

Optimizing inoculation strategies with *Lachancea thermotolerans* and *Saccharomyces cerevisiae* for enhanced wine acidity management and fermentative performance

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ABSTRACT

Maintaining adequate acidity in wine is essential for microbial stability, colour preservation, and sensory quality—factors increasingly challenged by climate change, which promotes grape ripening conditions that reduce acidity and raise pH. *Lachancea thermotolerans* offers a promising biotechnological solution through its capacity to produce lactic acid during alcoholic fermentation. This study evaluated how different inoculation strategies—namely co-inoculation and sequential inoculation—using varying ratios of *L. thermotolerans* and *Saccharomyces cerevisiae* affected wine acidification and fermentative efficiency. Co-inoculations with higher proportions of *L. thermotolerans* led to significant increases in total acidity, achieving acidification levels comparable to those of sequential inoculation, without impairing fermentation kinetics. However, excessive acid production was associated with slower fermentations and elevated residual sugar levels. In addition to lactic acid, co-inoculation impacted the concentrations of other organic acids (e.g., malic and succinic acids), as well as glycerol content, contributing to the final acidity and mouthfeel of the wine. Notable shifts in volatile composition, including esters and higher alcohols, were also observed. These findings highlight the importance of optimising inoculation ratios in mixed fermentations to achieve a balance between acidification and fermentation performance, offering practical guidance for biological acidification under warming climate conditions.

1. Introduction

The wine sector has been facing multiple challenges in recent decades, with loss of acidity and increased sugar concentration being among the major concerns affecting grape must (Gutiérrez-Gamboa et al., 2021; Rasilla et al., 2024; Volschenk et al., 2006). Variations in both parameters are associated with different factors, including grape variety, vineyard management, and annual vintages. However, recent climatic conditions, particularly in the Mediterranean basin, where viticulture plays a vital role in various economic and cultural aspects, have led to more frequent alterations in these two key parameters (Ubeda et al., 2020).

The wine sector has developed several strategies to address these issues, implementing different measures, from vineyard management to

the ageing of the resulting wines (Gutiérrez-Gamboa et al., 2021; Ubeda et al., 2020). A lack of acidity is associated both with microbial and organoleptic alterations, while increased sugar content is linked to the development of spoilage microorganisms and stuck fermentations. Nevertheless, the options to be applied during alcoholic fermentation are preferred, given the broad range of technical, physicochemical, and biological options that are easier to implement at this stage (e.g. (Del-Bosque et al., 2023; Delrot et al., 2020; Garrigós et al., 2024; González-Arenzana et al., 2020; Keller, 2023)). Among the biological options, several have been studied, particularly the use of yeasts that influence wine acidity, including those with the ability to acidify and deacidify, which have been widely researched and previously reviewed (Vicente et al., 2022; Vilela, 2019; Vion et al., 2024). Among the acidifying options, *Lachancea thermotolerans* stands out due to its oenological

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aptitude and its ability to produce high triter of lactic acid, a characteristic that is not common among wine-related yeasts (Comitini et al., 2011; du Plessis et al., 2017; Hranilovic et al., 2018; Kapsopoulou et al., 2005; Porter et al., 2019).

The use of *Saccharomyces cerevisiae* (or other *Saccharomyces* species) during wine fermentation is essential for achieving a successful conclusion of alcoholic fermentation. Other wine-related yeasts, such as *L. thermotolerans*, which are classified under the generic term non-*Saccharomyces* yeasts, are unable to consume all the sugars present in grape must and, therefore, cannot complete the fermentative process effectively. To harness the positive contributions of both groups to wine fermentation, mixed inoculum strategies have been developed and widely tested over the past few decades (Jiranek, 2024; Jolly et al., 2014; Morata et al., 2023).

There are two main strategies for inoculating both groups of microorganisms in wine fermentation: co-inoculation, which involves inoculating both species simultaneously, and sequential inoculation, where there is a gap between the inoculation of one yeast and the other. The most common strategy employs one non-*Saccharomyces* strain and one *S. cerevisiae* strain. However, recent winemaking trends have included more than two species, multiple strains from each species in mixed fermentations or different microorganisms consortia (De Gioia et al., 2022; Planells-Cárcel et al., 2024; Pretorius, 2020; Vicente, Wang et al., 2024). The use of different species or strains, whether of the same or different species, requires adequate compatibility between them, meaning that their interactions should be at least non-negative, if not synergistic. Previous studies have extensively characterised the mechanisms of yeast-yeast interactions, defining both yeast compatibility and the resulting fermentation outcomes from an industrial perspective as an adequate compatibility between species or strains is necessary to secure the fermentation outcome (Conacher et al., 2021; Ruiz et al., 2023; Vicente, Ruiz et al., 2023).

The interaction between *L. thermotolerans* and *S. cerevisiae* has been shown to be complex. Most studies indicate significant competition between these two species, particularly for limiting nutrients such as nitrogen and vitamins (Shekawat et al., 2020). This competition is further intensified by physical contact between the cells (Luyt et al., 2024). Co-culture assays using different strains of each species have demonstrated a lower growth rate of *S. cerevisiae* in co-culture with *L. thermotolerans* compared to pure cultures of *S. cerevisiae*, affecting growth rate, maximum population, and latency phase of the *S. cerevisiae* strain Pourcelot et al. (2023). Furthermore, recent studies have described spontaneous laboratory-scale fermentations dominated by *L. thermotolerans*, with a relative abundance exceeding 90 % instead of *S. cerevisiae* (de Celis et al., 2024).

