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Effect of the inoculation strategy of non-*Saccharomyces* yeasts on wine malolactic fermentation

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ABSTRACT

Interest in some non-*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 *Torulaspora delbrueckii* Biodiva and *Metschnikowia pulcherrima* Flavia were evaluated by co-inoculation and sequential fermentation with *S. cerevisiae* QA23. A fermentation with *S. cerevisiae* as a single starter was also performed as a control, then MLF was performed inoculating *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 *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 *T. delbrueckii*.

KEYWORDS

malolactic fermentation, *Metschnikowia pulcherrima*, non-*Saccharomyces*, *Oenococcus oeni*, *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 °C. To obtain the inocula, a colony was picked from the plates and grown in liquid media YPD at 28 °C (yeasts) and MRSmf at 27 °C in a 10 % CO_2 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 ° Brix; Mostos Españoles S.A., Tomelloso, Spain) and sterile MilliQ purified water to obtain a sugar concentration of 200 ± 10 g/L.

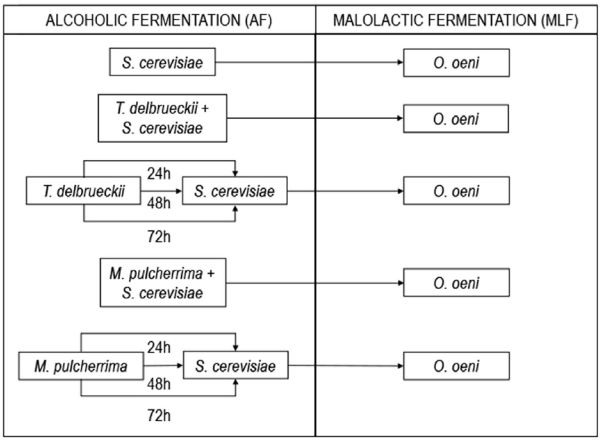

FIGURE 1. Diagram of the experimental fermentations. Each one was carried out by triplicate.

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 ºC 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™ 0.45 µm, Merck Millipore, Madrid, Spain).

Next, each wine (100 mL) was inoculated with *O. oeni* for a population of 2×10^7 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).

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 ≤ 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).

TABLE 1. Alcoholic (AF) and malolactic (MLF) fermentations duration and speed.

	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^{a}	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^{abc}	0.29 ± 0.01^{cd}
Td.48 h	25 ± 1^{f}	16.80 ± 1.07^{a}	4 ± 0^{ab}	0.48 ± 0.02^{ab}
Td.72 h	18 ± 0^{de}	19.29 ± 1.29^{ab}	4 ± 0^{ab}	0.53 ± 0.02^{a}
Mp-Sc	11 ± 0^{ab}	37.00 ± 2.08^{de}	4 ± 0^{ab}	0.40 ± 0.02^{bc}
Mp.24 h	14 ± 0^{cd}	30.40 ± 0.60^{c}	5 ± 2^{bcd}	0.33 ± 0.03^{cd}
Mp.48 h	14 ± 1^{abc}	37.80 ± 0.83^{e}	6 ± 1^{bcd}	0.35 ± 0.01^{cd}
Mp.72 h	14 ± 0^{bc}	33.15 ± 1.65^{cd}	7 ± 2^{cd}	0.32 ± 0.04^{cd}

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], vales are significantly different at $p \leq 0.05$, according to a Tukey post-hoc comparison test.

