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Comparative study of inoculation strategies of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on the performance of alcoholic and malolactic fermentations in an optimized synthetic grape must

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ABSTRACT

Progress in oenological biotechnology now makes it possible to control alcoholic (AF) and malolactic (MLF) fermentation processes for the production of wines. Key factors in controlling these processes and enhancing wine quality include the use of selected strains of non-*Saccharomyces* species, *Saccharomyces cerevisiae*, and *Oenococcus oeni*, as well as the method of inoculation (co-inoculation or sequential) and the timing of inoculation.

In the present work, we investigated the effects of different inoculation strategies of two *Torulaspora delbrueckii* (Td-V and Td-P) strains followed by *S. cerevisiae*. Times (two, four, and six days) and types (co-inoculation and sequential) of inoculation were evaluated on the AF of a synthetic grape must. Furthermore, this synthetic medium was optimized by adding linoleic acid and β-sitosterol to simulate the natural grape must and facilitate reproducible results in potential assays. Subsequently, the wines obtained were inoculated with two strains of *Oenococcus oeni* to carry out MLF. Parameters after AF were analysed to observe the impact of wine composition on the MLF performance. The results showed that the optimization of the must through the addition of linoleic acid and β-sitosterol significantly enhanced MLF performance. This suggests that these lipids can positively impact the metabolism of *O. oeni*, leading to improved MLF efficiency. Furthermore, we observed that a 4-day contact period with *T. delbrueckii* leads to the most efficient MLF process and contributed to the modification of certain AF metabolites, such as the reduction of ethanol and acetic acid, as well as an increase in available nitrogen. The combination of Td-P with Oo-VP41 for 4 or 6 days during MLF showed that it could be the optimal option in terms of efficiency. By evaluating different *T. delbrueckii* inoculation strategies, optimizing the synthetic medium and studying the effects on wine composition, we aimed to gain insights into the relationship between AF conditions and subsequent MLF performance. Through this study, we aim to provide valuable insights for winemakers and researchers in the field of wine production and will contribute to a better understanding of the complex interactions between these species in the fermentation process.

1. Introduction

The use of different yeast species in wine alcoholic fermentation (AF) has been an interesting subject of study in recent years (Beltran et al., 2002; Bordet et al., 2020; Englezos et al., 2022). In the past, it was common to inoculate only *Saccharomyces cerevisiae* into the fermentation process. However, in recent years, the use of non-*Saccharomyces* starter cultures and spontaneous fermentation has become more widespread (Bordet et al., 2020; Fazio et al., 2023; Jolly et al., 2014; Roudil et al., 2019). Currently, there are different species, or mixtures of species, available as commercial active dry yeast (ADY) that are used in the wine industry, e.g., *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans* or *Pichia kluyveri* (Fazio et al., 2023; Roudil et al., 2019; Viana et al., 2008; Vicente et al., 2021). Their use is an excellent biotechnological solution for better process control and a more stable product apart from other organoleptic improvements

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(Benito, 2018; Roudil et al., 2019).

Regarding *T. delbrueckii*, it has been demonstrated that, among other benefits, its use improves aromatic complexity (Belda et al., 2017; Carpena et al., 2021; Minnaar et al., 2015), reduces acetic acid production (Bely et al., 2008; Canonico et al., 2019) and improves the red wine colour (Balmaseda et al., 2021a; Du Plessis et al., 2017a, 2017b; Minnaar et al., 2018). However, this effect depends on the grape variety used (Minnaar et al., 2015) found that *T. delbrueckii* can decrease phenolic compounds in Pinotage wine but decrease in Cabernet franc wines. Furthermore, researchers have been dedicating more attention to understanding the compatibility of non-*Saccharomyces* with malolactic fermentation (MLF) (Du Plessis et al., 2017a, 2017b; Russo et al., 2020; Yılmaz and Gökmen, 2021). Especially, the species *T. delbrueckii* has shown positive impacts enhancing the efficiency and effectiveness of the process (Balmaseda et al., 2021a, 2022; Ferrando et al., 2020). MLF has a great impact on wine characteristics, mainly in overall acidity but also in flavour and aroma, due to the decarboxylation of L-malic acid into L-lactic acid by lactic acid bacteria (LAB), with *Oenococcus oeni* being the main bacteria responsible for this process in winemaking (Arnink and Henick-Kling, 2005; Lonvaud-Funel, 1999).

Yeast-bacteria synergy is strain-dependent (Benito, 2018); that is, different strains of each species may interact differently and produce different metabolites (Ruiz-de-Villa et al., 2023). In addition, the timing of inoculation of non-*Saccharomyces* into the must before the inoculation of *S. cerevisiae* can influence the outcome of the fermentation process (Hranilovic et al., 2020; Snyder et al., 2021; Zhao et al., 2022). During AF, non-*Saccharomyces* species are typically present in the early stages, while *S. cerevisiae* dominates and completes the fermentation (Ribéreau-Gayon et al., 2006). Industrially, non-*Saccharomyces* can be inoculated as co-inoculation or sequential inoculation with *S. cerevisiae*. Consequently, these strategies can have different effects on the final wine and the subsequent behaviour of *O. oeni* during MLF (Martín-García et al., 2020; Zhao et al., 2022). In order to investigate the microbiological interactions effectively, it is necessary to utilize reproducible media that allow for the analysis of fermentation behaviours under controlled conditions, without relying on the complex composition of natural grape must. However, the composition of designed synthetic grape musts sometimes does not lead to successful MLF outcomes in the corresponding obtained wines. Linoleic acid and β-sitosterol are two of the main lipids found in grape skin, between polyunsaturated fatty acid and phytosterols, respectively (Guittin et al., 2021; Le Fur et al., 1994). Therefore, it has been proposed to improve previously developed synthetic grape must by incorporating these lipids, with the aim of improving MLF assays.

The aim of this work was, firstly, to optimize a synthetic grape must by incorporating linoleic acid and β-sitosterol to achieve reproducible conditions for other possible tests in this context. Secondly, we evaluated the effect of various inoculation strategies and the timing involving different strains of *T. delbrueckii* and *S. cerevisiae* on the outcome of the AF products. Lastly, the third objective was to provide a comprehensive understanding of how wine composition, resulting from different AF conditions, affects the progression and efficiency of MLF with two *O. oeni* strains. According to these three objectives, we sought to study the influence of this optimized grape must on AF and MLF performance.

2. Materials and methods

2.1. Microorganisms and media

The yeast strains used in this study were a commercial *Saccharomyces cerevisiae* Lalvin-QA23 (Sc-QA) from Lallemand Inc. (Montreal, Canada) and two *Torulaspora delbrueckii* strains: Viniflora Prelude (Chr. Hansen Holding AS, Hoersholm, Denmark) (Td-P) and NSA1 Viniferm NSDT (Agrovin, Spain) (Td-V). Two strains of *Oenococcus oeni* were used: VP41 from Lallemand Inc. (Montreal, Canada) and CH11 from Christian Hansen A/S (Hørsholm, Denmark). These strains were chosen considering the results obtained in a previous work by Ruiz-de-Villa et al. (2023), which aimed to study the interactions between different strains of *S. cerevisiae*, *T. delbrueckii* and *O. oeni*. In this way, the strains that showed the best results in different parameters, such as AF and MLF kinetics, were selected for this study.

