Effect of the inoculation strategy of non-\textit{Saccharomyces} yeasts on wine malolactic fermentation

Alba Martín-García, Aitor Balmaseda, Albert Bordons* and Cristina Reguant

Grup de Biotecnologia Enológica, Departament de Bioquímica i Biotecnologia, Facultat d’Enologia, Universitat Rovira i Virgili, c/ Marcel·lí Domingo s/n, 43007 Tarragona, Catalonia, Spain

*corresponding author: albert.bordons@urv.cat

\textbf{ABSTRACT}

Interest in some non-\textit{Saccharomyces} yeasts has increased recently, because they have been associated with an improvement in wine quality. Nevertheless, little attention has been paid to the effect that the use of these yeasts may have on malolactic fermentation (MLF). In this study, the strains \textit{Torulaspora delbrueckii} Biodiva and \textit{Metschnikowia pulcherrima} Flavia were evaluated by co-inoculation and sequential fermentation with \textit{S. cerevisiae} QA23. A fermentation with \textit{S. cerevisiae} as a single starter was also performed as a control, then MLF was performed inoculating \textit{Oenococcus oeni} PSU-1 in all wines. Finally, the wines obtained after alcoholic fermentation and MLF were characterised. The results of the coinoculated fermentations were similar to those of the \textit{S. cerevisiae} control fermentations. Nevertheless, significant differences were observed in sequential fermentations in terms of lower content of acetic, L-malic and succinic acids. These differences were particularly noticeable in fermentations carried out with \textit{T. delbrueckii}.

\textbf{KEYWORDS}

malolactic fermentation, \textit{Metschnikowia pulcherrima}, non-\textit{Saccharomyces}, \textit{Oenococcus oeni}, \textit{Torulaspora delbrueckii}, wine
INTRODUCTION

There has been an increasing interest in inoculating grape musts with non-Saccharomyces yeasts to complement the traditional usage of Saccharomyces cerevisiae as a sole starter, as they improve product quality and complexity (Ciani et al., 2010; Comitini et al., 2011; Contreras et al., 2014; Jolly et al., 2003, 2014; Whitener et al., 2015; Zott et al., 2011). These other yeast species have little or moderate fermentation power and S. cerevisiae must be inoculated to finish the alcoholic fermentations (AF) (Benito et al., 2015). Thus, a new trend in winemaking uses mixed starter cultures of non-Saccharomyces and S. cerevisiae (Belda et al., 2015; Ciani et al., 2010) or a sequential inoculation of S. cerevisiae after non-Saccharomyces (Contreras et al., 2014; González-Royo et al., 2015). Among the different species of non-Saccharomyces, Torulaspora delbrueckii and Metschnikowia pulcherrima show the most promising results for global wine quality, such as low production of volatile acidity (Renault et al., 2009) or a notable mannoprotein release ability, which increases the mouthfeel properties of wine (Belda et al., 2016).

Malolactic fermentation (MLF) consists of the decarboxylation of L-malic acid to L-lactic acid by the lactic acid bacteria (LAB), mainly Oenococcus oeni. In addition to decreasing wine acidity, MLF induces other changes such as microbiological stability or organoleptic improvement (Bartowsky, 2005).

The performance of MLF by LAB is affected by the intrinsic properties of wine, which are mostly determined by yeasts (Balmaseda et al., 2018). The effects of yeasts on MLF can be either inhibitory, for example the production of ethanol or the nutrient exhaustion (Arnink and Henick-Kling, 2005), or stimulating, such as the production of citric and pyruvic acids (Liu et al., 2016). These effects depend on the concentration of the compounds in wine, which, in turn, depends on species and strains (Balmaseda et al., 2018).

The aim of this study was to determine the effect of the species of non-Saccharomyces with interesting oenological traits (T. delbrueckii and M. pulcherrima) on the MLF, by evaluating and comparing the inoculation strategies of co-inoculation (non-Saccharomyces and S. cerevisiae), or their sequential inoculation at different times.