Consequently, the inoculation ratio and strategy employed when using both species could be crucial, particularly for studies aimed at increasing wine acidity through lactic acid production by *L. thermotolerans*. Gobbi et al. (2013) demonstrated that acidification is favoured when a higher proportion of *L. thermotolerans* is used in co-inoculated fermentations compared to *S. cerevisiae*. Additionally, the use of both species contributes to a reduction in the ethanol levels of the resulting wines and increases the production of certain higher alcohols (e.g. phenylethyl alcohol) and esters, thereby enhancing the aroma and complexity of the wine (Vicente et al., 2021).

This study focused on optimising the acidification of final wines using *L. thermotolerans* and analysed the effects of different inoculation strategies and ratios on total acidity and pH. It describes the most appropriate ratio of both species to achieve effective acidity control, while ensuring a successful alcoholic fermentation. Additionally, the study underscores the importance of managing different strains of species in mixed fermentations and highlights how the effective application of biological acidification techniques could jeopardise the successful conclusion of fermentation.

2. Materials and methods

2.1. Yeasts strains and vinification assays

For mixed fermentations, the industrial strains *Lachancea thermotolerans* CR311 and *Saccharomyces cerevisiae* CRR0416 were used. All strains are deposited in the CYC (Complutense Yeast Collection. Complutense University of Madrid, Spain), cryopreserved in 25 % glycerol, and propagated on Sabouraud agar for routine handling. Tempranillo grape must (*Vitis vinifera* L. cultivar Tempranillo) from Ribera del Duero appellation of origin (Peñafiel, Spain) was used in all fermentation assays. Grapes were destemmed, crushed, and introduced into a fermentation tank. Before inoculation, free running grape juice was obtained and preserved at -20°C until further use. Grape must was supplemented with 50 g/L of Actimax Natura (Agrovin, Spain) before use. The initial parameters of the grape juice were: 264 g/L fermentable sugars, pH 3.68, 95 mg/L primary amino nitrogen, 115 mg/L ammonia nitrogen, 210 mg/L nitrogen from free amino acids (NFA), 7 ppm free SO_2 . The initial concentrations of lactic and acetic acids were below 0.05 g/L. Fermentation assays were conducted in 1.00 L borosilicate glass bottles with polypropylene caps, allowing CO_2 release, filled with 750 mL of grape must. All conditions were tested in triplicate at 25°C .

The preculture of the strains was carried out in YMB medium (5 g/L proteose peptone, 3 g/L malt extract, 3 g/L yeast extract and 10 g/L glucose) for 24 h at 25°C and shaking at 100 rpm. The optical density of the precultures was determined to calculate the final cell density (0.2 O. D. $\approx 1 \times 10^6$ cells/mL). Different fermentation strategies were followed (Table 1). Fermentation monitoring was performed by controlling fermentation density (DMA 35 Basic, Anton Paar, Austria).

2.2. Microbial populations monitoring

At different time points during the fermentation, from day 0 to 14, samples were taken, serially diluted 10-fold, and plated on malt extract agar and lysine agar (CM0191, Oxoid, UK), incubated at 28°C for total viable and non-*Saccharomyces* viable counts, respectively. On day 14 of fermentation, to verify strain prevalence, five colonies were selected from each plate, and DNA was extracted following a previously described protocol (Querol & Barrio, 1990). For *S. cerevisiae* isolates, interdelta polymorphism fingerprinting was performed (Legras & Karst, 2003), while for *L. thermotolerans*, microsatellite fingerprinting was used (J. Vicente, Navascués et al., 2023). Each PCR reaction used 100 ng of total DNA and DreamTaq Green DNA polymerase 2 \times (ThermoFisher, USA). DNA electrophoresis was performed using 15 μL of PCR product on a 1.6 % (w/v) agarose gel in $1 \times$ TAE buffer at 70 V for 110 min. DNA was stained with $1 \times$ GelRed[®] solution (Biotium, USA) in 0.1 M NaCl, and a 100–3000 bp DNA weight marker (VWR, USA) was used for band sizing. Gel images were captured with a Gel Analyzer System (Axygen Scientific, USA) and fingerprinting profiles were compared to the inoculated strains to calculate implantation percentages.

Table 1
Inoculation strategies followed in the study.

Inoculation strategy	Fermentation	Cell density (CFU/mL)	
		<i>L. thermotolerans</i> CR311	<i>S. cerevisiae</i> CRR0416
–	Control	–	10^6
Sequential* Co-inoculated	Sequential	10^6	10^6
	Co-6 + 6	10^6	10^6
	Co-6 + 5	10^6	10^5
	Co-6 + 4	10^6	10^4
	Co-6 + 3	10^6	10^3
	Co-3 + 6	10^3	10^6

* In the sequential fermentations, *S. cerevisiae* CRR0416 was inoculated 96 h after *L. thermotolerans* CR311.

