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Impact of *Lachancea thermotolerans* strain and lactic acid concentration on *Oenococcus oeni* and malolactic fermentation in wine

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ABSTRACT

The yeast *Lachancea thermotolerans* can produce lactic acid during alcoholic fermentation (AF) and thereby acidify wines with insufficient acidity. However, little is known about the impact of *L. thermotolerans* on *Oenococcus oeni*, the primary lactic acid bacterium used in malolactic fermentation (MLF). This study explored the impact of sequential cultures of *L. thermotolerans* and *Saccharomyces cerevisiae* on MLF performance in white and red wines. Four *L. thermotolerans* strains were tested in Sauvignon blanc with sequential *S. cerevisiae* inoculation, compared to an *S. cerevisiae* control and the initially un-inoculated treatments. The *L. thermotolerans* wines showed large differences in acidification, and progression of MLF depended on lactic acid production, even at controlled pH. The highest and lowest lactic acid producing strains were tested further in Merlot fermentations with both co-inoculated and sequentially inoculated *O. oeni*. The low lactic acid producing strain enabled successful MLF, even when this failed in the *S. cerevisiae* treatment, with dramatically quicker malic acid depletion in *O. oeni* co-inoculation than in sequential inoculation. In contrast, a high lactic acid producing strain inhibited MLF irrespective of the *O. oeni* inoculation strategy. In a follow-up experiment, increasing concentrations of exogenously added lactic acid slowed MLF and reduced *O. oeni* growth across different matrices, with 6 g/L of lactic acid completely inhibiting MLF. The results confirm the inhibitory effect of lactic acid on *O. oeni* while highlighting the potential of some *L. thermotolerans* strains to promote MLF and the others to inhibit it.

KEYWORDS

Lachancea thermotolerans, lactic acid, wine acidification, *Oenococcus oeni*, malolactic fermentation

Supplementary data can be downloaded through: https://oeno-one.eu/article/view/4657

INTRODUCTION

Because of climate change, historic wine regions across the world are warming rapidly (Jones and Davis, 2000; Moriondo *et al.,* 2013). This commonly results in insufficient acidity in grapes at harvest, leading to high pH wines with increased risks of microbial spoilage and sensory imbalances (Mira de Orduña, 2010). Acidification is therefore a common practice in warmer regions/vintages and is generally achieved through the addition of tartaric acid, and less commonly by using other organic acids and ion exchange techniques (Waterhouse *et al.,* 2016). Due to the additional costs of external inputs, and their potential rejection by consumers, the alternative of microbial modulation of wine acidity is of great interest; particularly via yeast species other than *Saccharomyces cerevisiae* (Benito *et al.,* 2019a; Varela, 2016; Vilela, 2019).

Of the non-*Saccharomyces* yeasts, *Lachancea thermotolerans* shows great potential for bio-acidification (Hranilovic *et al.,* 2017) due to its ability to produce L-lactic acid concurrently with alcoholic fermentation (AF). This occurs via lactate dehydrogenase activity from pyruvate obtained through glycolysis (breakdown of sugars) and thus is an alternative pathway to ethanol production (Hranilovic *et al.,* 2017; Sgouros *et al.,* 2020). Concomitant decreases in ethanol content represent another potential benefit of *L. thermotolerans* modalities, as warm-climate wines are often overly alcoholic (Hranilovic *et al.,* 2021). However, L-lactic acid production varies between *L. thermotolerans* strains, ranging from negligible in some to over 10 g/L in others, although the molecular mechanisms of these differences is still under investigation (Hranilovic *et al.,* 2018; Sgouros *et al.,* 2020).

One challenge in using *L. thermotolerans* is its inability to complete fermentation in grape juice (Hranilovic *et al.,* 2018; Morata *et al.,* 2018), therefore requiring either co-inoculation or sequential inoculation with *S. cerevisiae* or other robust yeast to reach dryness. Of the two strategies, sequential inoculation generally results in a greater impact of *L. thermotolerans* on wine chemical composition, including acidification (Gobbi *et al.,* 2013; Hranilovic *et al.,* 2021). Importantly, from the winemakers' perspective, chemical and sensory profiling of wines co-fermented with *L. thermotolerans* revealed positive characteristics as compared to their respective *S. cerevisiae* controls, including lower concentrations of ethanol, total SO_2, and volatile acidity, and higher concentrations of ethyl esters and terpenes (Benito *et al.,* 2016; Binati *et al.,* 2020; Hranilovic *et al.,* 2018). These sequentially fermented wines were also described by tasters as being "fresher" and "crisper" than wines fermented with *S. cerevisiae* controls (Hranilovic *et al.,* 2021). Following the substantial research on the use of *L. thermotolerans* as starter cultures, several strains are now commercially available for winemaking (Roudil *et al.,* 2020).

L. thermotolerans is actually not the primary source of L-lactic acid in wine. In most fermentations, it arises due to the activity of lactic acid bacteria (LAB), chiefly *Oenococcus oeni*, which decarboxylate L-malic acid to L-lactic acid (Bartowsky *et al.,* 2015). This process, known as malolactic fermentation (MLF), increases the pH of wines and affects their aroma and flavour (Antalick *et al.,* 2012; Sumby *et al.,* 2010; Sumby *et al.,* 2013), but may reduce colour intensity in reds (Abrahamse and Bartowsky, 2012; Burns and Osborne, 2013). MLF is often conducted to decrease wine acidity but it also increases microbial stability as L-malic acid could otherwise be metabolized by wine spoilage organisms (Edwards and Jensen, 1992; Lonvaud-Funel, 1999). However, in wines from warmer climates that already lack sufficient acidity, a further reduction in acidity via MLF may not be desired (Davis *et al.,* 1985).

Typically, MLF is conducted post-AF with sequentially inoculated *O. oeni*. Because of the importance of *O. oeni* to winemaking, much research has been undertaken to understand and improve their resistance to common wine stressors (Jiang *et al.,* 2018; Sumby *et al.,* 2019). Four major stressors that inhibit the growth of LAB in wine are high ethanol, low pH, high SO_2 and extreme temperatures, which can act synergistically to prevent the completion of MLF (Betteridge *et al.,* 2015). To increase the likelihood of successful MLF completion, there is growing interest in co-inoculation of *O. oeni* and yeasts during AF (Bartowsky *et al.,* 2015). Such an approach can be advantageous due to a more favourable physiochemical environment (*e.g.*, lower ethanol) and greater availability of nutrients (Edwards and Beelman, 1989; Zapparoli *et al.,* 2009). Accordingly, LAB co-inoculation was found to result in more rapid completion of MLF without negative impacts on AF performance (Abrahamse and Bartowsky, 2012; Jussier *et al.,* 2006). However, the uptake of co-inoculation by wineries remains

limited because of the potential for increased acetic acid due to the heterofermentative metabolism of *O. oeni* (Bartowsky *et al.,* 2015), even though this remains uncommon under winemaking conditions (Abrahamse and Bartowsky, 2012; Jussier *et al.,* 2006). Co-inoculation of LAB was also found to alter the chemical and sensory properties of wine in comparison to sequential inoculation, in a manner dependent on yeast and LAB strain (Antalick *et al.,* 2013).

