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Simulated lees of different yeast species modify the performance of malolactic fermentation by *Oenococcus oeni* in wine-like medium

Aitor Balmaseda [a], Nicolas Rozès [b], Albert Bordons [a], Cristina Reguant [a,*]

[a] Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Enològica, C/ Marcel·lí Domingo 1, 43007 Tarragona, Catalonia, Spain
[b] Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Grup de Biotecnologia Microbiana dels Aliments, C/ Marcel·lí Domingo 1, 43007 Tarragona, Catalonia, Spain

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ABSTRACT

The use of non-*Saccharomyces* yeast together with *S. cerevisiae* in winemaking is a current trend. Apart from the organoleptic modulation of the wine, the composition of the resulting yeast lees is different and may thus impact malolactic fermentation (MLF). Yeasts of *Saccharomyces cerevisiae, Torulaspora delbrueckii* and *Metschnikowia pulcherrima* were inactivated and added to a synthetic wine. Three different strains of *Oenococcus oeni* were inoculated and MLF was monitored. Non-*Saccharomyces* lees, especially from some strains of *T. delbrueckii*, showed higher compatibility with some *O. oeni* strains, with a shorter MLF and a maintained bacterial cell viability. The supplementation of lees increased nitrogen compounds available by *O. oeni*. A lower mannoprotein consumption was related with longer MLF. Amino acid assimilation by *O. oeni* was strain specific. There may be many other compounds regulating these yeast lees-*O. oeni* interactions apart from the well-known mannoproteins and amino acids. This is the first study of MLF with different *O. oeni* strains in the presence of *S. cerevisiae* and non-*Saccharomyces* yeast lees to report a strain-specific interaction between them.

1. Introduction

Malolactic fermentation (MLF) is a biotransformation that occurs in fermented beverages like wine and cider (Davis et al., 1985). This metabolism is a survival adaptation of lactic acid bacteria (LAB) under the stress conditions present in those media, such as low nutrient availability, low pH and high ethanol content. MLF is the decarboxylation of L-malic acid in L-lactic acid with a small increase in pH. It is desirable in those white wines with high acidity and for all red wines in general. Of all the LAB species present in wine, *Oenococcus oeni* is the most important since it is the one that best adapts to the conditions found in wine (Lonvaud-Funel 1999).

In a spontaneous winemaking process, oenological yeasts through alcoholic fermentation (AF) first ferment the sugars of the grape must. There is a high diversity of yeast species at the beginning of the AF. When the concentration of sugar starts to decrease producing ethanol, that diverse yeast group of non-*Saccharomyces* is rapidly replaced by *S. cerevisiae* (Beltran et al., 2002). As a result of AF, the grape must becomes a poor nutrient medium with low pH and a high concentration of ethanol. Consequently, the yeast-*O. oeni* compatibility is a key factor for successful MLF since this fermentation typically takes places after the AF (Balmaseda et al., 2018). Traditionally, *S. cerevisiae* has been inoculated to ensure a controlled AF, but nowadays there is increasing interest in the possible advantages of using selected non-*Saccharomyces* strains together with *S. cerevisiae* (Padilla et al., 2016). This opens up a new scenario of metabolic activities and chemical modulation in the wines produced, also modifying the media for the subsequent MLF (Balmaseda et al., 2021).

During AF, LAB from the grape surface or cellar equipment are present in a very low population (González-Arenzana et al., 2012). As time goes by, the yeast lees remaining in the wine begin to lose viability and undergo an autolysis process due to the low metabolic activity and high ethanol concentration. Under these conditions the yeasts lyse and release their intracellular content into the wine (Martínez-Rodriguez et al., 2001), promoting LAB growth (Reguant et al., 2005).

The main changes generally attributed to the lysis of yeasts are the increases in mannoproteins and nitrogen compounds, which are commonly related to a stimulation of MLF performance (Guilloux-Benatier et al., 1995; Alexandre et al., 2004; Balmaseda et al., 2018). These released macromolecules can be hydrolysed (Manca De Nadra et al.,

* Corresponding author.
E-mail address: cristina.reguant@urv.cat (C. Reguant).

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1999; Folio et al., 2008) by *O. oeni* and assimilated as a nitrogen source, stimulating its metabolism. However, the presence of higher concentrations of yeast extracts is not always linked to a higher protease activity by *O. oeni* (Remize et al., 2006).

The increase in released compounds will depend on the yeast strain. Indeed, some non-*Saccharomyces* such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* are reported to increase mannoprotein concentrations in wine (González-Royo et al., 2015; Ferrando et al., 2020), especially in ageing (Belda et al., 2016). In addition, there are other compounds released during the autolysis process that may have a negative rather than stimulatory effect (Patynowski et al., 2002; Herrero et al., 2003). However, there is little knowledge about the effect of yeast lees on MLF.

In this work we aim to evaluate the MLF performance of some selected *O. oeni* strains in the presence of yeast lees of different species. In particular we consider those compounds in relation to the stimulation of MLF under yeast lees in a defined synthetic wine and the L-malic acid consumption rate and viability of *O. oeni*.

2. Materials and methods

2.1. Experimental fermentations

Fermentations were performed in 250 mL flasks containing 250 mL of sterile wine-like medium (WLM) static at 20 °C. The WLM was prepared following Bordas et al. (2015), with 12% ethanol (v/v), 2 g/L of L-malic acid and pH 3.4, but with half nitrogen composition than in Bordas et al. (2015): 1.25 g/L of Bacto™ casamino acids (BD, France) and 1.25 g/L of peptone (Panreac, Química SLU, Castellar del Vallès, Spain). Yeasts for supplementation of WLM in the form of simulated yeast lees were grown in sterile concentrated must (65.4° Brix; Mostos Españoles S.A., Tomelloso, Spain) diluted to a concentration of 200 g/L of glucose and fructose (Martín-García et al., 2020). Seven yeasts belonging to the species *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* of different origins were used (Table 1). After 2 weeks' incubation, the yeast population was counted in a Neubauer chamber. An appropriate volume of the fermenting must was centrifuged (8500 rpm, 10') to achieve a final concentration of 10^7 CFU/mL in 2 L of WLM, which corresponded to an average biomass of 1.9 ± 0.39 mg/mL (wet weight) in the synthetic medium. The resulting pellet was resuspended in 50 mL of WLM. At this point the collected yeast biomass was inactivated by heating in three cycles of 1 min at 90 °C with 1 min in ice bath between each. The cells were then disrupted using a One Shot disruptor (Constant Systems Ltd., United Kingdom) at 5 °C, applying 2.5 kbar pressure (Margalef-Català et al., 2016). The aim of the disruption process was to simulate the yeast cell status in the final stages of AF, when some of them are already lysed but others are still intact with low viability ($<10^3$ CFU/mL). YPD agar medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 17 g/L agar, Panreac) was used to calculate the total number of viable yeast cells after incubation at 28 °C for 48 h.