Total yeast population kinetics and inocula were monitored by counting viable cells from YPD plates containing 10 g/L yeast extract (Panreac, Barcelona, Spain), 20 g/L peptone (Panreac), 20 g/L glucose (Panreac) and 20 g/L agar (Panreac). Similarly, the viability of *T. delbrueckii* was determined on a lysine medium (lysine agar 6.6 % (w/v), 2 mL/L potassium lactate 10 % (v/v) and 5 mL/L lactic acid (v/v)) (Sigma-Aldrich, Barcelona, Spain).

The *O. oeni* strains were precultured in an MRS broth medium (De Man et al., 1960) (Difco Laboratories, Detroit, MI, USA) supplemented with 4 g/L DL-malic acid (Sigma–Aldrich) and 5 g/L D-fructose (Panreac) at a pH of 5. The inocula were incubated at 28 °C in a CO_2 (10 %) incubator. The same conditions and counting medium (MRS plates) were used to evaluate the *O. oeni* populations.

2.2. Alcoholic fermentation

Two grape musts were used to perform AF; the first one (Sc-Lip-) was described by Ruiz-de-Villa et al. (2023), and the second one (Sc-Lip+) had the same composition but 10 mg/L β-sitosterol (ref 85451, Sigma–Aldrich) and 200 mg/L linoleic acid (ref 62240, Sigma–Aldrich) was added.

Before the inoculation of the synthetic grape must, the yeast strains were rehydrated from ADY according to the manufacturer's indications: 37 °C for 30 min for the *S. cerevisiae* and 30 °C for 30 min for the *T. delbrueckii* strains. The initial population was 2.5×10^6 cells/mL for both yeast species.

AF was carried out in triplicate with the synthetic must without lipids (Sc-Lip-) and in the presence of lipids (Sc-Lip+, chosen as a control throughout the study) in 500 mL bottles containing 450 mL of the must at 22 °C with agitation at 120 rpm in an Innova 42 incubator shaker (New Brunswick Scientific, Madrid, Spain). The closures allowed carbon dioxide to escape and sampling. AF kinetics were monitored by measuring density with an electronic densimeter (Densito 30PX, Mettler-Toledo, Barcelona, Spain). In addition, the population dynamics of the total yeast and *T. delbrueckii* were controlled with YPD and a lysine media, respectively. The AF was considered finished when residual sugars (glucose + fructose) were under 2 g/L.

Four inoculation strategies were tried: a co-inoculation at the same time point of *S. cerevisiae* and *T. delbrueckii* and three sequential inoculations with different time points of *T. delbrueckii* contact followed by the inoculation of *S. cerevisiae*: 2, 4 and 6 days. The different inoculation strategies were tested with both *T. delbrueckii* strains.

2.3. Malolactic fermentation

When AF was finalized, the wines were stabilized for 7 days at 4 °C. Then, they were centrifuged at 1500 ×*g* for 20 min at 4 °C and filtered with 0.22 μm filters (Merck, Darmstadt, Germany). To avoid biological differences, the replicates were mixed and then divided into tubes with 50 mL of wine by triplicate during static fermentation at 20 °C. The MLFs were inoculated with one of the two *O. oeni* strains described previously at a population of 2×10^7 cells/mL. The wines were not supplemented with L-malic acid before MLF. The process was monitored by measuring the consumption of L-malic acid enzymatically every day using the Y15 analyser (Biosystems S.A., Barcelona, Spain). The MLFs were considered finished when the L-malic acid concentration was <0.1 mg/L. Viable populations were determined by plating serial dilutions in an MRS media.

2.4. Calculation of the area under the curve (AUC)

The measurement of the area under the curve (AUC), the decrease in AF density or L-malic consumption in the MLF, was used as an indicator of fermentation performance which allowed us to overcome the total times and fermentation kinetics. The AUCs were calculated by integrating either the decreasing density during the AF or the decreasing L-malic acid consumption during the MLF between two consecutive times. The calculation formula is the sum of consecutive AUCs $= \Sigma\ [((d_2 + d_1) / 2) * (t_2 - t_1) + \ldots + ((d_n + d_{n-1}) / 2) * (t_n - t_{n-1})]$ where d_1, d_2, … d_{n-1}, d_n are the densities of the grape must at times 1, 2, n − 1 and n, respectively.

2.5. Chemical analysis

2.5.1. General oenological metabolites

The general parameters of the wines were analysed after the AF. Glycerol, ethanol, and citric acid were determined with high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) (Zhu et al., 2020). Sample pre-treatment consisted of filtering the samples with 0.22 μm pore filters (Agilent Technologies). The column used was a Hi-Plex H (300 mm × 7.7 mm) column inside a 1260 MCT (Infinity II Multicolumn Thermostat). The mobile phase consisted of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The HPLC was equipped with two detectors, an MWC detector (G1365B multiwavelength detector) and a RID detector (1260 Infinity II refractive index detector, Agilent Technologies). Glycerol, ethanol and citric acid concentrations were calculated from external calibration curves of known standards.

Ammonia, α-amino nitrogen (PAN), acetic acid and residual sugars were quantified with Biosystem's enzymatic kits. While succinic acid (Megazyme, Wicklow, Ireland) was analysed by an enzymatic kit using a microplate reader (POLARstar Omega, BMG LABTECH, Ortenberg, Germany).

The extraction of mannose to analyse the number of mannoproteins was performed as described by (Balmaseda et al., 2021c). Quantification was performed with a D-mannose and D-glucose enzymatic assay kit (Megazyme).

2.5.2. Volatile compound analysis

The volatile compounds of the wines obtained after alcoholic fermentation were analysed by liquid/liquid extraction with a methyl tert-butyl ether/hexane mixture (1/1). Briefly, 50 μL of H_3PO_4 (1/3) and 25 μL of internal standards (octanol-3, 1.98 g/L; heptanoic acid, 3.33 g/L and heptadecanoic acid, 1.03 g/L) were added to 5 mL of wine. To extract the volatile compounds, 400 μL of MTBE/hexane was added, and the mixture was stirred for 2 min by vortexing. After that, the wines were centrifuged at 5200 ×*g* for 5 min at room temperature. The organic extract was placed in an insert placed inside the vial to then injected into a GC-FID chromatograph. The chromatographic conditions were as follows: injection volume, 2 μL; injection mode: spitless; inlet and detector temperatures, 250 °C; column: HP-FFAP (30 m × 250 μm 0.25 μm, Agilent). The concentrations of the volatile compounds were calculated from external calibration curves of known standards. The volatile compounds determined were fusel alcohols acetates (isobutyl acetate, isoamyl acetate, 2-phenylethaol acetate), fusel alcohols (FA: isoamyl alcohol, hexanol, *cis*3-hexenol, 2-phenylethanol), ethyl esters of fatty acids (ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl dodecanoate), short-chain fatty acids (SCFA: propanoic, butanoic (butyric acid) and pentanoic (valeric acid)), medium-chain fatty acids (MCFA: hexanoic, octanoic, decanoic acids), and long-chain fatty acids (LCFA: tetradecanoic (myristic acid), hexadecanoic (palmitic acid), octadecanoic (stearic acid), and 9,12-octadecadienoic acid (linoleic acid)).