There is still much to understand about yeast and bacterial interactions and compatibility in wine. Yeast can produce metabolites such as ethanol, medium-chain fatty acids (MCFA), SO_2 and peptides that are inhibitory to LAB (Bartle *et al.,* 2019). Recent research reported that interactions between strains of *S. cerevisiae* and LAB during co-inoculation can have significant impacts on wine metabolite production (Englezos *et al.,* 2020). Even less is known about interactions between non-*Saccharomyces* yeasts and *O. oeni*, but recent reports described variable compatibility between different strains (Nardi *et al.,* 2019; Ferrando *et al.,* 2020; Martín-García *et al.,* 2020). While the acidification abilities of *L. thermotolerans* are well documented, much less is known about their interactions with *O. oeni* and how this impacts the completion of MLF. Exogenously added L-lactic acid was found to be inhibitory to *O. oeni* (Morata *et al.,* 2020) but whether this is due to the presence of L-lactic acid or the lower pH of the wine remains unclear.

This study aimed to explore the impact of the use of *L. thermotolerans* during alcoholic fermentation on *O. oeni* MLF performance. Furthermore, this study sought to determine if levels of L-lactic acid produced by *L. thermotolerans* could be responsible for inhibition of *O. oeni* and if a co-inoculation strategy could overcome this inhibition.

MATERIALS AND METHODS

1. Yeast and bacteria strains

The yeast strains used in this study included three commercially available L. thermotolerans: LaktiaTM (LAK; Lallemand, Canada); Levulia® Alcomeno (LEV; AEB, Italy); ConcertoTM (CON; CHR Hansen, Denmark) and one strain (UNIFIG18; UNI) characterized previously (Hranilovic et al., 2018). S. cerevisiae strain Zymaflore® Spark (SC, Laffort, France) was the control and used as a sequential inoculum. All strains were stored at -80 °C in 25 % (v/v) glycerol before streaking onto YPD agar plates (yeast extract 1 % w/v, peptone 2 % w/v, glucose 2 % w/v, agar 2 % w/v) and grown for 3 days at 22 °C prior to culture inoculation. The bacterium used in this study was VP41 (Lallemand, Canada) isolated from a commercial freeze-dried preparation. All *L. thermotolerans* strains were used in sequential inoculation with Zymaflore® Spark after 48 h (designated: …SC). An initially uninoculated treatment (IUN) was also included to account for the impact of indigenous microorganisms before inoculation with SC after 48 h. All treatments were triplicated.

2. Inoculum preparation

A single colony of each yeast (LAK, CON, LEV, UNI, SC) was inoculated into a sterile mix (1:1 ratio) of YPD broth (yeast extract 1 % w/v, peptone 2 % w/v, glucose 2 % w/v) and natural grape juice before overnight growth at 28 °C with agitation. Viable and total cell numbers were determined by flow cytometry (Guava® easyCyte™ 12HT, Merck Millipore, Massachusetts, USA) using 0.1 mg/mL propidium iodide in phosphate buffered saline. Cells were inoculated at 2 x 10^6 viable cells/mL. After 48 h, all *L. thermotolerans* and the uninoculated fermentations were inoculated with SC at 10^6 viable cells/mL following the procedure above.

3. Sauvignon blanc winemaking with *L. thermotolerans*

Fermentations were conducted in 2020 Sauvignon blanc (Adelaide Hills, SA, Australia; Supplementary Table 1). Sauvignon blanc fermentations were incubated at 17 °C and fermentation progress monitored daily via weight loss. Fermentations were deemed finished when weight loss was < 0.1 g over 24 h, centrifuged (4,400 x *g*) and the supernatant used for immediate pH and TA measurements. The remaining supernatant was stored with minimal headspace at 4 °C until further use. To explore if differences in MLF performance were due to the pH of the wine or other yeast modalities, MLF was initiated in pH non-adjusted and adjusted wines. Thus 10 ml were adjusted with 10 % tartaric acid solution to pH 3.37 (matching the lowest pH obtained after AF) and another portion was left at the post-fermentation pH. Both treatments were then filter sterilised (0.22 µm), aliquoted into 10 mL test tubes and inoculated with VP41 at 1 g/hL following the manufacturer's instructions.MLF was conducted at 22 °C and monitored enzymatically as described below.

4. Merlot winemaking with *L. thermotolerans*

The highest and lowest (UNI and CON, respectively) among the selected *L. thermotolerans* strains (Hranilovic *et al.,* 2018) were used in red (Merlot) fermentations. These strains were also inoculated sequentially with SC after 48 hours. An SC-only control and IUN…SC treatments were also included. To investigate if the timing of LAB inoculation influenced successful completion of MLF, two *O. oeni* inoculation strategies were concurrently investigated: co-inoculation (CO) at 48 h and sequential inoculation (SQ) post-alcoholic fermentation. In co-inoculation, *O. oeni* were inoculated into the must at the same time as the sequential SC yeast inoculation (48 hours). Sequential inoculation with *O. oeni* occurred at the completion of alcoholic fermentation (19 days). Both CO and SQ inoculation treatments used *O. oeni* VP41 at a rate of 1 g/hL as per the manufacturer's instructions. All yeast-bacteria treatment combinations were performed in triplicate.

Fermentations were conducted in 2015 Merlot juice (Coombe vineyard, Adelaide SA; Supplementary Table 1) that had been frozen for 5 years. Inoculation cultures were prepared as described above and inoculated at 2 x 10^6 cells/mL. After 48 h, the *L. thermotolerans* and IUN fermentations were inoculated with SC at 10^6 cell/mL. At this point, 450 mg/L of diammonium phosphate (DAP) were added to increase yeast assimilable nitrogen (YAN) to 260 mg/L. Fermentation kinetics were monitored by weight loss, and fermentations were considered complete when weight loss was < 0.2 g over 24 h. At the completion of alcoholic fermentation, wines were racked off of gross lees into 50 mL test tubes. MLF was continually monitored as outlined below until complete (< 0.1 g/L of L-malic acid) or until the end of the experiment. Both AF and MLF were conducted in a 22 °C controlled temperature room.

Samples were taken at Days 2, 5, and 8 to quantify yeast and bacterial growth. For yeast growth, non-Saccharomyces and Saccharomyces were tentatively differentiated by comparing the morphology of colonies developing on WL Nutrient Agar (CM 0309, Oxoid, ThermoFisher Scientific, Massachusetts, USA) after 3 days at 28 °C. To quantify LAB growth, samples were diluted up to 10-3 and spotted (2 µL) onto plates of MRSAJ (MRS, AM 103 and SP 437, Amyl Media, Victoria, Australia, with 20 % filtered apple juice (0.22 µm) and 2 % (w/v) agar. For wines that were not sterile filtered, MRSAJ was supplemented with 10 mg/L of cycloheximide immediately before plating to inhibit the growth of *S. cerevisiae* and *L. thermotolerans* yeast (Kurtzman *et al.,* 2011). Plates were incubated at 30 °C with a 20 % CO2 concentration for seven days prior to colony counting.

5. Impact of L-lactic acid on MLF

To determine if the inhibitory impact of some *L. thermotolerans* strains was due to L-lactic acid or other compositional alterations by *L. thermotolerans, O. oeni* performance was tested in wines spiked with increasing levels of lactic acid. Besides Sauvignon blanc and Merlot CON…SC wines, this experiment was conducted in fermented Chemically Defined Grape Juice Medium (CDGJM; Jiranek *et al.,* 1995) containing 200 g/L sugar and 350 mg/L YAN. CDGJM was fermented by the same *L. thermotolerans* modalities with the addition of 5 % w/v of GrapeEX (Tarac Technologies, Australia), a commercial tannin preparation, to create Red Chemically Defined Wine (RCDW). The wines (Supplementary Table 2) were spiked with 0, 1.5, 3, 6, and 9 g/L of L-lactic acid (~40 % solution), and adjusted to pH 3.60 (5M NaOH or HCl), to assess the impact of L-lactic acid on *O. oeni* separately of pH. The 10 mL aliquots were inoculated with *O. oeni* VP41 as per above. Bacterial growth was monitored as above, and L-malic acid concentration was monitored as outlined below.