2.3. Analytical determinations

Through the entire fermentative process, lactic acid production was followed by sampling at several time points, and its concentration was measured enzymatically (Y15 Autoanalyzer, Biosystems, Barcelona, Spain). Additionally, residual sugars, glycerol, acetic, and malic acids were enzymatically measured at the end of the fermentation, whereas succinic acid was determined by FTIR (Bacchus 3, Tecnología de Difusión Ibérica, Spain). At the end of the fermentation ethanol was determined by NIR absorption (Alcolyzer 3001, Anton Paar, Austria), and pH using a pH meter (pH Basic20, Crison, Spain). The analysis of the volatile compounds from the resulting wines was performed according to a previous methodology (Vicente, Kelanne, Rodrigo-Burgos et al., 2023). In brief, samples were analysed in triplicate using headspace solid phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS). The retention times (RIs) of the volatiles were calculated via co-injection with an alkane mixture (C7-C30, Sigma-Aldrich, St. Louis, MO). Volatiles were identified by matching the obtained mass spectra with the NIST Mass Spectral Search Program (v. 2.4, 2020) and by comparing the RIs to those of the compounds reported in the literature and the NIST Mass Spectral Search Program or Webbook (<https://webbook.nist.gov/chemistry/>). Moreover, the identification of a selected number of volatile compounds was confirmed by comparing the retention indices and mass spectra with those of the authentic reference compounds.

2.4. Statistics and data analysis

All statistical analyses were performed using R software version 4.4.1 (R Core Team, 2024). Analysis of variance (ANOVA) and Tukey post-hoc tests were applied to compare the different groups and values using *agricolae* library (v1.3–7). The significance level was set at $P < 0.05$. Principal Component Analysis (PCA) was calculated using the mean values of all the oenological parameters of the fermentations using *factoextra* library (v1.0.7). Linear regression models between different parameters were constructed using the stats package (v.4.3.1).

3. Results

3.1. Fermentative kinetic and yeasts performance

The shortest fermentation times were observed in the co-inoculated treatments, Co-6 + 6 and Co-3 + 6, both lasting 14 days, followed by the control fermentation, which lasted 16 days. The remaining fermentations, including the sequential inoculation, lasted 20 days (Fig. 1). All fermentations displayed an initial lag phase, more or less pronounced, during the first day, after which they followed a similar fermentation kinetic until the third day. At this point, the Co-6 + 6, Co-3 + 6, Co-6 + 5, and control fermentations exhibited a more vigorous fermentation kinetics, reaching density values close to 990 g/L by day 10, compared to the other fermentations, which had density values near 1005 g/L at the same time. The sequential inoculation of *L. thermotolerans*, followed by *S. cerevisiae* after 96 h, resulted in a moderate decrease in density compared to the control and co-inoculations, with this treatment exhibiting the most prolonged and steady fermentation.

In general, co-inoculation or sequential inoculation treatments tended to exhibit slower fermentation than the pure *S. cerevisiae* control, albeit at varying rates. The most divergent inoculation ratio, favouring *S. cerevisiae*, Co-3 + 6, produced the fermentation profile most similar to that of the control fermentation.

Regarding the yeast populations involved in fermentation, an overall positive development of the inoculated strains was observed, particularly in *L. thermotolerans* (Fig. S1). In all fermentations that included this non-*Saccharomyces* strain, its implantation was nearly 100 % among all analysed isolates. Only the control and Co-3 + 6 fermentations exhibited non-*Saccharomyces* yeast counts below 1×10^5 CFU/mL, while the remaining co-inoculated fermentations showed non-*Saccharomyces* yeast counts approaching 1×10^8 CFU/mL by the second day.

In contrast, total yeast counts followed similar kinetics across all fermentations, exceeding 1×10^7 CFU/mL within the first 24 h. However, *S. cerevisiae* displayed varying implantation rates depending on the inoculation strategy and ratio employed. Sequential fermentation, in particular, impacted the performance of the selected *S. cerevisiae* strain CRR00416, with final implantation rates of approximately 60 %. In the

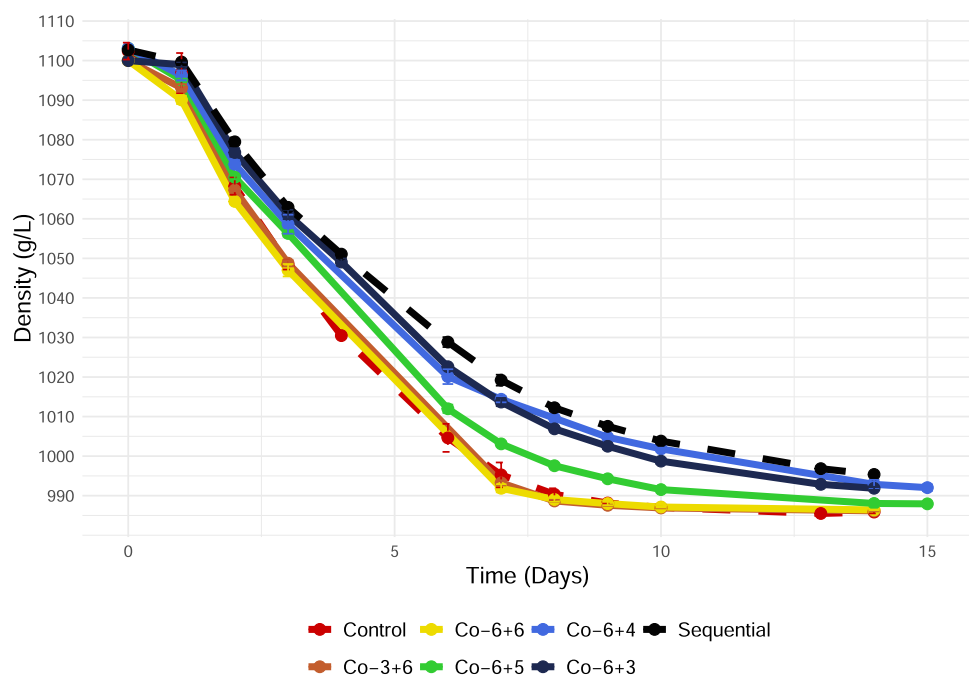


Fig. 1. Fermentative kinetics (density vs. time) of the fermentations under each condition (Table 1). Data points represent the mean values of biological replicates, with error bars indicating standard deviations. The colour legend within the figure distinguishes treatments: Sequential and Control fermentations are represented with dashed lines, while co-inoculated fermentations are shown with solid lines.