2.6. Statistical analysis

The fermentation was performed by triplicate biological samples. The statistical software used was XLSTAT version 2021.5.1 (Addinsoft, Paris, France). The data were analysed with two-way ANOVA with a post hoc Tukey test (Honesty Significant Difference) with a confidence interval of 95 % and significant results with a p-value < 0.05. Principal component analysis was also performed to describe the effects of the yeast and bacterial strains on the alcoholic and malolactic performance. For this, we used the AUC calculated from the AF and MLF kinetics.

3. Results and discussion

3.1. Alcoholic fermentation

3.1.1. Fermentation kinetics

The synthetic grape must (Sc-Lip+) used in this study was modified from the version previously published (Sc-Lip-) in the work by Ruiz-de-Villa et al. (2023). To improve the performance of AF and MLF, linoleic acid and β-sitosterol were added. These two components are among the main lipids found in the skin of grapes (Beltran et al., 2008; Guittin et al., 2021; Le Fur et al., 1994). Linoleic acid is the major polyunsaturated fatty acid in grapes (Liu et al., 2018) described concentrations which varies from trace to 280 mg/L depending on the cultivar and wine-making conditions, for instance the Ugni Blanc cultivar contains approximately 12.7 mg/g dry matter variety (Guittin et al., 2021). β-sitosterol is the main phytosterol of grapes, and according to previous studies, it can be 70 % (Le Fur et al., 1994) or 84 % (Guittin et al., 2021) of the total phytosterols.

The original synthetic must (Sc-Lip-) and the modified must (Sc-Lip+) were compared to observe possible modifications in the AF and MLF. The duration of the AF was shorter when using the Sc-Lip+ must (Fig. 1), finishing the AF in 16 days compared to 18 days for the original synthetic must (Sc-Lip-). The addition of linoleic acid and β-sitosterol to the Sc-Lip+ must appeared to have a positive impact on the alcoholic fermentation performance. In previous studies, it was observed that the addition of linoleic acid and β-sitosterol was observed to increase yeast viability values throughout the AF (Beltran et al., 2008). Since *S. cerevisiae* cannot synthesize unsaturated fatty acids (UFAs) in the absence of molecular oxygen, their presence in grape must is essential for yeast growth (Casu et al., 2016). The incorporation of β-sitosterol can be detected in *S. cerevisiae* biomass, suggesting that it is incorporated by yeast and used for growth (Luparia et al., 2004).

However, it has been reported that the addition of phytosterols, especially β-sitosterol, to fermentative media may lead to stuck fermentation in the absence of oxygen (Luparia et al., 2004). However, in the present study, we found that the addition of β-sitosterol at a concentration of 10 mg/L under our experimental conditions, without entry of oxygen and agitation, did not result in stuck fermentation, and the AF was completed successfully. These results suggest that the specific concentration of β-sitosterol used in our study may have played a role in the successful performance of the AF.

On the other hand, the use of *T. delbrueckii* in the co-inoculation or sequential inoculation with *S. cerevisiae* impacted the duration of the AF differently (Fig. 1). The co-inoculation AF kinetics resulted in shorter fermentation times with respect to the control, particularly when using the Td-P strain (Fig. 1A), which took 14 days to complete the fermentation. In comparison, the Td-V strain took 16 days to complete fermentation, the same amount of time as the Sc-Lip+ condition (Fig. 1B).

When considering sequential fermentations, the total fermentation time was increased given both species are major competitors for the nitrogenous nutrients within the grape must (Zilelidou and Nisiotou, 2021). Comparing the results in Fig. 1A and B, Td-V (B) fermentations tended to be shorter in duration than Td-P (A) fermentations. Moreover, the data suggest that the duration in which Td was present during the

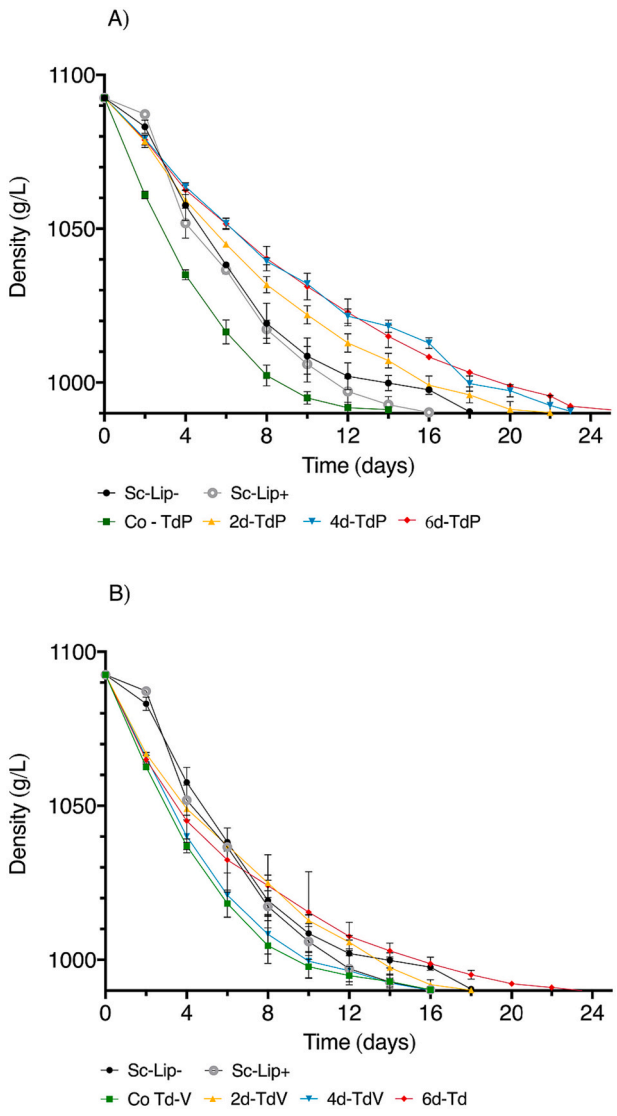

Fig. 1. Evolution of the alcoholic fermentation kinetics: Fermentations with A) *T. delbrueckii* Prelude + *S. cerevisiae* QA23 and B) *T. delbrueckii* Viniferm + *S. cerevisiae* QA23. Sc-Lip- and Sc-Lip+ correspond to the *S. cerevisiae* control fermentations with the original synthetic must and the modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations. 2, 4 and 6 days correspond to the sequential fermentations with *T. delbrueckii* and *S. cerevisiae* inoculated at these times. ● Sc-Lip-, ● Sc-Lip+, ■ Td-Co, ▲ 2d-Td, ▼ 4d-Td and ♦ 6d-Td.

fermentation process was directly proportional to the duration of the fermentation process. In the case of Td-P, 2, 4 or 6 days of its presence within the must resulted in 22, 23, or 26 days of AF, respectively. Similarly, in the case of Td-V, 2, 4 or 6 days of its presence within the must resulted in 16, 18, or 24 days of AF, respectively. These findings highlight the importance of considering the specific strain of Td and its duration within the AF process.

Regarding the areas under the curve (AUCs) calculated from the density decrease in AF kinetics in Fig. 1, there were significant differences that supported the AF kinetics results (Table 1). The AUCs made it possible to indirectly associate the duration of fermentation with the rate of sugar consumption. Among these differences, the co-inoculation conditions had the lowest AUC, followed by the single cultures of *S. cerevisiae*, with a faster AF for Sc-Lip+ than for Sc-Lip-. Moreover, the Td-V strain decreased the AF duration in the sequential inoculation compared to the co-inoculation treatment, while for the Td-P strain, the opposite was observed (Table 1).