6. Wine chemical analysis

Primary amino nitrogen, ammonia, residual sugar (RS), acetic acid and glycerol were determined enzymatically using appropriate kits following the manufacturer's instructions (SKU: 4B110, 4B120, 4B140, Vintessential® Laboratories, Australia; K-ACETRM and K-GCROLGK, Megazyme, Ireland). L-malic acid was determined using reagents (GOT, MDH, and NAD+) obtained from Megazyme following the protocol in Bartle (2020). L-lactic acid was determined enzymatically (SKU: 4A150, Vintessential® Laboratories) with modifications (all reagent volumes reduced 10-fold) for use in a microplate spectrometer.

Titratable acidity was determined with a Mettler Todelo T50 Autotitrator where 10 mL of wine were titrated with 0.33 M NaOH. Free and total SO_2 were measured using the aspiration-titration method (Iland *et al.,* 2013). Ethanol was determined using HPLC (Hranilovic *et al.,* 2018) for white wines and an Alcolyser (Anton Paar, Graz, Austria) for red wines.

7. Statistical analysis

Chemical parameters in Sauvignon blanc and Merlot wines were analysed using a one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) to determine the impact of the treatment groups. Principal component analysis (PCA) was performed on the entire chemical data set for each wine. Statistical analyses were run in XLSTAT (Addinsoft, Paris, France) with significance thresholds set at 5 %. GraphPad Prism (San Diego, CA, USA) was used for the visual representation of the data.

RESULTS

1. Alcoholic Fermentation in Sauvignon blanc

Fermentations were regularly monitored for fermentation and acidification kinetics (Figure 1A). All sequentially inoculated treatments resulted in 3–5 days slower fermentation compared to *S. cerevisiae* alone (Figure 1A). Among *L. thermotolerans* fermentations, CON completed before the remaining *L. thermotolerans* and IUN treatments. The fermentations greatly differed in their rate and extent of acidification. At 48 h, the largest drop in pH (~0.25 units) was seen for UNI…SC and LEV…SC. For others, the initial decrease appeared more modest, ahead of increases in pH over the remainder of the experiment in all cases except UNI…SC (Figure 1B). The final pH of SC, CON…SC, and IUN…SC was marginally higher (pH 3.77–3.83) than the initial pH of the juice (*i.e.*, 3.67).

All Sauvignon blanc fermentations progressed to dryness (< 4 g/L RS; Table 1). UNI…SC trended toward the lowest ethanol content (12.16 % v/v; Table 1), compared to the SC control (12.71 % v/v), although differences were not statistically significant. Differences ($P < 0.001$) were, however, found in pH/TA of wines (Table 1), but these were not always clearly linked to the 60-fold variation in yield of lactic acid. Certainly, the highest producers of L-lactic acid (UNI…SC and LEV…SC) showed the lowest pH (3.44 and 3.54, respectively). In the case of IUN…SC, which contained a relatively modest 0.9 g/L of lactic acid, the pH (3.83) was actually higher than the SC wines (3.77), likely due to a concomitant, 12 % reduction in malic acid content (Table 1). The SC wine also contained the highest amount of total SO_2 (23 mg/L), with comparable amounts present only in the initially un-inoculated wine (Table 1).

TABLE 1. Main oenological parameters of Sauvignon blanc wines produced with different strains of *L. thermotolerans* sequentially inoculated with *S. cerevisiae* after 48 hours.

Yeast treatment	glucose-fructose (g/L)	ethanol (% vol/vol)	pH	titratable acidity (g/L of H_2T)	L-lactic acid (g/L)	L-malic acid (g/L)	acetic acid (g/L)	total SO_2 (mg/L)	glycerol (g/L)
SC	0.5 ± 0.2^{a}	12.71 ± 0.34^{a}	3.77 ± 0.02^{a}	$7.1\ 0.3^{d}$	0.1 ± 0.1^{c}	4.72 ± 0.20^{a}	0.17 ± 0.01^{a}	23 ± 5^{a}	5.9 ± 0.3^{b}
LAK…SC	0.1 ± 0.1^{bc}	12.24 ± 0.31^{a}	3.66 ± 0.02^{b}	8.7 ± 0.2^{c}	1.5 ± 0.4^{bc}	4.24 ± 0.01^{ab}	0.11 ± 0.03^{bc}	12 ± 5^{bc}	6.9 ± 0.2^{ab}
CON…SC	0.0 ± 0.0^{c}	12.55 ± 0.25^{a}	3.77 ± 0.01^{a}	7.1 ± 0.0^{d}	0.2 ± 0.0^{c}	4.59 ± 0.24^{ab}	0.13 ± 0.01^{ab}	8 ± 2^{c}	6.9 ± 0.4^{ab}
LEV…SC	0.2 ± 0.0^{bc}	12.38 ± 0.31^{a}	3.54 ± 0.02^{c}	10.4 ± 0.2^{b}	2.8 ± 0.5^{b}	4.23 ± 0.08^{ab}	$<0.10 \pm 0.00^{c}$	9 ± 2^{bc}	6.9 ± 0.8^{ab}
UNI…SC	0.2 ± 0.2^{bc}	12.16 ± 0.36^{a}	3.44 ± 0.06^{d}	13.3 ± 1.2^{a}	6.1 ± 1.8^{a}	4.17 ± 0.01^{b}	0.10 ± 0.02^{bc}	5 ± 3^{c}	7.2 ± 0.5^{a}
IUN…SC	0.4 ± 0.1^{ab}	12.47 ± 0.18^{a}	3.83 ± 0.02^{a}	6.3 ± 0.1^{d}	0.9 ± 0.4^{bc}	3.50 ± 0.31^{c}	0.10 ± 0.02^{bc}	17 ± 1^{ab}	6.4 ± 0.6^{ab}

*Error values represent one standard deviation of 3 replicates. Letters represent statistically significant similar groupings determined by Tukey's HSD test. Parameters measured at the end of alcoholic fermentation.

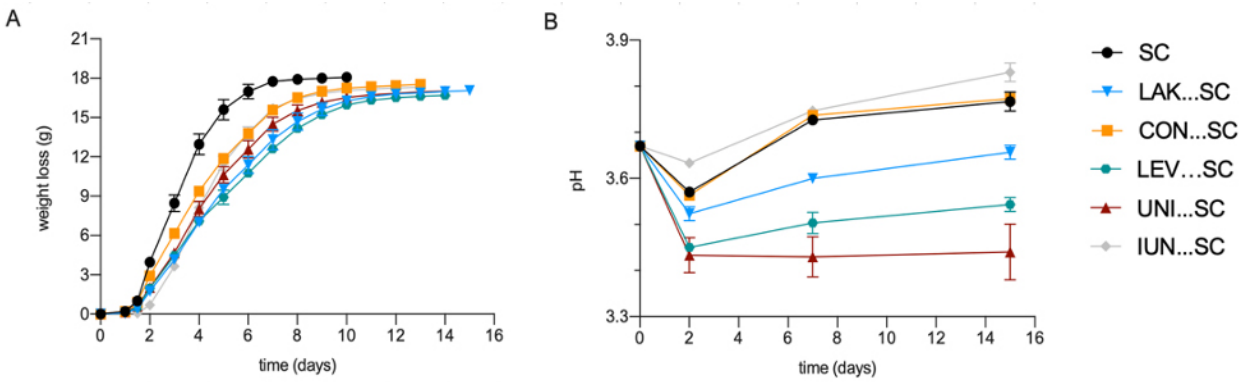

FIGURE 1. Alcoholic fermentation kinetics (A) and pH (B) of Sauvignon blanc fermented with different *L. thermotolerans* yeast modalities.

