2 and acetic acid. Another group is the one with *M. pulcherrima* sequential fermentations, due to their lower pH and NH_2 and higher SO_2 , acetic acid and MLF

speed, in comparison with the first group. However, both *T. delbrueckii* and *M. pulcherrima* mixed fermentations are not clustered with their respective species. In *T. delbrueckii*'s mixed fermentation, it has similar values for MLF speed, SO₂ and acetic acid, but pH and NH₂ are lower compared to sequential fermentations. As for *M. pulcherrima* mixed fermentation, it shows more acetic acid content and MLF speed, generally being characterized more like *S. cerevisiae*.

4. DISCUSSION

As previously introduced, there is an increasing interest in the use of non-*Saccharomyces* yeasts, in mixed and sequential fermentations with *S. cerevisiae* (Ciani and Maccarelli, 1998; Padilla et al., 2016; Lu et al., 2017). Due to their moderate fermentative power, it is needed to inoculate *S. cerevisiae* to finish the AF (Benito et al., 2015). These non-*Saccharomyces* yeasts tend to improve wine quality by releasing metabolites in different concentration than *S. cerevisiae* single cultures do, improving the following MLF and *O. oeni*'s growth. For example, they can produce wines with less ethanol content (Contreras et al., 2014) or increase its citric acid content (Giaramida et al., 2013). But non-*Saccharomyces* yeasts can also modify other compounds that can affect negatively the MLF and *O. oeni*, like a reduction on the L-malic acid content after AF (Belda et al., 2015). Therefore, the use of non-*Saccharomyces* yeasts as a starter culture can influence the chemical composition of the wine as well as the subsequent MLF. Knowing that, the main subject of this work was to evaluate the effect of non-*Saccharomyces* yeasts, with different times of inoculation for *S. cerevisiae*, on *O. oeni* and malolactic fermentation.

To study the interactions between non-*Saccharomyces* yeasts and *O. oeni* and MLF, nine fermentations with two different non-*Saccharomyces* yeasts and different times of inoculation of *S. cerevisiae* were performed **Figure 1**. Also, a control fermentation with *S. cerevisiae* as a single starter culture was carried out.

AF was monitored by following the yeasts sugar consumption. *S. cerevisiae* control fermentations finished in 12 days and a $31.33 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ sugar consumption rate. It was expected to be the fastest due to competition between yeasts in mixed and sequential fermentations. Even though, *T. delbrueckii* and *M. pulcherrima* mixed fermentations were fast, finishing in 11 days and an AF speed of $26.25 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$ and $27.00 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$, respectively. This can be related to the early imposition of *S. cerevisiae* over the non-*Saccharomyces* yeasts, even though *M. pulcherrima* remained viable until the end of the fermentation. However, sequential fermentations took more time to finish and with less speed than the control, as expected. *T. delbrueckii* sequential fermentations were the ones that took more time, getting to 18 days of fermentation for Td.72h, 20 days for Td.24h and 25 days for Td.48h. This can happen due to yeast competition for nutrients. But it can also be described as a consequence of *T. delbrueckii*'s lower tolerance for ethanol and SO_2 , compared with *S. cerevisiae* (Henick-Kling et al., 1998). In addition, it has been suggested that *S. cerevisiae* can produce killer toxins and other unknown metabolites than can affect negatively non-*Saccharomyces* viability (Albergaria et al., 2010; Ciani et al., 2010). Regarding *M. pulcherrima* sequential fermentations, they took place in 14 days each, with similar AF speed, and not significantly different from the *S. cerevisiae* control fermentation.

In regard to MLF and *O. oeni*, *T. delbrueckii* fermented wines promoted its growth (up to 4×10^7 CFU/mL), except for mixed fermentations with *S. cerevisiae*. Even so, L-malic degradation kinetics showed different MLF performances for both *T. delbrueckii* and *M. pulcherrima* wines, by slowing down. Contrary to AF, MLF of Td-Sc wines was the one that took longer (8 days) and was the slowest ($0.23 \pm 0.04 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$). This contrasts in conducting MLF could be related with different chemical composition (**Table 3**) in ethanol, organic acids and SO_2 of wines after AF.