3.1.2. Viable yeast population

With regard to the population of the viable cells of the two fermentations (S-lip- and S-lip+), the results showed that the populations of the viable cells of *S. cerevisiae* were higher in the presence of lipids than in their absence (Fig. SD1). This trend suggests that the addition of β-sitosterol and linoleic acid was beneficial for the growth and proliferation of the yeast under the conditions of the control fermentations, as has been reported in previous studies (Beltran et al., 2008; Casu et al., 2016).

In Fig. 2 is shown the evolution as a percentage of the viable cells of the two yeasts, *Torulaspora* and *Saccharomyces*, determined by the use of a selective solid growth media during the AF. Total population of both yeast species is shown in Fig. SD3. Regarding the co-inoculated AF, we observed that during the AF, the strain Td-P had a slightly higher abundance than Td-V (Fig. 2A and B). On the other hand, when comparing the abundance of the species in the co-inoculation with sequential AF, as previously reported (Bordet et al., 2020; Lleixà et al., 2016), *S. cerevisiae* dominated the fermentation from the middle to the end of the process. However, we found that in the co-inoculation, *S. cerevisiae* was more prevalent at the end of the AF process, while in the sequential fermentations, *T. delbrueckii* maintained a higher percentage at the end of the process, particularly in the Td-P strain, which comprised of 40–30 % of the total population (Fig. 2C, E and G). Renault et al. (2015) also observed more growth of *T. delbrueckii* in sequential inoculation than in co-inoculation. This higher presence of Td-P during the fermentation may be related to the longer duration of the AF, while in Td-V 2-Days and 4-Days, it was shorter, as in the co-inoculation AF (Fig. 1). Regarding the differences between the sequential conditions, it appears that during the fermentation with 4 days of *T. delbrueckii* contact, the population was higher than that at 2 and 6 days (Fig. 2E and F).

Other studies have also observed the impacts of the inoculation strategy on the relative abundance of *T. delbrueckii* and *S. cerevisiae* during AF. For example, testing other strains, Roca-Mesa et al. (2022) found that in a sequential AF at 48 h, *T. delbrueckii* was the dominant species, with 60 % at the end of the fermentation. In the case of co-inoculation, with a 1:1 proportion of the two species, they observed an imposition of *S. cerevisiae*, but *T. delbrueckii* still made up 40 % of the total population. Taillandier et al. (2014); Zhu et al. (2021) also reported the dominance of *S. cerevisiae* in co-inoculated fermentations at the end of AF.

Overall, our results and those of other studies suggest that the inoculation strategy can impact the relative abundance of *T. delbrueckii* and *S. cerevisiae* during AF. Additionally, the proportion of *T. delbrueckii* to *S. cerevisiae* appears to be strain dependent.

3.2. General oenological parameters analysed

The metabolic composition of the wines after the AF changed among the conditions. The original and modified controls showed significant differences in their metabolic compositions. Regarding the pH of the final wines, Sc-Lip+ presented a higher pH than Sc-Lip- (Table 1). On the other hand, L-malic acid had a decreasing trend in the Sc-Lip+ wines. The ethanol content was higher in the wines containing lipids (Lip+) (Table 1). As has been described, ethanol can increase up to 1 % vol. with the presence of linoleic acid in the fermentative medium (Liu et al., 2018). Furthermore, there was a decrease in the concentration of acetic acid in the modified synthetic must (Table 1). These results are consistent with previous findings, which have demonstrated that the presence of linoleic acid and β-sitosterol can lead to a reduction in acetic acid concentrations (Beltran et al., 2008).

The *T. delbrueckii* wines showed significant differences in pH and the concentration of ethanol and α-amino nitrogen (Table 1). The pH of the wines produced under the different fermentation conditions was found to vary significantly (Table 1). The Sc-Lip+ wines and the co-inoculated wines had lower pH values at the end of the AF compared to the sequential fermentation conditions. Also, there were significant

Table 1
Analytical parameters of the wines after AF. Mean and standard deviation (SD) (n = 3). Different uppercase letters indicate a significant difference between the values of inoculation strategies and must conditions, and lowercase letters indicate a significant difference between the values of the different strains using the Tukey (HSD) test at $p < 0.05$. Sc-Lip- and Sc-Lip+ correspond to *S. cerevisiae* control fermentations with original synthetic must and modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae*. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times.

	AUC	pH	L-malic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (%, v/v)	Eq-mannose (g/L)	α-Amino nitrogen (mg N/L)
Sc-Lip-	$18,495 \pm 54^{h}$	3.32 ± 0.03^{aA}	1.72 ± 0.03^{aA}	0.95 ± 0.06^{cA}	7.94 ± 0.25^{cB}	10.98 ± 0.15^{cdB}	0.07 ± 0.03^{aA}	5.67 ± 1.15^{bA}
Sc-Lip+	$16,460 \pm 31^{f}$	3.45 ± 0.07^{bcA}	1.65 ± 0.03^{bA}	0.84 ± 0.05^{bcA}	7.67 ± 0.49^{cB}	11.66 ± 0.56^{dB}	0.08 ± 0.05^{aA}	6.67 ± 1.53^{bA}
Co-TdP	$15,269 \pm 29^{i}$	3.49 ± 0.03^{abA}	1.53 ± 0.04^{aB}	0.37 ± 0.11^{aB}	4.37 ± 0.10^{aA}	9.49 ± 0.06^{aB}	0.20 ± 0.07^{bB}	4.00 ± 1.00^{aB}
Co-TdV	$17,281 \pm 15^{g}$	3.35 ± 0.01^{abA}	1.61 ± 0.04^{aA}	0.45 ± 0.13^{aA}	4.69 ± 0.34^{aB}	9.13 ± 0.12^{aA}	0.16 ± 0.01^{bB}	4.00 ± 0.01^{aA}
2 days- TdP	$22,565 \pm 23^{d}$	3.51 ± 0.01^{cA}	1.72 ± 0.04^{abB}	0.79 ± 0.04^{bB}	5.91 ± 0.42^{bA}	11.71 ± 0.29^{bcB}	0.14 ± 0.03^{abB}	9.33 ± 0.58^{bB}
4 days-TdP	$23,708 \pm 24^{c}$	3.66 ± 0.03^{cA}	1.78 ± 0.03^{bB}	0.82 ± 0.05^{bB}	6.02 ± 0.57^{bcA}	11.54 ± 0.50^{bcB}	0.17 ± 0.02^{bB}	9.33 ± 1.53^{bB}
6 days-TdP	$26,682 \pm 42^{a}$	3.65 ± 0.03^{cA}	1.71 ± 0.03^{abB}	0.84 ± 0.08^{bB}	4.63 ± 0.49^{bA}	11.36 ± 0.13^{bB}	0.13 ± 0.02^{abB}	11.33 ± 1.53^{bB}
2 days-TdV	$19,436 \pm 25^{e}$	3.56 ± 0.02^{dA}	1.62 ± 0.03^{bA}	0.62 ± 0.08^{bA}	5.85 ± 0.26^{bB}	10.51 ± 0.08^{bcA}	0.10 ± 0.01^{abB}	6.33 ± 0.58^{bA}
4 days-TdV	$17,312 \pm 52^{g}$	3.64 ± 0.02^{dA}	1.60 ± 0.04^{abA}	0.55 ± 0.13^{bA}	6.82 ± 0.90^{bcB}	10.53 ± 0.12^{bcA}	0.18 ± 0.02^{bB}	8.33 ± 1.53^{bA}
6 days-TdV	$24,413 \pm 100^{b}$	3.62 ± 0.03^{dA}	1.61 ± 0.04^{aA}	0.68 ± 0.07^{bA}	6.44 ± 0.22^{bB}	10.04 ± 0.12^{bA}	0.13 ± 0.04^{abB}	7.00 ± 1.00^{bA}