Co-6 + 3 fermentation, where the inoculated cell density of *S. cerevisiae* was 1000 times lower than that of the *L. thermotolerans* strain, the CRR00416 strain achieved mean implantation values of around 40 %. This indicates that when *L. thermotolerans* is present in higher proportions, there is a corresponding decrease in the implantation rates of *S. cerevisiae* CRR00416.

3.2. Basic oenological characterization of the resulting wines

Regarding the basic oenological parameters of the resulting wines, important differences could be observed. The PCA analysis reveals clear clustering of the different inoculation strategies based on their metabolic outcomes. Two main variables, total acidity and residual sugars account for more than the 94 % of the showed variation across samples. And, among them, sequential fermentation is strongly associated to higher residual sugars, whereas co-inoculations as Co-6 + 4 and Co-6 + 5 are linked to increased total acidity and lactic acid production (Fig. S2; Table 2).

3.2.1. Ethanol, glycerol and residual sugars

In addition to differences in fermentation kinetics, variations in the final levels of ethanol and residual sugars were observed across the different inoculation strategies. While the control fermentation exhibited the highest ethanol concentration (15.6 %), followed by Co-3 + 6 (15.4 %), the remaining fermentations showed values around 15.2 % (Co-6 + 6, Co-6 + 5) or below 15.0 %, such as the sequential, Co-6 + 3 (Fig. 2, Table 2).

Regarding residual sugars, only Co-3 + 6 showed no residual sugars, along with Co-6 + 6 and the control fermentations, which exhibited residual sugar levels below 0.5 g/L. The other fermentations showed increased levels of residual sugars, with Co-6 + 5 around 2 g/L, Co-6 + 3 and Co-6 + 4 around 4 g/L, and the sequential fermentation at approximately 7 g/L (Table 2). These results suggest that higher residual sugar levels correspond with a favoured presence of *L. thermotolerans*, although under certain co-inoculation conditions, complete elimination of residual sugars can occur when *L. thermotolerans* is present at lower cell density.

The different inoculation strategies affected glycerol production, with the highest values, around 7 g/L, observed in the control, sequential, Co-3 + 6, Co-6 + 3, and Co-6 + 4 fermentations. In contrast, Co-6 + 5 and Co-6 + 6 exhibited the lowest glycerol values, around 6.2 g/L. Therefore, no specific contribution to glycerol production can be attributed to either of the species employed, as fermentations where *S. cerevisiae* was favoured (e.g., control, Co-3 + 6) showed similar values to those where the role of *L. thermotolerans* was favoured (e.g., sequential, Co-6 + 3) (Table 2).

3.2.2. Organic acids content, total acidity and pH

The presence of *L. thermotolerans* is associated with an increase in total acidity and a decrease in final pH. Sequential and Co-6 + 4 fermentations exhibited the highest total acidity, reaching values around 9 g/L (compared to the control fermentation, which reached around 4 g/L), with a pH reduction of approximately 0.3 units, down to 3.3 (control

fermentation: pH 3.6). However, other treatments also showed significant increases in total acidity, such as Co-6 + 5, which reached total acidity values close to 7 g/L, and a corresponding decrease in pH from 3.6 to 3.4 compared to the control fermentation (Fig. 2; Table 2).

The increase in total acidity and subsequent pH reduction in *L. thermotolerans* fermentations is directly linked to the lactic acid production of this yeast. The kinetics of l-lactic acid production were monitored throughout the fermentation process in the different treatments (Fig. 3). As observed, between days 1 and 2, there was a sharp increase in lactic acid concentration, particularly when the presence of *L. thermotolerans* was favoured. The lactic acid levels reached in the first 48 h were then maintained throughout the fermentation, with no further increases or decreases.

In terms of the final lactic acid values, the fermentations could be grouped into three categories: those reaching values around 5 g/L, with the sequential fermentation showing the highest levels, along with Co-6 + 3 and Co-6 + 4; fermentations with lactic acid levels around 3.5 g/L, such as Co-6 + 5; and those with 1 g/L or less, including the fermentations where, despite the presence of *L. thermotolerans*, *S. cerevisiae* was favoured (Co-6 + 6, Co-3 + 6, and the control fermentation) (Table 2). Thus, as the presence of *L. thermotolerans* is favoured, higher levels of lactic acid are produced, leading to increased total acidity and lower pH values. Nevertheless, a significant correlation was found with residual sugars ($\beta = 1.07$, $p\text{-value} = 1.33 \cdot 10^{-10}$), what could suggest an inhibition of the fermentative capacity of the yeasts community.

Other organic acids of oenological relevance, contributing to both wine acidity and pH, also showed variations across the different fermentative conditions. In the case of malic acid, significant reductions in concentration were observed in several fermentations, such as sequential, Co-6 + 5, Co-6 + 4, and Co-6 + 3, with reductions of nearly 40 % from the initial malic acid concentration (1.0 g/L) (Table 2). Reductions in malic acid content were also observed in the other fermentations, but with lower percentages (around 30 %), with the *S. cerevisiae* control fermentation showing the highest malic acid concentration (around 0.75 g/L). However, despite the consumption of malic acid, the high lactic acid production counteracts the pH increase consequence of the pH removal, ultimately resulting in lower pH values.