Each WLM condition (250 mL), including the yeast lees, was then inoculated with one of the three *O. oeni* strains (Table 1) for a population of around 2×10^7 CFU/mL. All the strains were pre-cultured in MRSmf broth at 28 °C for three days (Margalef-Català et al., 2017) before inoculation in each WLM. These fermentations were carried out in triplicate at 20 °C. Samples were taken every 24 h to monitor the evolution of L-malic acid consumption and the bacterial population. Samples were plated on MRSmf (Margalef-Català et al., 2017) and incubated at 27 °C in a 10% CO_2 atmosphere for 7 days. MLF was considered to have finished when the L-malic acid was below 0.1 g/L.

Table 1
Microorganisms used in this work.

Abbreviation	Species	Strain name	Source
ScQA23	*S. cerevisiae*	Lalvin-QA23	Lallemand S.L.
Sc3D	*S. cerevisiae*	Viniferm-3D	Agrovin S.A.
TdBiodiva	*T. delbrueckii*	Biodiva	Lallemand S.L.
TdViniferm	*T. delbrueckii*	Viniferm NS-TD	Agrovin S.A.
TdTDP	*T. delbrueckii*	CECT 13135	BE-URV[a]
MpFlavia	*M. pulcherrima*	Flavia	Lallemand S.L.
MpMPP	*M. pulcherrima*	CECT 13131	BE-URV[a]
PSU-1	*O. oeni*	ATCC BAA-331	ATCC[c]
CH11	*O. oeni*	Viniflora-CH11	Chr. Hansen S.L.
1Pw13	*O. oeni*	CECT 8893	BE-URV[b]

BE-URV: Biotecnologia Enològica research group at the Universitat *Rovira i Virgili*, Tarragona, Catalonia, Spain.

[a] From Padilla et al., 2016.

[b] From Franquès et al., 2017.

[c] ATCC: American Type Culture Collection.

2.2. Synthetic wines characterisation

The synthetic wines were characterised after supplementation with the yeast lees (initial: t_0 MLF) and after MLF completion of each *O. oeni* strain. Samples were centrifugated (8500 rpm, 10′) and kept frozen at −20 °C until analysis. pH was measured before freezing (Crison micropH 2002, Hach Lange, L'Hospitalet, Spain) and various compounds (acetic acid, citric acid, L-lactic acid, L-malic acid, succinic acid and glucose + fructose) were analysed using a Miura One multianalyser (TDI SL, Gavà, Spain).

The total soluble protein concentration was determined by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) (Sigma150 Aldrich, St Louis, MO, USA) and dye reagents (Bio-Rad, Hercules, CA, USA) for the calibration curve.

The mannoprotein content of the WLM before and after MLF was quantified using a D-mannose and D-glucose assay kit K-MANGL (Megazyme, Wicklow, Ireland). Briefly, 25 mL of 95% (v/v) ethanol was added to 5 mL of each sample, vortexed and precipitated overnight at 4 °C. Each pellet obtained was washed twice with 10 mL of 95% ethanol and centrifugated (4500 rpm, 10′). The pellets were then transferred to 2 mL tubes and dried at 30 °C for 30′ in vacuum (Concentrator Plus, Eppendorf, Hamburg, Germany). Afterwards they were resuspended in 1 mL of 5 M H_2SO_4, incubated at 90 °C for 1h and neutralised with 1 mL of 10 M NaOH. Finally, the sample was centrifugated (8500 rpm, 5′) and the supernatant kept for analysis. The free sugar (D-glucose, D-fructose and D-mannose) content was then determined in accordance with the manufacturer's instructions.

2.3. Amino acid composition analysis

The amino acid and ammonium content was analysed by HPLC following the method described by Gómez-Alonso et al. (2007). 400 μL of sample was filtered through 0.22 μm syringe filter into a vial. Afterwards, 700 μL of 1M borate buffer at pH 9 (adjusted with NaOH 10N), 300 μL of methanol, 10 μL of internal standard (L-2-aminoadipic acid, 1 g/L) and 15 μL of DEEM (diethyl ethoxymethylenemalonate, Fluka, Germany) were added. The vial was encapsulated and vortexed for 30 s. The samples were then derivatised for 2 h at 80 °C. After that, 50 μL of each sample was directly injected into the HPLC. The HPLC (Agilent 1100, Agilent Technologies, Germany) was equipped with a DAD ultraviolet (Agilent Technologies, Germany), and separation was performed on a Hypersil ODS C18 column (Agilent Technologies, Germany) with a particle size of 5 μm (250 mm × 4.6 mm) and thermostated at 20 °C. The mobile phase (Buffer A) consisted of 2.05 g/L of sodium acetate anhydrous and 0.2 g/L of sodium azide with MilliQ water (Millipore Q-PODTM Advantage A10) adjusted to pH 5.8 with glacial acetic acid, while the mobile phase (Buffer B) consisted of 80% (v/v) acetonitrile and 20% (v/v) methanol (Panreac, Spain). The concentration of each amino acid was calculated with an external standard curve and the signal of each sample was normalised with the area of the

internal standard. The amino acid and ammonium content was transformed into yeast assimilable nitrogen (YAN, expressed as mg N/L) depending on the proportion of nitrogen atoms of each amino acid.

2.4. Statistical analysis

The statistical software used was XLSTAT version 2020.2.3 (Addinsoft, Paris, France). The data obtained underwent a two-way ANOVA (yeast lees and *O. oeni* strain as qualitative variables) with a subsequent analysis using the Tukey test, with a confidence interval of 95% and significant results with a p-value ≤ 0.05. Optimisation indexes (OI) for MLF performance were calculated based on Borrull et al. (2016) for each *O. oeni* strain in the 8 synthetic wines. The variables used for this calculation were MLF duration, MLF rate, pH, mannose content, ammonium content and total amino acid content. Values were normalised using the highest value for each parameter as (x/reference value). In the case of MLF duration, where the highest value represents the worst performance, the calculation was 1-(x/reference value). Principal component analysis (PCA) was performed with the obtained OI using the same statistical software.

3. Results and discussion

3.1. Fermentations

The duration of MLF in the control condition (WLM) was the same with *O. oeni* 1Pw13 and CH11 (11 days) whereas with *O. oeni* PSU-1 it was 4 days shorter (Table 2, Suppl. Fig. 1). The addition of yeast lees influenced MLF duration depending on the yeast strain used to obtain the lees and the *O. oeni* strain inoculated. In most of the fermentations with PSU-1 and 1Pw13 strains slight delays were observed in MLF with respect to the control condition (WLM). However, the addition of Sc3D lees with PSU-1 and 1Pw13, and QA23 lees with 1Pw13, caused a remarkable slowdown in MLF. Treatments with WLM-Td Biodiva were the only ones showing a shorter MLF with PSU-1 and 1Pw13 with respect to the control WLM. Instead, CH11 showed a completely different behaviour compared to the other *O. oeni* strains. Most of the fermentations of CH11 with lees were shorter than the control condition, except in WLM-TdTDP, that showed no statistical differences (Table 2, Suppl. Fig. 1). The performance in WLM-Sc3D clearly illustrates the differences among *O. oeni* strains. In WLM-Sc3D, the MLF with PSU-1 was stuck at around 0.3 g/L of L-malic acid and with 1Pw13 MLF took 30 days. Meanwhile, CH11 showed the fastest MLF among all conditions (4.3 days) with Sc3D lees (Table 2, Suppl. Fig. 1). Globally, *O. oeni* 1Pw13 was the slowest fermenting strain of the three, with the exception of its performance in WLM-TdBiodiva and WLM-TdTDP, whereas the most positively affected strain by supplementation with yeast lees was *O. oeni* CH11.

