Modulation of a defined community of *Oenococcus oeni* strains by *Torulaspora delbrueckii* and its impact on malolactic fermentation

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Abstract

Background and Aims: *Torulaspora delbrueckii* is being used increasingly as a starter for alcoholic fermentation (AF) because of its chemical modulation of wine. Previous studies on this yeast in a natural must have shown a different *Oenococcus oeni* population by the end of MLF. In this study we aim to evaluate this aspect in a defined *O. oeni* strain consortium in a sterile grape must during winemaking.

Methods and Results: Before commencing AF with either *S. cerevisiae* or both *T. delbrueckii* and *S. cerevisiae*, the must was inoculated with a defined population of *O. oeni* strains. The use of *T. delbrueckii* determined the bacterial population at the end of MLF. Also, the inoculation of a selected strain after AF produced wines with different chemical composition to those fermented with the initial bacterial community.

Conclusions: Different yeast inoculation strategies modulate the *O. oeni* population, and this has an impact on the chemical composition of the wines. Moreover, the inoculation of a small *O. oeni* population in must leads to a process similar to spontaneous MLF.

Significance of the Study: *Torulaspora delbrueckii* can be used as a tool to modulate the *O. oeni* population and enhance the aromas related to MLF.

Keywords: *malolactic fermentation, non*-Saccharomyces, Oenococcus oeni, *population dynamics,* Torulaspora delbrueckii

Introduction

Oenococcus oeni is the main species of lactic acid bacteria (LAB) that carries out MLF in alcoholic fermented beverages such as wine and cider (Wibowo et al. 1985, Lonvaud-Funel 1999). Malolactic fermentation consists of the decarboxylation of L-malic acid into L-lactic acid, which is related to an increase in pH, improvements in microbial stability and the production of aroma compounds (Lonvaud-Funel 1999). This metabolism is vital for LAB survival under the stressful conditions found in wine, such as low pH, high ethanol concentration and low nutrient availability (Bech-Terkilsen et al. 2020).

These LAB that participate in the MLF come from the grapes and the must and may also be part of the cellar's resident microbiota (González-Arenzana et al. 2012, Portillo et al. 2016, Franquès et al. 2017). As the grapes are transformed into must and then into wine the LAB population becomes more restricted, with the main significant species being *O. oeni*. As the predominance of this bacterial species often involves more than one strain, there can be several dominant strains (Reguant et al. 2005).

In this oenological context the population of *O. oeni* will be greatly affected by the grape cultivar (Portillo et al. 2016) and health status of the berries (Lleixà et al. 2018), the location of the vineyards and cellar practices (González-Arenzana et al. 2013) and the fermenting yeasts involved in the alcoholic fermentation (AF). At this final point the selected yeast strains inoculated into the must ready to undergo AF have a considerable impact (Alexandre et al. 2004, Balmaseda et al. 2018). Traditionally *S. cerevisiae* has been used as a starter culture in winemaking (Fleet 2008). Current research, however, in non-*Saccharomyces* yeasts in connection with the first stages of fermentation suggests the use of these non-*Saccharomyces* species to modulate the chemical and sensory characteristics of wines (Padilla et al. 2016). These non-*Saccharomyces* yeasts can also have an impact on the *O. oeni* community developed in those wines as a result of that chemical modulation (Balmaseda et al. 2018).

Torulaspora delbrueckii is a non-*Saccharomyces* yeast that has been proposed as a microbial tool to improve wine characteristics (Benito 2018). It is of special interest in red winemaking because of its enhancement of the colour parameters and volatile compounds (Belda et al. 2017, Escribano-Viana et al. 2019). In addition, it reduces the concentration of various compounds associated with an inhibitory effect on *O. oeni*, such as ethanol, SO_2 and succinic acid, and promotes certain stimulatory changes, such as an increase in mannoprotein concentration and pH (Belda et al. 2016, Benito 2018, Ferrando et al. 2020, Martín-García et al. 2020). Recent studies have also reported differences in the *O. oeni* strain imposition at the end of MLF associated with the use of *T. delbrueckii* when compared to wines fermented only with *S. cerevisiae* (Balmaseda et al. 2021a,b). This strengthens the importance of the concept of yeast–*O. oeni* strain compatibility for a successful MLF performance. In addition, the use of

doi: 10.1111/ajgw.12526

T. delbrueckii enabled spontaneous MLF in red wines of high concentration of phenolic compounds, which was not possible in wine fermented with *S. cerevisiae* (Balmaseda et al. 2021b).

In the present study we aimed to evaluate how the use of *T. delbrueckii* influences the evolution of a defined community of *O. oeni* strains. To this end we inoculated a sterile must with a selection of *O. oeni* strains and carried out the AF with *T. delbrueckii* in sequential inoculation with *S. cerevisiae*. Two strains of *T. delbrueckii* were used in order to evaluate the strain-dependent characteristics. The impact of this inoculation strategy on the *O. oeni* community was evaluated at the end of MLF and several relevant oenological parameters were measured over the course of the fermentation process.

Materials and methods

Microorganisms

The yeast strains used were *T. delbrueckii* Biodiva (Lallemand, Montréal, QC, Canada) (TdB), *T. delbrueckii* Viniferm NS-TD (Agrovin, Alcázar de San Juan, Spain) (TdV) and *S. cerevisiae* Lalvin-QA23 (Lallemand) (Sc). For the defined *O. oeni* community, henceforth referred to as the consortium, four strains isolated from wines fermented with *T. delbrueckii* in previous studies were used together with the commercial strain Viniflora-CH11 (Chr. Hansen, Hørsholm, Denmark). These four strains were isolated from vintage 2018—M25 and MCS5—(Balmaseda et al. 2021a) and from vintage 2019—AiB9 and AiB14 (Balmaseda et al. 2021b). *Oenococcus oeni* CH11 presented the best MLF performance in *T. delbrueckii* fermented wines (Balmaseda et al. 2021a,b). As a starter culture for MLF after AF, *O. oeni* PSU-1 (ATCC BAA-331) (PSU-1) was chosen. This strain was selected because of the wide knowledge that our group has of its behaviour from previous studies. The experimental design of the inoculation strategies is shown in Figure 1.