MATERIALS AND METHODS

1. Microorganisms and inocula

The yeast strains used were T. delbrueckii Biodiva (Td), M. pulcherrima Flavia (Mp) and S. cerevisiae Lalvin-QA23 (Sc), all from Lallemand Inc. (Montréal, Canada). Strain O. oeni PSU-1 (ATCC BAA-331) was used for the MLF. Yeasts were maintained on YPD plates (2 % glucose, 2 % bacto-peptone, 1 % yeast extract, 2% agar, w/v) and bacteria on MRSmf (Margalef-Català et al., 2017) plates, and all were stored at 4 ºC. To obtain the inocula, a colony was picked from the plates and grown in liquid media YPD at 28 ºC (yeasts) and MRSmf at 27 ºC in a 10 % CO₂ atmosphere (O. oeni). Then, aliquots of 400 μL of these preinocula were inoculated in 40 mL of the same fresh liquid media.

2. Experimental fermentations

Fermentations were performed in 500 mL flasks containing 400 mL of sterile must, prepared using white grape concentrated must (65.4 º Brix; Mostos Españoles S.A., Tomelloso, Spain) and sterile MilliQ purified water to obtain a sugar concentration of 200 ± 10 g/L.

Alcoholic fermentations (AF) were carried out with two non-Saccharomyces strains and the inoculation of S. cerevisiae was performed in different time regimes: co-inoculation (Td-Sc; Mp-Sc), after 24 h (Td.24 h; Mp.24 h), 48 h (Td.48 h; Mp.48 h) and 72 h (Td.72 h; Mp.72 h). A control fermentation was also performed, with S. cerevisiae as the sole starter (Sc) (Figure 1).
Each yeast was inoculated for a population of \(10^6\) cells/mL. All fermentations were carried out in triplicate. Samples were taken every 24 h to monitor the evolution of sugar consumption and yeast population. YPD agar was used to calculate the total number of yeast cells, and lysine agar medium (Oxoid LTD, England) was used to quantify the non-Saccharomyces (Wang et al., 2016) after incubation at 28 °C for 48 h. AF was considered to have finished when the sugar concentration was below 1 g/L. To eliminate all yeasts, the resulting wines were filtered (MF-Millipore \textsuperscript{TM} 0.45 μm, Merck Millipore, Madrid, Spain).

Next, each wine (100 mL) was inoculated with \(O.\) oeni for a population of \(2 \times 10^7\) cells/mL. These fermentations were also carried out in triplicate. Samples were taken every 24 h to monitor the evolution of L-malic acid consumption and the bacterial population. Samples were plated on MRSmf and incubated at 27 °C in a 10 % CO\(_2\) atmosphere for 7 days. MLF was considered to have finished when L-malic acid was below 0.05 g/L.

### 3. Wine characterisation

After AF, ethanol content was determined by enzymatic assay (R-Biopharm AG, Darmstadt, Germany). On completion of AF and MLF, pH was measured (Crison micropH 2002, Hach Lange, L’Hospitalet, Spain) and various compounds (acetic acid, citric acid, L-lactic acid, L-malic acid, ammonium, α-NH\(_2\), succinic acid, glycerol, glucose+fructose, total and free SO\(_2\)) were analysed with the multianalyser Miura One (TDI SL, Gavà, Spain).

| Yeast Type | AF Duration (d) | AF Speed* (g/L·d) | MLF Duration (d) | MLF Speed* (g/L·d) |
|------------|----------------|-----------------|----------------|-----------------|
| Sc         | 10 ± 3\(^a\)  | 31.25 ± 2.04\(^c\) | 3 ± 0\(^b\) | 0.56 ± 0.11\(^a\) |
| Td-Sc      | 11 ± 1\(^ab\) | 29.24 ± 1.53\(^c\) | 7 ± 1\(^d\) | 0.23 ± 0.02\(^d\) |
| Td.24 h    | 20 ± 0\(^e\)  | 22.05 ± 0.31\(^b\) | 5 ± 0\(^bc\) | 0.29 ± 0.01\(^ed\) |
| Td.48 h    | 25 ± 1\(^f\)  | 16.80 ± 1.07\(^a\) | 4 ± 0\(^b\) | 0.48 ± 0.02\(^b\) |
| Td.72 h    | 18 ± 0\(^bc\) | 19.29 ± 1.29\(^ab\) | 4 ± 0\(^b\) | 0.53 ± 0.02\(^a\) |
| Mp-Sc      | 11 ± 0\(^ab\) | 37.00 ± 2.08\(^de\) | 4 ± 0\(^b\) | 0.40 ± 0.02\(^bc\) |
| Mp.24 h    | 14 ± 0\(^ol\) | 30.40 ± 0.60\(^c\) | 5 ± 2\(^bd\) | 0.33 ± 0.03\(^ed\) |
| Mp.48 h    | 14 ± 1\(^abc\) | 37.80 ± 0.83\(^e\) | 6 ± 1\(^bd\) | 0.35 ± 0.01\(^ed\) |
| Mp.72 h    | 14 ± 0\(^bc\) | 33.15 ± 1.65\(^ed\) | 7 ± 2\(^d\) | 0.32 ± 0.04\(^ed\) |

