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Ana HRANILOVIC [A, B, *]; ***Warren ALBERTIN*** [C]; ***Dimitra Liacopoulos CAPONE*** [B,D]; ***Adelaide GALLO*** [B]; ***Paul R. GRBIN*** [B,D]; ***Lukas DANNER*** [B]; ***Susan E.P. BASTAIN*** [B]; ***Isabelle MASNEUF-POMAREDE*** [A,E]; ***Joana COULON*** [F]; ***Marina BELY*** [A]; ***Vladimir JIRANEK*** [B, D]

[A] UR Oenologie EA 4577, USC 1366 INRAE, Bordeaux INP, Université de Bordeaux, Bordeaux, France [B]; Department of Wine Science, School of Agriculture, Food and Wine, The University of Adelaide, Urrbrae, SA, 5064, Australia; [C] ENSCBP, Bordeaux INP, 33600, Pessac, France; [D] The Australian Research Council Training Centre for Innovative Wine Production, Urrbrae, SA, 5064, Australia; [E] Bordeaux Sciences Agro, 33170, Gradignan, France; [F] BioLaffort, 33270, Floirac, France

* Corresponding author: Email: ana.hranilovic@adelaide.edu.au; Phone: +61490431022; Address: Department of Wine Science, School of Agriculture, Food and Wine, The University of Adelaide, Urrbrae, SA, 5064, Australia

Co-authors' emails: warren.albertin@u-bordeaux.fr; dimitra.capone@adelaide.edu.au; adelaidegallo6@gmail.com; paul.grbin@adelaide.edu.au; lukas.danner@adelaide.edu.au; sue.bastian@adelaide.edu.au; isabelle.masneuf@agro-bordeaux.fr; joana.coulon@laffort.com; marina.bely@u-bordeaux.fr; vladimir.jiranek@adelaide.edu.au

Abstract

Wines from warm(ing) climates often contain excessive ethanol but lack acidity. The yeast *Lachancea thermotolerans* can ameliorate such wines due to partial conversion of sugars to lactic acid during alcoholic fermentation. This study compared the performance of five *L. thermotolerans* strains in two inoculation modalities (sequential and co-inoculation) to *Saccharomyces cerevisiae* and un-inoculated treatments in high sugar/low acidity Merlot fermentations. The pH and ethanol levels in mixed-culture dry wines were either comparable, or significantly lower than in controls (decrease of up to 0.5 units and 0.90 % v/v, respectively). The analysis of volatile compounds revealed marked differences in major flavour-active yeast metabolites, including up to a thirty-fold increase in ethyl lactate in certain *L. thermotolerans* modalities. The wines significantly differed in acidity perception, alongside 18 other sensory attributes. Together, these results highlight the potential of some *L. thermotolerans* strains to produce 'fresher' wines with lower ethanol content and improved flavour/balance.

Keywords

Lachancea thermotolerans; fermentation; wine acidification; lactic acid; wine aroma; ethyl lactate; RATA sensory analysis

1. Introduction

Merlot is one of the most important grapevine varieties on a global scale. After Cabernet Sauvignon, it is the second most-planted variety destined for winemaking, nowadays grown on 266,000 hectares across 37 countries (OIV, 2017). Merlot originates from the Bordeaux wine region, where it is commonly used in blends with Cabernet Sauvignon and, to a lesser extent, Cabernet Franc (Boursiquot, Lacombe, Laucou, Julliard, Perrin, Lanier, et al., 2009). It contributes to 'softness' and 'fruitiness' of Bordeaux blends, juxtaposed to more tannic and 'green' character of the remaining components (Robinson, Harding, & Vouillamoz, 2013). Besides its versatility as a blending grape, Merlot is also used in the production of mono-varietal reds. In fact, some of the world's most iconic wines, such as Petrus and Le Pin, are made exclusively from Merlot.

Despite its popularity and potential to make premium wines, Merlot is a challenging variety, as it is characterised by high sugar content and low to medium acidity of musts (Boursiquot, et al., 2009). It is therefore extremely sensitive to optimal harvest timing, and its tendency to over-ripen in warm areas (OIV, 2017) is further exacerbated through accelerated phenological development in the context of climate change (Schultz & Jones, 2010). Consequently, Merlot wines often contain overly high ethanol levels but lack acidity. Such profiles are detrimental for wine chemical and sensory 'balance', microbial stability and, given the rising demand for 'fresher' styles, consumer acceptance and marketability (Morata, Escott, Banuelos, Loira, Fresno, Gonzalez, et al., 2019; Varela, Dry, Kutyna, Francis, Henschke, Curtin, et al., 2015).

Winemakers can address these inadequacies through a range of external inputs and/or interventions. Excessive ethanol in wines can be moderated via different approaches implemented across the whole grape and wine production chain; from altered vineyard practices to partial physical dealcoholisation of wines (Varela, et al., 2015). Acidity is most commonly adjusted through addition of tartaric acid, and less so with other organic acids and ion exchange techniques (Waterhouse, Sacks,

66 & Jeffery, 2016). Albeit effective, these interventions can be costly, complicated and detrimental for
67 wine quality and/or consumer perception. Microbiological solutions are therefore in high demand, in
68 particular, the use of an acidifying lower-ethanol yielding yeast to conduct fermentation.

69 One yeast with such potential is *Lachancea thermotolerans* (LT), a ubiquitous species that
70 occupies a range of ecological niches worldwide (Hranilovic, Bely, Masneuf-Pomarede, Jiranek, &
71 Albertin, 2017). It is a common constituent of grape/wine microbiota, and has thus been explored for
72 its application in oenology (Jolly, Varela, & Pretorius, 2014; Mora, Barbas, & Mulet, 1990). Under
73 oenological conditions, LT strains can ferment to about 10 % v/v ethanol (Hranilovic, et al., 2018), and
74 therefore require simultaneous or sequential addition of another co-starter to 'complete' wine
75 fermentation (i.e., deplete all sugars). The co-starters are typically strains of *Saccharomyces cerevisiae*
76 (SC), although recent research also proposed the use of *Schizosaccharomyces pombe* that role (S.
77 Benito, 2018), and in fact, several LT strains are now commercially available for such mixed-starter
78 fermentations (Roudil, Russo, Berbegal, Albertin, Spano, & Capozzi, 2020).

79 The major metabolic contribution of LT is L-lactic acid production from sugars during alcoholic
80 fermentation. The maximal reported concentration of lactic acid formed during LT wine fermentations
81 is 16.6 g/L (Banilas, Sgouros, & Nisiotou, 2016), which by far exceeds that recorded for any other non-
82 GM yeast (Sauer, Porro, Mattanovich, & Branduardi, 2010). By comparison, SC strains produce very
83 little, if any, lactic acid (Sauer, Porro, Mattanovich, & Branduardi, 2010). In practical oenological terms,
84 lactic acid is both physicochemically and microbially stable, unlike other permitted wine acidulants
85 (i.e., tartaric, malic or citric acid) (Waterhouse, Sacks, & Jeffery, 2016). The LT strains, however, greatly
86 vary in their lactic acid production (i.e., bio-acidification) capacity. For example, concentrations of
87 lactic acid formed in fermentations of the same grape juice by 94 different LT strains ranged between
88 1.8 to 12 g/L, and significantly affected the wine pH (3.2 – 3.8) (Hranilovic, Gambetta, Schmidtke, Boss,
89 Grbin, Masneuf-Pomarede, et al., 2018). In mixed cultures of LT and SC, lactic acid production depends
90 on the LT strain but also on the yeast inoculation regime. Due to antagonistic activities of SC towards

LT, mediated by mechanisms of cell-cell contact and secretion of antimicrobial peptides (Kemsawasd, Branco, Almeida, Caldeira, Albergaria, & Arneborg, 2015), co-inoculation generally results in lower levels of lactic acid compared to sequential inoculation (Gobbi, Comitini, Domizio, Romani, Lencioni, Mannazzu, et al., 2013; Kapsopoulou, Mourtzini, Anthoulas, & Nerantzis, 2007; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020). The extent of wine acidification in LT modalities is thus variable; from comparable, to about 0.5 units lower pH, relative to the SC control (Gobbi, et al., 2013; Morata, Bañuelos, Vaquero, Loira, Cuerda, Palomero, et al., 2019; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020).