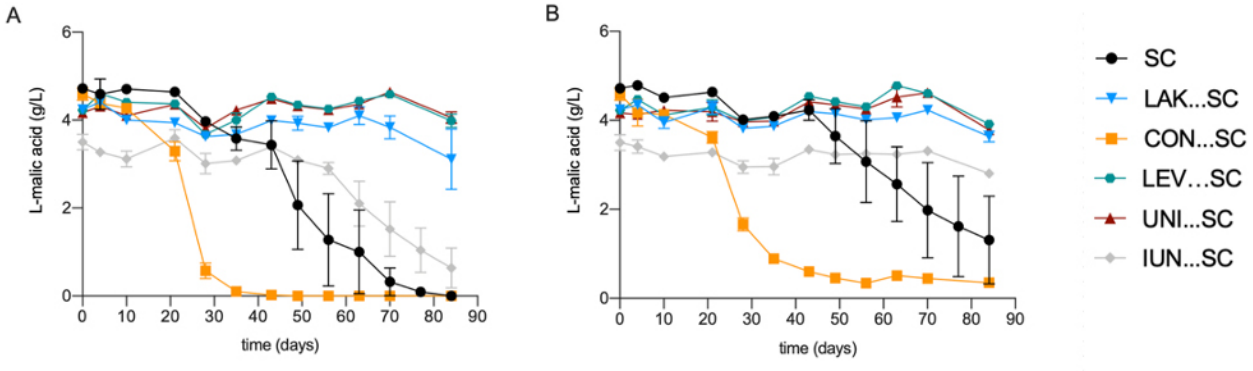

FIGURE 2. Progression of MLF in Sauvignon blanc wines produced with different *L. thermotolerans* strains at initial pH (A) and an adjusted pH of 3.4 (B).
Error bars represent the standard error of three replicates.

All *L. thermotolerans* wines had lower total SO_2 concentrations than initially present in the juice (22 mg/L; Table S1). Glycerol varied between 5.9 and 7.2 g/L in SC and UNI…SC wines, respectively, while acetic acid concentrations remained low (< 0.2 g/L) across all treatments (Table 1).

2. Impact of L. thermotolerans yeast on MLF in Sauvignon blanc

The impact of yeast modalities on MLF performance with sequentially inoculated *O. oeni* was evaluated with and without pH standardisation. One set of Sauvignon blanc wines remained at the pH value attained at the end of AF (Table 1), while the other was adjusted to 3.4 (the lowest attained pH) using tartaric acid. Large differences were evident in MLF progress depending on the yeast treatment, with MLF completing in some wines and no evidence of MLF in others (Figure 2). Among the former, CON…SC and SC were first and second, respectively, to complete MLF in the pH un-adjusted wines and showed the greatest malic acid consumption in the pH standardised wines (Figure 2). These wines contained the lowest initial concentrations of L-lactic acid (Table 1). At the other extreme, bio-acidified wines with the highest L-lactic acid content, *i.e.*, UNI…SC and LEV…SC, showed no change in malic acid (Figure 2).

3. AF and MLF in Merlot wines

Fermentations in Merlot investigated four yeast treatments: sequential inoculation with the *L. thermotolerans* strains showing the highest and the lowest lactic acid production (UNI and CON, respectively), alongside the SC and initially un-inoculated control (IUN). For each yeast treatment, two LAB inoculation strategies were trialled: co-inoculation (CO) and sequential inoculation (SQ). As seen in Sauvignon blanc, the SC treatment was the first to finish alcoholic fermentation (Figure 3A), while the *L. thermotolerans* treatments exhibited slower fermentation rates (Figure 2B). Decreases in pH occurred in all treatments, but the largest decreases were seen for UNI…SC, whether under co- or sequential bacteria inoculation (Figure 3B).

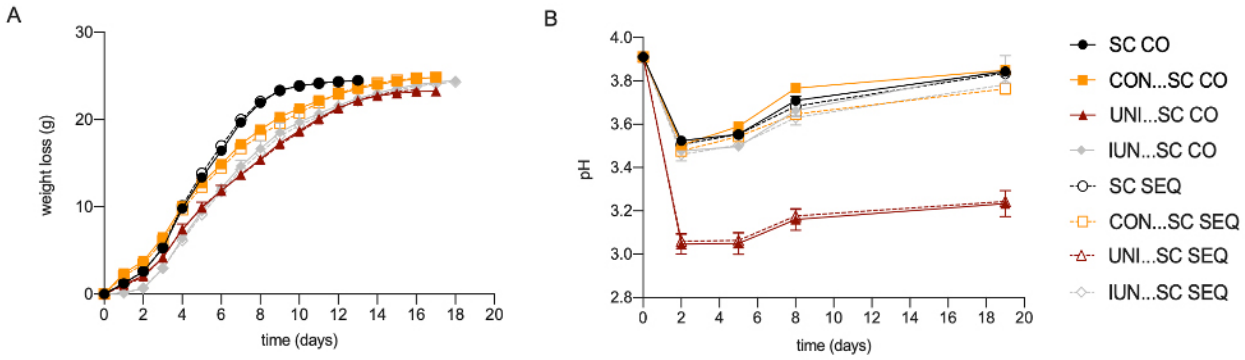

FIGURE 3. Fermentation kinetics (A) and pH (B) of Merlot fermented with different *L. thermotolerans* yeast and LAB modalities.

Error bars represent the standard deviation of three replicates.

To better understand the cause of the observed trends, several parameters were analysed at the point of sequential SC inoculation and LAB co-inoculation (48 h). Fermentation with CON progressed further than those with SC (Table 2), UNI…SC was slower, while the IUN treatment was slowest (Table 2; Figure 2A). Interestingly, significant differences in YAN levels were not detected between treatments, IUN...SC included (Table 2). Large differences in pH between NI…SC and the other yeast treatments were seen (Figure 3B; Table 2), linked to 40-fold differences in L-lactic acid concentrations between SC (0.2 g/L) and UNI…SC (8.0 g/L). The UNI…SC fermentations also contained up to 0.2 g/L less L-malic acid at this time point than CON and UN fermentations (Table 2).

All treatments completed AF (< 4 g/L of RS; Table 3) with ethanol content lower in UNI…SC Merlot wines (15.1 % v/v) compared with the SC control (16.2 % v/v). UNI…SC wines also had low pH (< 3.30) and high TA (> 13 g/L), associated with over 10 g/L of L-lactic acid. In comparison, SC, CON…SC, and IUN…SC all had pH values ≥ 3.80, only < 6 g/L TA and < 2 g/L L-lactic acid. Glycerol ranged from 8.2 to 11.6 g/L, while acetic acid did not exceed ~0.7 g/L, irrespective of *O. oeni* treatment. Total SO_2 levels were low (< 3 g/L) across all treatments.

Because Sauvignon blanc wines fermented with high L-lactic acid producing *L. thermotolerans* strains failed to complete MLF when sequentially inoculated with *O. oeni*, co-inoculation was explored in Merlot. Interestingly, neither SC treatment finished MLF, while in *L. thermotolerans* treatments the effectiveness of co-inoculation for MLF depended on the *L. thermotolerans* strain (Figure 4). For the low L-lactic acid producer, CON, co-inoculated treatments finished MLF by Day 8, before the end of AF (Figure 4A). These treatments also had the highest population of bacteria (> 10^7 cfu/mL on Day 8; Figure 4B). Two of three replicates of sequentially inoculated CON…SC wines also finished MLF, albeit in a delayed manner (> 50 days). For UNI, initial decreases in L-malic acid occurred up to 8 days after *L. thermotolerans* inoculation and remained stable thereafter at about 1.2 g/L (Figure 4A). These trends were seen in both *O. oeni* treatments, with no bacteria recoverable after Day 5 (Figure 4B). CO IUN…SC fermentations also finished MLF (54 days), unlike the SQ IUN…SC ones (Figure 4A).