Succinic acid levels of up to 1.30 g/L were observed in sequential fermentations with *L. thermotolerans* and *S. cerevisiae*, which differed from those in co-inoculated or control fermentations. Co-inoculated fermentations and the *S. cerevisiae* control showed final succinic acid levels barely reaching 1 g/L, with Co-6 + 6 and Co-6 + 5 showing the lowest values, around 0.8 g/L (Table 2).

Finally, acetic acid, which is usually more relevant to the organoleptic perception of wines than to acidity regulation and pH management, also showed statistically significant differences. However, all final concentrations remained well below the negative sensory threshold for acetic acid in wine (0.8 g/L; (Ruiz et al., 2019)). Concentrations of acetic acid ranged from approximately 0.25 to 0.45 g/L, with the highest values observed in fermentations with a strong influence of *L. thermotolerans* (Co-6 + 4, Co-6 + 3, Co-6 + 5, and sequential), whereas the remaining fermentations (control, Co-3 + 6, and Co-6 + 6) showed the lowest values (Table 2).

Table 2
Summary of final basic chemical analyses of fermentations from Tempranillo must.

Fermentation	Ethanol (%, v/v)	pH	Total acidity	Acetic acid	Malic acid	Lactic acid	Succinic acid	Residual sugars	Glycerol
Control	15,58±0,12a	3,59±0,03a	4,22±0,08e	0,26±0,03d	0,78±0,05a	0,45±0,05d	0,99±0,08bc	0,44±0,28e	7,05±0,25a
Sequential	14,92±0,06bc	3,26±0,01d	8,79±0,04a	0,37±0,02c	0,64±0b	4,98±0,05a	1,27±0,06a	7,3 ± 0,5a	6,9 ± 0,02a
Co-6 + 6	15,16±0,05bc	3,53±0,01b	4,67±0,01d	0,23±0,02de	0,76±0,07ab	1 ± 0,06c	0,79±0,07d	0,25±0,08e	6,17±0,1c
Co-6 + 5	15,15±0,04bc	3,43±0,02c	6,94±0,11c	0,39±0,03bc	0,64±0,02b	3,48±0,13b	0,81±0,03cd	2,12±0,16d	6,28±0,17bc
Co-6 + 4	14,66±0,03bc	3,29±0,04d	8,78±0,55a	0,47±0,03a	0,64±0,02b	4,87±0,71a	1,09±0,23ab	4,21±0,14b	6,9 ± 0,39a
Co-6 + 3	15,11±0,04bc	3,29±0,01d	8,19±0,08b	0,44±0,01ab	0,64±0,02b	4,51±0,08a	1,06±0,02abc	3,42±0,34c	6,76±0,04ab
Co-3 + 6	15,37±0,04ab	3,57±0,01ab	4,29±0,04de	0,18±0,03e	0,77±0,05ab	0,32±0,02d	0,98±0,11bcd	0 ± 0e	6,92±0,09a

The results are the mean and standard deviation (SD) of the biological replicates. Different letters indicate statistical significance between groups. Unless specified, excluding pH, all parameters' units are g/L. Fermentation abbreviations correspond to those used in Table 1.

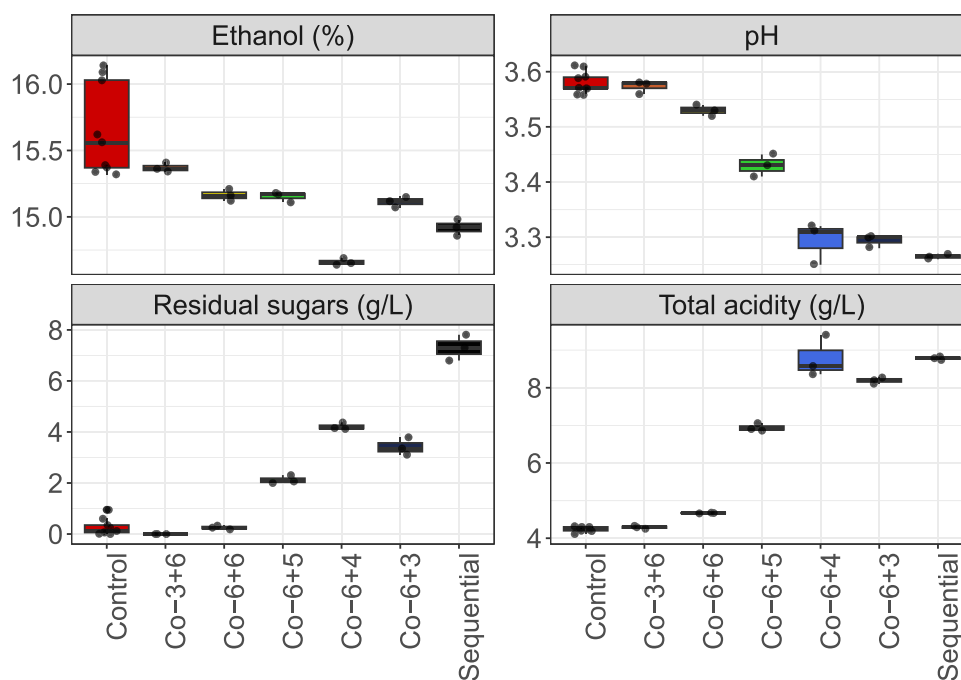


Fig. 2. Ethanol production (% v/v), residual sugars (g/L), pH, and total acidity (g/L) in experimental fermentations with different inoculum strategies and ratios between *L. thermotolerans* CR311 and *S. cerevisiae* CRR0416 (Table 1). Colour codes are the same as in employed in Fig. 1.