Lactic acid production by LT occurs via lactic acid dehydrogenase (LDH) activity from glycolysis-derived pyruvate (i.e., via breakdown of sugars), and hence is a carbon sink competing with ethanol. Depending on the strain and conditions, reports describe either similar or about 1% v/v lower ethanol concentrations in wines co-fermented with LT as compared to their respective SC monocultures (Binati, Lemos Junior, Luzzini, Slaghenaufi, Ugliano, & Torriani, 2019; Comitini, Gobbi, Domizio, Romani, Lencioni, Mannazzu, et al., 2011; Gobbi, et al., 2013; Morata, Bañuelos, et al., 2019; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020). Other compositional alterations in LT wines include increases in glycerol (Gobbi, et al., 2013; Kapsopoulou, Mourtzini, Anthoulas, & Nerantzis, 2007; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020), decreases in acetic acid (Á. Benito, Calderón, Palomero, & Benito, 2015; S. Benito, 2018; Comitini, et al., 2011; Kapsopoulou, Mourtzini, Anthoulas, & Nerantzis, 2007) and partial degradation of malic acid (Hranilovic, et al., 2018; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020; Whitener, Stanstrup, Carlin, Divol, Du Toit, & Vrhovsek, 2017). Previous work also reported modulation of a range of both grape- and yeast-derived aroma compounds in LT wines (Binati, Lemos Junior, Luzzini, Slaghenaufi, Ugliano, & Torriani, 2019; Gobbi, et al., 2013; Hranilovic, et al., 2018; Nisiotou, Mallouchos, Tassou, & Banilas, 2019; Whitener, Stanstrup, Carlin, Divol, Du Toit, & Vrhovsek, 2017) and their effect on wine colour (S. Benito, 2018). Besides chemical composition, sensory properties of LT wines were also studied (Á.

Benito, Calderón, Palomero, & Benito, 2015; Gobbi, et al., 2013; Morata, Bañuelos, et al., 2019; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020).

However, most previous studies were set up in grape varieties of a local rather than global importance with a limited number of LT strains; in fact, rarely more than one (S. Benito, 2018). It therefore remains unclear to what extent the reported alterations are affected by the variability of LT strains, as compared to inoculation regimes with SC. The current study therefore aimed to determine the performance of five genetically and phenotypically divergent LT strains in both co- and sequential inoculations with SC, alongside SC and un-inoculated treatments, in high sugar/low acidity Merlot fermentations. The treatments were compared for fermentation performance, and the resultant wines subject to comprehensive chemical and sensory profiling, with a focus on acidification extent, production of primary and secondary metabolites, and rating by wine experts, which together highlighted promising yeast modalities for winemaking in warming climates.

2. Materials and methods

2.1. Chemicals

Chemicals and consumables were purchased from commercial suppliers. Chemicals used for quantification of the volatiles were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) with the exception of ethyl 2-phenylacetate, which was purchased from Alfa Aesar (Ward Hill, MA, USA) and were all ≥97% pure as described in Wang, Capone, Wilkinson, and Jeffery (2016). Solvents (analytical grade) were obtained from Chem Supply (Gillman, SA, Australia). Deuterium-labelled internal standards were obtained from CDN isotopes (Pointe-Claire QC, CA, USA) or synthesised as previously reported in Wang, Capone, Wilkinson, and Jeffery (2016). Sodium chloride was purchased from Rowe Scientific (Lonsdale, SA, Australia). Water used was purified through a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Standards and internal standards were prepared as

previously reported (Wang, Capone, Wilkinson, & Jeffery, 2016) volumetrically in absolute ethanol. Stock solutions and working solutions were stored at -20 °C until required.

2.2. Grapes and winemaking

Merlot grapes (clone D3V14) were handpicked from the experimental Coombe vineyard (Waite Campus, University of Adelaide, South Australia) on the 7 March 2019. The grapes were stored in a cool room (0 °C) prior to destemming and crushing. Potassium metabisulfite (PMS; 100 mg/L) was added at crush to yield approximately 50 mg/L of total SO_2. Around 200 kg of crushed grapes were gently pressed (at approximately 0.5 bar for 10 min) using a basket press to separate the grape juice from the skins, and the total soluble solids (TSS) in the juice diluted from 16 to 14.5 °Bé using RO water. Each fermenter (5 L plastic buckets with a lid) was filled with 2.5 L of juice and 0.5 kg skins so as to ensure a consistent liquid-to-solid ratio across all treatments. Musts were acclimatised to ~24 °C (i.e., set room temperature for fermentation) before inoculation as described below. The initial pH was 3.9 and malic acid content 2.6 g/L. Diammonium phosphate (DAP, 10% aqueous solution) was added to each fermenter to increase the initial yeast assimilable nitrogen (YAN) to 180 mg/L. An additional 80 mg/L of YAN was supplemented as a combination of NUTRISTART (30 mg/L YAN; Laffort, France) and DAP (50 mg/L YAN) on the fifth day of fermentation. The cap was plunged once a day with concurrent monitoring of TSS and pH, using a digital density meter (DMA 35, Anton Paar, Austria) and a pH meter, respectively. After the TSS dropped below 0 °Bé, residual sugars (RS) were determined spectrophotometrically (Infinite 200 PRO, Tecan, Männedorf, Switzerland) using an enzymatic kit (K-FRUGL, Megazyme, Ireland) in a 96-well plate format. After 14 days of maceration, the wines were pressed off with a basket press into 2 L bottles and cold stabilised and stored at 0 °C until bottling. Wines were dosed with 30 mg/L PMS, bottled (0.75 L; crown seal) and stored at room temperature (~ 24 °C) ahead of further analysis. Dry ice was used at all stages of winemaking to minimise oxidation.

2.3. Yeast treatments and inoculation procedure

Twelve yeast treatments included five LT strains in two inoculation modalities, alongside a monoculture of an SC strain (Zymaflore® Spark, Laffort, France) and an un-inoculated treatment. The LT strains represented three commercially available starters (LT3, LT4, LT5) and their two experimental counterparts (LT1 and LT2). The commercial strains were sourced from different manufacturers, i.e., AEB, Italy; CHR Hansen, Denmark; Lallemand, Canada. The LT1 and LT2, also known as ISVV Ltyq 25 and UNIFG 18, respectively, were previously characterised and pre-selected as superior wine starters (Hranilovic, Bely, Masneuf-Pomarede, Jiranek, & Albertin, 2017; Hranilovic, et al., 2018). In co-inoculations, denoted with the symbol 'x' (e.g., LT1xSC), LT and SC strains were simultaneously inoculated at 3 x 10^6 and 1 x 10^6 cells/mL, respectively. In sequential inoculations, denoted with the symbol '…' (e.g., LT1…SC), LT strains were added at 2 x 10^6 cells/mL, followed 48 h later by SC at 1 x 10^6 cells/mL. The SC-only treatment was inoculated at 2 x 10^6 cells/mL, whereas any inoculation was omitted in the "UN" treatment. All fermentations were conducted in triplicate (i.e., biological replication). The inoculated strains were grown from cryo-cultures (-80 °C in 25% glycerol) on YPD plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar) at 24 °C. After 3 days of incubation, single colonies were transferred into YPD broth (50 mL in 200 mL flasks) for an overnight incubation at 24 °C. The filter-sterilised diluted grape juice (45% water, 5% YPD; 300 mL in 800 mL flasks) was then inoculated at 10^7 cell/mL, and incubated overnight (24 °C, 120 rpm) to reach the final inoculation rates reported above. Inoculations were performed directly from liquid cultures upon determination of cell densities via flow cytometry (Guava easyCyte 12HT, Merck, USA).