4. Multivariate analysis of chemical parameters of Sauvignon blanc and Merlot wines

Chemical parameters of Sauvignon blanc (Table 1) and Merlot (Table 3) wines were subjected to PCA. In Sauvignon blanc, the first component (PC1) explained 50.5 % of the variance and separated the SC control and the highest lactic acid-producing UNI…SC treatment (Figure 5A). The UN…SC was positioned closest to the SC while the remaining *L. thermotolerans* treatments were positioned in between based on their acidification extent. Accordingly, the separation along PC1 was driven by increases in L-lactic acid and TA, as opposed to high pH, total SO_2, acetic acid and ethanol (Figure 5B). The second component (PC2) separated CON…SC wine from the remaining treatments (Figure 5A), accounting for 18.3 % of the variance, and was positively correlated with glycerol and negatively correlated with residual sugar (Figure 5B).

TABLE 2. L-malic acid, L-lactic acid, pH, and weight loss of Merlot fermentations before sequential inoculation with yeast and bacteria (48 hours).

Yeast Treatment	L-lactic acid (g/L)	pH	L-malic acid (g/L)	YAN (mg/L)	CO_2 release (g)
SC	0.2± 0.0 [b]	3.51 ± 0.01 [a]	1.68± 0.40 [a]	28 ± 6 [a]	5.3 ± 0.1 [b]
CON…SC	0.8 ± 0.2 [b]	3.49 ± 0.01 [a]	1.76 ± 0.08 [a]	27 ± 4 [a]	6.3 ± 0.3 [a]
UNI…SC	8.1 ± 1.7 [a]	3.05 ± 0.03 [b]	1.57 ± 0.04 [b]	25 ± 9 [a]	4.2 ± 0.2 [c]
IUN…SC	0.3 ± 0.0 [b]	3.47 ± 0.02 [a]	1.75 ± 0.06 [a]	24 ± 6 [a]	2.9 ± 0.1 [d]

*Error values represent the standard deviation of 6 replicates as at this time point O. oeni inoculation had not occurred in any of the wines. Letters represent statistically significant groupings determined by Tukey's HSD test.

TABLE 3. Main oenological parameters of Merlot wines fermented with different yeast and bacteria modalities at the end of alcoholic fermentation.

Yeast Treatment	Bacteria Treatment	RS (g/L)	pH	titratable acidity (g/L)	L-lactic acid (g/L)	ethanol (% vol/vol)	L-malic acid (g/L)	acetic acid (g/L)	glycerol (g/L)
SC	CO	0.66 ± 0.46 [b]	3.81 ± 0.01 [ab]	5.21 ± 0.06 [b]	0.1 ± 0.1 [b]	16.27 ± 0.06 [a]	1.60 ± 0.03 [a]	0.27 ± 0.08 [bc]	9.9 ± 0.2 [abc]
	SQ	0.65 ± 0.36 [b]	3.83 ± 0.02 [ab]	4.88 ± 0.18 [b]	0.3 ± 0.1 [b]	16.21 ± 0.02 [a]	1.35 ± 0.04 [ab]	0.17 ± 0.05 [c]	9.0 ± 0.4 [bc]
CON…SC	CO	1.31 ± 0.61 [ab]	3.85 ± 0.01 [ab]	5.32 ± 0.31 [b]	1.9 ± 0.1 [b]	15.37 ± 0.20 [b]	0.00 ± 0.00 [c]	0.44 ± 0.09 [ab]	11.6 ± 0.7 [a]
	SQ	1.25 ± 0.26 [ab]	3.84 ± 0.05 [ab]	4.91 ± 0.46 [b]	1.4 ± 0.3 [b]	15.43 ± 0.09 [b]	0.15 ± 0.26 [c]	0.23 ± 0.03 [bc]	10.5 ± 0.1 [ab]
UNI…SC	CO	1.51 ± 0.55 [ab]	3.22 ± 0.05 [c]	14.63 ± 2.03 [a]	11.5 ± 4.1 [a]	15.09 ± 0.20 [b]	1.21 ± 0.05 [b]	0.39 ± 0.04 [bc]	8.3 ± 1.6 [bc]
	SQ	1.72 ± 0.57 [ab]	3.27 ± 0.02 [c]	13.59 ± 1.17 [a]	10.2 ± 1.8 [a]	15.14 ± 0.12 [b]	1.25 ± 0.05 [b]	0.28 ± 0.06 [bc]	8.2 ± 0.7 [c]
IUN…SC	CO	2.02 ± 0.80 [ab]	3.89 ± 0.03 [a]	4.53 ± 0.54 [b]	1.0 ± 0.2 [b]	16.12 ± 0.02 [a]	0.06 ± 0.09 [c]	0.69 ± 0.19 [a]	8.9 ± 0.4 [bc]
	SQ	2.37 ± 0.80 [a]	3.80 ± 0.01 [b]	5.25 ± 0.32 [b]	0.4 ± 0.1 [b]	16.07 ± 0.08 [a]	1.07 ± 0.15 [b]	0.66 ± 0.06 [a]	8.5 ± 1.0 [bc]

*Error values represent the standard deviation of 3 replicates. Letters represent statistically significant groupings determined by Tukey's HSD test.

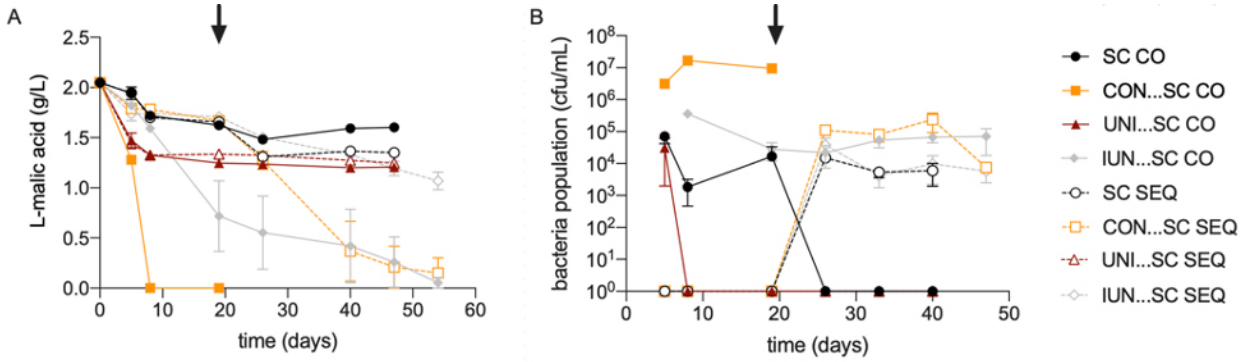

FIGURE 4. Malic acid consumption (A) and LAB population dynamics (B) in Merlot fermentations under different yeast and LAB modalities.

The arrow refers to the time point of sequential *O. oeni* inoculation (Day 19). Error bars represent the standard error of three replicates.