Regarding the yeast strains, the strongest negative impact on MLF duration observed was associated to *S. cerevisiae* lees addition, mainly with Sc3D, whereas treatments with *T. delbrueckii* Biodiva lees were the only ones causing a shortening of MLF with respect to the control condition in the three *O. oeni* strains studied, being significant in two of them.

The differences in MLF duration correlated well, in most of the cases, with the differences observed in L-malic acid consumption rate, being CH11 the strain showing the highest rates and 1Pw13 the strain with the lowest rates (Table 2). *O. oeni* PSU-1 showed, in general, intermediate L-malic acid consumption rates when compared to the other two strains.

As mentioned earlier, the yeast lees viability was low, at around 500 CFU/mL, and decreased during MLF to less than 100 CFU/mL in some cases (data not shown). Presumably, therefore, there was no inhibition due to viable yeast metabolic activity.

The presence of yeast lees during MLF can modulate MLF performance. The yeast metabolites released during autolysis can have a stimulatory or a negative effect on MLF depending on the *O. oeni* fermenting strain (Patynowski et al., 2002; Herrero et al., 2003). Patynowski et al. (2012) reported a longer lag phase of *O. oeni* growth in wines with longer contact with yeast lees. Also, supplementation with commercial yeast extracts in the MLF of cider is described as being possibly more stimulatory to *O. oeni* than supplementation with recovered yeast lees (Herrero et al., 2003). In the present work we confirm that the effect of yeast lees on MLF performance strongly depends on the fermenting *O. oeni* strain and on the yeast lees strain.

The different patterns observed in these MLF performances could be related to the specific nutritional requirements of each *O. oeni* strain and the compatibility between each yeast-*O. oeni* strain couple.

3.2. Viability

The changes observed in *O. oeni* viability at the end of MLF were variable depending on the added lees and the inoculated strain (Fig. 1). *O. oeni* PSU-1 lost around one logarithmic unit in most of the synthetic wines, with the exception of the control WLM and WLM-TdViniferm, where the viability was maintained. In WLM-Sc3D, *O. oeni* PSU-1 suffered a drastic decrease in viability that led to the unfinished MLF. On the other hand, a slight decrease was observed in viability of strains 1Pw13 and CH11 in most of the conditions. These two strains also showed some exceptions in which the viability was maintained or slightly increased: WLM-TdViniferm and WLM-TdTDP, for 1Pw13, and WLM-TdBiodiva and WLM-MpMPP, for CH11.

The observed variation in viability was not directly related to MLF duration or consumption rates (Table 2), with the exception of *O. oeni* PSU-1 in the WLM-Sc3D wine. In general, the bacterial population at the

Table 2
Malolactic fermentation (MLF) duration and the consumption rate of the three *O. oeni* strains (PSU-1, 1Pw13 and CH11) in the different wine-like media (WLM) with yeast lees. Values shown are the mean of triplicates ± SD. The duration of *O. oeni* PSU-1 in WLM-Sc3D was excluded from the analysis as that MLF did not finish.

	Duration (days)			Consumption rate (L-malic acid g/L·d)[a]		
	PSU-1	1Pw13	CH11	PSU-1	1Pw13	CH11
WLM	7 ± 0.0^{c}	11 ± 0.0^{e}	11 ± 0.0^{a}	0.35 ± 0.01^{deC}	0.24 ± 0.00^{bA}	0.30 ± 0.01^{eB}
WLM-ScQA23	6.5 ± 0.5^{cdA}	17.5 ± 0.5^{bC}	8.7 ± 0.6^{bB}	0.49 ± 0.00^{bC}	0.14 ± 0.00^{deA}	0.40 ± 0.01^{dB}
WLM-Sc3D	–	30 ± 0.0^{aB}	4.3 ± 0.6^{dA}	0.16 ± 0.00^{gB}	0.12 ± 0.01^{eA}	0.62 ± 0.06^{aC}
WLM-TdBiodiva	5.7 ± 0.6^{dA}	8 ± 0.0^{fB}	10.8 ± 0.3^{aC}	0.52 ± 0.02^{aB}	0.29 ± 0.01^{aA}	0.55 ± 0.00^{bcB}
WLM-TdViniferm	9 ± 0.0^{b}	12 ± 0.0^{d}	5 ± 0.0^{cd}	0.44 ± 0.01^{cB}	0.2 ± 0.01^{cA}	0.49 ± 0.01^{cC}
WLM-TdTDP	8.7 ± 0.6^{bA}	11.3 ± 0.6^{deB}	11.7 ± 0.6^{aB}	0.27 ± 0.00^{fB}	0.15 ± 0.00^{dA}	0.39 ± 0.01^{dC}
WLM-MpFlavia	11 ± 0.0^{aB}	12.8 ± 0.3^{cC}	8.7 ± 0.6^{bA}	0.34 ± 0.01^{eB}	0.19 ± 0.01^{cA}	0.35 ± 0.03^{deB}
WLM-MpMPP	9 ± 0.0^{bB}	12 ± 0.0^{dC}	5.7 ± 0.6^{cA}	0.36 ± 0.02^{dB}	0.14 ± 0.02^{deA}	0.57 ± 0.00^{abC}

[a–g] Values are significantly at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences among the values of the same *O. oeni* strain in the different WLM. Uppercase letters correspond to differences between values of the three strains in the same synthetic wine after MLF. The absence of uppercase letter in the duration of some synthetic wines is due to the lack of SD.

[a] Consumption rate of L-malic acid was calculated considering the period of exponential decrease of this acid.