Yeasts were maintained on YPD plates (2% glucose, 2% bacto-peptone, 1% yeast extract, 2% agar, all mass/volume) (Panreac Química, Castellar del Vallès, Spain) and bacteria on MRSmf plates (Martín-García et al. 2020); all were stored at 4°C.

Experimental fermentations

Natural concentrated must from the Airén cultivar (Mostos, Tomelloso, Spain) was used for fermentations. The must was diluted with sterile MilliQ water to a sugar concentration of 180 ± 10 g/L, which corresponded to an initial concentration of citric acid and L-malic acid of 0.32 and 2 g/L, respectively. It was supplemented with 0.4 g/L of Nutrient Vit Nature (Lallemand) and the pH was adjusted to 3.6. At this point the must contained 152 mg/L of primary amino acids (NOPA) and 74 mg/mL of NH_4. The must was then sterilised with 0.1% (v/v) of dimethyl dicarbonate (ChemCruz, Santa Cruz Biotechnology, Dallas, TX, USA) and stored overnight at 4°C.

Fermentations were carried out statically at 20°C in triplicate in 1 L flasks containing 1 L of must. First, the must was inoculated with the *O. oeni* consortium. Each strain was grown separately to a population of around 10^9 colony-forming units (CFU)/mL in MRSmf liquid medium consisting of MRS (De Man et al. 1960) (Difco, Fisher Scientific, Madrid, Spain) supplemented with 4 g/L D,L-malic acid and 5 g/L fructose. The pH was adjusted to 5.0 with 10 N NaOH. Then an equal population of each strain was added to a sterile saline solution [0.9% (m/v)]. The resulting mixture was serially diluted in saline solution and added to each must replicate for a theoretical total population of 500 CFU/mL, corresponding to around 100 CFU/mL for each strain. The intention was to emulate the level of indigenous LAB population found in must, which can vary between 10^2 and 10^4 CFU/mL (Lonvaud-Funel 1999).

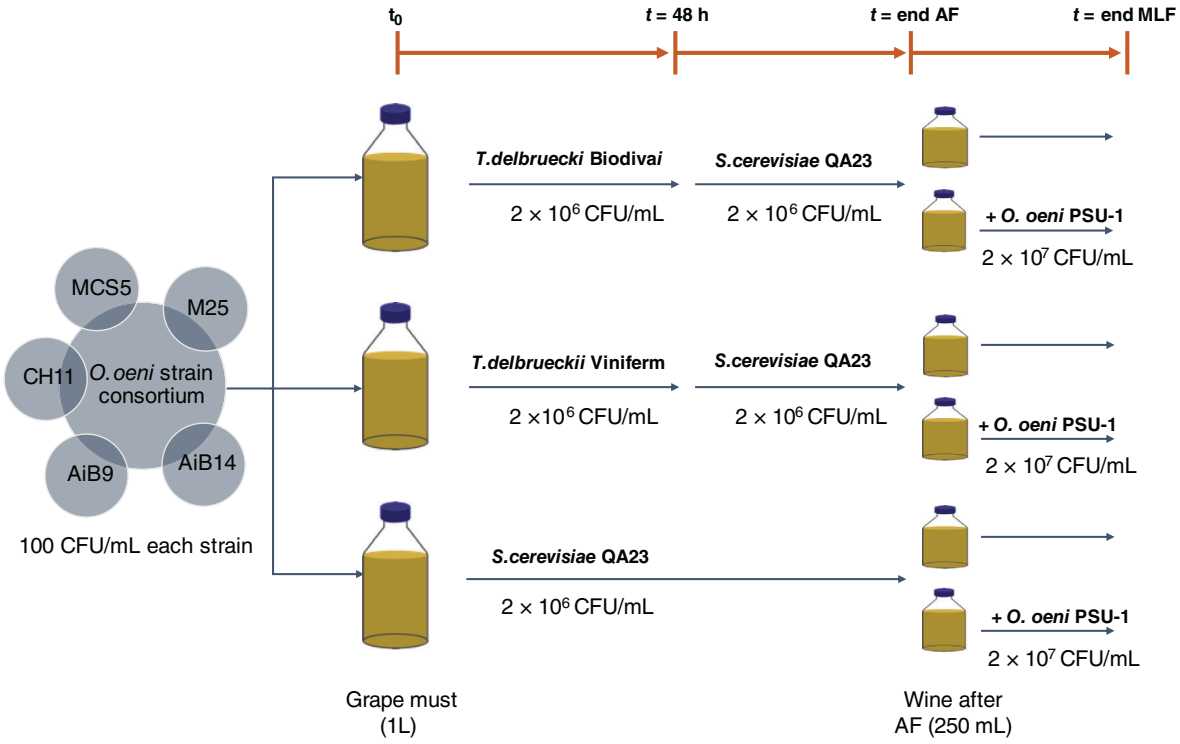

Figure 1. Schematic representation of the inoculation strategies used in this study.

Yeasts were inoculated at a population of 2×10^6 cells/mL to undergo the AF. In the case of sequential inoculation with *T. delbrueckii*, 48 h after the initial inoculation *S. cerevisiae* QA23 was inoculated to the same population. The decrease in density and yeast population was determined at least every 48 h. The total viable yeasts were counted on YPD agar medium, and lysine agar medium (Oxoid, Basingstoke, England) was used for the enumeration of non-*Saccharomyces*, after incubation at 28°C for 48 h. The AF was considered to have finished when the sugar concentration was below 2 g/L.