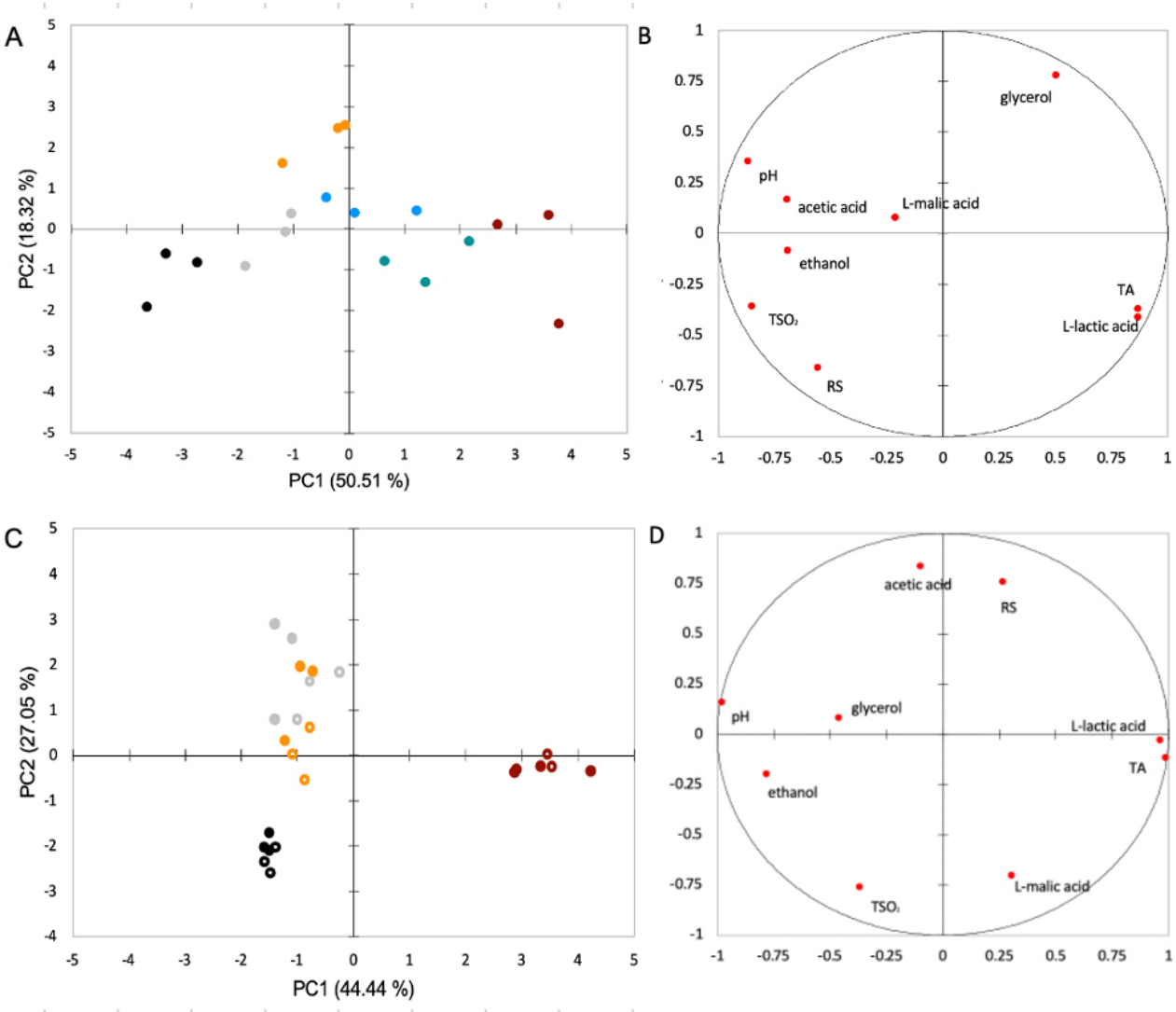

FIGURE 5. Principal component analysis of basic wine chemical parameters for Sauvignon blanc (A and B) and Merlot (C and D) wines.

Figures A and C are the experimental observations and B and D correlation circles. Yeast treatments: SC (black), CON…SC (orange), LAK…SC (blue), LEV…SC (green); UNI…SC (brown); and IUN…SC (grey). *O. oeni* co-inoculation and sequential inoculation are represented with open and closed circles, respectively.

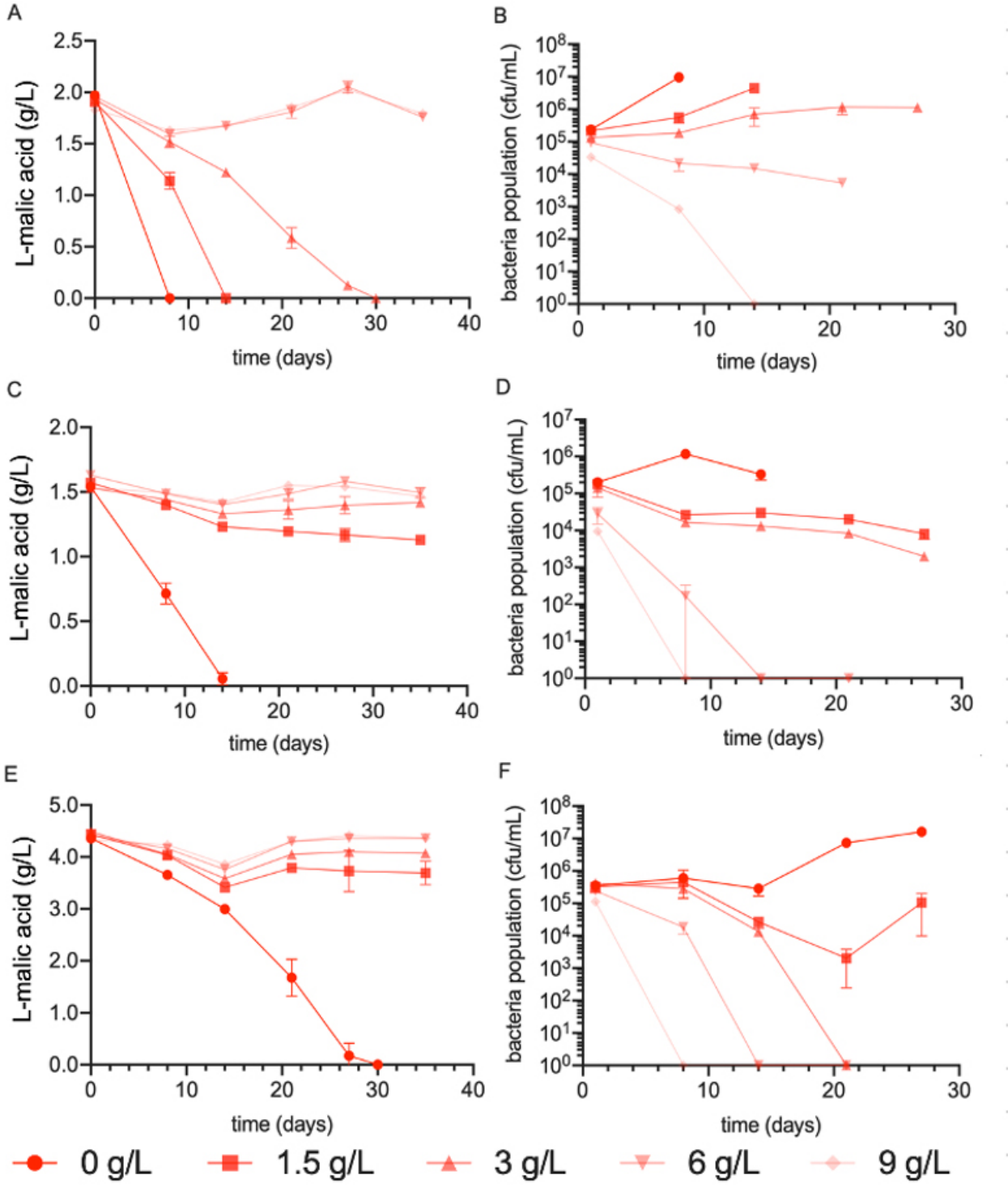

FIGURE 6. Impact of L-lactic acid concentration on L-malic acid consumption and LAB population dynamics in three wine or wine-like matrices: RCDW (A, B), Merlot (C, D), and Sauvignon blanc (E, F).