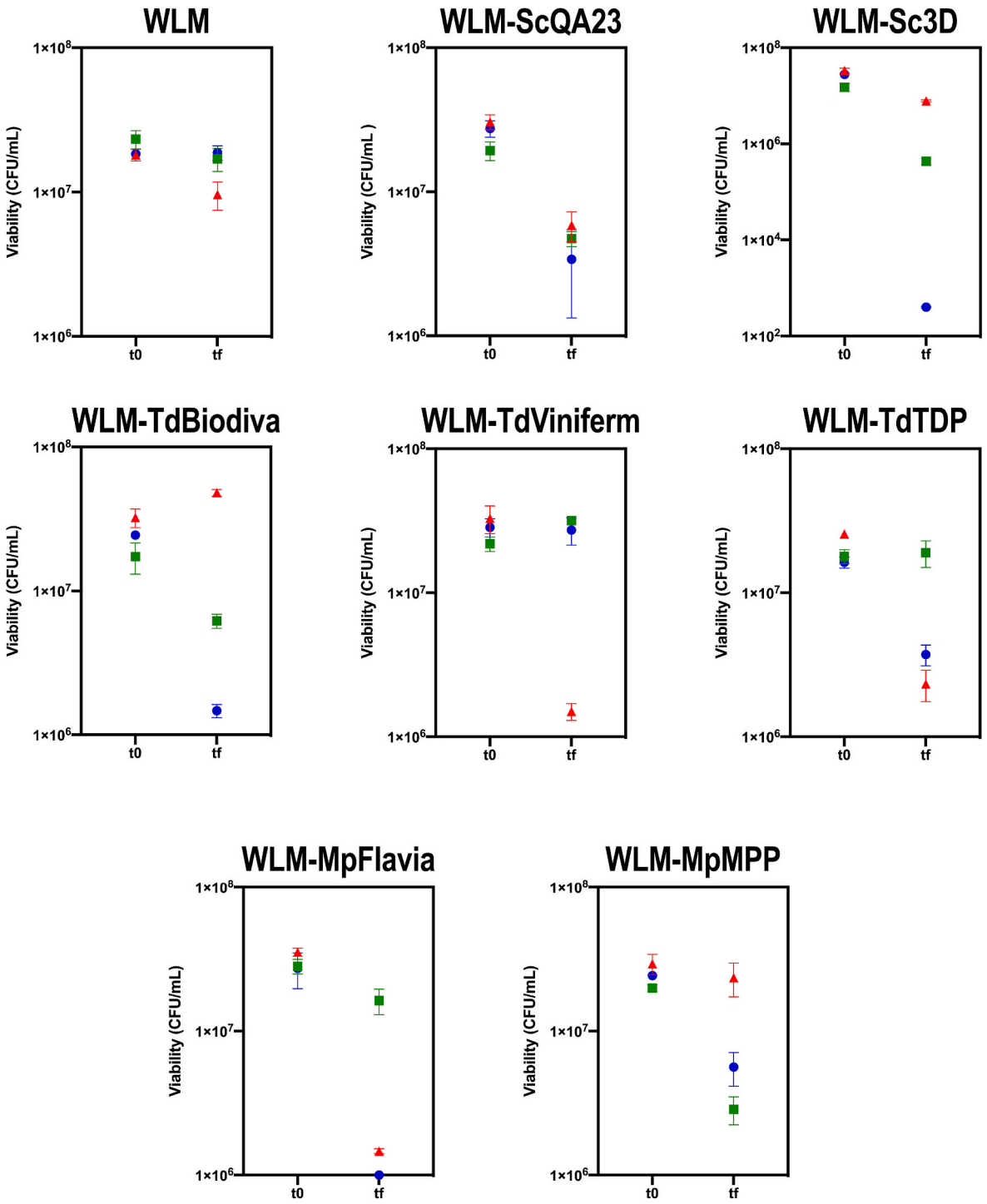

Fig. 1. Viability of the three studied *O. oeni* strains in the different wine-like media (WLM) at the beginning of MLF (t_0) and after MLF, when [L-malic acid] < 0.1 g/L (t_f). (●) *O. oeni* PSU-1, (■) *O. oeni* 1Pw13, (▲) *O. oeni* CH11.

end of MLF was enough (Reguant et al., 2005), i.e. higher than 10^6 CFU/mL, to ensure MLF performance (Table 2, Fig. 1). Consequently, the effect of yeast lees on MLF, excluding Sc3D, may be associated with an inhibition of MLF capacity and not with a loss of viability.

3.3. Chemical parameters

The supplementation with yeast lees changed significantly some parameters of WLM (Table 3). Some significant changes were observed in pH, such as in WLM-TdBiodiva and WLM-TdTDP, which increased by 0.18 and 0.22 pH units, respectively, compared to control. As the wine-like conditions represent a highly acidic environment in which *O. oeni* has to grow, any increase in that value may improve membrane integrity and cell survival (Tourdot-Maréchal et al., 2000). Higher initial pH values may be one of the stimulating factors regarding the duration of MLF in the case of WLM-TdBiodiva, but not the only one, since this is not observed with all strains of *O. oeni*. As a result of the increased initial pH value, the values of those synthetic wines after MLF were the highest (Table 3).

In most of the cases, sugars were not consumed by *O. oeni* during

Table 3
Oenological parameters of wine-like media (WLM) before *O. oeni* inoculation (Initial, after yeast lees addition in supplemented WLM) and after malolactic fermentation (MLF) of the three *O. oeni* strains (PSU-1, 1Pw13 and CH11). Values shown are the means of triplicates ± SD.