differences in pH among the different durations of *T. delbrueckii* contact: the wines fermented with 2 days of *T. delbrueckii* contact had a lower pH than those fermented with 4 or 6 days of contact. Among the *T. delbrueckii* strains, there were no significant differences in pH. The extended presence of *T. delbrueckii* resulted in wines with high pH values. Balmaseda et al. (2022), Chen et al. (2018), Martín-García et al. (2020) reported a pH increase of 0.1 or more in sequential wines with *T. delbrueckii* for 4 days with respect to the control with *S. cerevisiae*.

Significant differences in α-amino nitrogen were found at the end of the AF (Table 1). The remaining α-amino nitrogen was lower in the *S. cerevisiae* control wines and in the co-inoculated wines, which was significantly lower compared to the sequential fermentation wines. Among them, there were significant differences in the α-amino nitrogen remaining in the 4-Day and 6-Day *T. delbrueckii* contact conditions. When considering only the Sc-controls and *T. delbrueckii* strains, the Td-P wines displayed significantly higher α-amino nitrogen levels compared to the other wines (9.33–11.33 mg/L). Previous studies (Bely et al., 2008; Martín-García et al., 2020) have reported higher concentrations of residual α-amino nitrogen in wines fermented with *T. delbrueckii*. However, the extent of this increase can vary depending on the specific strain used, as they may differ in their ability to release amino acids, their nitrogen requirements (Benito, 2018) or the occurrence of *T. delbrueckii* autolysis.

This suggests that the choice of the *T. delbrueckii* strain and its interaction with the fermenting process can impact the levels of residual α-amino nitrogen in the resulting wine.

The glycerol concentration exhibited significant differences based on the timing of the inoculation strategy and the strain (Table 1). Literature reported that glycerol increases using *T. delbrueckii being* related to a decrease in ethanol (Balmaseda et al., 2022; Belda et al., 2015; González-Royo et al., 2015). However, the co-inoculated wines tended to have lower glycerol concentrations compared to sequential and control conditions in spite of the fact they also showed the lowest ethanol concentrations (Table 1). It has been described that the production of glycerol relies on the development of the glycero-pyruvic pathway (Benito, 2018) but in our experimental conditions, some results showed that as described by Zhu et al. (2020, 2021) and Rodrigues et al. (2016) that the reduction of glycerol in non-*Saccharomyces*/*Saccharomyces* sequential fermentations in a synthetic grape must did not necessarily imply that glycerol production was the main route of ethanol reduction in mixed fermentations in culture. Regarding ethanol content between conditions wines fermented only with *S. cerevisiae* (Sc-Lip+ and Lip-) presented the highest values, followed by the sequential fermentations, being the lowest values in 6-days condition (Table 1). The decrease was related not only to the time but also to the strain, and the Td-V wines had a larger reduction in ethanol, from 1 to 1.5 % (v/v). In synthetic must, Zhu et al. (2020) described a decrease of 0.29 or 0.47 % (v/v) depending on the strain. Other authors have also observed a reduction in alcohol degree in sequential fermentations, despite they had other conditions as natural grape must (Belda et al., 2015) or the presence of other species in the starter culture (Yılmaz and Gökmen, 2021).

L-malic acid consumption by yeast varies between 10 and 25 % depending on the species (Ribéreau-Gayon et al., 2006). The sequential Td-P and control wines consumed a lower amount of L-malic acid, ranging from 0.22 to 0.36 g/L, while the Td-V wines consumed slightly more, from 0.38 to 0.4 g/L. Finally, the co-inoculated wines had the highest consumption of L-malic acid (Table 1).

In addition, the higher decrease in acetic acid levels was found when using the co-inoculation strategy with a final concentration of 0.37–0.45 g/L (Table 1). The sequential fermentation conditions also resulted in lower levels of acetic acid compared to the control (Sc-Lip+). Furthermore, the Td-V wines had significantly lower concentrations of acetic acid. Similarly, Taillandier et al. (2014) reported a decrease of 0.3 g/L in synthetic grape must through the use of *T. delbrueckii* in sequential fermentations for 48 h. However, there are also variations between different yeast strains. Du Plessis et al. (2017a, 2017b) also reported in synthetic must with *T. delbrueckii* monoculture fermentations a reduction in volatile acidity with *T. delbrueckii* compared to *S. cerevisiae*.

The concentrations of citric and succinic acids did not show significant differences.

Consistent with previous research, the results for mannoproteins determined in eq-mannose showed a trend towards higher mannoprotein values in the wines fermented with *T. delbrueckii* (Belda et al., 2015; Ruiz-de-Villa et al., 2023). However, there were no significant differences between the Td strains (Table 1).

3.3. Volatile composition

Volatile composition after AF is showed in Table 2. Some authors described a relationship between the presence of UFA in the grape must and the formation of volatile compounds derived from yeast (Sumby et al., 2010). However, any differences were found between Lip- and Lip+ regarding volatile composition, thus the addition of linoleic acid at