Values shown are the means of triplicates ± SD. *Calculation based on consumption speed of sugar (AF) and L-malic acid (MLF) considering the period of exponential decrease of these compounds. \(^a-d\), values are significantly different at \(p \leq 0.05\), according to a Tukey post-hoc comparison test.

### 4. Statistical analysis

Statistical software XLSTAT version 2018.4.51298 (Addinsoft, Paris, France) was used. The data obtained was submitted to one-way ANOVA with subsequent analysis using the Tukey test, with a confidence interval of 95 % and significant results with a p-value of \(\leq 0.05\). Principal component analysis (PCA) was also performed to determine differences between the wines.

**RESULTS AND DISCUSSION**

1. Alcoholic fermentation

Control fermentation with only \(S.\) cerevisiae was completed in 10 days, with a sugar consumption rate of 31.25 g/L·day (Table 1). This was the fastest fermentation performed because, unlike coinoculated and sequential fermentations, there was neither synergy nor competition for the substrate between yeasts. Coinoculated Td-Sc and Mp-Sc fermentations was completed in 11 days with a lower consumption rate (Table 1).

Table 1 shows that sequential fermentations of \(T.\) delbrueckii (Td.24 h, 48 h, 72 h) took longer than the control fermentations, largely because the final stages were slower (Figure 2 left), while fermentations of \(M.\) pulcherrima had slow early stages but finished at the same time as the control. In fact, sugar consumption was not significant until \(S.\) cerevisiae was inoculated (Figure 2, right). Nevertheless, during the initial days the non-Saccharomyces populations were stable and did not decrease until the \(S.\) cerevisiae inoculation (data not shown).
2. Malolactic fermentation

No significant differences were observed in MLF between Sc and non-Saccharomyces wines (considering the exponential decrease in L-malic acid), except for Mp 72 h, which was slower (Table 1 and Figure 3). Nonetheless, there were significant differences in terms of MLF duration between non-Saccharomyces species. MLF was slower in wines produced from Mp sequential fermentations and Td-Sc.

3. Changes in wine composition

As expected, the inoculation strategy had an impact on wine composition after AF (Table 2). The most relevant changes were observed in sequential inoculation, which were related to the longer persistence of non-Saccharomyces populations, as introduced above. Td fermentations showed no significant differences in ethanol content with respect to the control. Although T. delbrueckii has been reported to produce less ethanol than S. cerevisiae (Contreras et al., 2014), several other authors have found almost no difference (Belda et al., 2015) and thus it may depend on the strain and the conditions (Benito, 2018).

On the contrary, Mp sequential fermentations were found to have significantly lower ethanol content, especially in Mp.24 h. The production of glycerol was observed to be similar in Sc and Td wines. However, Mp fermentations presented a significantly higher content at the end of AF, as has been observed elsewhere (Contreras et al., 2014). This may be due to the higher capacity of non-Saccharomyces yeasts (Mp) to use the glycopyruvic pathway instead of the usual pyruvate-to-ethanol pathway (Jolly et al., 2014).
TABLE 2. Characterisation of initial must and wines after alcoholic (AF) and malolactic (MLF) fermentations.