		Glucose + fructose (g/L)	Citric acid (g/L)	Acetic acid (g/L)	pH	Proteins (mg/mL)	Amino acids (mg N/L)	NH_4 (mg/L)
WLM	Initial	2.12 ± 0.00^{a}	0.48 ± 0.00^{a}	0.20 ± 0.00^{b}	3.40 ± 0.00^{e}	0.28 ± 0.00^{e}	123.70 ± 0.00^{f}	8.73 ± 0.00^{de}
	PSU-1	1.99 ± 0.07^{aA}	0.20 ± 0.01^{aB}	0.31 ± 0.01^{bcA}	3.69 ± 0.02^{cA}	0.21 ± 0.01^{cA}	117.86 ± 8.64^{bcA}	4.45 ± 1.11^{bA}
	1Pw13	2.01 ± 0.02^{aA}	0.29 ± 0.00^{abA}	0.28 ± 0.01^{abB}	3.65 ± 0.01^{dA}	0.24 ± 0.02^{abA}	117.13 ± 6.04^{bcA}	5.75 ± 0.08^{bcA}
	CH11	1.99 ± 0.02^{bcA}	0.20 ± 0.03^{abB}	0.33 ± 0.01^{bcA}	3.65 ± 0.04^{dA}	0.23 ± 0.01^{cA}	116.07 ± 3.95^{cA}	4.92 ± 0.25^{cA}
WLM-ScQA23	Initial	2.11 ± 0.00^{a}	0.44 ± 0.00^{b}	0.20 ± 0.00^{b}	3.35 ± 0.00^{g}	0.26 ± 0.00^{f}	142.39 ± 0.00^{c}	8.04 ± 0.00^{e}
	PSU-1	1.98 ± 0.05^{aA}	0.10 ± 0.03^{dB}	0.32 ± 0.02^{bA}	3.60 ± 0.02^{dA}	0.26 ± 0.01^{bcA}	107.65 ± 15.36^{bcAB}	4.33 ± 0.09^{bB}
	1Pw13	n.d.^{cB}	0.18 ± 0.03^{cA}	0.25 ± 0.05^{bA}	3.55 ± 0.01^{eB}	0.24 ± 0.04^{abA}	87.71 ± 3.11^{eB}	0.52 ± 0.17^{dC}
	CH11	2.00 ± 0.02^{bcA}	0.16 ± 0.01^{abAB}	0.32 ± 0.01^{bcdA}	3.60 ± 0.01^{eA}	0.22 ± 0.02^{cA}	118.05 ± 6.91^{cA}	6.14 ± 0.11^{cA}
WLM-Sc3D	Initial	2.00 ± 0.00^{d}	0.48 ± 0.00^{ab}	0.20 ± 0.00^{b}	3.45 ± 0.00^{c}	0.34 ± 0.01^{a}	142.88 ± 0.00^{c}	9.02 ± 0.00^{cd}
	PSU-1	0.10 ± 0.04^{cB}	0.17 ± 0.01^{abc}	0.27 ± 0.01^{dB}	3.62 ± 0.02^{dC}	0.26 ± 0.02^{bcA}	98.74 ± 1.58^{cAB}	n.d.^{cC}
	1Pw13	n.d.^{cB}	0.18 ± 0.04^{cA}	0.28 ± 0.01^{abB}	3.67 ± 0.01^{cdB}	0.23 ± 0.01^{bB}	104.56 ± 4.89^{cdA}	1.34 ± 0.25^{dB}
	CH11	1.96 ± 0.04^{cAA}	0.08 ± 0.00^{cB}	0.37 ± 0.01^{aA}	3.76 ± 0.01^{bA}	0.24 ± 0.01^{bcAB}	93.95 ± 2.70^{dB}	4.59 ± 0.30^{cA}
WLM-TdBiodiva	Initial	2.00 ± 0.00^{d}	0.48 ± 0.00^{ab}	0.21 ± 0.00^{b}	3.58 ± 0.00^{b}	0.29 ± 0.00^{d}	152.62 ± 0.00^{b}	10.00 ± 0.00^{bc}
	PSU-1	2.11 ± 0.02^{aA}	0.18 ± 0.01^{abC}	0.31 ± 0.01^{bcA}	3.92 ± 0.02^{bA}	0.38 ± 0.07^{aA}	118.76 ± 5.53^{bcA}	5.92 ± 0.68^{abAB}
	1Pw13	2.00 ± 0.04^{aB}	0.30 ± 0.00^{aA}	0.27 ± 0.02^{abB}	3.87 ± 0.01^{bB}	0.30 ± 0.06^{abA}	114.68 ± 8.90^{bcA}	4.59 ± 0.47^{cB}
	CH11	2.06 ± 0.02^{abAB}	0.2 ± 0.01^{aB}	0.3 ± 0.01^{cdeA}	3.9 ± 0.01^{aAB}	0.32 ± 0.01^{aA}	122.2 ± 6.28^{bcA}	7.67 ± 1.26^{cA}
WLM-TdViniferm	Initial	2.09 ± 0.00^{b}	0.50 ± 0.00^{a}	0.21 ± 0.00^{b}	3.42 ± 0.00^{d}	0.30 ± 0.02^{c}	134.39 ± 0.00^{d}	8.87 ± 0.00^{de}
	PSU-1	2.05 ± 0.03^{aA}	0.13 ± 0.00^{cdC}	0.30 ± 0.02^{bcdA}	3.71 ± 0.01^{cA}	0.31 ± 0.05^{abA}	126.01 ± 4.59^{bB}	7.20 ± 1.04^{aB}
	1Pw13	2.03 ± 0.03^{aAB}	0.24 ± 0.01^{bA}	0.25 ± 0.00^{bB}	3.68 ± 0.00^{cB}	0.33 ± 0.04^{aA}	129.62 ± 2.55^{bAB}	10.75 ± 0.18^{aA}
	CH11	1.96 ± 0.02^{cB}	0.19 ± 0.02^{abB}	0.27 ± 0.02^{eAB}	3.71 ± 0.01^{cA}	0.30 ± 0.02^{aA}	135.91 ± 1.45^{abA}	11.78 ± 0.42^{bA}
WLM-TdTDP	Initial	2.09 ± 0.00^{b}	0.50 ± 0.00^{a}	0.21 ± 0.00^{b}	3.62 ± 0.00^{a}	0.26 ± 0.01^{f}	124.45 ± 0.00^{e}	33.82 ± 0.00^{a}
	PSU-1	2.08 ± 0.07^{aA}	0.15 ± 0.00^{bcC}	0.31 ± 0.01^{bcA}	3.98 ± 0.02^{aA}	0.29 ± 0.03^{abcA}	153.59 ± 12.06^{aA}	n.d.^{cB}
	1Pw13	2.05 ± 0.07^{aA}	0.24 ± 0.01^{bA}	0.27 ± 0.01^{abB}	3.94 ± 0.01^{aB}	0.28 ± 0.00^{abA}	156.66 ± 8.71^{aA}	n.d.^{dB}
	CH11	2.07 ± 0.05^{abA}	0.19 ± 0.01^{abB}	0.29 ± 0.02^{deAB}	3.92 ± 0.02^{aB}	0.31 ± 0.03^{aA}	152.3 ± 7.42^{aA}	26.28 ± 3.13^{aA}
WLM-MpFlavia	Initial	2.09 ± 0.00^{b}	0.48 ± 0.00^{ab}	0.21 ± 0.00^{b}	3.33 ± 0.00^{h}	0.32 ± 0.01^{b}	162.22 ± 0.00^{a}	10.26 ± 0.00^{b}
	PSU-1	0.76 ± 0.47^{bB}	0.20 ± 0.03^{aAB}	0.28 ± 0.01^{cdB}	3.54 ± 0.01^{eB}	0.26 ± 0.03^{bcA}	95.92 ± 3.12^{cB}	n.d.^{cB}
	1Pw13	0.88 ± 0.54^{bB}	0.25 ± 0.00^{abA}	0.27 ± 0.01^{abB}	3.55 ± 0.01^{eB}	0.26 ± 0.02^{abA}	93.07 ± 4.01^{deB}	0.81 ± 0.45^{dB}
	CH11	1.96 ± 0.05^{cA}	0.16 ± 0.03^{bB}	0.32 ± 0.01^{bcdA}	3.59 ± 0.01^{eA}	0.28 ± 0.02^{abA}	117.89 ± 8.88^{cA}	7.88 ± 1.31^{cA}
WLM-MpMPP	Initial	2.06 ± 0.00^{c}	0.45 ± 0.00^{b}	0.23 ± 0.00^{a}	3.38 ± 0.00^{f}	0.28 ± 0.00^{e}	122 ± 0.00^{g}	33 ± 0.00^{a}
	PSU-1	2.06 ± 0.01^{aA}	0.16 ± 0.01^{abcB}	0.37 ± 0.01^{aA}	3.62 ± 0.00^{dA}	0.27 ± 0.02^{bcA}	125.49 ± 4.17^{bA}	6.73 ± 0.02^{aA}
	1Pw13	2.06 ± 0.01^{aA}	0.29 ± 0.02^{abA}	0.31 ± 0.02^{aB}	3.68 ± 0.00^{cA}	0.26 ± 0.03^{abA}	114.37 ± 5.89^{bcA}	6.32 ± 1.19^{bA}
	CH11	2.14 ± 0.05^{aA}	0.2 ± 0.01^{abB}	0.35 ± 0.01^{abA}	3.68 ± 0.00^{cdA}	0.28 ± 0.01^{abA}	112.85 ± 6.65^{cA}	5.67 ± 1.01^{cA}

[a–h] Values are significantly different at $p \leq 0.05$ according to a Tukey HSD post-hoc comparison. Lowercase letters correspond to differences among the values of the same *O. oeni* strain in the different WLM. Lowercase letters also apply to the comparison among the values of the initial WLM. Uppercase letters correspond to differences between values of the three strains in the same WLM after MLF.
n.d.: not detected.