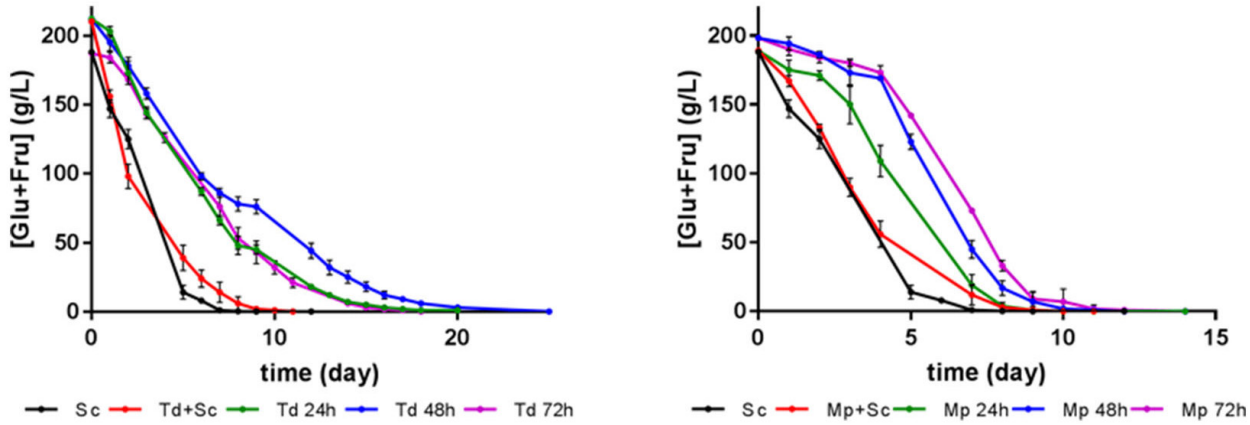

FIGURE 2. Evolution of alcoholic fermentation through sugar consumption by yeasts.
Left: *T. delbrueckii* fermentations (Td-Sc; Td.24 h; Td.48 h; Td.72 h) and control (Sc). Right: *M. pulcherrima* fermentations (Mp-Sc; Mp.24 h; Mp.48 h; Mp.72 h) and control (Sc).

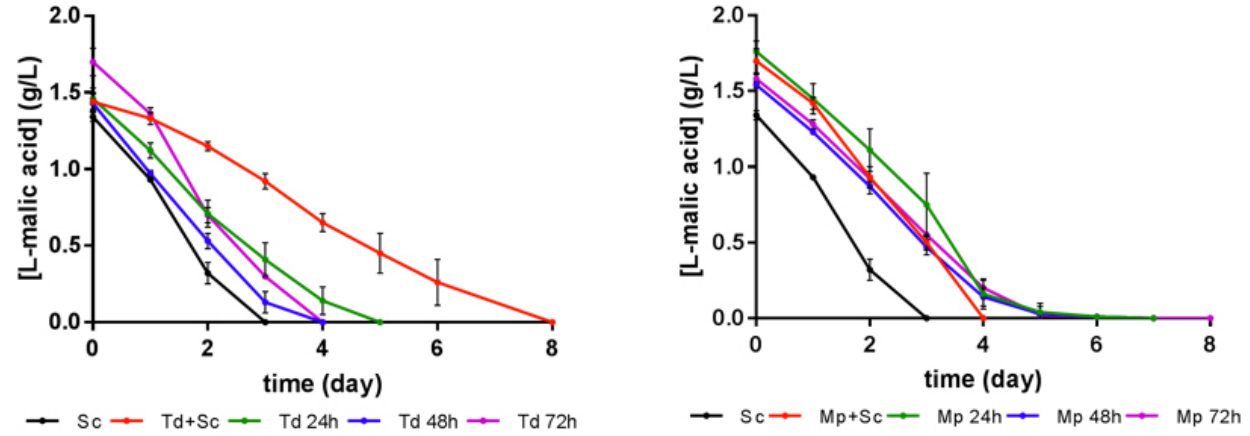

FIGURE 3. Evolution of malolactic fermentation after AF by monitoring the L-malic acid consumption by *O. oeni* PSU-1.
Left: wines fermented with *T. delbrueckii* (Td-Sc; Td.24 h; Td.48 h; Td.72 h) and control (Sc). Right: wines fermented with *M. pulcherrima* (Mp-Sc; Mp.24 h; Mp.48 h; Mp.72 h) and control (Sc).