| Sugar (g/L) | L-malic acid (g/L) | Citric acid (g/L) | α-NH₂ (mg/L) | Ammonium (mg/L) | pH  |
|------------|-------------------|-------------------|--------------|-----------------|-----|
| Initial must | 198.11 ± 10.72 | 2.14 ± 0.08 | 0.25 ± 0.05 | 153.90 ± 11.21 | 70.22 ± 4.44 | 3.94 ± 0.01 |

| Sugar (g/L) | Ethanol | L-malic acid (g/L) | pH | Citric acid (g/L) | Acetic acid (g/L) |
|------------|---------|-------------------|----|------------------|-------------------|
| AF | AF | MLF | AF | AF | MLF | AF | MLF |
| Sc | 0.11 ± 0.09 | 11.67 ± 0.29ᵃ | 1.36 ± 0.03ᵃ | 3.45 ± 0.03ᶜ | 3.86 ± 0.02ᵃ | 0.22 ± 0.02 | 0.01 ± 0.01ᶜ | 0.23 ± 0.04ᵇ | 0.41 ± 0.03ᵇᶜ |
| Td-Sc | 0.23 ± 0.21 | 11.12 ± 0.28ᵇ | 1.46 ± 0.06ᵈ | 3.56 ± 0.09ᵇᵉ | 3.74 ± 0.00ᵇ | 0.23 ± 0.03 | 0.17 ± 0.03ᵇ | 0.18 ± 0.02ᵈ | 0.28 ± 0.02ᵈ |
| Td.24 h | 0.63 ± 0.23 | 11.48 ± 0.08ᵃ | 1.46 ± 0.07ᵃ | 3.85 ± 0.05ᵃᵇ | 4.20 ± 0.02ᵃ | 0.28 ± 0.01 | 0.09 ± 0.01ᵇ | 0.12 ± 0.02ᵇ | 0.22 ± 0.01ᵇ |
| Td.48 h | nd | 11.51 ± 0.11ᵃ | 1.43 ± 0.06ᵃ | 3.93 ± 0.04ᵇ | 4.23 ± 0.00ᵃ | 0.24 ± 0.01 | 0.22 ± 0.01ᵇ | 0.25 ± 0.03ᵇ |
| Td.72 h | 0.10 ± 0.09 | 11.16 ± 0.28ᵃ | 1.70 ± 0.08ᵃ | 3.81 ± 0.01ᵇᶜ | 4.03 ± 0.02ᵇ | 0.23 ± 0.01 | 0.18 ± 0.02ᶜ | 0.28 ± 0.02ᶜ |
| Mp-Sc | 0.16 ± 0.05 | 11.64 ± 0.10ᵃ | 1.70 ± 0.08ᵃ | 3.49 ± 0.04ᵇ | 3.93 ± 0.01ᵇᶜ | 0.22 ± 0.02 | 0.01 ± 0.01ᶜᵈ | 0.35 ± 0.02 | 0.49 ± 0.02ᵇ |
| Mp.24 h | 0.23 ± 0.20 | 7.88 ± 0.21ᵈ | 1.76 ± 0.07ᵃ | 3.46 ± 0.00ᵇ | 3.97 ± 0.09ᵇ | 0.21 ± 0.03 | 0.10 ± 0.01ᵇ | 0.29 ± 0.01ᵇᶜ | 0.35 ± 0.06ᵇᶜ |
| Mp.48 h | 0.13 ± 0.09 | 9.96 ± 0.16ᶜ | 1.54 ± 0.02ᶜ | 3.73 ± 0.07ᵇᶜ | 3.88 ± 0.13ᵇᶜ | 0.23 ± 0.01 | 0.05 ± 0.04ᵇᶜ | 0.29 ± 0.03ᵇ | 0.35 ± 0.01ᵇᶜ|
| Mp.72 h | 0.25 ± 0.20 | 10.35 ± 0.18ᵇᶜ | 1.58 ± 0.02ᶜ | 3.65 ± 0.03ᵇᶜ | 3.81 ± 0.08ᶜ | 0.29 ± 0.04 | 0.03 ± 0.01ᶜᵈ | 0.19 ± 0.05ᶜᵈ | 0.30 ± 0.05ᵇᶜ |