Therefore, the results of this study showed that the use of non-*Saccharomyces* yeast in mixed and, particularly, sequential fermentations affect the wine final composition. Ethanol content presented significant differences between fermentations. Nowadays, there is a tendency towards lowering alcoholic content in wines. Some authors reported significant decrease in sequential fermentations with *T. delbrueckii* (Quirós et al., 2014; Puertas et al., 2017). But, contrary to those studies, in the tested conditions, sequential inoculation showed a slight increase in ethanol content. This may be consequence of yeast competition. *S. cerevisiae*, to make the environment more hostile for other yeasts like *T. delbrueckii*, produces ethanol. It is noteworthy that Td.72h presented higher ethanol content (11.5 ± 0.1 %(v/v)) than *S. cerevisiae* control wine (10.7 ± 0.2 %(v/v)), but lower than other *T. delbrueckii* sequential fermentations. Regarding Td.24h and Td.48h, ethanol values for this fermentations were considered erroneous due to the difference between other *T. delbrueckii* fermentations and available bibliography. Also, if probable ethanol content is calculated, it is not possible to obtain those values with a sugar initial concentration of 200 ± 10 g/L.

Regarding pH, as previously stated, there was a tendency for a higher pH the later *S. cerevisiae* was inoculated, highlighting *T. delbrueckii* fermentations. pH is another factor in the interactions between yeasts and LAB. pH tolerance has been widely reported (G-Alegría et al., 2004). Td.24h (3.85 ± 0.05) and Td.48h (3.93 ± 0.04) pH values can be one of the attenuating factors for ethanol content. Anyway, this pH values also mean that wine can be contaminated easily with other LAB with less acid pH tolerance. This could be negative for the final wine organoleptic quality.

L-malic acid consumption by yeasts agreed with other authors (Belda et al., 2015; du Plessis et al., 2017). Initial must contained 2.14 ± 0.08 g/L of L-malic acid. All fermentations showed consumption, being significantly higher for Td.24h (0.77 ± 0.07 g/L) and Td.48h (0.80 ± 0.06 g/L), in comparison to *S. cerevisiae* control (0.65 ± 0.03 g/L). Even though, after AF there was enough substrate to perform a successful MLF in all wines. According to some authors, non-*Saccharomyces* yeasts can produce slightly more citric acid than *S. cerevisiae*. In the present work, there were no significant differences between *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* citric acid production during AF. As for values after MLF, there were no significant differences in metabolization of citric acid by *O. oeni* among wines, except for Td-Sc (0.17 ± 0.03 g/L) and Td.24h (0.09 ± 0.01 g/L). Its consumption was total in Td.48h, Td.72h and Mp.24h wines. This consumption has a negative impact on wine, by contributing to acetic acid formation. Still, it can contribute to wine aroma as citric acid consumption by *O. oeni* also forms diacetyl (Bartowsky, 2005).

Concerning acetic acid production, it has been reported that non-*Saccharomyces* sequential fermentations can lower its content (Taillandier et al., 2014; Chen et al., 2018). Results of this study are in accordance, showing a tendency of lowering acetic acid production during AF when *S. cerevisiae* is inoculated later in time. Between *T. delbrueckii* and *M. pulcherrima* wines, the first had less production. On top of that, *M. pulcherrima* mixed fermentations produced more acetic acid than *S. cerevisiae* single culture. Data obtained for *M. pulcherrima* sequential fermentations can be due to the early imposition of *S. cerevisiae*. As for acetic acid during MLF, wines where *O. oeni* citric acid consumption was total, had significantly a higher final acetic acid concentration.

A similar glycerol production was observed between *S. cerevisiae* control fermentation and *T. delbrueckii* mixed and sequential fermentations. However, some authors reported that non-*Saccharomyces* yeast can produce more glycerol than *S. cerevisiae* (Ciani and Maccarelli, 1998; Belda et al., 2015). *M. pulcherrima* fermentations presented a significant higher glycerol content at the end of AF.