In Merlot, PC1 explained 44 % of the variation and differentiated the bio-acidified UNI…SC wine from all the other treatments (Figure 5C). Again, the separation of UNI…SC was driven by increases in L-lactic acid and TA, and decreases in pH and ethanol (Figure 5C). PC2 explained 27 % of the variance and separated the SC wines (lower left quadrant) from the IUN…SC and *L. thermotolerans* treatments (Figure 5C). The SC control was associated with higher L-malic acid and total SO_2, and lower acetic acid and residual sugars (Figure 5D). PCA resulted in the separation of yeast treatments, in particular SC control, UNI and co-inoculated CON and IUN treatments. However, within these groups, the two different *O. oeni* inoculation strategies remained unresolved (Figure 5C).

5. Impact of L-lactic acid on *O. oeni* growth and MLF

The impact of lactic acid on MLF was further tested in three matrices (RCDW, Merlot and Sauvignon blanc) produced with the same yeast treatment (CON…SC) and spiked with different amounts of L-lactic acid with pH standardization (pH 3.6). All unspiked treatments completed MLF in a matrix-dependent manner: 7 days in RCDW, 14 days in Merlot and 30 days in Sauvignon blanc (Figure 6). The concentration-dependent inhibition of MLF was particularly apparent in RCDW, and corresponded to slower MLF, with durations doubling relative to the unspiked control (7 days) with each additional 1.5 g/L of L-lactic acid (Figure 6A). Additions beyond this (*i.e.*, 6 and 9 g/L) resulted in complete inhibition of MLF in RCDW. In Merlot and Sauvignon blanc, the addition of only 1.5 g/L of L-lactic acid prevented MLF completion over this time frame. It appears that some malic acid metabolism occurred in Merlot and Sauvignon blanc wines spiked with 1.5 g/L of L-lactic acid (Figures 6C and 6E); however, this drop was no more than 21 % (SB) and 26 % (Merlot) in comparison to the starting malic acid levels, showing that even a relatively small concentration of lactic acid can inhibit MLF completion.

Trends in LAB population dynamics further supported the inhibitory effect of L-lactic acid on *O. oeni*. In RCDW without added L-lactic acid, rapid MLF completion corresponded to the highest LAB population density, 10^7 cfu/mL by Day 7. Upon addition of 1.5 g/L and 3 g/L L-lactic acid, LAB growth was delayed and/or reduced (Figure 6B), while 6 g/L and 9 g/L of L-lactic acid elicited a decline in the LAB population, with the latter leading to complete loss of culturable cells by Day 14 (Figure 6B). In unspiked Merlot and Sauvignon blanc wines, the LAB population increased, whereas the addition of L-lactic acid lead to a decline in inoculation numbers (Figures 6D and 6F). This decline was steepest in wines spiked with the highest concentrations of L-lactic acid (Figure 6D and 6F).

DISCUSSION

In the wine industry, there is a growing number of non-*Saccharomyces* strains available for use as starter cultures to modulate chemical and sensory parameters of wines, including acidity (Benito *et al.,* 2019b). However, limited knowledge exists on interactions between non-*Saccharomyces* yeast and the LAB responsible for MLF, chiefly *O. oeni* (Englezos *et al.,* 2020; Martín-García *et al.,* 2020). Interactions between LAB and *L. thermotolerans* are particularly interesting as *L. thermotolerans* strains are capable of producing L-lactic acid and thereby markedly lowering the pH of the wine. Low pH has a negative impact on LAB growth and can slow the rate of MLF, as shown in numerous studies (Costello *et al.,* 2012; Davis *et al.,* 1986; Rosi *et al.,* 2003). Inhibitory effects of L-lactic acid on LAB have been reported (Hsiao and Siebert, 1999; Morata *et al.,* 2020a; Nakai and Siebert, 2004). For example, 2.83 g/L of lactic acid was defined as the inhibitory threshold for a strain of *O. oeni* (Nakai and Siebert, 2004), albeit in a synthetic medium and at a pH (5.25) exceeding winemaking conditions. More recent research confirmed inhibition of MLF upon lactic acid addition to wine, but it was unclear whether this inhibition occurred due to lactic acid or the pH decrease (Morata *et al.,* 2020). Up to now, published work on *L. thermotolerans* has not found evidence of MLF inhibition (Du Plessis *et al.,* 2017; Fairbairn *et al.,* 2021). However, there is large variability among *L. thermotolerans* strains in terms of L-lactic acid production (Hranilovic *et al.,* 2017, 2018), and information on MLF performance in the presence of high L-lactic acid producers is lacking.

This study confirmed the strain-specific performance of sequential inoculations of *L. thermotolerans* strains, which corresponded to their characterisation in pure cultures (Hranilovic *et al.,* 2018) and co-cultures (Hranilovic *et al.,* 2021), alike. In particular, a large variation in L-lactic acid production (and the consequent pH/TA modulation) was observed across the strains studied here (Figure 6).

The pH and the TA of the CON wines were comparable to the SC controls, while the UNIF wines were more than 0.3 and 0.5 units lower than the SC controls in Sauvignon blanc and Merlot, respectively. Besides a marked effect on wine acidification, Merlot wines also differed in ethanol content, with up to 1.1 % v/v less ethanol in the LT than the SC control wines (Table 2). All inoculated wines produce similar amounts of acetic acid and glycerol, while the total SO_2 only increased in the SC control, remaining constant or decreasing in *L. thermotolerans* inoculations, as observed previously (Benito *et al.,* 2016; Hranilovic *et al.,* 2021). Post-AF, Sauvignon blanc wines were sequentially inoculated for MLF with and without pH standardisation, while both co- and sequential inoculation of LAB were investigated in Merlot.

In Sauvignon blanc, the success of MLF with sequentially inoculated LAB was related to the L-lactic acid content of the wines. Treatments with high concentrations (LEV…SC, UNI…SC) did not start MLF, while wines with the lowest L-lactic acid (CON…SC and SC) completed MLF. At intermediate L-lactic acid contents (IUN…SC and LAK…SC), partial consumption of L-malic acid was seen. Within the same yeast treatment, lower pH negatively impacted MLF, which agrees with multiple previous studies (Betteridge *et al.,* 2015, Liu and Gallander, 1983). However, the concentration-dependent effect of L-lactic acid on MLF inhibition was also apparent in the pH-adjusted wines, suggesting that failed MLF cannot be explained solely by lower pH due to L-lactic acid production.