| Glycerol (g/L) | Succinic acid (mg/L) | α-NH₂ (mg/L) | Ammonium (mg/L) | Total sulfite (mg/L) | Free sulfite (mg/L) |
|---------------|-------------------|--------------|-----------------|---------------------|-------------------|
| AF | MLF | AF | MLF | AF | MLF | AF | MLF | AF | MLF |
| Sc | 5.06 ± 0.20ᵃ | 5.80 ± 0.44 | 331.94 ± 2.98ᵇ | 32.54 ± 1.24ᵃ | 32.23 ± 2.48ᵇ | 3.00 ± 0.71 | nd | 45.50 ± 0.71ᵃᵇ | 13.67 ± 2.31ᵃᵇ | 4.00 ± 1.00ᵇ |
| Td.24 h | 6.28 ± 0.09ᵃ | 6.52 ± 0.02 | 311.53 ± 3.96ᵇ | 65.73 ± 5.54ᵃ | 61.57 ± 2.93ᵃ | 2.50 ± 0.71 | 3.00 ± 1.00 | 26.67 ± 3.05ᵃᵇ | 4.67 ± 3.79ᵃᵇᶜ | 3.33 ± 0.58ᵇ |
| Td.48 h | 5.74 ± 0.27ᵃ | 5.92 ± 0.07 | 309.66 ± 1.81ᶜ | 73.06 ± 5.38ᵃ | 66.37 ± 2.71ᵃ | 2.50 ± 0.71 | 5.50 ± 0.71 | 16.00 ± 2.82ᵇ | 5.00 ± 1.41ᵃᵇ | 1.67 ± 0.58ᵇ |
| Td.72 h | 5.87 ± 0.27ᵃ | 4.59 ± 0.01 | 314.20 ± 1.15ᵃ | 63.80 ± 3.52ᵃ | 60.47 ± 0.74 | 1.00 ± 0.00 | 3.50 ± 0.71 | 18.00 ± 0.00ᵃ | 7.00 ± 0.00ᵃᵇ | 1.50 ± 0.71ᵇ |
| Mp.24 h | 6.19 ± 0.21ᵃ | 4.97 ± 0.01 | 320.28 ± 1.28ᵇ | 26.98 ± 4.02ᵃ | 28.90 ± 1.24ᵃ | 3.50 ± 1.41 | 2.00 ± 0.00 | 45.67 ± 0.00ᵇ | 18.00 ± 2.83ᵃ | 3.67 ± 0.57ᵇ |
| Mp.48 h | 6.45 ± 0.14ᶜ | 5.29 ± 0.18 | 320.29 ± 7.01ᵇ | 20.26 ± 2.00ᵃ | 20.00 ± 3.70ᵃ | 2.00 ± 1.00 | 2.00 ± 1.00 | 28.00 ± 4.24ᵃᵇ | 15.33 ± 3.79ᵇ | 2.33 ± 0.58ᵇ |
| Mp.72 h | 7.27 ± 0.21ᵇ | 7.05 ± 0.16 | 315.53 ± 2.60ᵃ | 39.07 ± 8.30ᵇ | 38.77 ± 3.03ᵃ | 4.00 ± 0.00 | 3.50 ± 0.71 | 33.00 ± 2.00ᵃᵇ | 20.67 ± 3.79ᵇ | 2.33 ± 0.58ᵇ |
| Mp.72 h | 8.01 ± 0.25ᵃ | 7.50 ± 0.65 | 309.42 ± 1.69ᵇ | 28.32 ± 5.33ᵃ | 19.97 ± 3.87ᵃ | 7.00 ± 0.00 | 3.33 ± 1.53 | 29.67 ± 2.89ᵃᵇᶜ | 13.33 ± 1.53ᵃᵇᶜ | 2.33 ± 0.58ᵇ |

ᵃᵇᶜ, values are significantly different at p ≤ 0.05, according to a Tukey post-hoc comparison test, values without superscript letters did not show significant differences.
The pH was significantly higher in Td wines with sequential inoculation, and was closer to initial must pH. In addition, Mp.48 h and Mp.72 h showed significantly higher pH than control Sc. A higher pH can be an attenuating factor for the inhibitory effect of ethanol on *O. oeni*, but MLF was not shorter in Td sequential wines. Despite this, under non-sterile cellar conditions, a pH close to 4 or higher may promote the development of other LAB, such as *Pediococcus* spp. and threaten wine quality (Wade *et al.*, 2019).