It has been observed that some *S. cerevisiae* strains can produce significant concentrations of succinic acid (Caridi and Corte, 1997). This organic acid can act as a competitive inhibitor of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982). In the present work, it was observed a slight decrease in succinic acid production by non-*Saccharomyces* yeast, in accordance with other studies (Contreras et al., 2014). This differences were most remarkable in *M. pulcherrima* fermentations, like Mp.48h (315.53 ± 2.60 mg/L) or Mp.72h (309.42 ± 1.69 mg/L), in comparison with *S. cerevisiae* control fermentations (331.94 ± 2.98 mg/L).

Initial α -NH₂ must concentration was of $153,90 \pm 11,21$ mg/L. *T. delbrueckii* sequential fermentations were the ones that consumed less α -NH₂, with values of 88.17 ± 5.54 mg/L (Td.24h), 80.84 ± 5.38 mg/L (Td.48h) and 90.10 ± 3.52 mg/L (Td.72h). *S. cerevisiae* and *M. pulcherrima* fermentations consumed more α -NH₂, leaving wine medium with less nitrogen for *O. oeni* to use during MLF. Although *S. cerevisiae* imposed in early AF stages over *M. pulcherrima*, the non-*Saccharomyces* yeast remained viable until the end of the fermentation. Therefore, this data is in accordance with Gobert et al.(2017), who observed certain competition for nitrogen sources between *S. cerevisiae* and non-*Saccharomyces* yeasts. Anyway, this content was enough for *O. oeni* to perform a successful MLF.

Another metabolite acting as an antagonist, due to its antimicrobial activity, is SO₂ (Wells and Osborne, 2011; Benucci et al., 2016). It has been reported that some non-*Saccharomyces* strains can produce significant amounts of SO₂ (Fleet, 2003). In the present work, significant differences were found in *T. delbrueckii* sequential fermentations and *S. cerevisiae* and *M. pulcherrima* fermentations. Td.48h (16.00 ± 2.82 mg/L) and T.72h (18.00 ± 0.00 mg/L) were the wines with lower total SO₂ concentration. As for free SO₂, the same happened (T.48h 1.67 ± 0.58 mg/L; Td.72h 1.50 ± 0.71 mg/L). The non existing differences between *M. pulcherrima* and *S. cerevisiae* wines, may be due to the early imposition of the second in mixed and sequential fermentations. Usual SO₂ values in wine oscillate around 150-200 mg/L for total SO₂ and 60 mg/L for free SO₂ (Ribéreau-Gayon et al., 2006). So, even though *S. cerevisiae* and *M. pulcherrima* wines had higher SO₂ than *T. delbrueckii*, *O. oeni* was able to perform MLF in all of them successfully.

Finally, after a statistical analysis using ANOVA, it was able to determine which variables were significant in wine characterization after AF and MLF. Then, a Principal Component Analysis (PCA) was performed (**Figure 4** for AF; **Figure 5** for MLF) with the objective to observe a differentiation between species. After the analysis, both PCA show that there exists a difference between *T. delbrueckii* and *M. pulcherrima* sequential fermentations. However, it is not possible to group *T. delbrueckii* and *M. pulcherrima* mixed fermentations with their respective species. Besides, it is able to differentiate between species but not between time of inoculation of *S. cerevisiae*, since PCA clusters Td.24h, Td.48h and Td.72h in one group and Mp.24h, Mp.48h and Mp.72h in another one. This shows that there exist similarities between wines fermented with the same non-*Saccharomyces* species, independently from time of inoculation with *S. cerevisiae*.