2.4. Chemical analysis

Wine ethanol concentrations were determined with an alcolyser (Anton Paar, Austria), and pH and titratable acidity (TA) with a pH meter and an autotitrator (Mettler Toledo T50, OH, USA),

190 respectively. High performance liquid chromatography (HPLC) was used to measure the
191 concentrations of glycerol, lactic, malic and acetic acid. Before injection (20 µL), samples were pre-
192 filtered (0.45 µm) and diluted in deionised water (2:1; final volume 2 mL). The Agilent 1100 instrument
193 (Agilent Technologies, Santa Clara, CA, USA) was fitted with an HPX-87H column (300 mm × 7.8 mm;
194 BioRad, Hercules, CA, USA). The eluent was 2.5 mM H_2SO_4, at a 0.5 mL/min flow rate at 60 °C for a
195 35 min run time. Signals were detected using an Agilent G1315B diode array and G1362A refractive
196 index detectors. Analytes were quantified using external calibration curves ($R^2 > 0.99$) in ChemStation
197 software (version B.01.03). Acetaldehyde and pyruvic and succinic acid were measured using the
198 appropriate enzymatic kits in a 96-well plate format (K-PYRUV, K-ACHYD, K-SUCC, Megazyme, Ireland).
199 Concentrations of SO_2 were measured using an aspiration/titration method (Rankine & Pocock, 1970).
200 The analysis of volatile compounds was carried out as described in Wang, Capone, Wilkinson, and
201 Jeffery (2016). The wine sample (0.5 mL) was transferred to a glass vial (20 mL solid phase
202 microextraction (SPME) screw cap vial), and diluted with Milli-Q water (4.5 mL), spiked with a mixture
203 of deuterium labelled standards and sodium chloride (2 gm) was added. The samples were stored at
204 4 °C until ready for analysis. Analysis was carried out with a Gerstel MPS auto sampler (Lasersan
205 Australasia Pty Ltd. Robina, QLD, Australia) utilising head space SPME (HS-SPME) injection, with a
206 DVB/CAR/PDMS fibre (50/30 µm, 1 cm, 23 gauge) (Supelco, Bellefonte, PA). This was injected on an
207 Agilent 7890A gas chromatograph (GC) combined with a 5975C inert XL Mass Spectrometer (MS)
208 (Agilent Technologies, Santa Clara, USA), with conditions detailed in Wang, Capone, Wilkinson, and
209 Jeffery (2016).

210 ### 2.5. Sensory analysis

211 All studies were performed in accordance with the Ethical Guidelines for Scientific Research
212 at the University of Adelaide and approved by the Human Ethics Committee (H-2018-130). The wines
213 were first tasted by a panel of experts in order to assure the absence of faults and consistency within
214 replicates. The expert panel also defined a list of attributes to be used in the formal sensory evaluation
215 using Rate-All-That-Apply (RATA) methodology. RATA is a rapid sensory profiling method in which the

assessors are presented with a list of attributes and instructed to rate the intensity of those that they perceive in the samples (Danner, Crump, Croker, Gambetta, Johnson, & Bastian, 2018). Experienced wine tasters (n = 47, 62% females, average age 27.5 years) were recruited among the post-graduate students and staff in the Department of Wine Science at the University of Adelaide. Wines were equilibrated to room temperature (22-24 ˚C) before pouring, and the triplicates of each treatment were blended together given their consistency (as determined by the expert panel). Wine samples (25 mL) were presented in opaque ISO-standard glasses, labeled with randomised four-digit-codes, and covered with glass Petri dishes. Wines were served sequentially and monadically in a random order to overcome carryover effects. The assessors were instructed to use a seven-point scale (1 = extremely low, 4 = moderate intensity, 7 = extremely high) to rate the applicable aroma attributes (orthonasally), flavour attributes (retronasally), and attributes related to taste, mouthfeel and length upon expectoration. In addition, the assessors were asked to indicate which attribute best described the wine acidity profile; 'flat/flabby', 'crisp/fresh/bright', 'sour/tart' or 'harsh/acrid'. Assessors were given one-minute breaks between samples, during which they cleansed their palates with crackers and water. Wines were evaluated in individual booths at room temperature, and data was collected using RedJade online software (Redwood City, CA, USA).

2.6. Statistical analysis

Data was analysed with custom-made scripts in R (R Development Core Team, 2013). Fermentation and acidification dynamics were analysed using K-means clustering (*cutRepeatedKmeans* function; *ClassDiscovery* package). The chemical parameters of wines produced with the 12 yeast treatments were subjected to one-way ANOVA, followed by Tukey's post-hoc comparisons (*agricolae* package). The subset of 10 LT wines was then subjected to two-way ANOVA to examine the effect of five LT strains in two inoculation modalities. The sensory data were analysed using a two-way ANOVA with panellists as random and samples as fixed factors. The significance

thresholds for all ANOVA were set at 5%, and P-values were corrected for multiple tests (Benjamini-Hochberg correction). The acidity profiles were analysed by median test allowing multiple comparisons (*agricolae* package). Chemical dataset was subjected to principal component analysis (PCA), and the links between the chemical and sensory parameters (X and Y variables, respectively) that were significantly affected by the yeast treatment were analysed using partial least square regression (PLS-R) in XLSTAT (version 2020.4; Addinsoft, Paris, FR).

3. Results and discussion

Merlot grapes were fermented with 12 yeast treatments, including five LT strains in two inoculation modalities (co-inoculation and sequential inoculation), alongside the SC and un-inoculated controls. Albeit common for most reds, deliberate malolactic fermentation (MLF) was not conducted so as to better understand the impact of yeast treatments alone. Their performance was comprehensively characterised in terms of fermentation and acidification kinetics, and the resultant wines underwent detailed chemical and sensory analysis. The tested LT strains differed in their oenological phenotypes in pure cultures (Hranilovic, et al., 2018), and the current experimental design aimed to determine whether, and to what extent, the tested parameters were affected by LT strains and/or inoculation regimes with SC. The variation in measured parameters was analysed for the entire dataset, as well as the LT wines alone, and the use of both univariate and multivariate statistical tools highlighted pronounced effects of different yeast modalities on the profiles of the experimental Merlot wines.

3.1. Fermentation and acidification kinetics

The fermentation and acidification kinetics were subjected to K-means clustering, which resolved five and six profiles, respectively (Figure 1). Co-inoculations with LT1, LT3 and LT5 displayed the fastest fermentations (Profile 1'), followed by the SC and the remaining co-inoculations (Profile

2'). As typical for such modalities (Gobbi, et al., 2013; Morata, Bañuelos, et al., 2019; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020), sequential inoculations were comparatively slower (Figure 1). Sequential inoculations with LT1 and LT3 (Profile 3') progressed faster than those with LT2, LT4 and LT5 (Profile 5'). Despite the initial lag, common for un-inoculated fermentations in which early-prevailing non-*Saccharomyces* yeasts were subsequently overtaken by SC (Jolly, Varela, & Pretorius, 2014), the UN treatment (Profile 4') reached 0 °Bé prior to three sequential LT inoculations (Figure 1).

The trends in pH showed slight increases at the onset of all fermentations, possibly due to homogenisation between the liquid and solid phase (e.g., leaching of potassium from skins), followed by declines at different rates and extents (Figure 1). Upon the initial drop, pH in most LT treatments started to increase from day six (Figure 1). In both LT3 treatments (Profile 1), pH increased to comparable levels as in SC and UN (Profile 2). Co-inoculation and sequential inoculation with LT4 (Profile 3 and 4, respectively) resulted in higher pH than co-inoculations with LT1, LT2 and both LT5 treatments (Profile 5). The largest drop in pH of approximately 0.5 units was detected in sequential inoculations with LT1 and LT2 (Profile 6). Previous research also reported great variability in acidification capacity of LT modalities, including marginal decreases with LT3 as compared to other strains (Morata, Bañuelos, et al., 2019; Vaquero, Loira, Banuelos, Heras, Cuerda, & Morata, 2020).