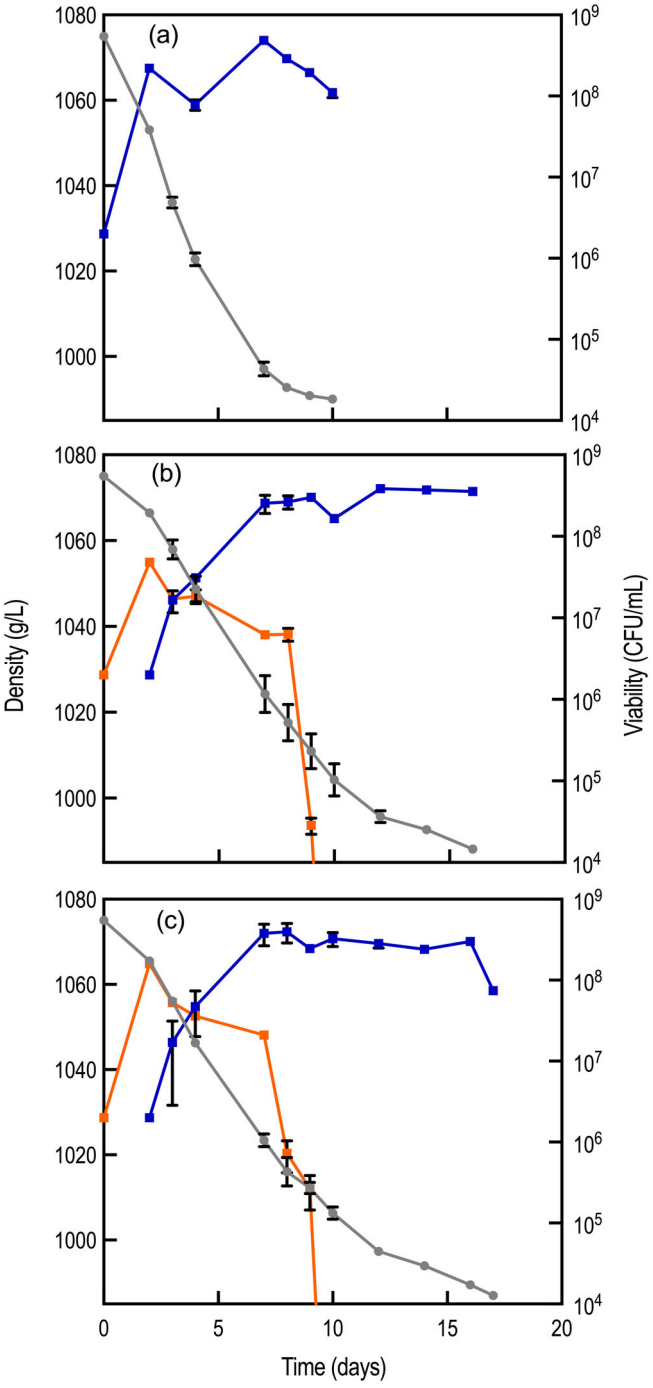

Figure 2. Evolution of alcoholic fermentation with (a) *Saccharomyces cerevisiae* alone and combined with (b) *Torulaspora delbrueckii* Biodiva and (c) *T. delbrueckii* Viniferm through the monitoring of density decrease (●) and yeast cell viability of *S. cerevisiae* (■) and *T. delbrueckii* (■).

At this point, 250 mL of each wine was transferred into two sterile 250 mL flasks. One flask of each wine was inoculated with *O. oeni* PSU-1 for a population of 2×10^7 CFU/mL in order to evaluate the impact of using an MLF starter on the evolution of the *O. oeni* consortium. The other flask of each wine was left without additional *O. oeni* inoculation. The two flasks were then incubated under the same conditions as in the AFs. These MLFs were also carried out in triplicate. Samples were taken every 24 h to monitor the consumption of L-malic acid and the evolution of the bacterial population in the wines inoculated with PSU-1, and at longer intervals in the others that contained only the bacterial consortium. Samples were plated on MRSmf and incubated at 27°C in a 10% CO_2 atmosphere for 7–15 days. The MLF was considered to have finished when the L-malic acid was below 0.1 g/L.

Oenococcus oeni *typing*

Once MLF was completed, ten isolates from the inoculated wines and 20 from the non-inoculated wines were randomly selected from the MRSmf plates for typing. The procedure of Balmaseda et al. (2021a) was followed for the DNA extraction and the typing procedure was based on the variable number of tandem repeat markers method described by Claisse and Lonvaud-Funel (2012, 2014). Samples were analysed using capillary electrophoresis by Eurofins Genomics Europe (Edersberg, Germany).

Wine characterisation

The concentration of sugar in the final stages of AF and of L-malic acid during MLF was determined using the Miura One multianalyser (TDI, Gavà, Spain). On completion of AF and MLF, pH was measured (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain).

Succinic acid after AF was determined using the succinic acid assay kit K-SUCC (Megazyme, Wicklow, Ireland). The glucose, glycerol, acetic acid, citric acid, lactic acid and ethanol of the wines after AF and MLF were determined by HPLC using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) as described in Zhu et al. (2020).

Analysis of wine volatile compounds

Wine samples (10 mL) were taken after AF and MLF. The volatile compounds were liquid/liquid extracted with 0.4 mL dichloromethane and 2.5 g $(NH_4)_2SO_4$, adding 40 µL of a solution of 4-methyl-2-pentanol (0.8 g/L) and heptanoic acid (0.7 g/L) as internal standards. Samples were analysed as described in Balmaseda et al. (2021a).