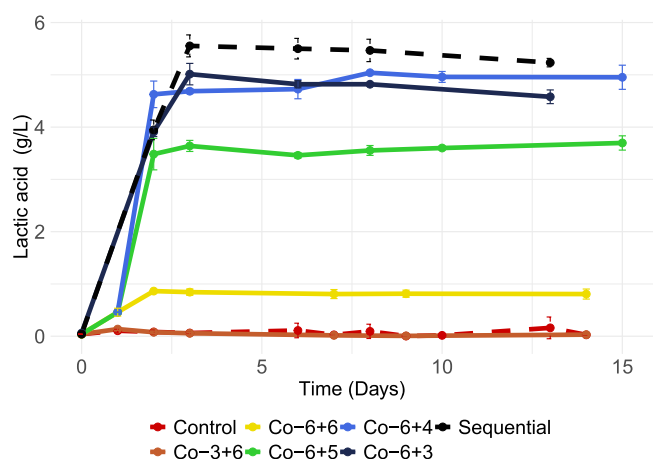


Fig. 3. l-Lactic acid production kinetics of the fermentations for each condition (Table 1). Data points represent the average values of the three biological replicates with standard deviation bars. The colour legend within the figure distinguishes treatments: Sequential and Control fermentations are represented with dashed lines, while co-inoculated fermentations are shown with solid lines.

3.2.3. Volatile profile of the resulting wines

Regarding the volatile profile of the fermentations, esters exhibited the greatest variation among the treatments, followed by higher alcohols and fatty acids (Table S1). These differences significantly impacted the aromatic profiles of the wines and were closely related to the inoculation strategies used. The PCA analysis clearly illustrated this distribution of volatile compounds, with PC1 explaining 93.77% of the total variance (Fig. S3). The ester profile among the fermentations revealed significant variation attributed to inoculation strategies and yeast ratios. Fermentations favouring *S. cerevisiae*, such as the control and Co-3 + 6, resulted in total ester concentrations close to 7 mg/L. These fermentations were particularly rich in key esters: hexyl acetate concentrations were up to seven times higher in the control (0.17 mg/L) compared to other treatments (0.02–0.08 mg/L), ethyl hexanoate showed a nearly

threefold increase in the control (0.6 mg/L) versus the sequential inoculation, and ethyl butanoate was also more abundant under *S. cerevisiae*-dominated conditions (0.13–0.17 mg/L) than in fermentations favouring *L. thermotolerans* (0.08–0.09 mg/L). The OAV values, calculated according to Gómez-Míguez et al. (2007), further support these differences, reaching approximately 150 for ethyl hexanoate and 530 for ethyl octanoate.

The sequential fermentation exhibited a volatile profile similar to that observed in fermentations with a higher inoculum of *L. thermotolerans*, both in terms of ester and higher alcohol concentrations. These fermentations also displayed distinctive patterns in acid-derived esters. Specifically, compounds associated with *L. thermotolerans* metabolism showed clear trends: ethyl lactate levels were higher, peaking at 0.99 mg/L in the sequential inoculation and ranging from 0 to 0.84 mg/L in co-inoculated fermentations, likely reflecting the influence of lactic acid accumulation.

While higher alcohols showed minor concentration differences, 3-methyl-1-pentanol, heptanol, and phenylethyl alcohol levels varied notably across fermentations. Higher *L. thermotolerans* ratios increased propanol, 3-methyl-1-pentanol, and heptanol levels, reaching twice the concentrations of the control. Propanol and hexanol were particularly enriched in Co-6 + 3 and sequential fermentations (0.11 and 0.16 mg/L, respectively) if compared to the control (0.05 and 0.08 mg/L). Conversely, *L. thermotolerans* negatively impacted phenylethyl alcohol, yielding lower concentrations (0.1–0.25 mg/L) in co-inoculations favouring *L. thermotolerans*, compared to *S. cerevisiae*-dominated fermentations (0.58–0.76 mg/L).

4. Discussion

Wine acidity control is a key challenge in winemaking, especially in warmer wine regions suffering from climate change, where the reduction in organic acids during grape maturation can lead to wines with undesirably high pH levels and low acidity, together with increased sugars levels (Ubeda et al., 2020; Volschenk et al., 2006). Controlling acidity is crucial for maintaining microbial stability, colour stability, and the sensory attributes of the wine. In recent years, there has been growing interest in the use of non-*Saccharomyces* yeasts, such as *L.*

thermotolerans, to modulate acidity during fermentation due to this yeasts ability of producing lactic acid (Vicente et al., 2022).

The present study examined the impact of different inoculation strategies, both co-inoculation and sequential inoculation, using various ratios of industrial *L. thermotolerans* and *S. cerevisiae* strains. The selected *L. thermotolerans* strain (CR311 -Lt253-) was chosen for its acidifying capacity (Vicente, Friedrich et al., 2025). In the sequential inoculation trials, *S. cerevisiae* was added after up to 96 h to allow *L. thermotolerans* sufficient time to express its acidification potential before competition for nutrients intensified under the presence of *S. cerevisiae* (Vicente, Kelanne, Rodrigo-Burgos et al., 2023). These approaches aimed to exploit the acidifying properties of *L. thermotolerans* while ensuring efficient fermentation through *S. cerevisiae*. By varying the inoculation strategies, the goal was to identify optimal combinations for achieving balanced acidity while maintaining adequate fermentative performance. Co-inoculation is usually the preferred method in industrial practice, as it allows for a single inoculation at the start of fermentation without the need for re-inoculations and ensures the dominance of *S. cerevisiae* by the end of fermentation (Englezos et al., 2022).