3.2. Chemical composition of Merlot wines

3.2.1. Basic oenological parameters

The SC and all co-inoculation treatments fermented to dryness (< 2 g/L residual sugars (RS); Table 1). Despite some RS, this was also the case with the UN and sequential inoculations with LT3 and LT2 (Table1). The remaining sequential inoculation treatments contained more RS, with the highest value in the LT1…SC wine (8.2 g/L), potentially suggesting negative interactions between certain LT strains and SC. In agreement with the glucophilic character of both yeast species (Jolly,

291 Varela, & Pretorius, 2014), the RS was mainly fructose (Table 1). The SC control resulted in the highest
292 concentration of ethanol (16.5 % v/v), comparable to those in the UN treatment, LT3 in both
293 inoculation modalities and the LT4 co-inoculation (Table 1). Co-inoculations with LT1, LT2 and LT5 had
294 up to 0.5 % v/v less ethanol than the SC control, and further decreases in ethanol were recorded in all
295 sequential inoculations except LT3 (Table 1). However, in the sequentially inoculated LT1 treatment,
296 the decrease of 1.5 % v/v was partially related to RS (Table 1). Sequential inoculation with LT2 had the
297 lowest ethanol content amongst the dry wines, i.e., 0.9 % v/v less than the SC (Table 1). This ethanol
298 decrease was lower than the largest one to date reported in an LT treatment, that of 1.6 % v/v,
299 achieved in sterile fermentations sequentially inoculated with SC at 1 % v/v ethanol (Sgouros,
300 Mallouchos, Filippousi, Banilas, & Nisiotou, 2020). However, in non-sterile fermentations, the same
301 strain and inoculation regime resulted in an ethanol decrease of only 0.3 % v/v (Sgouros, Mallouchos,
302 Filippousi, Banilas, & Nisiotou, 2020), highlighting potential effects of indigenous grape microbiota on
303 implantation and, in turn, metabolic contribution of yeast inocula.

304 Lower-ethanol content in mixed-culture wines is in line with the partial diversion of carbon
305 flux from ethanol to lactic acid in *L. thermotolerans*, the extent of which varies between the strains
306 (Banilas, Sgouros, & Nisiotou, 2016; Hranilovic, et al., 2018). Accordingly, wines with lower ethanol
307 content contained more lactic acid with maximum concentrations reached in LT2…SC (8.1 g/L; Table
308 1). Both LT3 treatments resulted in lactic acid levels that were comparable to those in SC (0.41 g/L)
309 and UN wine (1.66 g/L). In the latter, lactic acid content was likely related to the complete degradation
310 of malic acid by the indigenous microflora, which agrees with the stoichiometry of MLF (i.e., 0.67 g of
311 lactic acid yielded per 1 g of malic acid). Lactic acid concentrations significantly affected the pH and
312 TA levels in wines, and all LT treatments except LT3 resulted in wine acidification, i.e., a pH drop and
313 TA increase, compared to the SC and UN (pH 3.9 and TA ~5 g/L; Table 1). The sequential inoculations
314 with LT1 and LT2 had the lowest pH and the highest TA (3.4 and 11 g/L, respectively). Lactic acid
315 production and acidification capacities of LT strains in co-cultures reflected those determined in their
316 pure cultures (Hranilovic, et al., 2018). Of particular interest was the contrasting behaviour of LT3 and

317 LT2 strains, representatives of two genetically differentiated subpopulations, i.e., 'Domestic 1' and
318 'Domestic 2' (Hranilovic, Bely, Masneuf-Pomarede, Jiranek, & Albertin, 2017) characterised by low and
319 high lactic acid production, respectively (Hranilovic, et al., 2018). While LT strains had more effect on
320 lactic acid levels (71 % of explained variation), and the resultant pH and TA modulation (90 % and 76
321 % of explained variation, respectively), the inoculation modalities were also significant (Figure 2, Table
322 S1). In agreement with previous work (Gobbi, et al., 2013; Kapsopoulou, Mourtzini, Anthoulas, &
323 Nerantzis, 2007; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020), the delay in SC
324 inoculation allowed for a greater metabolic contribution of LT strains in terms of lactic acid production
325 and acidification (Table 1).

326 The concentrations of glycerol were lower in SC (8.3 g/L) than in UN (10.2 g/L) or any other LT
327 treatment (Table 1). Pure LT cultures do not necessarily produce more glycerol than SC (Gobbi, et al.,
328 2013; Kapsopoulou, Kapaklis, & Spyropoulos, 2005), and, as per the current dataset (Table 1, Figure
329 2), increases in sequential inoculations are larger than in co-inoculations (Gobbi, et al., 2013;
330 Kapsopoulou, Mourtzini, Anthoulas, & Nerantzis, 2007; Sgouros, Mallouchos, Filippousi, Banilas, &
331 Nisiotou, 2020). In SC, glycerol formation by glycerol 3-phosphate dehydrogenases (GPD) serves as a
332 redox valve to eliminate excess cytosolic NADH under anaerobic conditions, and the expression of
333 homologous genes *GPD1* and *GPD2* is induced by osmotic stress and anoxia, respectively (Ansell,
334 Granath, Hohmann, Thevelein, & Adler, 1997). It remains to be verified whether glycerol increases in
335 sequential cultures occurred as a response of SC being inoculated into a medium with depleted
336 oxygen, with potential links to acetic acid production, which generally accompanies glycerol formation
337 (Ansell, Granath, Hohmann, Thevelein, & Adler, 1997). In the current study, the lowest levels of acetic
338 acid were detected in SC wine (0.15 g/L), comparable to those in LT co-inoculations (Table 1).
339 Sequential inoculations contained significantly increased levels of acetic acid (Table 1), despite low
340 and rather invariant acetate production by LT strains alone (Hranilovic, et al., 2018). Acetic acid in all
341 LT wines was lower than in the UN treatment (0.67 g/L; formed by the un-inoculated yeasts and
342 bacteria alike), and remained within regular limits for red wines (Waterhouse, Sacks, & Jeffery, 2016).

343 The highest concentration of malic acid was present in the SC wine (2.4 g/L), while those in
344 the UN remained undetectable, likely due to spontaneous MLF and as discussed above (Table 1). The
345 LT co-inoculations contained between 0.3 and 0.7 g/L less malate than the SC, with further decreases
346 reached in sequential inoculations (Table 1). The inoculation modality accounted for 84% of the
347 variation in malic acid content, compared to 5 % explained by the LT strains (Figure 2, Table S1). Under
348 non-sterile conditions, the contribution of indigenous grape microbiota to these trends cannot be
349 excluded. Nonetheless, lower concentrations of malic acid in LT modalities agree with previously
350 reported partial degradation of malate in pure LT cultures (Hranilovic, et al., 2018) and co-cultures
351 (Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020; Whitener, Stanstrup, Carlin, Divol, Du Toit,
352 & Vrhovsek, 2017) alike.

353 Succinic acid concentrations ranged between 1.7 g/L and 3.9 g/L in the SC and LT1…SC wine,
354 respectively. Strain-derived differences in succinic acid production by LT were previously described (S.
355 Benito, 2018), but here, specific links between the tested strains and/or inoculation modality were
356 not obvious (Figure 2). The levels of acetaldehyde were relatively low, ranging from 10.3 in the UN to
357 18.7 mg/L in the LT1…SC wine (Table 1). Sequential LT inoculations generally contained more
358 acetaldehyde than the co-inoculations (Figure 2). The UN wine also had the lowest concentrations of
359 pyruvic acid (53.7 mg/L), SC intermediary (125.7 mg/L) and LT5…SC the highest (170 mg/L). The
360 concentrations of pyruvate were affected by LT strains, which aligns with previously reported inter-
361 strain variation of about 30 % (S. Benito, 2018), but not inoculation modalities (Figure 2). Albeit low,
362 the total SO_2 concentrations were the highest in the SC wine, which agrees with previous reports (Á.
363 Benito, Calderón, Palomero, & Benito, 2015; Binati, Lemos Junior, Luzzini, Slaghenaufi, Ugliano, &
364 Torriani, 2019), but requires further investigation as it is of potential interest in the production of
365 wines with lower SO_2 content.