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-NH_2 (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	(% (v/v))	AF	AF	MLF	AF	MLF	AF	MLF
Sc	0.11 ± 0.09	11.67 ± 0.29^{a}	1.36 ± 0.03^{d}	3.45 ± 0.03^{e}	3.86 ± 0.02^{bc}	0.22 ± 0.02	0.01 ± 0.01^{cd}	0.23 ± 0.04^{bc}	0.41 ± 0.03^{ab}
Td-Sc	0.23 ± 0.21	11.12 ± 0.28^{ab}	1.46 ± 0.06^{cd}	3.56 ± 0.09^{de}	3.74 ± 0.00^{de}	0.23 ± 0.03	0.17 ± 0.03^{a}	0.18 ± 0.02^{cd}	0.28 ± 0.02^{cd}
Td.24 h	0.63 ± 0.23	11.48 ± 0.08^{a}	1.46 ± 0.07^{cd}	3.85 ± 0.05^{ab}	4.20 ± 0.02^{a}	0.28 ± 0.01	0.09 ± 0.01^{b}	0.12 ± 0.02^{d}	0.22 ± 0.01^{d}
Td.48 h	nd	11.51 ± 0.11^{a}	1.43 ± 0.06^{cd}	3.93 ± 0.04^{a}	4.23 ± 0.00^{a}	0.24 ± 0.01	nd^{d}	0.12 ± 0.03^{d}	0.25 ± 0.03^{d}
Td.72 h	0.10 ± 0.09	11.16 ± 0.28^{a}	1.70 ± 0.08^{ab}	3.81 ± 0.01^{abc}	4.03 ± 0.02^{b}	0.22 ± 0.02	nd^{d}	0.10 ± 0.01^{d}	0.29 ± 0.01^{bcd}
Mp-Sc	0.16 ± 0.05	11.64 ± 0.10^{a}	1.70 ± 0.08^{ab}	3.49 ± 0.04^{de}	3.93 ± 0.01^{bc}	0.22 ± 0.02	0.01 ± 0.01^{cd}	0.35 ± 0.02^{a}	0.49 ± 0.02^{a}
Mp.24 h	0.23 ± 0.20	7.88 ± 0.21^{d}	1.76 ± 0.07^{a}	3.46 ± 0.00^{de}	3.97 ± 0.09^{bc}	0.21 ± 0.03	nd^{d}	0.31 ± 0.04^{bc}	0.35 ± 0.06^{abc}
Mp.48 h	0.13 ± 0.09	9.96 ± 0.16^{c}	1.54 ± 0.02^{c}	3.73 ± 0.07^{bc}	3.88 ± 0.13^{bc}	0.23 ± 0.01	0.05 ± 0.04^{bc}	0.29 ± 0.03^{ab}	0.35 ± 0.01^{abc}
Mp.72 h	0.25 ± 0.20	10.35 ± 0.18^{bc}	1.58 ± 0.02^{bc}	3.65 ± 0.03^{cd}	3.81 ± 0.08^{c}	0.29 ± 0.04	0.03 ± 0.01^{cd}	0.19 ± 0.05^{cd}	0.30 ± 0.05^{bcd}