5. CONCLUSIONS AND PERSPECTIVES

To conclude this study about the effect of different regimes of inoculation for *S. cerevisiae* and non-*Saccharomyces* yeasts on MLF and *O. oeni*, it can be resolved that:

- During AF *S. cerevisiae* usually overcomes non-*Saccharomyces* yeasts, limiting their impact on wine, especially in mixed fermentations
- The longer it takes to inoculate *S. cerevisiae*, non-*Saccharomyces* yeasts have more impact on MLF, *O. oeni* and wine. It affects certain parameters (L-malic acid, pH, citric acid during MLF, acetic acid, glycerol, succinic acid, α -NH₂ and SO₂)
- Mixed fermentations show no significant differences compared to fermentations with *S. cerevisiae* as a sole starter
- There is some variability in wines composition depending on non-*Saccharomyces* species
- There is not much variability between wines of the same species but with different time of inoculation with *S. cerevisiae*

To sum up, non-*Saccharomyces* yeasts have an impact on wine when sequential inoculation is used. In the tested conditions, *S. cerevisiae*'s time of inoculation is not as important as having non-*Saccharomyces* present to start AF and ferment on their own to have more or less production or consumption of certain compounds. However, there exist some differences between species. *T. delbrueckii* fermented wines should have a positive effect on *O. oeni* and MLF due to lower acidity, succinic acid and SO₂. Despite that, *O. oeni* performed MLF slower than in *S. cerevisiae* and *M. pulcherrima* fermented wines. Thus, there are other compounds that should be studied with a greater negative effect on *O. oeni*. The same may happen on *M. pulcherrima* fermented wines. Moreover, those effects would need to be studied at a molecular level, using approaches such as transcriptomic and/or proteomic studies. Finally, testing other *S. cerevisiae*, non-*Saccharomyces* and *O. oeni* strains should provide more information about yeast-LAB interactions.

6. FUNDING

This work was supported by grant AGL2015-70378-R awarded by the Spanish Ministry of Economy and Competitiveness. I would like to thank Universitat Rovira i Virgili for the scholarship granted to attend the Master in Fermented Beverages, that made possible the accomplishment of the present master thesis.

7. ACKNOWLEDGEMENTS

First, I would like to thank Albert Bordons and Cristina Reguant for giving me the opportunity to take part of their team and tutor my master thesis.

Also, this present master thesis would not be possible without the everyday support of my lactic teammates Nuria Ferrando and, especially, Aitor Balmaseda that worked hardly next to me and motivated and helped me when needed.

I also have to mention the support of my laboratory mates Adeline Vignault, Lucie Asbiro, Jordi Gombau, Pere Pons and Olga Pascual of Oenological Technology group.

Finally, I am very grateful to all Oenological Biotechnology team that made me fell like home. Especially, Maria Jesús Torija, Gemma Beltran, Braulio Esteve, Maria Martinez, Jessica Lleixà, Maria Ángeles Morcillo, Helena Roca and Mercè Sunyer.

References

- Albergaria, H., Francisco, D., Gori, K., Arneborg, N., and Gírio, F. (2010). *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Applied Microbiology and Biotechnology*, 86(3):965–972.
- Alexandre, H., Costello, P. J., Remize, F., Guzzo, J., and Guilloux-Benatier, M. (2004). *Saccharomyces cerevisiae*-*Oenococcus oeni* interactions in wine: Current knowledge and perspectives. *International Journal of Food Microbiology*, 93(2):141–154.
- Balmaseda, A., Bordons, A., Reguant, C., and Bautista-Gallego, J. (2018). Non-*Saccharomyces* in wine: Effect upon *Oenococcus oeni* and malolactic fermentation. *Frontiers in Microbiology*, 9:1–8.
- Bartowsky, E. J. (2005). *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. *Australian Journal of Grape and Wine Research*, 11(2):174–187.
- Belda, I., Navascués, E., Marquina, D., Santos, A., Calderon, F., and Benito, S. (2014). Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. *Applied Microbiology and Biotechnology*, 99(4):1911–1922.
- Belda, I., Navascués, E., Marquina, D., Santos, A., Calderon, F., and Benito, S. (2015). Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. *Applied Microbiology and Biotechnology*, 99(4):1911–1922.
- Beltran, G., Torija, M. J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J. M., Rozès, N., and Mas, A. (2002). Analysis of yeast populations during alcoholic fermentation: A six year follow-up study. *Systematic and Applied Microbiology*, 25(2):287–293.
- Bely, M., Stoeckle, P., Masneuf-Pomarède, I., and Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii*-*Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Microbiology*, 122(3):312–320.
- Benito, S., Hofmann, T., Laier, M., Lochbühler, B., Schüttler, A., Ebert, K., Fritsch, S., Röcker, J., and Rauhut, D. (2015). Effect on quality and composition of Riesling wines fermented by sequential inoculation with non-*Saccharomyces* and *Saccharomyces cerevisiae*. *European Food Research and Technology*, 241(5):707–717.
- Benucci, I., Cappannella, E., Liburdi, K., and Esti, M. (2016). Inhibitory effect of ethanol, sulphur dioxide and proanthocyanidinic tannins on lysozyme antimicrobial activity in model wine. *LWT - Food Science and Technology*, 73:320–325.
- Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M. G., Caldeira, J., and Albergaria, H. (2014). Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Applied Microbiology and Biotechnology*, 98(2):843–853.
- Britz, T. and Tracey, R. (1990). The combination effect of pH, SO₂, ethanol and temperature on the growth of *Leuconostoc oenos*. *Journal of Applied Bacteriology*, 68(1):23–31.