Co-inoculation with *O. oeni* was explored in Merlot with the expectation that at this stage the fermenting must may not be as inhibitory as post-AF, facilitating MLF. Importantly, neither of the two *O. oeni* inoculation strategies lead to MLF completion in the SC control, showcasing the erratic nature of MLF due to the sensitivity of *O. oeni* to a range of winemaking stressors (Betteridge *et al.,* 2015; Sumby *et al.,* 2019). In contrast, CON showed compatibility with the tested *O. oeni* strain, resulting in MLF completion under both *O. oeni* inoculation regimes (Figure 4). Lower ethanol concentrations in CON Merlot wines as compared to the SC control could partially explain the differences in MLF success. However, faster MLF in Sauvignon blanc CON wines in comparison to the SC control, despite similar ethanol levels, suggests that other compositional changes to the wine matrix are likely to play a role. One example is medium-chain fatty acids (MCFA), well-known inhibitors of *O. oeni* (Bartle *et al.,* 2019; Carreté *et al.,* 2006; Guilloux-Benatier *et al.,* 1998; Wibowo *et al.,* 1988). Recent work on these same strains found that sequential *L. thermotolerans* fermentations contained significantly lower concentrations of MCFA in comparison to the SC control (Hranilovic *et al.,* 2021). As for different *O. oeni* inoculation regimes, in CON wines, MLF duration was dramatically shortened in co-inoculation as compared to the sequential inoculation, which moreover was the only successful MLF strategy for the initially un-inoculated treatment (Figure 4). This aligns with the claimed benefits of *O. oeni* co-inoculation (Bartowsky *et al.,* 2015) but further experiments with continuous monitoring of a wider range of metabolites, coupled with transcriptomics, are required to understand the molecular mechanisms driving the differences in MLF performance with different LAB inoculation strategies within each yeast treatment.

In UNI treatments, MLF was inhibited in sequentially inoculated *O. oeni*, but also in co-inoculated *O. oeni* treatments (Figure 4). Initial decreases in malic acid were observed during AF irrespective of the bacteria inoculation regime and were thus linked to partial malic acid consumption by *L. thermotolerans* rather than *O. oeni*, as shown previously (Hranilovic *et al.,* 2018). The majority of L-lactic acid production occurred during the early stages of fermentation, prior to LAB co-inoculation (> 8 g/L; Table 2), which is in accordance with previous research (Benito *et al.,* 2016; Gatto *et al.,* 2020; Hranilovic *et al.,* 2021). *O. oeni* co-inoculation thus failed as a strategy to overcome unsuccessful MLF with a high L-lactic acid producer under the described conditions (Table 2).

Lactic acid was definitively shown to inhibit *O. oeni* and MLF across three matrices (RCDW, Merlot, and Sauvignon blanc; Figure 6). Increasing L-lactic acid concentrations were associated with impaired *O. oeni* growth, and in turn, slower MLF, in wines at a constant pH. However, the concentration at which MLF was completely inhibited varied depending on the matrix. For example, in RCDW, MLF completed even when the matrix was spiked with 3 g/L of L-lactic acid (for an initial concentration of 4.2 g/L), but did not complete when spiked with 6 g/L. In Merlot and Sauvignon blanc, MLF only completed the unspiked treatment despite partial MLF in the 1.5 g/L spiked treatments.

L-lactic acid contents of 6 g/L or more were enough to completely inhibit MLF in all matrices. This data suggests that, while MLF is inhibited by L-lactic acid, the concentration of L-lactic acid required to completely inhibit successful MLF varies depending on other matrix factors (*e.g.*, ethanol, SO_2, and nutrient availability) and further investigation would be useful to determine this threshold in a variety of wine contexts.

The inhibitory mechanisms of lactic acid on *O. oeni* remain largely elusive. There is some evidence that L-lactic acid may have an inhibitory impact during MLF due to its role in energy generation for *O. oeni*. During MLF, L-malic acid is directly decarboxylated to L-lactic acid and CO_2 and then transported out of the cell. However, if the concentration of lactic acid outside the cell is too high, lactic efflux could be inhibited (Henick-Kling, 1993). In addition, lactic acid from outside the cell may be transported into the cell, decreasing the intracellular pH and adding additional stress. The genetic mechanisms for lactic acid transport in *O. oeni* are uncharacterised and further investigation would do much to elucidate the causes of the observed inhibition.

This paper describes the inhibition of *O. oeni* by both yeast-derived (Figures 3 and 4) and exogenously added L-lactic acid (Figure 5), which suggests that certain *L. thermotolerans* modalities could be used to prevent undesired MLF. While MLF remains common practice to ensure microbial stability and stylistic distinctness of certain wines (Bartowsky *et al.,* 2015), some winemakers and researchers question its necessity in warmer areas given the additional increases in pH/loss of acidity (Burns and Osborne, 2013; Davis *et al.,* 1985). This concept was recently revisited, with fumaric acid being added to the list of permitted wine additives for wine acidification and recent research showing its role in MLF inhibition (Morata *et al.,* 2020). Other common methods to prevent MLF and ensure microbial stability, chiefly the addition of SO_2 or sterile filtration, can be expensive, impractical in wines destined for long-term storage/ageing, and may negatively impact wine sensory profile and/or consumer acceptance (Bartowsky, 2009; Mierczynska-Vasilev & Smith, 2015). This study shows that certain *L. thermotolerans* modalities can produce sufficient quantities of L-lactic acid to inhibit *O. oeni* and MLF even in the presence of little or no SO_2, thereby offering reduced processing time and preservative use.

Optimization of this technique to prevent MLF requires added investigation. First, it remains to be seen if high L-lactic acid production associated with some *L. thermotolerans* strains is maintained in large volume industrial settings with precisely defined inoculation regimes (*e.g.*, inoculation densities and timing). Second, it remains to be seen if L-lactic acid inhibition of MLF is consistent across different *O. oeni* strains, as it could vary in the same way that ethanol and SO_2 sensitivity do (Sumby *et al.,* 2019). While L-lactic acid may inhibit *O. oeni*, it is still unknown if it has the same impact on other LAB species with the ability to metabolize malic acid, such as *Lactiplantibacillus plantarum*. Deletions of aquaglyceroporins GlpF1 and GlpF4 in the oenologically relevant species *L. plantarum* showed growth delay under lactic acid stress. Furthermore, while the genes for these proteins were found to be conserved within many *Lactobacillales*, they were absent in *O. oeni* (Bienert *et al.,* 2013). This may indicate that other species of LAB have different responses to lactic acid stress than seen in *O. oeni*. It is also important for winemakers to ensure that the inhibition of MLF by *L. thermotolerans* does not leave the wine more susceptible to spoilage by other organisms (*e.g.*, *Acetobacter* spp*., Brettanomyces bruxellensis*). Finally, while lactic acid production by *L. thermotolerans* has generally been regarded as beneficial by increasing the acidity of warmer climate wines, excessive production of lactic acid is likely to result in overly acidic wines. Finding the right balance between lactic acid required for MLF inhibition and desirable sensory profiles thus remains to be explored.

CONCLUSION

This paper investigates the impact of sequential inoculation of different *L. thermotolerans* strains on *O. oeni* and the success of MLF. The results highlighted the contrasting behaviour of *L. thermotolerans* strains not only in terms of bio-acidification but also their impact on MLF. The use of low lactic acid producing strain, CON, was conducive to successful and timely MLF, even when prolonged or unsuccessful in the SC monoculture. Conversely, high lactic producing strain UNI inhibited MLF irrespective of the *O. oeni* inoculation strategy (co-inoculation vs. sequential inoculation). Further investigation confirmed that the inhibitory impact of lactic acid was not merely due to the associated lower pH. The concentration of lactic acid required to inhibit MLF varied depending on the matrix; while 1.5 g/L additional lactic acid prevented MLF in Merlot and Sauvignon blanc wines, in RCDW,

MLF finished when lactic acid concentrations were over 3 g/L. These results suggest that high lactic acid producing strains of *L. thermotolerans* could be used to inhibit MLF, while lower lactic acid producing strains could promote it.

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