Table 1. Chemical composition of Merlot wines fermented with 12 yeast treatments. Values are the mean of winemaking triplicates (µg/L, unless otherwise indicated) and letters denote significance groups (ANOVA; Tukey's post-hoc α = 5 %). Volatile compounds in italics were detected below their sensory threshold in all wines. Compounds in italics and bold were in some wines below, and in others, above, their sensory threshold (Table S2).

Compounds	Yeast treatment											
	SC	**LT1xSC**	**LT1…SC**	**LT2xSC**	**LT2…SC**	**LT3xSC**	**LT3…SC**	**LT4xSC**	**LT4…SC**	**LT5xSC**	**LT5…SC**	**UN**
Basic oenological parameters												
Glucose (g/L)	nd c	nd c	2.0 a	0.1 bc	0.1 bc	nd c	0.2 bc	0.1 bc	0.7 b	nd c	0.3 bc	nd c
Fructose (g/L)	nd d	0.2 cd	6.3 a	0.3 cd	1.2 cd	nd d	1.2 cd	0.1 cd	3.1 b	0.2 cd	1.8 bc	1.2 cd
Ethanol (% v/v)	16.5 a	16.0 c	15.0 e	16.1 bc	15.6 d	16.4 ab	16.2 abc	16.3 abc	15.6 d	16.1 c	15.7 d	16.2 abc
pH	3.86 a	3.50 d	3.37 e	3.49 d	3.36 e	3.85 a	3.90 a	3.71 b	3.58 c	3.55 cd	3.51 cd	3.89 a
TA (g/L)	5.0 f	8.9 bc	11.0 a	8.2 cd	11.1 a	5.2 f	5.1 f	6.2 e	8.1 d	7.6 d	9.1 b	4.7 f
Lactic acid (g/L)	0.4 e	5.4 b	7.6 a	3.7 c	8.1 a	0.6 e	1.0 e	1.8 de	3.4 cd	3.6 c	5.8 b	1.7 e
Glycerol (g/L)	8.3 g	9.2 de	9.6 cd	8.8 ef	10.2 b	8.7 fg	9.9 bc	9.4 d	11.6 a	9.2 def	10.1 b	10.2 b
Acetic acid (g/L)	0.15 g	0.21 fg	0.29 defg	0.22 fg	0.54 ab	0.29 cdef	0.47 bcd	0.17 fg	0.49 abc	0.29 efg	0.45 bcde	0.67 a
Malic acid (g/L)	2.4 a	1.7 c	1.2 ef	1.9 bc	1.2 ef	2.1 b	1.1 f	2.0 b	1.3 de	1.9 bc	1.5 d	0 g
Succinic acid (g/L)	1.7 b	2.6 ab	3.9 a	2.9 ab	2.8 ab	3.1 ab	3 ab	2.9 ab	3.2 ab	3.6 a	3.1 ab	2.7 ab
Acetaldehyde (mg/L)	14.3 abc	15 abc	18.7 a	12.7 abc	16.3 abc	12.7 abc	11.7 bc	10.7 c	16.7 abc	11.7 bc	18 ab	10.3 c
Pyruvic acid (mg/L)	126 bc	86 ef	129 bc	93 de	83 efg	152 ab	60 fg	94 de	111 cde	119 cd	170 a	54 g
Total SO_2 (mg/L)	13.3 a	2.1 cd	2.1 cd	8.5 abc	9.1 ab	3.7 bcd	0.5 d	6.4 bcd	5.9 bcd	5.3 bcd	4.8 bcd	3.7 bcd
Volatile compounds												
Ethyl acetate	31898 e	40099 de	40224 de	41644 de	57151 c	41906 de	45618 cde	48926 cd	79191 b	49502 cd	51260 cd	166893 a

Ethyl lactate	***6070 d***	***95379 b***	***184449 a***	***68060 c***	***185507 a***	***8224 d***	***10617 d***	***21673 d***	***57646 c***	***57671 c***	***111069 b***	***18539 d***
Ethyl propanoate	*194 c*	*196 c*	*111 e*	*211 bc*	*167 cd*	*191 c*	*131 de*	*293 a*	*284 a*	*256 ab*	*183 cd*	*255 ab*
Ethyl 2-methyl propanoate	116 d	197 c	280 b	155 cd	250 b	161 cd	182 c	164 c	327 a	184 c	257 b	152 cd
Ethyl butanoate	216 a	141 cd	97 e	162 bcd	125 de	178 abc	135 de	186 ab	163 bcd	183 ab	125 de	194 ab
Ethyl 2-butenoate	43 bc	27 de	19 e	29 de	29 de	37 bcd	50 b	43 bc	65 a	34 cd	28 de	47 bc
Ethyl 2-methylbutanoate	9 de	13 ab	11 bcd	11 bcd	13 bc	11 bcd	8 e	12 bc	16 a	12 bc	10 cde	8 e
Ethyl 3-methylbutanoate	9 abc	9 abc	8 c	9 abc	9 abc	6 d	5 d	8 bc	10 a	9 abc	8 bc	9 ab
Ethyl hexanoate	736 a	506 bc	204 e	531 b	275 de	577 b	279 de	523 b	288 d	496 bc	239 de	431 c
Ethyl octanoate	638 a	325 b	204 b	389 b	238 b	375 b	245 b	388 b	230 b	374 b	210 b	357 b
Ethyl decanoate	*93 a*	*74 bc*	*67 c*	*80 abc*	*69 c*	*83 abc*	*81 abc*	*81 abc*	*71 c*	*88 ab*	*75 bc*	*90 ab*
Diethyl succinate	*nd f*	*97 cd*	*281 a*	*43 ef*	*295 a*	*nd f*	*nd f*	*nd f*	*114 c*	*53 de*	*181 b*	*31 ef*
Σ Ethyl esters	40022 f	137063 cd	225954 a	111323 d	244125 a	51749 ef	57350 ef	72298 e	138403 cd	108862 d	163645 bc	187004 b
Isoamyl acetate	1542 c	1549 c	1529 c	1508 c	1780 bc	1630 bc	1590 bc	1707 bc	2339 a	1785 bc	1884 b	2253 a
Hexyl acetate	*20 a*	*20 a*	*16 bc*	*19 a*	*18 ab*	*18 ab*	*16 bc*	*14 c*	*15 c*	*15 c*	*14 c*	*18 ab*
2-phenylethyl acetate	*172 ef*	*133 fg*	*254 bc*	*117 g*	*254 bc*	*116 g*	*200 de*	*102 g*	*302 a*	*129 fg*	*220 cd*	*289 ab*
Σ Acetate esters	1734 cd	1702 d	1800 bcd	1644 d	2052 bc	1764 cd	1806 bcd	1824 bcd	2656 a	1929 bcd	2119 b	2559 a
1-Propanol	32139 bcd	37558 a	31554 bcd	30914 cd	29556 d	33070 abcd	31168 bcd	35438 abc	35699 ab	32581 bcd	29002 d	28665 d
1-Butanol	*2322 b*	*2285 b*	*1761 de*	*1534 ef*	*2139 b*	*2051 bc*	*2743 a*	*1767 de*	*1837 cd*	*1647 de*	*1652 de*	*1302 f*
Isobutanol	***21899 g***	***31737 cd***	***34500 bc***	***22130 fg***	***30497 cd***	***23414 efg***	***29252 cde***	***28705 cdef***	***45601 a***	***27046 defg***	***29207 cde***	***40712 ab***
3-Methyl-1-butanol	338766 bc	361172 ab	296268 d	307278 cd	310335 cd	284955 d	291554 d	350278 b	395572 a	312927 cd	284626 d	307923 cd