MLF. However, a significant decrease in sugars was observed at the end of MLF with PSU-1 and 1Pw13 in WLM-Sc3D and WLM-MpFlavia, and also with 1Pw13 in WLM-ScQA23 (Table 3). No increase in acetic acid was observed in all these assays (Table 3), which could be linked to sugar consumption by *O. oeni*. Moreover, these MLF were slower than the rest of the fermentations for these two *O. oeni* strains. Altogether, it may be possible that the remaining viable yeast in the lees were responsible for sugar consumption in those MLF that *O. oeni* had a weak malolactic activity.

Under stress conditions, *O. oeni* can consume citric acid (Davis et al., 1986). In this study, the initial citric acid was consumed approximately between 40 and 80% at the end of the MLF depending on the fermentation (Table 3). In general, the *O. oeni* 1Pw13 was the lowest consumer of this organic acid. WLM-Sc3D was the only synthetic wine in which all three strains consumed more citric acid (less than 0.2 g/L was quantified after MLF). In all cases, acetic acid increased after MLF as a consequence of citric acid consumption (Table 3). In addition, succinic acid, a possible MLF inhibitor (Balmaseda et al., 2018), was also analysed but not detected in the synthetic wines (data not shown).

As for the substrate and product of the MLF, the concentration of each was homogeneous in all the synthetic wines with the exception of those stuck fermentations in which remaining traces of L-malic acid were detected (Suppl. Fig. 1) and less L-lactic acid was quantified (data not shown).

One of the metabolisms of interest in the fermentations tested was the assimilation of mannoproteins. As a result of yeast lees supplementation, increased concentrations of mannose (mannoproteins) were quantified in the initial WLM (Fig. 2). Some studies have reported a higher mannoprotein concentration in aged wines with non-*Saccharomyces* yeast as regards *S. cerevisiae*, specially *T. delbrueckii* and *M. pulcherrima* (González-Royo et al., 2015; Belda et al., 2016; Benito 2018; Benito et al., 2019). In this study, the highest concentrations of mannoproteins were observed in two of the three tested *T. delbrueckii,* Biodiva and Viniferm, whereas *M. pulcherrima* strains showed lower concentrations than *S. cerevisiae* strains (Fig. 2). Surprisingly, although *S. cerevisiae* Viniferm-3D is described as an overproducer of mannoproteins (Belda et al., 2016), under our fermentation conditions it only produced 80 mg eq. mannose/L.

Under oenological conditions *O. oeni* can hydrolyse mannoproteins, and the released products (Jamal et al., 2013), e.g. mannose, can be assimilated as a carbon source by the bacterium (Cibrario et al., 2016). The use of mannose depended on the *O. oeni* strain and the medium it was fermenting (Fig. 2). In most of the cases, *O. oeni* PSU-1 and CH11 showed high consumption of this monosaccharide whereas *O. oeni* 1Pw13 showed lower assimilation of mannose. Indeed, no consumption of mannose was observed when *O. oeni* 1Pw13 fermented in WLM-MpMPP. In contrast, all three strains consumed nearly all the mannose content of WLM-Sc3D.

It seems that mannoprotein consumption may be strain specific with complex regulation (Cibrario et al., 2016), since each strain behaves quite differently in the different wines and not always proportionally to the mannoprotein concentration (Remize et al., 2005). In view of our results, we could not relate a higher consumption of mannoproteins to a quicker MLF. Nevertheless, we did observe that *O. oeni* 1Pw13, which

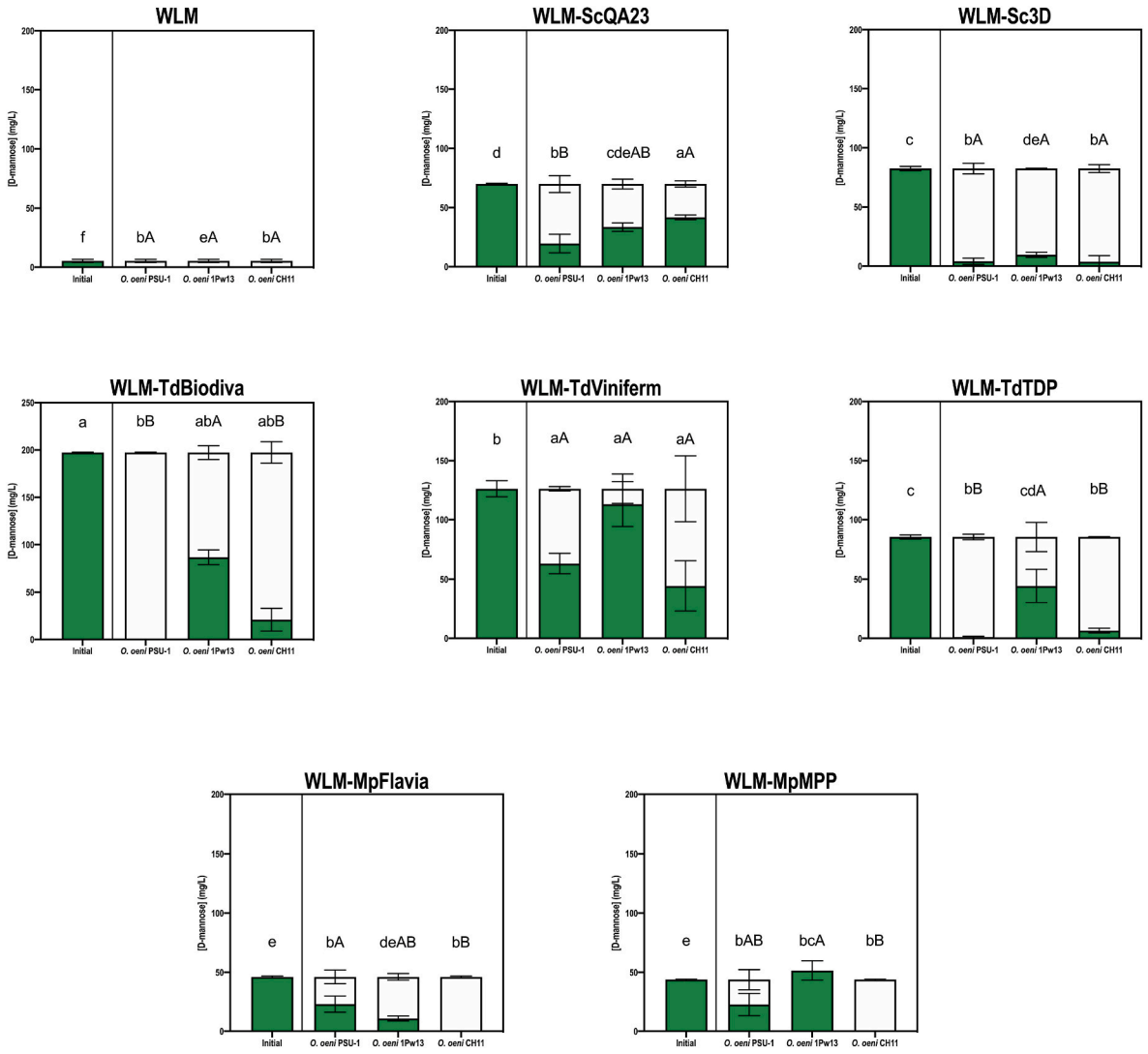

Fig. 2. D-mannose concentration detected in wine-like media (WLM) at the beginning of MLF (Initial) and after MLF for *O. oeni* PSU-1, 1Pw13 and CH11. Histograms reflect the consumption of D-mannose during MLF by *O. oeni* as (■) detected D-mannose and (□) consumed D-mannose as the difference between the initial concentration and the concentration detected after MLF. Values shown are the means of triplicates ± SD. Lowercase letters correspond to differences among the values of the same *O. oeni* strain in the different WLM. Lowercase letters also apply to the comparison among the values of the initial synthetic wines. Uppercase letters correspond to differences between values of the three strains in the same WLM after MLF.

presented the lowest mannoprotein consumption, had the worst MLF performance of the strains tested. Moreover, the highest initial concentration of mannoproteins, as well as the highest mannose consumption, was detected in WLM-TdBiodiva, where the MLF were faster for all the *O. oeni* strains with respect to the control (WLM). Considering altogether, the ability of mannoproteins utilization may play a role in the stress response of *O. oeni* in oenological conditions.