Statistical analysis

The statistics software XLSTAT version 2020.2.3.65345 (Addinsoft, Paris, France) was used for all the statistical analyses of the results. To test for differences between samples, a one-way ANOVA was performed using Tukey's honest significant difference post-hoc test at a *P*-value of 0.05.

Results and discussion

Fermentations and microbial growth parameters

The duration of AF was dependent on the inoculation strategy (Figure 2). The sequential inoculation with non-*Saccharomyces* increased the time of AF in synthetic or natural media as has already been reported (Martín-García et al. 2020, Balmaseda et al. 2021a). Sequential inoculation with *T. delbrueckii* increased the duration of AF by about 50% compared to the *S. cerevisiae* Control fermentation

Table 1. Fermentative parameters of alcoholic and malolactic fermentations and growth parameters of *Oenococcus oeni*.

Yeast and bacterial strains		AF duration (days)	MLF lag phase (days)	MLF (days)	Duration of AF and MLF (days)	Consumption rate [L-malic acid g/(L·day)]†	Growth phase (days)‡	Maximum biomass (CFU/mL)	Growth rate (/day)
PSU-1	Sc	10 ± 0	n.d.	4 ± 0a	14 ± 0a	0.7 ± 0.15b	n.d.	$6.3 \times 10^7 \pm 6.7 \times 10^6$a	
	TdB	16 ± 0	n.d.	4 ± 0a	20 ± 0a	0.54 ± 0.04b	n.d.	$9.6 \times 10^7 \pm 9 \times 10^6$a,b	
	TdV	17 ± 0	n.d.	5 ± 0a	22 ± 0a	0.25 ± 0.01a	n.d.	$3.7 \times 10^7 \pm 4.8 \times 10^6$a	
Consortium	Sc	10 ± 0	66 ± 3b	11 ± 1c	87 ± 4b	0.19 ± 0.01a	20 ± 1c	$7.6 \times 10^7 \pm 3.3 \times 10^7$a	0.46 ± 0.01a
	TdB	16 ± 0	70.5 ± 1b	8.5 ± 1b	95 ± 2b	0.12 ± 0.01a	14 ± 1b	$5 \times 10^8 \pm 4 \times 10^8$b	0.58 ± 0.01b
	TdV	17 ± 0	87 ± 31b	7.5 ± 1b	111.5 ± 32b	0.24 ± 0.07a	19 ± 4c	$2.7 \times 10^8 \pm 2.7 \times 10^8$a,b	0.47 ± 0.03a

Values shown are the mean of triplicates ± SD; lower case letters indicate a significant difference at $P \leq 0.05$ according to a Tukey post-hoc comparison test; n.d., not detected. †Calculation based on consumption rate of L-malic acid considering the period of exponential decrease of these values; growth phase refers to the time needed to reach sufficient *O. oeni* population to start consuming L-malic acid since viable cells were detected. AF, alcoholic fermentation; Consortium, contains *O. oeni* strains CH11, AiB9, MCS5 and M25; PSU-1, *O. oeni* PSU-1 strain; MLF, malolactic fermentation; Sc, wines fermented with *Saccharomyces cerevisiae*; TdB, wines fermented with *Torulaspora delbrueckii* Biodiva and *S. cerevisiae*; TdV, wines fermented with *T. delbrueckii* Viniferm and *S. cerevisiae*.

(Figure 2, Table 1). During the fermentation process the two *T. delbrueckii* strains remained viable until the middle fermentation stage. Afterwards their viability was lost on Lys plates ($<10^4$ CFU/mL). After AF, because the yeast lees were maintained in wine, they continued to be viable during MLF. Indeed, the viable yeast population remained at around 2–4 $\times$ 10^5 CFU/mL in all the wines, including those not inoculated with *O. oeni* PSU-1 (data not shown).

The *O. oeni* consortium evolved differently depending on the yeast inoculation strategy used for AF (data not shown). From the calculated 500 CFU/mL inoculated, after 1 h of incubation the population detected in all the wines was around 300 CFU/mL. After 2 days from the *S. cerevisiae* inoculation in Sc wines, the viable bacterial population was undetectable in the MRSmf plating. However, 2 days after *T. delbrueckii* fermentation in the TdB and TdV wines, the population of the *O. oeni* consortium remained at around 300 CFU/mL. Nevertheless, at fermenting day 4—2 days after the *S. cerevisiae* inoculation—the viable population was undetectable in these wines. This clearly shows a positive interaction between *T. delbrueckii* and *O. oeni* that allowed the bacterial consortium population to be maintained during the first 2 days. Probably the high fermentation capacity of *S. cerevisiae* resulted in the loss of the consortium's viability when it was inoculated. This can be seen in the decrease in density (Figure 2). *Torulaspora delbrueckii* underwent a less active fermentation process than *S. cerevisiae*, and this may be related to more gradual changes that would allow *O. oeni* to remain viable, although it did not support bacterial growth.

Few differences were observed in the overall fermentation process stemming from the use of *T. delbrueckii* in the MLFs inoculated with *O. oeni* PSU-1 (Table 1). As a result of the extended duration of the *T. delbrueckii* AF and the similar MLF duration, the total fermentation process was longer in *T. delbrueckii* wines. Of these fermentations, that of the TdV wine resulted in the longest fermentation with the lowest consumption rate and the lowest maximum biomass (Table 1). No significant differences were observed in the Sc and TdB wines.