4-Methyl-2-pentanol	*23 h*	*38 b*	*41 a*	*32 cd*	*35 c*	*26 g*	*27 fg*	*29 ef*	*31 de*	*31 de*	*30 de*	*26 g*
1-Hexanol	*1162 a*	*1052 abc*	*772 cde*	*920 abcd*	*906 abcd*	*605 e*	*580 e*	*806 bcde*	*794 cde*	*807 bcde*	*712 de*	*1077 ab*
2-Ethyl-1-hexanol	*5 ab*	*6 ab*	*5 ab*	*6 ab*	*4 ab*	*4 b*	*6 ab*	*6 ab*	*5 ab*	*5 ab*	*4 ab*	*6 a*
1-Octanol	***7 a***	***3 c***	***1 f***	***2 de***	***1 f***	***3 c***	***2 cd***	***3 c***	***1 f***	***1 ef***	***1 f***	***6 b***
2-Phenylethanol	101647 a	105621 a	96433 b	91683 b	97492 b	91110 ab	101427 ab	99736 ab	115629 ab	85810 b	85547 b	82981 a
Benzyl alcohol	*127 abc*	*125 ab*	*102 abc*	*103 bc*	*102 abc*	*114 bc*	*120 abc*	*113 abc*	*113 a*	*105 bc*	*101 bc*	*127 c*
Σ Higher alcohols	498097 bcd	539596 ab	461437 cde	454590 cde	471065 cde	435350 de	456879 cde	516879 bc	595281 a	460961 cde	430882 e	462825 cde
Butyric acid	***901 a***	***376 bc***	***164 d***	***413 bc***	***221 d***	***373 bc***	***290 bcd***	***420 b***	***292 bcd***	***366 bc***	***205 d***	***273 cb***
Isobutyric acid	***1417 f***	***4065 c***	***5017 b***	***2642 de***	***5568 b***	***3270 cd***	***4961 b***	***2777 de***	***7032 a***	***2858 de***	***3748 c***	***2143 ef***
Hexanoic acid	***858 a***	***396 bc***	***nd c***	***219 bc***	***nd c***	***428 b***	***nd c***	***61 bc***	***nd c***	***112 bc***	***nd c***	***nd c***
Octanoic acid	10959 a	4810 b	1157 f	5744 b	1603 ef	5106 b	2527 de	5688 b	1512 ef	4604 bc	943 f	3457 cd
Decanoic acid	*565 a*	*299 bc*	*164 cd*	*267 bcd*	*145 d*	*321 b*	*548 a*	*196 bcd*	*151 cd*	*266 bcd*	*224 bcd*	*206 bcd*
Σ Acids	14699 a	9946 b	6502 ef	9286 bc	7537 de	9497 bc	8326 cd	9141 bc	8987 bc	8207 cd	5119 f	6078 f
Linalool	***14 d***	***17 a***	***15 abcd***	***15 abcd***	***16 ab***	***15 bcd***	***15 abcd***	***15 abcd***	***16 abc***	***16 abc***	***16 ab***	***14 cd***

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3.2.2. Volatile profiles

A total of thirty one volatile compounds, predominantly represented by yeast-derived metabolites was quantified, and included those that previously been identified as the main contributors to the aroma of Merlot wines (Zhao, Qian, He, Li, & Qian, 2017). Besides their concentrations, these were also analysed for their odour active values (OAV), which, despite perception interactions, serve as indicators for the contribution of each compound to wine aroma (Zhao, Qian, He, Li, & Qian, 2017).

The esters that were predominant in the wines were either ethyl acetate or ethyl lactate (Table 1). The lowest concentration of ethyl acetate was detected in the SC wine (32 mg/L), while those in LT modalities were either comparable or up to 2.5-times higher (e.g., LT4…SC, Table 1). Despite the increases, ethyl acetate concentrations in LT wines did not exceed the point where it is seen as faulty rather than 'fruity'/'complexing' (150 mg/L; Sumby et al. 2010), which was the case in the UN wine alone (Table 1). Ethyl acetate is generally the most abundant ester formed during AF, while the concentrations of ethyl lactate increase upon MLF (Sumby, Grbin, & Jiranek, 2010). The LT modalities, however, are conducive to increases in ethyl lactate due to the availability of lactic acid as its precursor. As a result, the sequential inoculations of LT1 and LT2 were about 30-times higher in ethyl lactate than the SC control (185 and 6 mg/L, respectively; Table 1). In these LT wines, ethyl lactate surpassed its relatively high sensory threshold as compared to the ethyl esters of fatty acids (Waterhouse, Sacks, & Jeffery, 2016), which was thus far not recorded in the LT modalities. The only treatment that completed MLF, UN, contained 19 mg/L of ethyl lactate, and even lower levels were detected in LT wines with moderate lactic acid production (Table 1). Production of ethyl acetate and ethyl lactate alike was more affected by the LT strains than the inoculation modalities (Figure 2, Table S2).

As a result of ethyl lactate increases, LT1…SC and LT2…SC contained the highest levels of total ethyl esters, and SC the lowest levels (Table 1, Figure 3). However, certain esters with high OAV values,

i.e., ethyl esters of straight-chain fatty acids (ethyl butanoate, ethyl hexanoate, ethyl octanoate, alongside ethyl decanoate) were the highest in the SC wine (Table 1, Table S3). These ethyl esters were intermediary in co-inoculations, and further decreased in sequential inoculations (Table 1). The levels of ethyl esters of medium-chained fatty acids (MCFA) predominantly depend on the availability of their respective precursors (butanoic, hexanoic, octanoic and decanoic acid; Dennis et al. 2012), which, accordingly, followed the same trend (Table 1, Figure 2, Figure 3). Such observations were in general agreement with some studies on the oenological characterisation of LT strains (Comitini, et al., 2011; Gobbi, et al., 2013; Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020), but in contrast with others (Binati, Lemos Junior, Luzzini, Slaghenaufi, Ugliano, & Torriani, 2019; Nisiotou, Mallouchos, Tassou, & Banilas, 2019). The MCFA are by-products of yeast lipid metabolism produced from acetyl-CoA through the fatty acid synthase (FAS) complex (Waterhouse, Sacks, & Jeffery, 2016). They can be released from the FAS complex to partake in ethyl ester formation by condensation of MCFA-CoA with ethanol (Waterhouse, Sacks, & Jeffery, 2016). Interestingly, in our study, lower levels of MCFA and their ethyl esters in LT co-inoculations than in the SC control, and their further drops in sequential inoculations, were apparent for all LT strains despite their major phenotypic variability (Table 1, Figure 2). These observations invite further research on investigating the differences between LT and SC in the biosynthesis of fatty acids and/or release of medium-chain intermediates available for esterification, and their modulations in response to co-culturing.

Trends in ethyl 2-methylpropanoate (ethyl isobutyrate) and isobutyric acid were opposite to those seen for MCFA and their ethyl esters, i.e., they were higher in sequential inoculations than in co-inoculations, and at their lowest in the SC control (Table 1, Figure 2). Higher production of these compounds in sequential LT cultures, previously reported elsewhere (Sgouros, Mallouchos, Filippousi, Banilas, & Nisiotou, 2020; Whitener, Stanstrup, Carlin, Divol, Du Toit, & Vrhovsek, 2017), occurred irrespective of the LT strain, with inoculation modality explaining 60% and 57% of the variation in isobutyric acid and its ethyl ester, respectively (Figure 2, Table S1). High OAV values of ethyl 2-methylpropanoate (range 7.7 in SC and 21.8 LT4…SC) indicated its contribution in shaping the

aroma/flavour profiles of the analysed wines (Table S3). As the branched-chain fatty acid, isobutyric acid is formed from valine via the Ehrlich pathway (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008), this could suggest differences in amino acid catabolism between LT and SC. The remaining quantified ethyl esters that surpassed their sensory threshold, i.e., ethyl 2-methyl butanoate, ethyl 3-methyl butanoate and ethyl 2-butenoate, were detected in the highest concentrations in LT4…SC wines. Besides UN, LT4…SC also had the highest levels of isoamyl acetate and total acetate esters (Table 1, Figure 4), which depended more on the LT strains than the inoculation modalities (Figure 2, Table S1). In contrast to ethyl esters, the concentrations of acetate esters depend more on the enzymatic activities than substrate availability (Waterhouse, Sacks, & Jeffery, 2016), potentially suggesting differences in acetyltransferase enzymes between the strains.