The rapid production of lactic acid by *L. thermotolerans* in the first 48–72 h has been previously shown (Battjes et al., 2023; Vicente, Benito et al., 2025). This condition could be advantageous, as it allows microbial stabilization through managing pH and total acidity content in the first stages of the fermentation, what reduces the possibility of deviations during the fermentation, as all those related with the emergence of undesired microbial groups (Vion et al., 2024). Organic acids have a more inhibitory effect than pH on microorganisms growth. In the fermentative media, organic acids are present in its undissociated form, being able to permeate through the cellular membrane, and inside the cell, in a neutral pH, this organic acid dissociates, releasing protons, altering intracellular pH and accumulating inside the cell, requiring a carrier to be exported out of the cell (Mira et al., 2010). Additionally, pH reduction contributes to increases in colour intensity, favouring anthocyanins polymerization, as well as increases in tartaric and protein stability of the resulting wines (Vicente et al., 2022).

In the particular case of *L. thermotolerans* producing lactic acid in mixed fermentative conditions together with *S. cerevisiae*, it has been shown to be linked to the ratio and moment of inoculation of the former yeast. It has been reported that sequential fermentation between *L. thermotolerans* and *S. cerevisiae* are associated with higher levels of lactic acid compared to co-inoculation at equal initial inocula (Vicente et al., 2021). This study demonstrated that co-inoculations with higher proportions of *L. thermotolerans* can achieve increases in total acidity and other acidity parameters comparable to those obtained with sequential inoculation. Additionally, we have showed that the inoculum ratio could be employed as an additional strategy to modulate wine acidity, as it allows a moderate degree of acidification control. Previous studies have shown increases in lactic acid concentration when *L. thermotolerans* and *S. cerevisiae* inoculum ratio was 10:1 if compared to equal inoculum concentration, being this factor the one with highest relevance as far as lactic acid production and pH regulation in concerned (Joran et al., 2022). Additionally, other studies assaying co-inoculation at a similar ratio in mixed cultures in natural grape musts, showed that the effects of the interaction between *L. thermotolerans* and *S. cerevisiae* on fermentation (decreases in ethanol and pH, increase in lactic acid compared to a pure culture of *Sc*) were as well affected by the fermentative temperature, being more significant at 20 °C than at 30 °C (Gobbi et al., 2013).

Other acids apart from lactic acid showed variations as well. Several studies have highlighted the strain-dependent behaviour of *L. thermotolerans* regarding malic acid degradation, ranging from 20 to 50 % (Benito, 2018). This ability has been previously studied in mixed fermentations, with a strain specifically selected by this character together with its lactic acid production, both combined with *S. cerevisiae* (Vicente, Kelanne, Rodrigo-Burgos et al., 2023) and *Sch. pombe* (J. Vicente, Kelanne, Navascués et al., 2023). Other acids, such as succinic acid, despite its lower concentrations, contribute not only to final pH but

also to the organoleptic perception of wines, enhancing the mineral character of wines and its overall quality by increasing structure and sensory complexity (Baron & Fiala, 2012; Zaldívar Santamaría et al., 2019).

Our results revealed that the degree of acidification by *L. thermotolerans* could be modulated by adjusting the co-inoculum ratio relative to *S. cerevisiae* and the timing of inoculation. However, excessive acidification negatively impacted the fermentative performance of the yeast community. Specifically, significant residual sugar levels (>4 g/L) (OIV, 2021) were observed at the end of fermentations when total acidity exceeded 8 g/L, corresponding to lactic acid levels above 4.5 g/L. This fact could be related to competition or interaction between the microbial species, or the new physicochemical conditions imposed by the high lactic acid titre.

Previous works regarding co-inoculated fermentations between *L. thermotolerans* and *S. cerevisiae* have shown that the ratio in which both species drive the fermentative process determine its length, despite the influence of this ratio is lower to the impact of other fermentative parameters such as oxygenation or fermentative temperature (Joran et al., 2022). Previous studies have shown that co-inoculating both species at a similar ratio slows fermentation kinetics compared to sequential fermentation at 24 and 48 h (Gobbi et al., 2013). Additionally, using sequential fermentations with five different strains led to higher residual sugar levels than in the *S. cerevisiae* control in all cases (Vicente, Kelanne, Rodrigo-Burgos et al., 2023). These differences could not be solely explained due to the high competitiveness of *L. thermotolerans* under fermentative conditions against *S. cerevisiae*, a fact that has been widely documented (de Celis et al., 2024; Pourcelot et al., 2023), but with the possible role that the increase in lactic acid is playing on the main fermentative species employed in all the cases, *S. cerevisiae*, as the physicochemical conditions of the fermentation are being modified. Consequently, both factors can explain the reduction in the implantation percentages of the selected *S. cerevisiae* under certain co-inoculation ratios showed in this work.

The competition between both species is influenced by various biological factors, including nutrient competition (Roca-Mesa et al., 2020; Shekhawat et al., 2020) and physical contact between cells of different species (Luyt et al., 2024). Organic nitrogen plays an essential role in modulating both species interaction, as the presence of *S. cerevisiae* has been shown to be related with an increase in amino acids metabolism in *L. thermotolerans* (Shekhawat et al., 2020), showing strain-specific requirements as far as nitrogen nutrition is concerned (Roca-Mesa et al., 2020). At the same time, that amino acids scarcity seems to be regulating lactic acid production, as an increase in yield has been observed when this nitrogen source is limiting (Battjes et al., 2023). Nevertheless, not only nitrogen plays an essential role in inter-species competition, as other minor nutrients, such as vitamins, especially, thiamine, plays an important role in this interaction, specially under physical interaction conditions (Conacher et al., 2022; Luyt et al., 2024).