The MLFs undergone by the consortium without *O. oeni* inoculation after AF had long lag phases (Table 1). Spontaneous MLF usually needs a long time to reach a high enough population, around 10^5 CFU/mL, for L-malic consumption to begin (Reguant et al. 2005). Interestingly, there was no difference in the observed lag phases arising from the use of different yeasts (Table 1). Nevertheless, the high heterogeneity of the results achieved in the TdV wine masked the fact that one of the replicates of this wine had a lag phase of 65 days, similar to the average lag phase in the Sc and TdB wines. The impact of the use of *T. delbrueckii* was noticeable in the duration of MLF, considered as the time taken for L-malic acid consumption, disregarding the initial lag phase (Table 1). In this regard, the use of both *T. delbrueckii* strains reduced the time of MLF. This is in line with the previously reported effect of this yeast on MLF when *O. oeni* is inoculated or when a reduction in the time of spontaneous fermentation is observed (Balmaseda et al. 2021b).

Oenococcus oeni *strain population at the end of MLF*

To understand the impact of *T. delbrueckii* on *O. oeni* strain population diversity we defined the consortium with five strains: four autochthonous *O. oeni* strains isolated from *T. delbrueckii* fermented wines and one commercial CH11

strain with enhanced MLF performance in *T. delbrueckii* wines (Balmaseda et al. 2021a,b). In addition, we inoculated PSU-1 after AF so as to have a control condition in which *O. oeni* is inoculated in the wine after AF.

The dynamics of the *O. oeni* strain populations in the wines varied as a consequence of the yeast inoculation strategy employed (Figure 3). Two tendencies were observed in the bacterial population at the end of MLF. One clustered the populations of Sc and TdB wines, while the other corresponded to TdV wines.

In the Sc and TdB wines the imposition of PSU-1 was complete, with no other strain from the initial consortium being detected (Figure 3). In previous studies we have observed the imposition capacity of PSU-1 when used as an MLF starter culture in the cellar (Balmaseda et al. 2021a,b). In those wines without PSU-1 inoculation the dominant strain from the consortium at the end of MLF was the commercial CH11, which corresponded to up to 80% in TdB wine and 90% in Sc wine on average (Figure 3). The only other strain detected in these wines was AiB9. The presence of this particular strain was significantly greater in TdB wine compared to Sc wine, suggesting that *T. delbrueckii* can modulate *O. oeni* strain predominance.

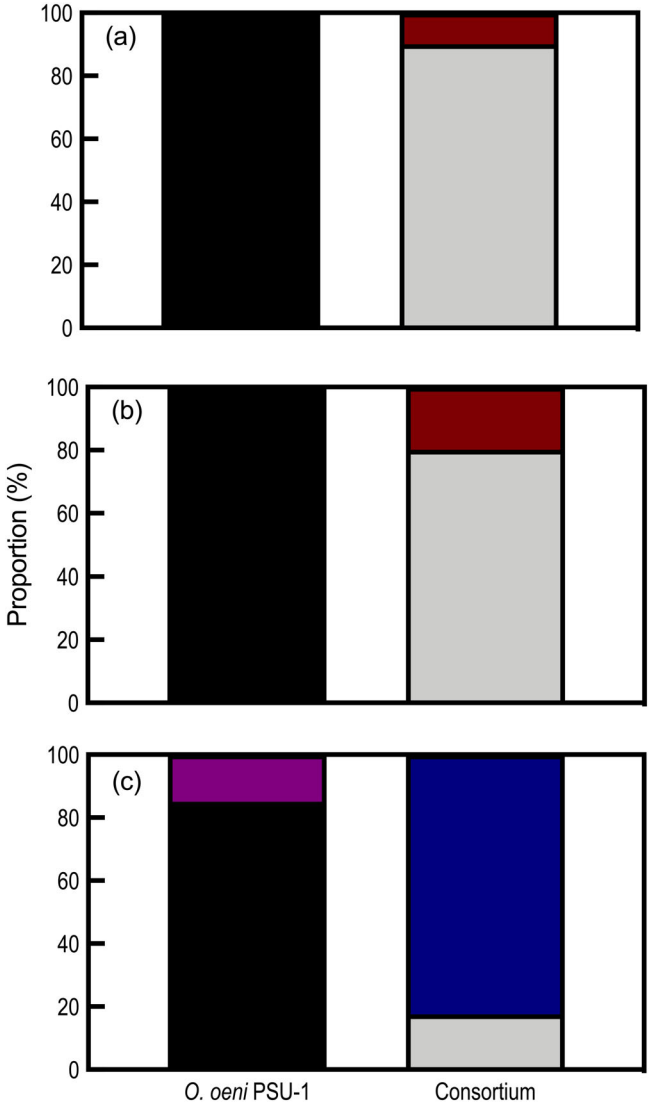

Figure 3. Proportion of imposition of the different variable number of tandem repeat markers (VNTR) profiles of *Oenococcus oeni* at the end of malolactic fermentation in wines inoculated after alcoholic fermentation with PSU-1 (■) or fermented with the initial bacterial population (consortium) containing *O. oeni* strains CH11 (■) AiB9 (■) MCS5 (■) and M25 (■). (a) Wine inoculated only with *Saccharomyces cerevisiae* and combined with (b) *Torulaspora delbrueckii* Biodiva and (c) *T. delbrueckii* Viniferm. Proportion values are the mean of three replicates.

A different pattern was observed in the TdV wine (Figure 3). First, in the wine inoculated with PSU-1, the MSC5 strain was detected. Unlike the other wines, the dominance of PSU-1 in the TdV wine was incomplete, and MCS5 corresponded to approximately 15% on average of the detected population. The wine not inoculated with PSU-1 presented a different population at the end of MLF compared to the Sc and TdB wines. Surprisingly, the presence of CH11 was poor because it represented just 20% on average. In this wine the dominant strain was M25.