- Capucho, I. and San Romão, M. V. (1994). Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. *Applied Microbiology and Biotechnology*, 42(2):391–395.
- Caridi, A. and Corte, V. (1997). Inhibition of malolactic fermentation by cryotolerant yeasts. *Biotechnology Letters*, 19(8):723–726.
- Carreté, R., Vidal, M., Bordons, A., and Constantí, M. (2002). Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. *FEMS Microbiology Letters*, 211(2):155–159.
- Chen, K., Escott, C., Loira, I., del Fresno, J. M., Morata, A., Tesfaye, W., Calderon, F., Suárez-Lepe, J. A., Han, S., and Benito, S. (2018). Use of non-Saccharomyces yeasts and oenological tannin in red winemaking: Influence on colour, aroma and sensorial properties of young wines. *Food Microbiology*, 69:51–63.
- Ciani, M., Comitini, F., Mannazzu, I., and Domizio, P. (2010). Controlled mixed culture fermentation: A new perspective on the use of non-Saccharomyces yeasts in winemaking. *FEMS Yeast Research*, 10(2):123–133.
- Ciani, M. and Maccarelli, F. (1998). Oenological properties of non-Saccharomyces yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology*, 14(2):199–203.
- Ciani, M., Morales, P., Comitini, F., Tronchoni, J., Canonico, L., Curiel, J. A., Oro, L., Rodrigues, A. J., and Gonzalez, R. (2016). Non-conventional yeast species for lowering ethanol content of wines. *Frontiers in Microbiology*, 7:1–13.
- Cinquanta, L., De Stefano, G., Formato, D., Niro, S., and Panfili, G. (2018). Effect of pH on malolactic fermentation in southern Italian wines. *European Food Research and Technology*, page 1.
- Comitini, F., Ferretti, R., Clementi, F., Mannazzu, I., and Ciani, M. (2005). Interactions between *Saccharomyces cerevisiae* and malolactic bacteria: Preliminary characterization of a yeast proteinaceous compound(s) active against *Oenococcus oeni*. *Journal of Applied Microbiology*, 99(1):105–111.
- Contreras, A., Hidalgo, C., Henschke, P. A., Chambers, P. J., Curtin, C., and Varela, C. (2014). Evaluation of non-Saccharomyces yeasts for the reduction of alcohol content in wine. *Applied and Environmental Microbiology*, 80(5):1670–1678.
- du Plessis, H. W., du Toit, M., Hoff, J. W., Hart, R. S., Ndimba, B. K., and Jolly, N. P. (2017). Characterisation of non-Saccharomyces yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation. *South African Journal of Enology and Viticulture*, 38(1):46–63.
- Englezos, V., Torchio, F., Cravero, F., Marengo, F., Giacosa, S., Gerbi, V., Rantsiou, K., Rolle, L., and Cocolin, L. (2016). Aroma profile and composition of Barbera wines obtained by mixed fermentations of *Starmerella bacillaris* (synonym *Candida zemplinina*) and *Saccharomyces cerevisiae*. *LWT - Food Science and Technology*, 73:567–575.