Despite the importance of the biological interaction between strains, the indirect modification of the physicochemical conditions of the fermentative media should be considered too. In our study, *L. thermotolerans* produced significant amounts of lactic acid, reaching nearly 5 g/L, with pH reductions of about 0.35 units and positive correlation with residual sugars, suggesting that higher lactic acid levels may be inhibiting fermentation and affecting the performance of *S. cerevisiae*. It has been shown that high lactic acid concentrations do not inhibit *S. cerevisiae* growth, but, however, lactic acid, even at low concentrations, can modulate the fermentative capacity and efficiency of this yeast (Tanabe et al., 2023; Walker et al., 2020). This effect is strain-dependent and evolutionarily associated with a conserved prion-like element, [GAR⁺] (Jarosz, Brown et al., 2014; Jarosz, Lancaster et al., 2014). This element allows *S. cerevisiae* cells to circumvent glucose repression, showing a basal lower fermentative capacity than prion-deficient cells, and lactic acid has been shown to contribute to the establishment of this metabolic switching systems in the yeast (Tanabe

et al., 2023; Walker et al., 2020).

Other parameters of oenological interest are as well influenced by the different inoculation strategies and the presence of *L. thermotolerans*. Differences in glycerol may be attributed to the negative interaction between glycerol and lactic acid production previously observed in *L. thermotolerans*. Higher lactic acid levels have been associated with lower glycerol and ethanol production, all fermentative products derived from pyruvate (Vicente, Benito et al., 2025); as well as with the high intraspecific diversity related to this trait (Vicente, Vladic et al., 2024). Variations in glycerol content in the resulting wines could impact mouthfeel by reducing viscosity and roundness (Araujo et al., 2021; Pissoni et al., 2023).

Thus, to find an equilibrium between wine acidification and a proper fermentative kinetic, several strategies could be employed. According to our results, the optimum combination of strains is the co-inoculation in a 10:1 ratio (Co-6 + 5), since optimum lactic acid levels are reached, allowing a decrease in pH in around 0.3 units, at the same time that residual sugars are not drastically increased (2 g/L). This inoculum ratio has been previously assayed with successful results (Gobbi et al., 2013). Nevertheless, other approaches could be followed to successfully address this problem, as deep strain selection programs (Alvarez et al., 2023; Gardner et al., 2023; Jiranek, 2024), involving a characterization of strains compatibility (Pourcelet et al., 2023) or from different genetic backgrounds for both species, as well as the analysis of specific nutritional requirements during the fermentation, supporting and enhancing *S. cerevisiae* specific fermentative performance. In the specific case of *S. cerevisiae* including the [GAR⁺] phenotype in the strain selection panels could allow a better compatibility with *L. thermotolerans* strains selected by its lactic acid production for pH and acidity management during alcoholic fermentation (Tanabe et al., 2023; Walker et al., 2020).

The inoculation strategy and the proportion of each yeast strain had a clear influence on the volatile profile of the wines, affecting their aromatic expression. Fermentations with a higher presence of *S. cerevisiae* promoted ester-rich profiles associated with fruity and floral aromas, while *L. thermotolerans* enhanced lactic and fermentative notes, including increased ethyl lactate and specific higher alcohols (J. Vicente et al., 2023; Hranilovic et al., 2021, 2022; Nisiotou et al., 2019). These variations indicate that the combination of yeast strains affects not only acidification and fermentation, but also the sensory characteristics of the final product. Consequently, the aromatic profile is modulated by both the inoculation strategy and the species ratio. Adjusting strain proportions can therefore serve as a tool to tailor wine style according to climate conditions and target market preferences. Nevertheless, to properly evaluate the impact on the sensory profile, larger-scale fermentations should be conducted and assessed by a trained panel to determine their effect on the organoleptic characteristics.

The increase in acidity achieved through the use of *L. thermotolerans* effectively addresses pH-related challenges in wine fermentation but can occasionally lead to deviations in fermentative dynamics. Under controlled conditions, this study provides a solid foundation, supported by statistically significant results, for the potential scaling of these strategies to industrial applications. The findings underscore the importance of strain management in mixed fermentations, where achieving a balance between acidification, pH control, and fermentation performance depends on the careful adjustment of yeast ratios. Beyond highlighting the role of inoculation ratios, this work advocates for a comprehensive approach to fermentation strategy design—one that integrates not only the primary objective, such as acidity regulation, but also other factors influencing the process, beginning with targeted strain selection programmes. To fully characterise the impact of the proposed fermentative strategies, it is necessary to conduct industrial-scale fermentations and sensory panel evaluations in order to accurately assess the fermentation outcomes.

Ethical statement - studies in humans and animals

Not applicable.

CRedit authorship contribution statement

Javier Vicente: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eva Navascués:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization. **Niina Kelanne:** Writing – review & editing, Investigation. **Antonio Santos:** Writing – review & editing, Project administration, Methodology, Investigation, Conceptualization. **Baoru Yang:** Writing – review & editing, Methodology, Investigation. **Santiago Benito:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2025.101306.

Data availability

All data used has been included in the manuscript or Supplementary Material

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