	Glycerol (g/L)		Succinic acid (mg/L)	α-NH_2 (mg/L)		Ammonium (mg/L)		Total sulfite (mg/L)		Free sulfite (mg/L)
	AF	MLF	AF	AF	MLF	AF	MLF	AF	MLF	AF
Sc	5.06 ± 0.20^{d}	5.80 ± 0.44	331.94 ± 2.98^{a}	32.54 ± 1.24^{b}	32.23 ± 2.48^{b}	3.00 ± 0.71	nd	45.50 ± 0.71^{ab}	13.67 ± 2.31^{ab}	4.00 ± 1.00^{a}
Td-Sc	5.56 ± 0.38^{cd}	6.17 ± 0.19	323.82 ± 3.66^{ab}	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}
Td.24 h	6.28 ± 0.09^{cd}	6.52 ± 0.02	311.53 ± 3.96^{bc}	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}
Td.48 h	5.74 ± 0.27^{cd}	5.92 ± 0.07	309.66 ± 1.81^{c}	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}
Td.72 h	5.87 ± 0.27^{cd}	4.59 ± 0.01	314.20 ± 1.15^{bc}	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}
Mp-Sc	6.19 ± 0.21^{cd}	4.97 ± 0.01	320.28 ± 1.28^{bc}	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}
Mp.24 h	6.45 ± 0.14^{bc}	5.29 ± 0.18	320.29 ± 7.01^{bc}	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}
Mp.48 h	7.27 ± 0.21^{b}	7.05 ± 0.16	315.53 ± 2.60^{bc}	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}
Mp.72 h	8.01 ± 0.25^{a}	7.50 ± 0.65	309.42 ± 1.69^{c}	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}

$^{a-d}$, values are significantly different at $p \leq 0.05$, according to a Tukey post-hoc comparison test, values without superscript letters did not show significant differences.

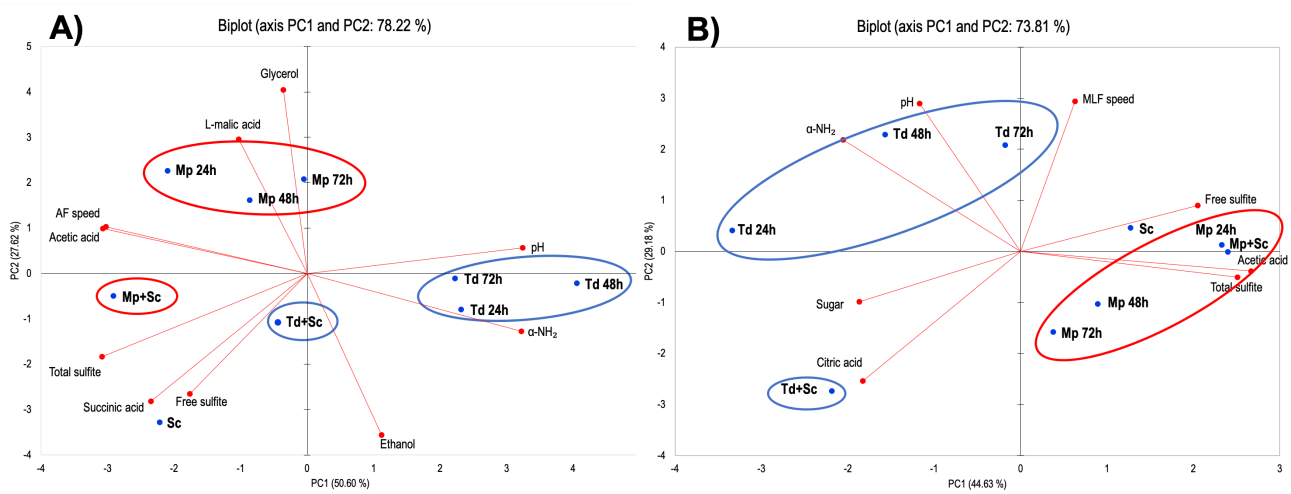

FIGURE 4. Principal component analysis (PCA) biplot of wines obtained at the end of (A) alcoholic fermentation and (B) malolactic fermentation.
The values shown are the mean of triplicates

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-NH_2 - 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 SO_2 (Nehme *et al.*, 2008). Some non-*Saccharomyces* strains can produce significant amounts of SO_2 (Wells and Osborne, 2011). In this study, differences were found between Td wines and the others. Sequential Td wines showed that total SO_2 production was lower and the content of free SO_2 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_2 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 coinoculated 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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