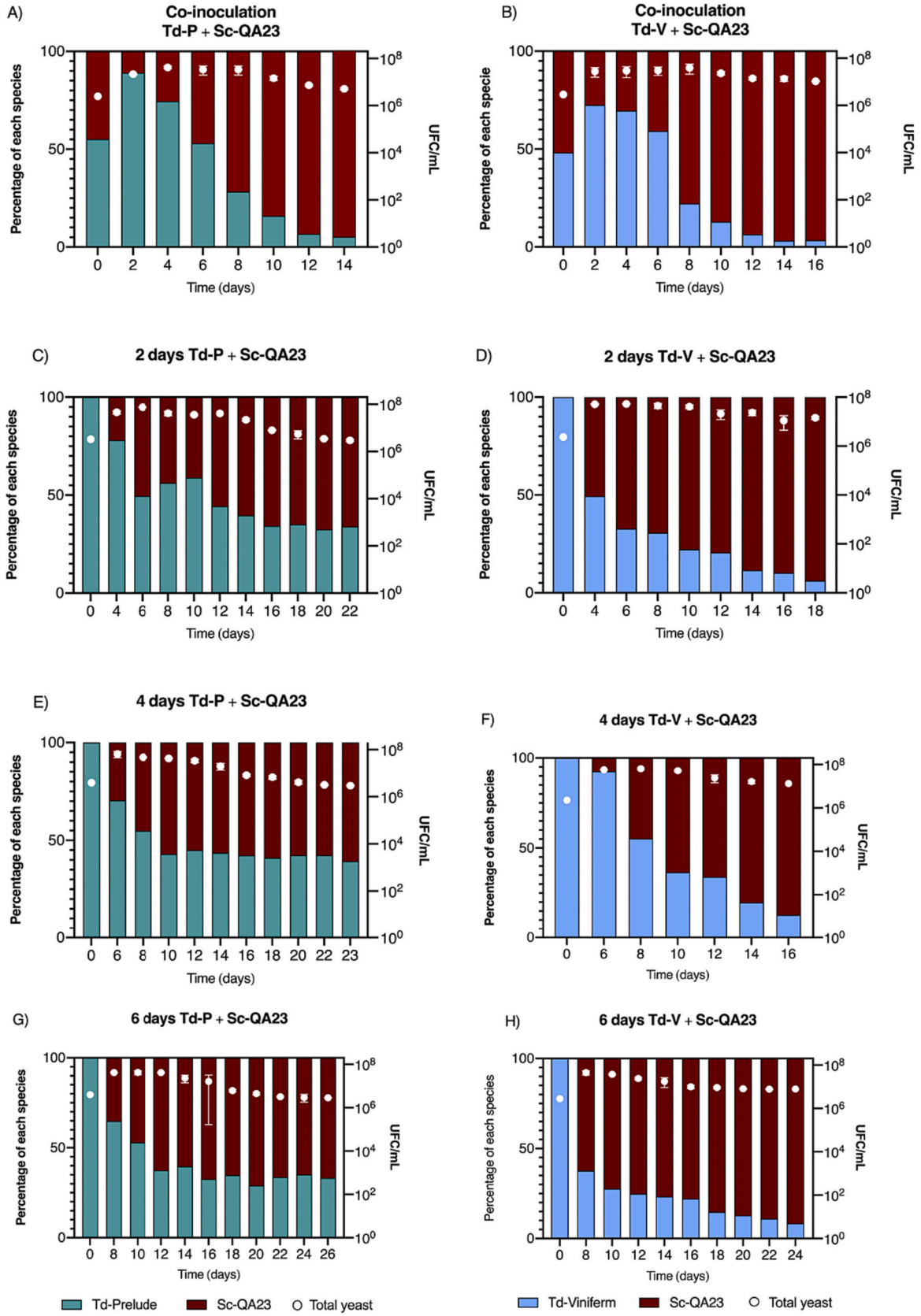

Fig. 2. Total yeast viability (CFU/mL) and percentage of each species (*Saccharomyces cerevisiae* and *Torulaspora delbrueckii*) in co-inoculated and sequential fermentations at 2, 4 or 6 days. Sc-QA23, Td-P and Td-V correspond to *S. cerevisiae*, *T. delbrueckii* Prelude and *T. delbrueckii* Viniferm, respectively.

Table 2
Volatile compounds of the wines after AF. Σ: Fusel alcohol acetates (isobutyl acetate, isoamyl acetate and 2-phenylethanol acetate), ethyl esters of FA (ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl dodecanoate), fusel alcohols (isoamyl alcohol, 1-hexanol, cis-3-hexen-1-ol, 2-phenylethanol), SCFA (propionic, butyric and valeric acids), MCFA (octanoic and decanoic acids), LCFA (myristic acid, palmitic acid and stearic acid). Mean and standard deviation (SD) (n = 3). Different lowercase letters indicate a significant difference between conditions using the Tukey (HSD) test at $p < 0.05$. Sc-Lip- and Sc-Lip+ correspond to *S. cerevisiae* control fermentations with original synthetic must and modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae*. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times.

	∑ Fusel alcohol acetates	∑ Ethyl esters of FA acetates	∑ Fusel alcohols	∑ SCFA	∑ MCFA	∑LCFA
Sc-Lip-	1.58 ± 0.26^{a}	1.09 ± 0.17^{a}	334.06 ± 52.71^{ab}	2.65 ± 0.56^{a}	0.83 ± 0.31^{bc}	4.70 ± 1.77^{a}
Sc-Lip+	1.67 ± 0.17^{a}	0.92 ± 0.18^{a}	333.28 ± 26.75^{ab}	2.89 ± 0.76^{a}	0.72 ± 0.12^{bc}	4.24 ± 1.24^{a}
Co-TdP	1.69 ± 0.09^{a}	1.04 ± 0.25^{a}	251.30 ± 70.97^{a}	2.68 ± 1.63^{a}	1.20 ± 0.39^{c}	4.96 ± 1.36^{a}
Co-TdV	1.52 ± 0.32^{a}	0.96 ± 0.03^{a}	285.73 ± 68.58^{ab}	2.87 ± 1.83^{a}	1.20 ± 0.50^{c}	3.72 ± 0.01^{a}
2 days-TdP	1.02 ± 0.12^{a}	1.10 ± 0.09^{a}	368.66 ± 24.72^{ab}	10.30 ± 0.57^{b}	0.31 ± 0.03^{b}	4.47 ± 1.22^{a}
4 days-TdP	1.33 ± 0.27^{a}	0.87 ± 0.13^{a}	325.24 ± 20.81^{ab}	8.25 ± 1.47^{b}	0.56 ± 0.03^{ab}	6.47 ± 1.94^{a}
6 days-TdP	1.15 ± 0.23^{a}	0.66 ± 0.06^{a}	354.67 ± 70.75^{ab}	10.82 ± 3.50^{b}	0.34 ± 0.02^{ab}	5.78 ± 1.09^{a}
2 days-TdV	1.43 ± 0.10^{a}	1.04 ± 0.20^{a}	348.99 ± 24.05^{ab}	7.23 ± 1.40^{ab}	0.33 ± 0.10^{ab}	6.86 ± 0.21^{a}
4 days-TdV	1.07 ± 0.54^{a}	1.07 ± 0.17^{a}	364.47 ± 55.31^{ab}	8.39 ± 0.44^{b}	0.08 ± 0.07^{b}	6.33 ± 2.05^{a}
6 days-TdV	1.21 ± 0.16^{a}	1.02 ± 0.30^{a}	411.70 ± 39.59^{b}	11.20 ± 0.96^{b}	0.09 ± 0.05^{b}	7.39 ± 0.36^{a}

this concentration did not affect the volatile composition.

The most significant differences between inoculation strategies were found in the content of SCFA and MCFA (Table 2). SCFAs with the sequential fermentations showing remarkably higher concentrations than the co-inoculation and Sc-Lip+, with butyric acid being the main factor responsible for this difference.

Conversely, in MCFA, there was a significant decrease in the levels of octanoic acid and decanoic acid in wines with sequential presence of *T. delbrueckii*, regardless of the inoculation time, compared to the co-inoculated wines. Additionally, an increasing trend was observed in Sc-Lip+ and Lip- wines compared to the sequential wines. Literature also describes a decrease in MCFA content resulting from the presence of *T. delbrueckii* during sequential fermentations of natural grape must (Balmaseda et al., 2018, 2021a).

In terms of ethyl esters of FA and fusel alcohol, no significant differences were found. However, there was an increasing trend observed in sequential wines, particularly in the 6 days-TdV condition, with respect to fusel alcohols. Several authors have also reported an increase in fusel alcohol content, primarily in wines derived from natural must (Azzolini et al., 2015; Belda et al., 2015; Ruiz-de-Villa et al., 2023).