Overall, although both *T. delbrueckii* strains showed a similar persistence during AF in sequential inoculation with *S. cerevisiae* QA23, the use of the Biodiva strain did not significantly modify the *O. oeni* population behaviour compared to the Control with only *S. cerevisiae* QA23, whereas the Viniferm strain completely changed it. When we relate these data to MLF performance (Table 1), few correlations can be found. Nevertheless, it is interesting to note that the growth rate associated with the TdB wine was significantly higher than that of the Sc wine, being composed of the same *O. oeni* population. Also, the duration of MLF—considering only the exponential consumption of L-malic acid—increased when the proportion of CH11 imposition decreased. Some authors have reported variable L-malic consumption rates by different *O. oeni* strains, which may be related (Nehme et al. 2010). Even so, MLF is a complex microbial process in which the dominant strains may change over time, and therefore other strains apart from those detected at the end of MLF could have participated in the fermentation.

Wine chemical composition

The chemical composition of the wines obtained depended on the inoculation strategy used (Table 2). Ethanol had a similar concentration of 10.5% (v/v) in all the wines after AF and MLF.

The substrate of the MLF—L-malic acid—was significantly reduced (to about 0.2–0.3 g/L on average) when the two *T. delbrueckii* strains were inoculated (Table 2). In the *T. delbrueckii* wines after MLF was inoculated, a higher concentration of lactic acid was observed (Table 2) compared to that of the *S. cerevisiae* Control wine. This may be related to the traces of glucose and fructose detected in the *T. delbrueckii* wines after AF (data not shown). As well as consuming L-malic acid, *O. oeni* can metabolise traces of sugar found in wine, thereby increasing D-lactic acid (Lonvaud-Funel 1999). Thus, we can observe an increase in these wines because of the contribution of the D-lactic acid isomer to the total lactic acid concentration.

The citric acid concentration was similar in the wines after AF (Table 2). The non-*Saccharomyces* modulation of this particular acid is heterogeneous, because some species increase it (Ferrando et al. 2020) while others produce quantities similar to *S. cerevisiae* (Belda et al. 2017, Martín-García et al. 2020). Under oenological conditions *O. oeni* metabolises this acid as an energy source and therefore its concentration is lower by the end of MLF (Davis et al. 1986). It is interesting to note that this consumption was observed in the wines inoculated after AF but not in

Table 2. Oenological parameters and some volatile compound families of wines after alcoholic and malolactic fermentations.

	L-Malic acid (g/L)	Lactic acid (g/L)	Citric acid (g/L)	Acetic acid (g/L)	Succinic acid (mg/L)	Ethanol (% v/v)	Glycerol (g/L)	pH	Σ SCFA (mg/L)	Σ MCFA (mg/L)	Σ Fusel alcohols (mg/L)	Σ Fusel alcohol acetates (mg/L)
Sc												
AF	1.51 ± 0.1c	n.d.	0.68 ± 0.03d	0.30 ± 0.04a,b	375 ± 4c	10.5 ± 0.2a	4.18 ± 0.67a	3.42 ± 0.03a	2.72 ± 0.32a,b	24.57 ± 0.94e	94.43 ± 0.9a	4.19 ± 0.45a,b
PSU-1	n.d.	1.45 ± 0.01b	0.19 ± 0.02a	0.48 ± 0.07d		10.7 ± 0.1a	6.03 ± 0.07b,c,d	3.65 ± 0.01b,c,d	4.01 ± 0.08b,c	22.66 ± 0.46e	94.35 ± 2a	3.73 ± 0.3a
Consortium	n.d.	1.40 ± 0.01b	0.54 ± 0.1c	0.31 ± 0.01a,b,c		10.4 ± 0.1a	5.83 ± 0.06b,c	3.59 ± 0.03b	5.5 ± 1.51c	23.97 ± 0.12e	97.03 ± 6.3a	4.44 ± 1a,b
TdB												
AF	1.35 ± 0.01b	n.d.	0.76 ± 0.0d	0.24 ± 0.04a	197 ± 8b	10.2 ± 0.2a	5.26 ± 0.04b	3.57 ± 0.0b	1.06 ± 0.34a	6.92 ± 0.79a	109.57 ± 2.9b	4.25 ± 0.17a,b
PSU-1	n.d.	2.04 ± 0.02d	0.28 ± 0.03a,b	0.41 ± 0.01c,d		10.5 ± 0.1a	6.15 ± 0.33c,d	3.73 ± 0.0c,d	4.51 ± 0.34b,c	12.8 ± 1.1b	103.09 ± 2.9a,b	4.2 ± 0.8a,b
Consortium	n.d.	1.8 ± 0.07c	0.79 ± 0.0d	0.37 ± 0.02b,c,d		10.2 ± 0.1a	6.02 ± 0.05b,c,d	3.63 ± 0.09b,c	3.82 ± 1.19b,c	16.74 ± 0.6c	99. 5 ± 3a,b	5.16 ± 1.3a,b
TdV												
AF	1.28 ± 0.06b	n.d.	0.78 ± 0.02d	0.24 ± 0.04a	183 ± 2a	10.5 ± 0.1a	5.97 ± 0.03b,c,d	3.55 ± 0.0b	3 ± 0.44a,b	8.84 ± 0.07a	101.4 ± 4.3a,b	5.96 ± 0.7b,c
PSU-1	n.d.	2.04 ± 0.13d	0.32 ± 0.01b	0.39 ± 0.02b,c,d		10.3 ± 0.3a	6.71 ± 0.18d	3.76 ± 0.01d	3.14 ± 0.16a,b	17.45 ± 0.53c,d	99.52 ± 3.3a,b	7.38 ± 0.52c
Consortium	n.d.	1.73 ± 0.01c	0.66 ± 0.01c,d	0.38 ± 0.02b,c,d		10.5 ± 0.2a	6.63 ± 0.02c,d	3.61 ± 0.08b,c	4.66 ± 0.65b,c	19.53 ± 1.76d	119.3 ± 4.2b	5.75 ± 0.06a,b,c