The most prevalent higher alcohol in all of the wines (>64% of total higher alcohols) was 3-methyl-1-butanol (isoamyl alcohol), detected at the highest levels in LT4…SC (Table 1, Figure 3), followed by 2-phenylethanol. An increase in 2-phenylethanol is generally attributed to mixed fermentations with LT strains but not necessarily their respective monocultures (Comitini, et al., 2011; Gobbi, et al., 2013; Morata, Bañuelos, et al., 2019), potentially due to its role as a signalling molecule (Avbelj, Zupan, Kranjc, & Raspor, 2015). The levels of 2-phenylethanol in presently analysed LT modalities were, however, either comparable or lower than in the SC and UN wines (Table 1). Albeit present at lower concentrations than 3-methyl-1-butanol and 2-phenylethanol, propanol had comparatively superior OAV values, and was detected at the highest levels in LT1xSC (Table 1, Table S3). The LT strains accounted for more variation in the content of most of the analysed higher alcohols compared to the inoculation modalities (Figure 2, Table S1). Interestingly, strain-derived differences were noticeable in both fermentation-derived higher alcohols formed as by-products of yeast amino acid metabolism through the Ehrlich pathway (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008), and grape-derived higher alcohols (e.g., 1-hexanol), as previously confirmed for pure culture LT fermentations (Hranilovic, et al., 2018). In mixed-culture LT wines, limited research identified links between the concentrations of certain amino acids and their corresponding higher alcohols (Á. Benito,

Calderón, Palomero, & Benito, 2015; S. Benito, 2018), however, the inter-strain LT variation in amino acid metabolism requires further attention. Overall, relative to the SC control (498 mg/L), the sum of quantified higher alcohols was higher in LT4…SC (595 mg/L), lower in LT5…SC (431 mg/L) and comparable in all other treatments (Table 1, Figure 3). The LT strains also had a significant effect on the concentrations of linalool, which were generally higher in LT wines as compared to SC and UN controls (Table 1, Figure 2), possibly due to differences in β-glucosidase activities between LT strains (S. Benito, 2018; Comitini, et al., 2011).

3.2.3. Multivariate analysis of the chemical parameters

Besides the univariate analysis, the chemical dataset was also subjected to PCA. The first principal component (PC1) separated the SC monoculture from the remaining treatments and accounted for 38% of the explained variance (Figure 4). The SC wines were associated with higher concentrations of ethanol, 1-octanol, MCFA and their ethyl esters (Figure 4). The co-inoculations had an intermediate location along PC1, in between the SC and all LT sequential inoculations, except LT3. The separation of the sequential inoculations was driven by the increases in lactic acid and, in turn, TA and ethyl lactate, as well as certain basic oenological parameters (residual sugars, acetaldehyde, acetic acid, glycerol and succinic acid) and volatile compounds (diethyl succinate, 4-methyl-pentanol, isobutyric acid and its ethyl ester; Figure 4). Sequential inoculation with LT4 was further differentiated from the remaining treatments on the second principal component (PC2, upper right quadrant), as was the case with UN (upper left quadrant). The separation on PC2, which explained 16 % of variance, was primarily affected by higher production of isoamyl acetate, ethyl acetate, ethyl-2-butenoate and isobutanol (Figure 4).

3.3. Sensory profiles of Merlot wines

A large number of studies have explored the use of non-*Saccharomyces* yeasts in oenology, but are often devoid of wine sensory analysis (Tempere, Marchal, Barbe, Bely, Masneuf-Pomarede, Marullo, et al., 2018). This study delivers extensive sensory profiles of the experimental wines scored on 43 attributes, by 47 experienced panelists using RATA methodology. Previous research showed that RATA profiles are comparable to those obtained by the costlier and lengthier Descriptive Analysis (Danner, Crump, Croker, Gambetta, Johnson, & Bastian, 2018). RATA profiling revealed significant differences in 18 sensory attributes with, unsurprisingly, the largest variation detected in wine 'acidity' (range of ratings 3.2 – 5.4; Table S4). The highest acidity was recorded for LT2…SC wine followed by LT1…SC and LT5…SC (Figure 5B, Table S4). The SC wine was rated as the least acidic, alongside UN and both LT3 treatments (Figure 5B, Table S4). These wines also scored high in 'sweetness', 'bitterness', 'hotness' and 'body' (Table S4). The intensity and length of acidity were congruent with the pH/TA levels in the wines, while the sweetness ratings did not correspond to the residual sugar levels and were instead largely affected by low acidity (Table 1, Table S4). For example, despite significantly higher residual sugars, LT1…SC scored lower in 'sweetness' than the SC, UN and LT3 wines (Table 1, Table S4). The wines significantly differed in eleven aroma and flavour attributes (Table S4). Six of these attributes were fault-related (i.e., 'cooked vegetables', 'medicinal/rubbery', 'VA' and 'oxidised'), and perceived at highest intensities in the UN wine (Table S4). Importantly, the ratings of the faulty attributes in LT wines were comparable to those of the SC control, with the exception of the highest score in 'oxidised' aroma of LT1…SC wine (Figure 5B, Table S4). Of further note were the lower intensities of 'red fruit' and 'herbaceous' aroma/flavour in UN wine, and highest scores in 'dark fruit' aroma and 'chocolate' flavour in LT3…SC and LT3xSC wines, respectively (Table S4).

The PLS-regression was performed to elucidate the links between the chemical parameters as explanatory (X) and sensory profiles as dependent (Y) variables that were significantly affected by the yeast treatment (ANOVA; $p < 0.05$; Table 1, Table S4). The first two components distinguished the yeast treatments and accounted for 57% and 63% percent of variation in wine chemical and sensory

profiles, respectively (Figure 5B). Along the first component the SC control was separated from the co-inoculations, UN and LT3…SC wines, with further divergence of the remaining LT sequential inoculations (Figure 5B). The UN, and to a lesser degree LT4…SC, was separated from the remaining treatments along the second component (Figure 5B). The acidity intensity and length corresponded to increases in lactic acid and TA, which, alongside pH and ethyl lactate, contributed the most to the separation along the first component as seen from the highest VIP values (Supplementary Figure 1). The configuration of attributes further highlighted the links between high ethanol and perceptions of 'hotness', 'bitterness' and 'body', which were in agreement with previous sensory studies (Pham, Ristic, Stockdale, Jeffery, Tuke, & Wilkinson, 2020; Schelezki, Suklje, Boss, & Jeffery, 2018). These parameters showed negative correlation with the first factor, as did the ethyl esters with high OAV (Figure 5A). However, an increased abundance of these esters did not enhance the fruity character of wines, potentially suggesting their masking by high ethanol concentrations (Pham, Ristic, Stockdale, Jeffery, Tuke, & Wilkinson, 2020). Similar masking effects were arguably exerted upon red fruit attributes by fault-related ones, as seen in the UN wine (Figure 5B). Their grouping on the second component was driven by the increases in ethyl acetate, acetate esters and acetic acid as opposed to higher malic acid content (Figure 5B).

Sensory analysis further focused on characterising the acidity profiles of the experimental wines. For that purpose, during RATA evaluation the panelists were instructed to indicate which attribute best described the acidity (i.e., 'flat/flabby', 'fresh/crisp/bright', 'sour/tart' or 'harsh/acrid'). The median test of the responses revealed six different acidity profiles (Figure 5C, Table S5). The SC UN and LT3xSC were described as 'flat/flabby' by ~50% of panellists as was LT3…SC. Both LT4 wines were denoted as 'flat/flabby', 'fresh/crisp/bright' and 'sour/tart' by a comparable number of tasters and the LT1 and LT5 wines were predominantly perceived as 'sour/tart'. This was also the case with LT2xSC, while the acidity of LT2…SC was denoted as 'harsh/acrid' by 40% of the panellists (Figure 5C, Table S5).

4. Conclusion

Excessive ethanol levels and insufficient acidity are of increasing concerns for the wine sector, and LT properties show potential to address these issues. In mixed cultures of LT and SC, applicable for wine production, compositional alterations of wines depend on the LT strains but also on the yeast inoculation regime. This work delivers extensive oenological characterisation of Merlot wines fermented with five LT strains in two inoculation regimes, alongside the SC and un-inoculated treatments.