Both *S. cerevisiae* and non-*Saccharomyces* consumed some L-malic acid during AF (Table 2). In Mp sequential fermentations, consumption was higher when it took longer to inoculate Sc, although all non-*Saccharomyces* consumed less L-malic acid than Sc. Nevertheless, high values of L-malic acid tend to ensure a good MLF. L-lactic acid production depended on L-malic acid consumption (data not shown).

Citric acid content did not vary during AF. No differences were found in its metabolisation by *O. oeni*, except for Td-Sc and Td.24 h, in which it was not completely consumed, unlike other fermentations. In Td fermentations, MLF was slower when *O. oeni* did not totally consume the citric acid.

Acetic acid concentration after AF was up to 60 % lower in Td sequential AF wines. It has been observed elsewhere that some non-*Saccharomyces* can decrease acetic acid concentration (Chen *et al.*, 2018). Data obtained from Mp wines appeared to be similar to the control data, although Mp-Sc was higher, probably due to the early imposition of Sc. After MLF, as expected, the acetic acid concentration was higher due to citrate consumption.

In agreement with other studies, succinic acid production decreased in non-*Saccharomyces* AF (Contreras *et al.*, 2014). These differences were most noticeable in Mp fermentations, in which succinic acid decreased by up to 10 % more than Sc fermentation. Succinic acid can act as a competitive inhibitor of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad, 1982), which has a negative effect on MLF, although this inhibition has not been observed in the present study.

Td sequential fermentations consumed the least α-NH₂ - the free alpha-amino nitrogen that is equivalent to available amino acids (Table 2). The coexistence of the two yeast populations may have resulted in higher nitrogen consumption. This data is in agreement with other studies reporting competition for nitrogen sources between yeasts (Gobert *et al.*, 2017). No significant differences in ammonium consumption by yeasts were observed here.

One of the main products of the antagonistic interactions between yeasts and *O. oeni* is SO₂ (Nehme *et al.*, 2008). Some non-*Saccharomyces* strains can produce significant amounts of SO₂ (Wells and Osborne, 2011). In this study, differences were found between Td wines and the others. Sequential Td wines showed that total SO₂ production was lower and the content of free SO₂ was similar. The lack of any difference between Mp and Sc wines may be the consequence of an
early imposition of the latter. The values of total SO$_2$ were clearly lower after MLF than before it (Table 2). This could be explained by the known reduction of bound SO$_2$ levels due to degradation of acetaldehyde and other binding compounds by *O. oeni* during MLF (Davis et al., 1985; Jackowetz and Mira de Orduña, 2012).

Considering the variables studied, the PCA (Figure 4) confirmed the differences between yeast species. It can be observed that in wines after AF (Figure 4 A), Td sequential fermentations are clustered in one group and Mp sequential fermentations are grouped in another. The first group consumes less α-NH$_3$ and has a higher pH, while the second has a lower ethanol but higher glycerol content. This shows that there are similarities between wines fermented with the same non-*Saccharomyces* species, regardless of the time of inoculation with *S. cerevisiae*.

After MLF (Figure 4 B), the clusters were maintained with slight differences. Mp-Sc wine is clustered with the other sequential fermentations of Mp. In addition, all wines are closer in the PCA, indicating a homogenisation of wines after MLF due to the metabolism of the *O. oeni* strain used.

**CONCLUSIONS**

This study shows that the impact of non-*Saccharomyces* was greater on sequential AF than on co-inoculated AF. Differences were observed between *T. delbrueckii* and *M. pulcherrima*. When *T. delbrueckii* was used, it had a positive effect on *O. oeni* and MLF due to lower acidity, succinic acid and SO$_2$, even though MLF was slightly slower than in *S. cerevisiae* wines. *M. pulcherrima* decreased ethanol content during AF, which minimised its negative effect on *O. oeni*, yet MLF was slower than in control wines. Thus, other compounds must have a negative effect on *O. oeni*.

Further research is required for a better understanding of the impact of non-*Saccharomyces* on MLF

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