- Fleet, G. H. (2003). Yeast interactions and wine flavour. *International Journal of Food Microbiology*, 86(1-2):11–22.
- Fleet, G. H., Lafon-Lafourcade, S., and Ribereau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology*, 48(5):1034–1038.
- G-Alegría, E., López, I., Ruiz, J. I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., and Ruiz-Larrea, F. (2004). High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiology Letters*, 230(1):53–61.
- Giaramida, P., Ponticello, G., Di Maio, S., Squadrito, M., Genna, G., Barone, E., Scacco, A., Corona, O., Amore, G., Di Stefano, R., and Oliva, D. (2013). *Candida zemplinina* for production of wines with less alcohol and more glycerol. *South African Journal of Enology and Viticulture*, 34(2):204–211.
- Gobert, A., Tourdot-Maréchal, R., Morge, C., Sparrow, C., Liu, Y., Quintanilla-Casas, B., Vichi, S., and Alexandre, H. (2017). Non-*Saccharomyces* Yeasts nitrogen source preferences: Impact on sequential fermentation and wine volatile compounds profile. *Frontiers in Microbiology*, 8(NOV):1–13.
- Gockowiak, H. and Henschke, P. A. (2003). Interaction of pH, ethanol concentration and wine matrix on induction of malolactic fermentation with commercial 'direct inoculation' starter cultures. *Australian Journal of Grape and Wine Research*, 9(3):200–209.
- Guerzoni, M. E., Sinigaglia, M., Gardini, F., Ferruzzi, M., and Torriani, S. (1995). Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos*: Modelling of the malolactic activity. *American Journal of Enology and Viticulture*, 46(3):368–374.
- Guilloux-Benatier, M., Guerreau, J., and Feuillat, M. (1995). Influence of Initial Colloid Content on Yeast Macromolecule Production and on the Metabolism of Wine Microorganisms. *American Journal of Enology and Viticulture*, 46(4):486–492.
- Henick-Kling, T., Edinger, W., Daniel, P., and Monk, P. (1998). Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *Journal of Applied Microbiology*, 84:865–876.
- Izquierdo Cañas, P. M., García-Romero, E., Heras Manso, J. M., and Fernández-González, M. (2014). Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. *European Food Research and Technology*, 239(2):279–286.
- Liu, S. Q. (2002). Malolactic fermentation in wine - Beyond deacidification. *Journal of Applied Microbiology*, 92(4):589–601.
- Lonvaud-Funel, A. and Strasser de Saad, A. M. (1982). Purification and Properties of a Malolactic Enzyme from a Strain of *Leuconostoc mesenteroides* Isolated from Grapes.