Values shown are the means of triplicates ± SD; lower case letters indicate a significant difference at $P \leq 0.05$ according to a Tukey post-hoc comparison test; n.d., not detected. Σ MCFA, medium chain fatty acids; Σ SCFA, short-chain fatty acids; AF, alcoholic fermentation; Consortium, contains *O. oeni* strains CH11, AiB9, MCS5 and M25; PSU-1, *O. oeni* PSU-1 strain; Sc, wines fermented with *Saccharomyces cerevisiae*; TdB, wines fermented with *Torulaspora delbrueckii* Biodiva and *S. cerevisiae*; TdV, wines fermented with *T. delbrueckii* Viniferm and *S. cerevisiae*.

the case of the MLF carried out by the consortium (Table 2). The consumption of citric acid by *O. oeni* is reported in the literature as strain specific and generally occurs when L-malic acid is depleted (Bartowsky and Henschke 2004). It appears that under the conditions studied the *O. oeni* strains from the consortium exhibited a low metabolism of citric acid (Table 2), even though the fermentation duration was much longer (Table 1).

Acetic acid is a volatile compound that can be produced by LAB as a result of citric acid or other sugar consumption (Davis et al. 1986). Similar values were observed after AF with a slight increase after MLF, mainly in the Sc wine with *O. oeni* PSU-1 (Table 2). Acetic acid is just one possible compound produced as a consequence of citric acid consumption (Bartowsky and Henschke 2004). Thus, those fermentations that exhibited a higher consumption of citric acid—as observed when inoculating *O. oeni* PSU-1—could contribute to an increase in acetic acid concentration.

Succinic acid, which is a competitive inhibitor of L-malic acid for the active site of the malolactic enzyme (Lonvaud-Funel and Strasser de Saad 1982), can be regarded as a potential inhibitor of MLF in wine. Previous studies have associated the use of *T. delbrueckii* with a decrease in succinic acid concentration (Martín-García et al. 2020, Balmaseda et al. 2021a). In concordance with those studies, in this experiment we observed a significant decrease in the sequential inoculations with *T. delbrueckii* (Table 2). Nevertheless, the concentration of succinic acid detected is still quite low to have a direct impact on MLF performance.

Glycerol was increased by the use of *T. delbrueckii* in the wines after AF (Table 2). Indeed, this yeast is usually associated with a higher production of glycerol than *S. cerevisiae* under oenological conditions (González-Royo et al. 2015, Belda et al. 2016). Also, a small but significant increase in this compound was observed after MLF in the Sc and TdB wines. To the best of our knowledge there is no published study that has found an increase in glycerol after MLF. Nevertheless, the higher concentration of this compound should correspond to its release from the yeast lees because no direct relation with *O. oeni* is known. Thus, we can suggest from these results that fermenting over yeast lees may contribute to an increase in glycerol concentration in some cases.

The pH was also dependent on the AF inoculation strategy (Table 2). The *T. delbrueckii* wines had significantly higher values at the end of AF than those observed in the *S. cerevisiae* wine as found in previous studies (Martín-García et al. 2020). This can be related to a better MLF performance, because acid pH is one of the most well-known inhibitor factors for *O. oeni* in wine. As expected, the pH value increased after MLF in all wines. The pH was higher in those wines inoculated with PSU-1 than in the consortium-fermented wines. This could be related to a higher amount of citric acid in these wines at the end of MLF (Table 2).

The volatile composition of the different wines was quite similar (Table 2). Some changes, however, were observed, for instance the increase in short-chain fatty acids (SCFA) after MLF in the Sc and TdB wines. Some slight differences were noted at the end of AF, when the wine TdB had the lowest SCFA concentration. In all cases the higher amount of SCFA quantified was a result of the accumulation of isobutyric acid (Table S1).

The concentration of medium chain fatty acids (MCFAs) was significantly reduced by the use of *T. delbrueckii* after AF (Table 2). Some authors have already reported a decrease in this family of compounds when using *T. delbrueckii*. In this study we detected hexanoic (C6), octanoic (C8) and decanoic (C10) acids. Hexanoic acid was not detected at the end of AF in the *T. delbrueckii* wines. This acid was responsible for the reduction in total MCFA composition (Table S1) in the TdB and TdV wines. Interestingly, after MLF the concentration of MCFA was similar in the *S. cerevisiae* wines and had increased in the *T. delbrueckii* wines. The concentration after MLF in *T. delbrueckii*, however, was never as high as that detected in *S. cerevisiae* (Table 2).

The fusel alcohol concentration was significantly higher in the TdB wines than in the *S. cerevisiae* wines after AF (Table 2). No significant differences, however, were observed after MLF with the exception of the TdV consortium wine, that showed a slight increase. A higher concentration of the fusel alcohol acetates was detected in TdV PSU-1 wine after MLF compared to the other wines (Table 2). The production of fusel alcohol acetates is highly dependent on the enzymatic capacities of the fermenting strains (Ugliano and Moio 2005) and is not regulated by substrate availability (fusel alcohols). After MLF, any increase in the concentration of this family of compounds may depend on the *O. oeni* strain (Ugliano and Moio 2005). As we detected different *O. oeni* strains by the end of MLF (Figure 3), we can associate higher enzymatic capacity with the dominance of the M25 strain in this particular medium.

Figure 4 shows the changes in a selection of volatile compounds in connection with MLF. Ethyl lactate is one of the most abundant volatile compounds produced during MLF. Its production has been linked to the inoculated *O. oeni* strain (Malherbe et al. 2012). Our results showed a similar ethyl lactate production in the Sc and TdB wines, which had similar *O. oeni* strain compositions (Figure 3). Nevertheless, the TdV associated with different fermenting strains at the end of MLF had a higher concentration in the consortium-fermented wines, while a slight reduction was observed in the PSU wine. Thus, we can relate the M25 strain to higher production of this compound.