3.4. Protein and amino acid content

Supplementation with yeast lees can increase the soluble proteins in WLM due to the autolytic process (Martínez-Rodriguez et al., 2001). Four out of the seven synthetic wines with added lees showed a significant increase of protein concentration (Table 3), the highest being those of *S. cerevisiae* 3D, *T. delbrueckii* Viniferm and *M. pulcherrima* Flavia.

During MLF, protein concentrations can increase as a consequence of the progress of yeast autolysis or decrease as *O. oeni* hydrolyses proteins to smaller peptides or amino acids (Manca De Nadra et al., 1999; Folio et al., 2008). Consequently, the variation in the quantification of proteins (Table 3) and amino acids (Table 3, Fig. 3) is the sum of (i) the protein release from autolytic yeast lees, except for the control WLM, (ii) the hydrolysis of proteins and release of amino acids, and (iii) the assimilation of amino acids by *O. oeni*. In the synthetic wines with *S. cerevisiae* and *M. pulcherrima* lees, the protein concentration decreased slightly during MLF, as well as in the control WLM. However, a mild increase in protein concentration was observed in the synthetic wines with *T. delbrueckii* lees at the end of MLF (Table 3). In general, these changes were the same whatever the *O. oeni* strain inoculated.

The addition of yeast lees increased the amino acid concentration in most of the cases, with the exception of WLM-MpMPP (Table 3, Fig. 3A). After MLF, there was a decrease of the amino acid concentration with the exception of WLM-TdTDP, in which the amino acid concentration increased (Table 3). We could not correlate the higher amino acid release in WLM-TdTDP during MLF to the decrease in protein concentration. A lower consumption of amino acids by *O. oeni* in these fermentations, or a higher peptidase activity, may have been the cause of the higher concentration of free amino acids with respect to the rest of the conditions.

The increase in YAN concentration before MLF ranged in mean value from 8.5% (WLM-TdViniferm) to 30% (WLM-MpFlavia). In most of the

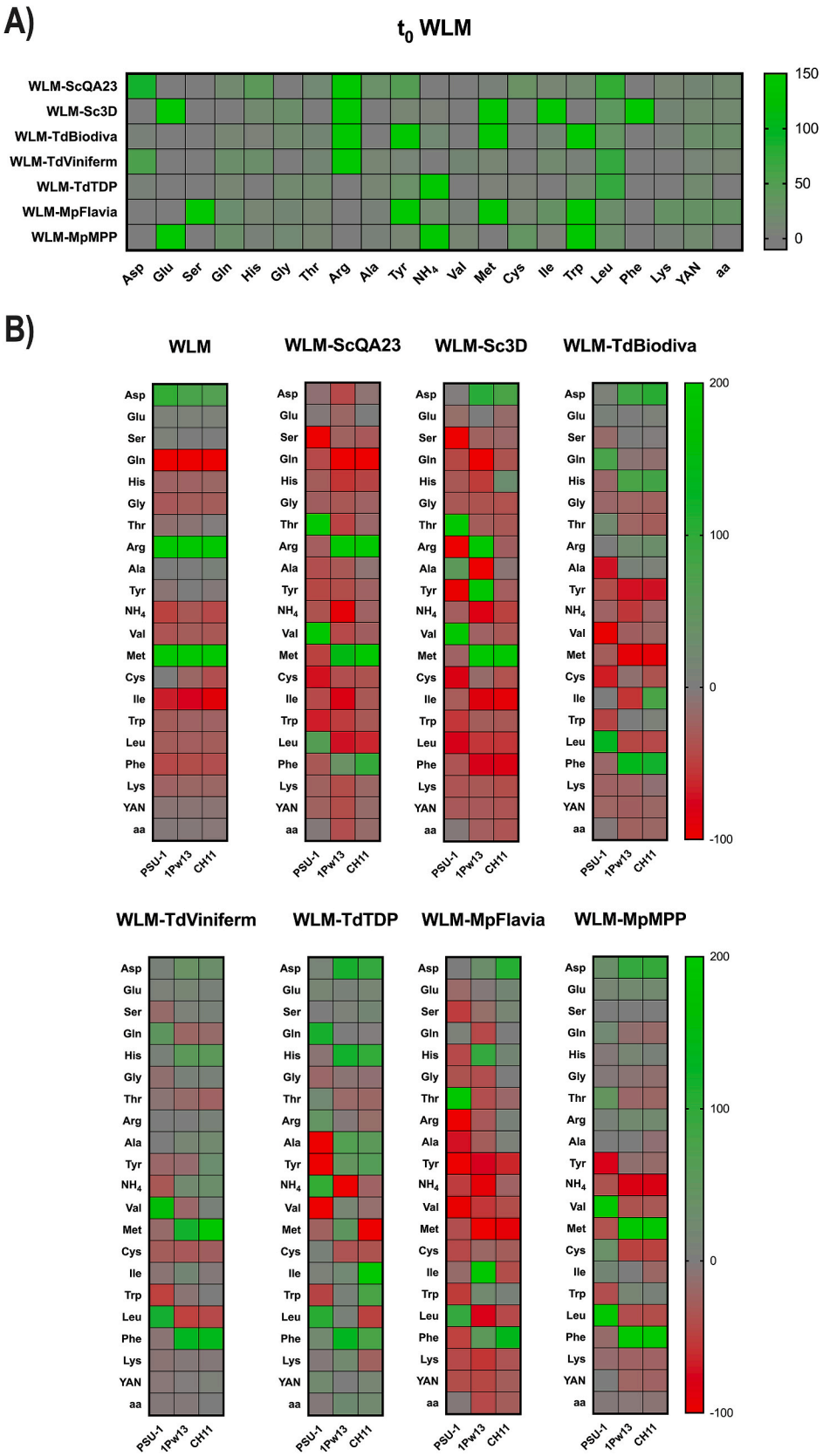

Fig. 3. Amino acid analysis of the wine-like media (WLM). A) Enrichment of amino acids in each WLM supplemented with the yeast lees expressed as a percentage of the increase at the beginning (t_0) of malolactic fermentation (MLF). B) Variation in amino acid concentration as a result of MLF represented as a percentage of the increase (green) or decrease (red). No variation (black) is used as the baseline. YAN and total amino acid are also represented in these figures (A and B).

cases, the increase was due to certain amino acids and not to an increase in ammonium concentration. Arginine and tryptophan were the most increased amino acids (Fig. 3A). Nevertheless, the TdTDP and MpMPP strains significantly increased the ammonium concentration with respect to the control condition without yeast lees addition (Table 3).