- Lu, Y., Voon, M. K. W., Chua, J. Y., Huang, D., Lee, P. R., and Liu, S. Q. (2017). The effects of co- and sequential inoculation of *Torulaspora delbrueckii* and *Pichia kluyveri* on chemical compositions of durian wine. *Applied Microbiology and Biotechnology*, 101(21):7853–7863.
- Magyar, I., Nyitrai-Sárdy, D., Leskó, A., Pomázi, A., and Kállay, M. (2014). Anaerobic organic acid metabolism of *Candida zemplinina* in comparison with *Saccharomyces* wine yeasts. *International Journal of Food Microbiology*, 178:1–6.
- Martínez-Rodríguez, A. J., Carrascosa, A. V., and Polo, M. C. (2001). Release of nitrogen compounds to the extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. *International Journal of Food Microbiology*, 68(1-2):155–160.
- Mendoza, L. and Farías, M. E. (2010). Improvement of wine organoleptic characteristics by non-*Saccharomyces* yeasts. *Applied Microbiology*, pages 908–919.
- Mendoza, L. M., de Nadra, M. C. M., and Farías, M. E. (2010). Antagonistic interaction between yeasts and lactic acid bacteria of oenological relevance. Partial characterization of inhibitory compounds produced by yeasts. *Food Research International*, 43(8):1990–1998.
- Padilla, B., Gil, J. V., and Manzanares, P. (2016). Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Frontiers in Microbiology*, 7:411.
- Patynowski, R. J., Jiranek, V., and Markides, A. J. (2002). Yeast viability during fermentation and sur lie ageing of a defined medium and subsequent growth of *Oenococcus oeni*. *Australian Journal of Grape and Wine Research*, 8(1):62–69.
- Petruzzi, L., Capozzi, V., Berbegal, C., Corbo, M. R., Bevilacqua, A., Spano, G., and Sinigaglia, M. (2017). Microbial resources and enological significance: Opportunities and benefits. *Frontiers in Microbiology*, 8:1–13.
- Puertas, B., Jiménez, M. J., Cantos-Villar, E., Cantoral, J. M., and Rodríguez, M. E. (2017). Use of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in semi-industrial sequential inoculation to improve quality of Palomino and Chardonnay wines in warm climates. *Journal of Applied Microbiology*, 122(3):733–746.
- Quirós, M., Rojas, V., Gonzalez, R., and Morales, P. (2014). International Journal of Food Microbiology Selection of non- *Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *International Journal of Food Microbiology*, 181:85–91.
- Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., and Lonvaud, A. (2006). The Use of Sulfur Dioxide in Must and Wine Treatment. In *Handbook of Enology*, chapter 8, pages 193–221. Wiley-Blackwell.
- Sumby, K. M., Grbin, P. R., and Jiranek, V. (2014). Implications of new research and technologies for malolactic fermentation in wine. *Applied Microbiology and Biotechnology*, 98(19):8111–8132.

- Taillandier, P., Lai, Q. P., Julien-Ortiz, A., and Brandam, C. (2014). Interactions between *Torulopsis delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. *World Journal of Microbiology and Biotechnology*, 30(7):1959–1967.
- Terrade, N. and Mira de Orduña, R. (2009). Determination of the essential nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus*. *International Journal of Food Microbiology*, 133(1-2):8–13.
- Versari, A., Parpinello, G. P., Cattaneo, M., San, V., Bologna, G., Brece, V., and Ancona, B. (1999). *Leuconostoc oenos* and malolactic fermentation in wine: a review. *Journal of Industrial Microbiology & Biotechnology*, 23:447–455.
- Wells, a. and Osborne, J. P. (2011). Production of SO₂ Binding Compounds and SO₂ by *Saccharomyces* during Alcoholic Fermentation and the Impact on Malolactic Fermentation. *South African Journal of Enology and Viticulture*, 32(2):267–279.

Appendices

Appendix I: Microorganisms, culture medium, solutions and reagents

I. Microorganisms

The microorganisms used in the present study are described in the table .4, those being three yeasts strains, divided in two groups: *Saccharomyces* and non-*Saccharomyces*; and one lactic acid bacteria strain.

Table .4: Microorganisms used in the experimental fermentations

	Species	Strain	Origin
Yeast	<i>Saccharomyces cerevisiae</i>	Lalvin-QA23	Lallemand
	<i>Torulasporea delbrueckii</i>	BIODIVA	Lallemand
	<i>Metschnikowia pulcherrima</i>	FLAVIA	Lallemand
Lactic acid bacteria	<i>Oenococcus oeni</i>	PSU-1	Pennsylvania State University, USA

II. Culture media

II. I. Yeast-Peptone-Dextrose (YPD) liquid medium

Volume: 1L
 20g Glucose
 20g Peptone
 10g Yeast Extract

Once the medium was hydrated, it was sterilized (121 °C, 20min.), and stored at room temperature.