3.4. Influence of T. delbrueckii inoculation strategy on malolactic fermentation

To perform the MLF, the wines produced from different alcoholic fermentations were inoculated with two *O. oeni* strains, Oo-VP41 and Oo-CH11. Our results showed that with the modified synthetic must (Sc-Lip+), the MLF process was completed one day earlier than the original must (Fig. 3). In a previous study (Ruiz-de-Villa et al., 2023), where the same synthetic must was used but without the addition of linoleic acid and β-sitosterol, different strains were tested in sequential fermentation and resulted in some cases of MLF failure. However, this study's conditions successfully allowed all wines to complete the MLF process. This suggests that adding β-sitosterol and linoleic acid to synthetic grape must enhances the efficiency of the MLF process. It is hypothesised, as for the yeast viability described above, that LAB can improve the fermentation performance in the presence of linoleic acid and/or β-sitosterol. Furthermore, this synthetic must proposed has a simpler formulation than the one proposed by Costello et al. (2003) and showed similar or even better results in relation to MLF efficiency (Du Plessis et al., 2017a, 2017b).

When the two yeast species were co-inoculated, the MLF process was as slow as the Sc-control fermentations (Fig. SD4) but lasted longer than the wines produced with sequential fermentation (Fig. 3). Martín-García et al. (2020) described a similar effect in the case of co-inoculations with these species. This behaviour could be linked to the lower abundance of *T. delbrueckii* during alcoholic fermentation (AF), where *S. cerevisiae* dominated the fermentation. Thus, affecting the metabolic profiles of the resulting wines, which in this case, as previously described, the co-inoculated wines had the highest concentration of MCFA, compounds toxic to *O. oeni* (Capucho and San Romão, 1994) via the destabilization of their membrane (Sereni et al., 2020). Furthermore, these wines exhibited the lowest concentration of α-amino nitrogen, which is an important nutrient for the growth and metabolism of *O. oeni* (Remize et al., 2006).

The positive impact of *T. delbrueckii* on the MLF process (Balmaseda et al., 2022; Balmaseda et al., 2021a; Ferrando et al., 2020) can be observed in sequential fermentations, particularly when using the Oo-VP41 strain, which completed the MLF process faster than the Oo-CH11 strain in all the experimental conditions (Fig. 3), agreeing with previous results (Ruiz-de-Villa et al., 2023). In the 2-Day condition, it was observed that the time of MLF and L-malic acid consumption rate improved compared to the control condition, especially with the Td-P strain. For the 4-Day conditions, the improvement was even greater, with the Oo-VP41 strain enhancing the MLF performance by three days (with lower AUCs) and the Oo-CH11 strain by two days (Fig. 3). Both the Td-P and Td-V strains reduced the L-malic consumption rate compared to the *S. cerevisiae* wines. The positive effects observed under the 4-Day conditions may be attributed to the higher percentage of *T. delbrueckii* during AF, particularly with Td-P. As previously mentioned, the resulting wines presented lower alcohol content and a slightly higher pH, which creates a more favourable environment for the metabolism of LAB. Additionally, the elevated nitrogen composition in the form of mannoproteins and α-amino nitrogen (Table 1) can further promote the performance of MLF, because *O. oeni* can utilize these nutrients as a source of energy and carbon (Alexandre et al., 2004; Balmaseda et al., 2021b; Diez et al., 2010). Additionally to this factor, the decreased concentrations of MCFAs in this condition may have indirectly facilitated the progression and efficiency of MLF by creating an environment more favourable to the activity of the selected *O. oeni* strains, as discussed earlier. Furthermore, these two factors could be related since the presence of higher amount of mannoproteins (Table 1) could be related with an absorption of MCFA detoxifying the media (Lafon-Lafourcade et al., 1984).

Finally, in the 6-Day condition, the MLF performance was improved by two days with the Oo-VP41 strain, specifically with Td-P, but with the Oo-CH11 strain, the MLF performance was slower than the control.

To summarize the findings of this study, a PCA was conducted using the AUC values obtained for both AF and MLF (Fig. 4). This analysis allows us to visualize the relationship between AF and MLF, as depicted in Fig. 2. Interestingly, when the AUC for AF was higher, the AUC for MLF was lower, indicating an inverse correlation. This suggests that a more prolonged and gradual AF results in a shorter and faster MLF. This pattern was particularly evident in the Td-P wines, especially after 4 and