As far as the variation in YAN composition during MLF is concerned, some compounds decreased as *O. oeni* assimilated them, while others increased (Fig. 3B). As observed in protein concentration, it is difficult to assess the assimilation patterns of each amino acid since the extracellular quantification is the sum of the assimilation of amino acids and the hydrolysis of macromolecules. There is no general agreement on amino acid metabolism for *O. oeni* during MLF. According to the available literature, its patterns are strain specific and, as observed in the present study (Fig. 3), also depend on the media. However, some amino acids such as asparagine and histidine seem to undergo less change after MLF (Pozo-Bayón et al., 2005).

In the control condition (WLM), all three *O. oeni* strains had similar patterns of amino acid metabolism. The most consumed amino acids were glutamine and isoleucine, whereas asparagine, arginine and methionine increased their concentrations (Fig. 3B). These latter amino acids were probably released due to protease activity and not assimilated by *O. oeni* while the other released products were incorporated. The preference of amino acids in *O. oeni* has not been as widely studied as it has been in wine yeasts. Nevertheless, the consumed glutamine and usually the isoleucine are classified as good nitrogen sources for yeast (Roca-Mesa et al., 2020), and this could explain their consumption in the control WLM. As regards the supplemented WLMs, the correlation is not very clear as a consequence of the protease activity of *O. oeni*. Overall, the WLM with the two *S. cerevisiae* strains and *M. pulcherrima* Flavia presented higher amino acid incorporation since their extracellular concentration decreased. In contrast, WLM-TdViniferm generally showed an increase in all the analysed compounds.

3.5. Optimisation indexes for MLF performance

To better determine the suitability of the yeast lees for each *O. oeni* strain, the optimisation indexes (OI) were calculated. This allowed us to integrate the yeast lees' contribution to the chemical composition of the synthetic wines with the output of *O. oeni* in the MLF performance (Fig. 4). In general, the OI increased in WLMs supplemented with yeast lees with respect to the control WLM. This behaviour was noticeable in *O. oeni* 1Pw13 and CH11 (Fig. 4A). Meanwhile the variation as regards the *O. oeni* PSU-1 OI presented higher heterogeneity depending on the yeast lees. The OI only decreased in WLM-Sc3D for this *O. oeni* strain. Also, little variation was observed in the *M. pulcherrima* supplemented WLMs. The greatest increases in OI for all the *O. oeni* strains were obtained with the *T. delbrueckii* yeast lees, remarkably for Biodiva strain (Fig. 4A). According to the PCA of the OIs (Fig. 4B), the parameters of MLF performance (MLF duration and MLF rate) are related to higher concentrations of mannoproteins (mannose eq.), where the OIs of *T. delbrueckii* Biodiva and Viniferm for all the *O. oeni* strains are plotted. The opposite side of the PCA (Fig. 4B) is where the OIs with the lowest values are plotted, relating to *S. cerevisiae, M. pulcherrima* and the control WLM.

4. Conclusions

The present study on MLF performance in the presence of simulated yeast lees in synthetic medium has shown that MLF may be positively or negatively affected by the presence of yeast lees and that this is strongly dependent on the *O. oeni* strain used. The highly heterogenous behaviour observed shows complex compatibility patterns between the yeast lees and *O. oeni*, as it occurs with wines fermented by different yeasts. The duration of MLF can be modified in the presence of yeast lees. In this study, the best MLF performance was observed in fermentations supplemented with one *T. delbrueckii* strain. This could be related to more favourable conditions for MLF associated to the addition of this yeast lees, such as a higher pH and a higher mannoprotein concentrations. In this regard, mannoprotein concentrations were increased with the addition of *T. delbrueckii* lees with respect to *S. cerevisiae* lees. In some cases, the consumption of mannoproteins could be related to a better malolactic performance. The protein and amino acid metabolism of each *O. oeni* strain comes about in response to the particular characteristics of the wine. In conclusion, further research is needed to understand yeast-bacteria strain compatibility, which is key to propose oenological practices to improve MLF performance.

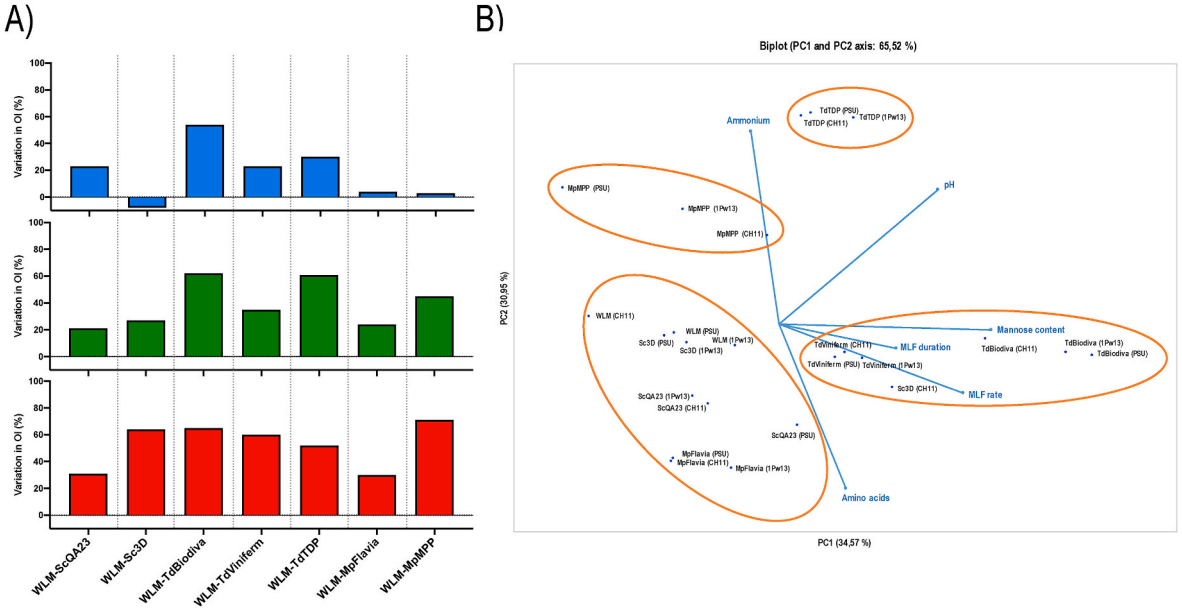

Fig. 4. Optimisation indexes. A) Percentage variation in the optimisation index (OI) with respect to the control WLM for the three *O. oeni* strains: PSU-1 (blue), 1Pw13 (green) and CH11 (red). B) Principal component analysis (PCA) biplots of varimax rotated PCA of OI for each *O. oeni* strain in the different WLM on which observations and variables are plotted.

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Declaration of competing of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2021.103839.

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