II. II. Yeast-Peptone-Dextrose (YPD) agar medium

Volume: 1L
 20g Glucose
 20g Peptone
 10g Yeast Extract
 17g Agar

Once the medium was hydrated, the agar was added (in order to solidify the medium) and sterilized (121 °C, 20min.). Next, it was distributed in Petri dishes and, once gelled, it was stored at room temperature.

II. III. Man, Rogosa and Sharpe malic and fructose (MRS_{mf}) liquid medium

Volume: 1L
 55g MRS Broth (Difco™ Lactobacilli MRS Broth (Ref.: 288130))
 4g DL-Malic Acid
 5g D-Fructose

Once the medium was hydrated and adjusted at pH 5, it was sterilized (121 °C, 20min.), and stored at room temperature.

II. IV. Man, Rogosa and Sharpe malic and fructose (MRS_{mf}) agar medium

Volume: 1L

55g MRS Broth (DifcoTM Lactobacilli MRS Broth (Ref.: 288130))

4g DL-Malic Acid

5g D-Fructose

20g Agar

Once the medium was hydrated and at pH 5, it was added the agar (in order to solidify the medium) and sterilized (121 °C, 20min.). Next, it was distributed in Petri dishes and, once gelled, it was stored at room temperature.

II. V. Lysine agar medium (LYS)

Volume: 500mL

33g Lysine medium

5mL Potassium lactate solution

2mL Lactic acid

First, the lysine medium was hydrated and the potassium lactate solution was added. Next, it was sterilized (121 °C, 20min.) and, finally, in a sterile environment, the lactic acid was added. Then, it was distributed in Petri dishes and, once gelled, it was stored at room temperature.

II. VI. Fermentation must

Rectified Concentrated Must (RCM) (Mosto Concentrado Blanco, 65,4°Brix, Mostos Españoles, S.A.)

Sterile MilliQ purified water

The fermentation must was prepared from rectified concentrated must (RCM) and sterile MilliQ purified water. RCM and MilliQ water proportions were adjusted to obtain a must with a sugar concentration of 200±10g/L

III. Solutions

III. I. Saline solution

0.225g NaCl

250mL distilled water

Saline solution was sterilized (121 °C, 20min.) and stored at room temperature.

III. II. Hydroalcoholic solution 12% (v/v)

125mL Etanol 96% (v/v)

875mL distilled water

Hydroalcoholic solution was stored at room temperature.

IV. Reagents

In order to analyze the wine and must chemical compounds, it was used a multiparametric autoanalyzer, Miura One (ISE S.r.l., Ref.: 13310001200), and the following reagents and standards.

IV. I. Acetic acid enzymatic detection kit

TDI, Ref.: 2401

Stored at 4 °C.

IV. II. Aminic nitrogen enzymatic detection kit

TDI, Ref.: 2408

Stored at 4 °C.

IV. III. Amonia enzymatic detection kit

TDI, Ref.: 2407

Stored at 4 °C.

IV. IV. Citric acid enzymatic detection kit

TDI, Ref.: 2406

Stored at 4 °C.

IV. V. Free sulfite enzymatic detection kit

TDI, Ref.: 2409

Stored at 20 °C.

IV. VI. Glycerol enzymatic detection kit

TDI, Ref.: 2420

Stored at 4 °C.

IV. VII. Glucose/Fructose enzymatic detection kit

TDI, Ref.: 2404

Stored at 4 °C.

IV. VIII. L-lactic acid enzymatic detection kit

TDI, Ref.: 2403

Stored at 4 °C.

IV. IX. L-malic acid enzymatic detection kit

TDI, Ref.: 2402

Stored at 4 °C.

IV. XI. Total sulfite enzymatic detection kit

TDI, Ref.: 2410

Stored at 4 °C.

IV. XII. Multiparametric standard Enocal LD

TDI, Ref.: 2100D

Stored at 20 °C.

IV. XIII. Amonia standard

TDI, Ref.: 2108D

Stored at 4 °C.

V. Other Reagents

V. I. Succinic acid enzymatic detection manual kit

Megazyme, Ref.: K-SUCC

Stored at 4 °C.