The production of 2,3-butanediol in the consortium-fermented wines was much higher than in the wines inoculated with PSU-1. This compound may be related to the citric acid metabolism by *O. oeni* (Bartowsky and Henschke 2004). Also, wines in contact with yeast lees are associated with a higher concentration of butanediol (del Fresno et al. 2019). As mentioned earlier, in our study yeast viability was maintained throughout the fermentation process. As a result, the metabolically active lees could have increased the 2,3-butanediol concentration in consortium-fermented wines. In addition, a significantly higher 2,3-butanediol concentration was detected in the *T. delbrueckii* wines inoculated after AF, which corresponded to longer contact with the yeast lees (16 and 17 days) compared to the Sc wine (10 days).

The hexyl acetate concentration was significantly higher in the consortium-fermented wines than in the wines inoculated with PSU-1 (Figure 4). The production of this compound is usually related to the specific characteristics of the fermenting strain (Malherbe et al. 2012). We observed that the wines inoculated after AF with *O. oeni* PSU-1 had similar but lower values than those fermented with the consortium, which corresponded to another *O. oeni* population. The concentration of 2-phenylethyl acetate usually remains constant after MLF (Pozo-Bayón et al. 2005, Malherbe et al. 2012). Nevertheless, some authors have observed an increase after MLF (Ugliano and Moio 2005). Few differences were observed for this compound, with the exception of one wine. In the TdV wine inoculated with PSU-1 the

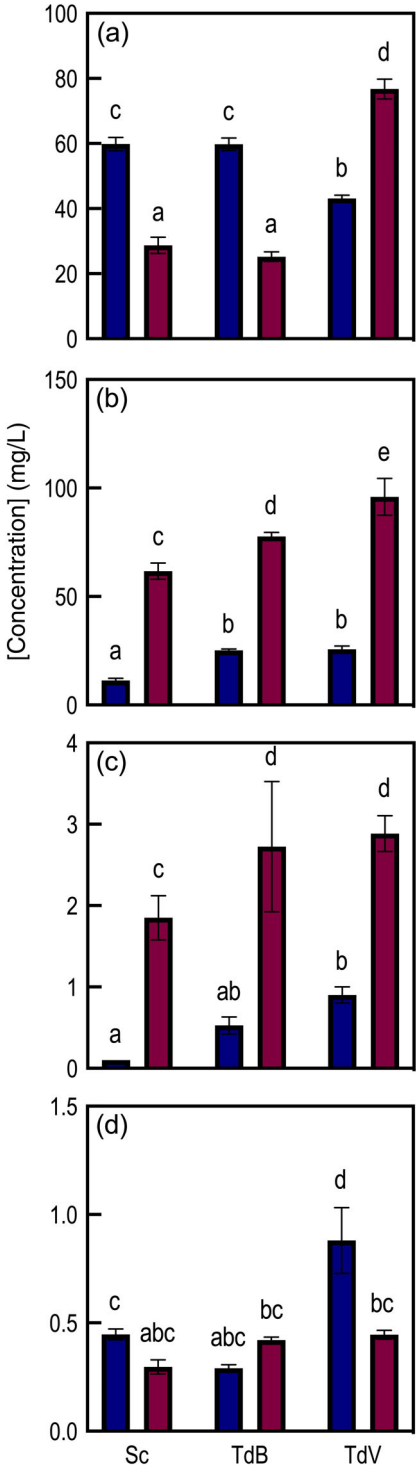

Figure 4. Concentration of (a) ethyl lactate, (b) 2,3-butanediol, (c) hexyl acetate and (d) 2-phenylethylacetate associated with malolactic fermentation with significant differences. Wines were inoculated with PSU-1 (■) after the alcoholic fermentation and were fermented with the initial consortium bacterial population (■). Values shown are the mean of triplicates ± SD. Sc, *Saccharomyces cerevisiae*; TdB, *Torulaspora delbrueckii* Biodiva and *S. cerevisiae*; and TdV, *T. delbrueckii* Viniferm and *S. cerevisiae* fermented wines.

concentration of this compound was significantly higher than in the other wines (Figure 4), reaching twice the concentration.

Conclusions

We have studied the effect of an inoculation strategy involving the use of *T. delbrueckii* during AF on a defined community of *O. oeni* strains. Under these controlled conditions we observed a direct impact on the *O. oeni* community by the inoculated yeasts. The dynamics of the *O. oeni* strain populations were significantly modified by one of the *T. delbrueckii* strains used, this being Viniferm. The other strain, Biodiva, did not modify the evolution of the *O. oeni* strains when compared to wines inoculated only with S. *cerevisiae*. Even when an effect on the population was noted, little impact was observed as to the duration of the MLF. As the community changed, the quantified wine parameters were also modulated. These data were compared to wines inoculated with a selected *O. oeni* strain at the end of AF. The volatile composition of wines fermented with the initial consortium was more complex and the aromas associated with MLF, such as 2,3-butanediol and hexyl acetate, were enhanced. Overall, these results present new data that highlight the impact of the inoculated yeast on the *O. oeni* population, which can be modulated through the use of non-conventional yeasts.

Acknowledgements

This work was supported by grants AGL2015-70378-R and PGC2018-101852-B-I00 (MCI/AEI/FEDER,UE). Aitor Balmaseda is grateful to the Catalan Government for the predoctoral fellowship (2018FI_B 00501).

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Manuscript received: 14 June 2021
Revised manuscript received: 3 August 2021
Accepted: 19 August 2021

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website: http://onlinelibrary.wiley.com/doi/10.1111/ajgw.12526/abstract.

Table S1. Composition of volatile compounds in the wines fermented with combinations of *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Oenococcus oeni*.