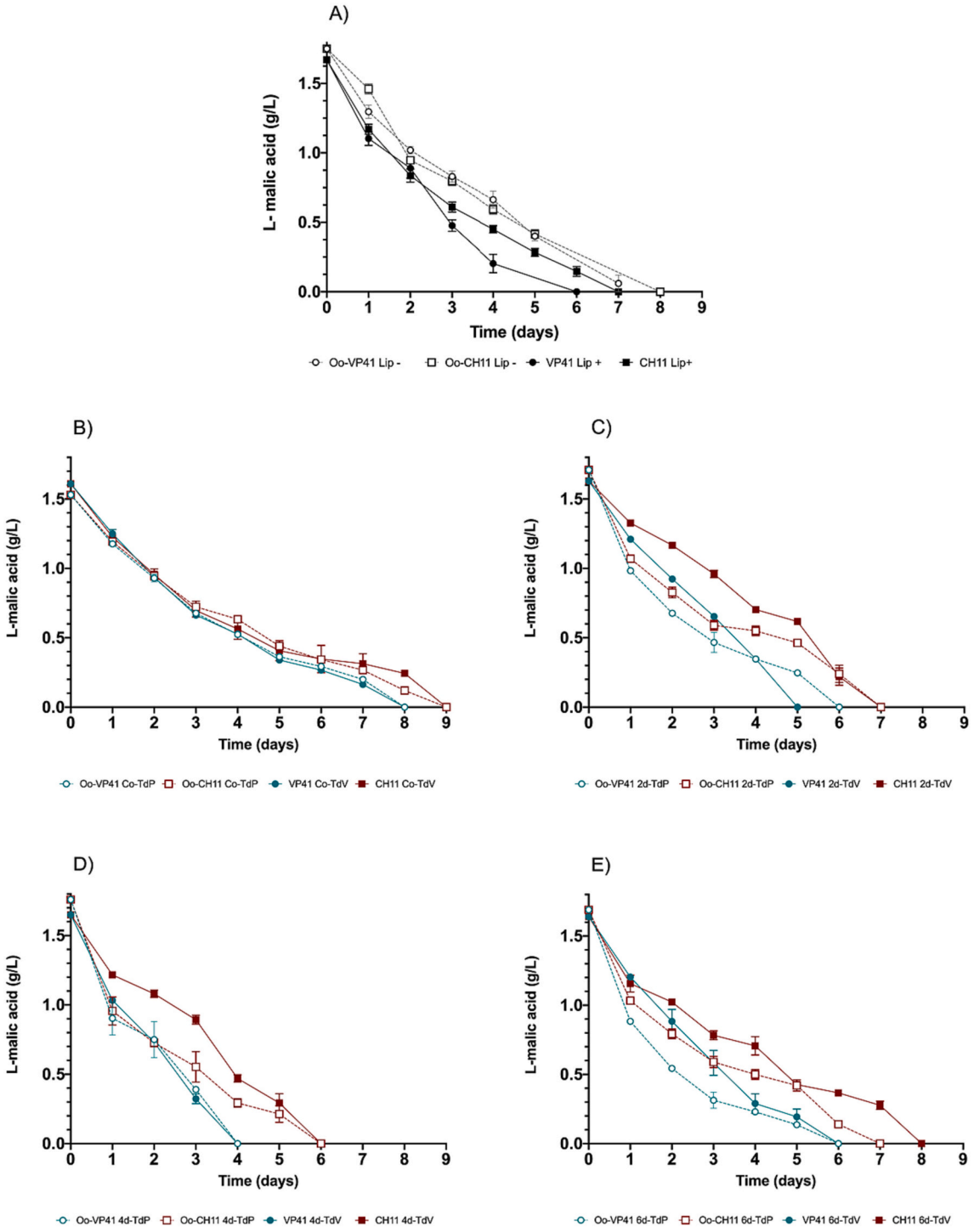

Fig. 3. Evolution of malolactic fermentation kinetics: A) Sc-Lip- and Sc-Lip+ correspond to the *S. cerevisiae* control fermentations with the original synthetic must and the modified synthetic must, respectively; B) Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae* QA23. C) 2, D) 4 and E) 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times. Oo-VP41 and Oo-CH11 correspond to the two strains of *O. oeni* VP41 and CH11. The values are expressed as the average of three biological replicates for each condition.

6 days of contact with *T. delbrueckii*, which is equivalent to inoculating *S. cerevisiae* at densities of approximately 1060 and 1050 g/L, respectively. Similarly, controlled fermentations conducted without lipids and co-inoculated wines, which exhibited significantly faster AF, demonstrated a slower and more protracted MLF, as previously described.

4. Conclusions

In summary, in relation to the synthetic fermentative medium, the optimization performed by adding linoleic acid and β-sitosterol was found to be effective in promoting good MLF performance, which suggests that these lipids can improve the metabolism of *O. oeni*. Regarding the co-inoculated wines, where *S. cerevisiae* dominated the AF, the

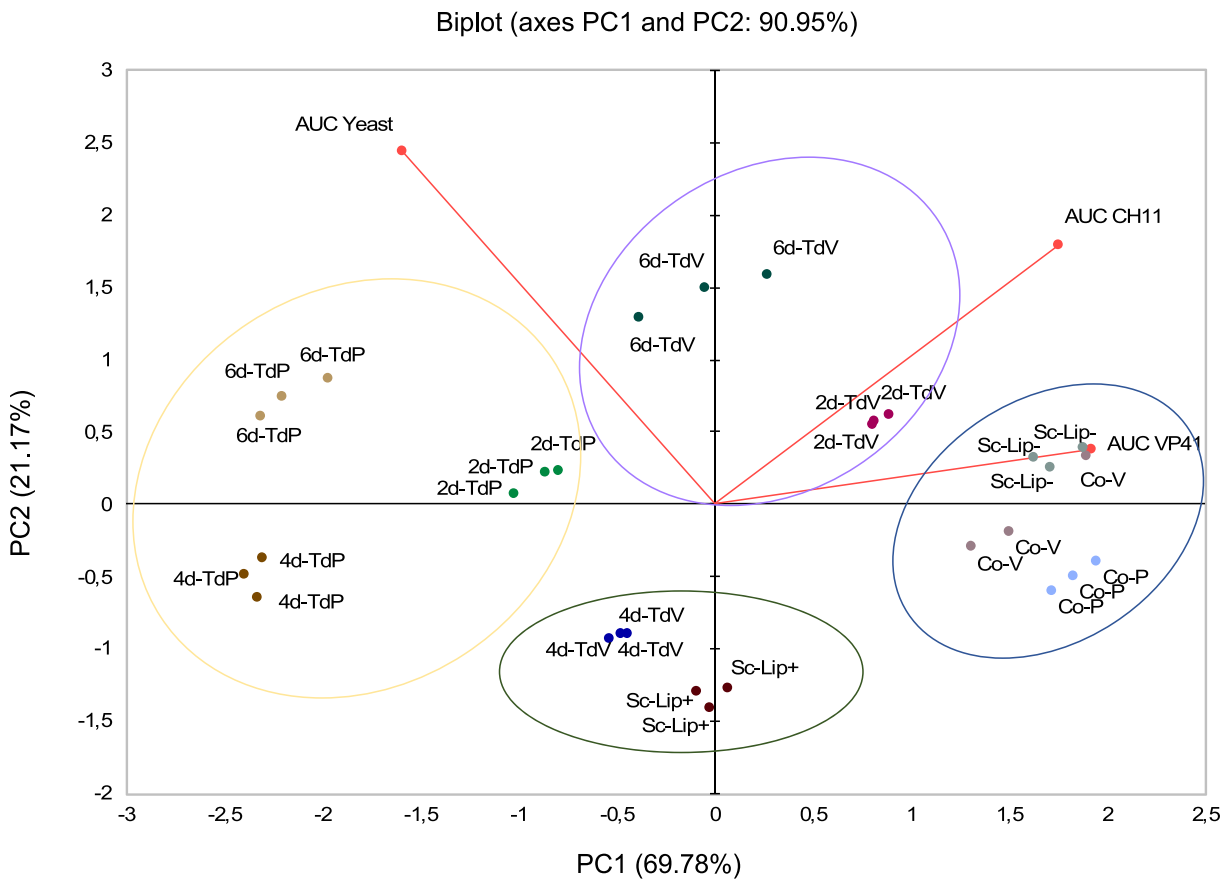

Fig. 4. Principal component analysis biplots built from the following variables: AUC of AF data and AUC of MLF data, MLF driven by the *O. oeni* strains VP41and CH11. The observations are Sc-Lip- and Sc-Lip+, which correspond to the *S. cerevisiae* control fermentations with the original synthetic must and the modified synthetic must, respectively; Co-TdP and Co-TdV correspond to the co-inoculated fermentations with *T. delbrueckii* Prelude or Viniferm + *S. cerevisiae* QA23. 2 days, 4 days and 6 days correspond to the sequential fermentations with *T. delbrueckii* (Prelude or Viniferm) and *S. cerevisiae* inoculated at these times.

performance of MLF was slower, which can be attributed to a higher concentration of MCFA and a low concentration of α-amino nitrogen.

Whereas sequential fermentation was a better option to obtain an efficient MLF process and improve AF metabolites, such as a reduction in ethanol or acetic acid, especially with Td-V. The 4 days of *T. delbrueckii* contact allowed a higher presence of these species during the AF, which, due to their positive synergy with *O. oeni*, improved the MLF. Even though the Td-V wines showed better oenological parameters, the use of Td-P for 4 or 6 days, when combined in the MLF with Oo-VP41, was the best option in terms of MLF efficiency. In addition, our results showed that there is a relationship between the duration and speed of AF and how it affects MLF. Overall, these findings highlight the importance of considering both the inoculation strategy and the specific strains to obtain a better understanding of the complex interactions between these species throughout the fermentation process.

CRediT authorship contribution statement

Candela Ruiz-de-Villa: Investigation, Methodology & Original draft preparation. **Montse Poblet**: Investigation, Methodology & Data curation. **Albert Bordons**: Supervision, Reviewing & Visualization. **Cristina Reguant**: Supervision, Writing, Reviewing & Funding acquisition. **Nicolas Rozès**: Supervision, Writing, Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110367.

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