The SC monoculture resulted in 'flat/flabby' high-alcohol wines in which the highest abundance of the ethyl esters of MCFA (highest OAVs) failed to enhance the 'fruity' character. The un-inoculated wines were also high in ethanol and low in acidity, and their fault-driven profiles (e.g., increased acetic acid, ethyl acetate, 'VA' and 'oxidised' sensory scores) highlighted the erratic nature of such fermentation modalities.

In LT treatments, the initial absence of SC allowed for the greater metabolic contribution of LT strains in sequential inoculations as compared to the co-inoculations. However, certain parameters were more affected by the LT strain; in particular, the production of lactic acid and the resultant pH/TA and ethyl lactate modulation. The behaviour of low-lactate producing strain LT3 was in stark contrast to the LT1 and LT2 strains, pre-selected for their acidifying character. Sequential inoculations of both strains resulted in 0.5 units lower pH than the controls, however the LT1…SC treatment led to an incomplete fermentation. Conversely, LT2…SC dry wine contained 0.9 % less ethanol than the SC control, in line with partial diversion of sugars away from ethanol. The extent of acidification by the remaining LT strains was intermediary, and the perceived acidity intensities/profiles mirrored such modulations. The bio-acidified wines scored lower in 'hotness', 'bitterness' and 'body', and their flavour profile was largely shifted towards the red fruit spectrum.

Together, these results provide information on the expression of LT phenotypic landscape in co-cultures with SC whilst highlighting the modalities that lend themselves as effective means to modulate wine acidity, ethanol and flavour balance upon fermenting grapes from warming climates.

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Appendix

Supplementary material.

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674

675

676 ## Figure captions

677 **Figure 1.** K-means clustering of acidification and fermentation kinetics in Merlot resolved six and five
678 profiles, respectively. The upper two panels show the mean values of K-means clustering profiles, and
679 the corresponding treatments (and number of replicates) are indicated below.

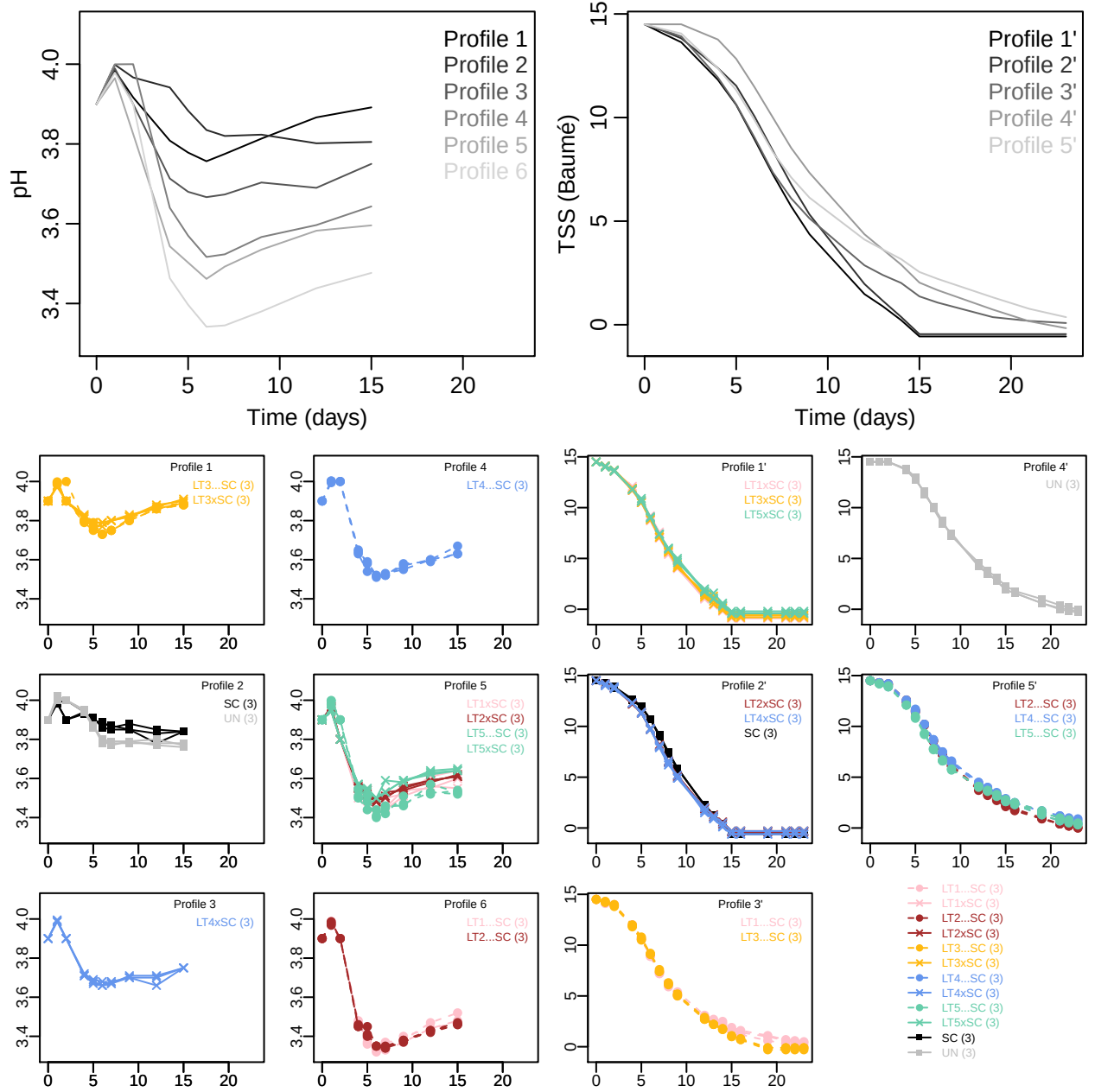
680

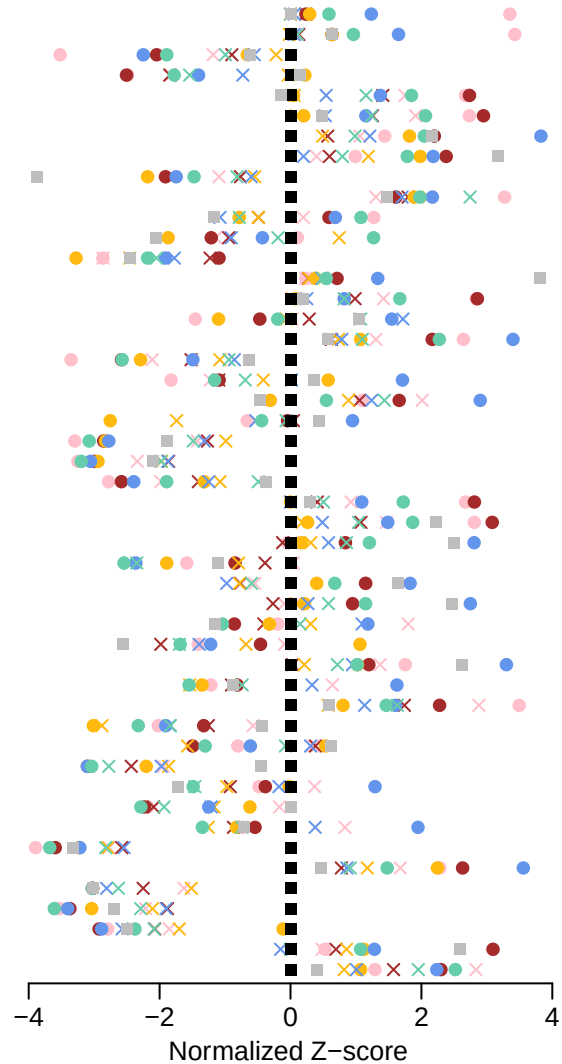
Figure 2. Variation in chemical composition of the experimental Merlot wines. Normalised Z-scores centered to SC wine (left). Percentages of variation in LT treatments explained by the LT strain (LT), inoculation modality (i.e., co-inoculation vs. sequential inoculation; INOC), their interaction (INTER) and residual (RES) as determined by 2-way ANOVA (right).

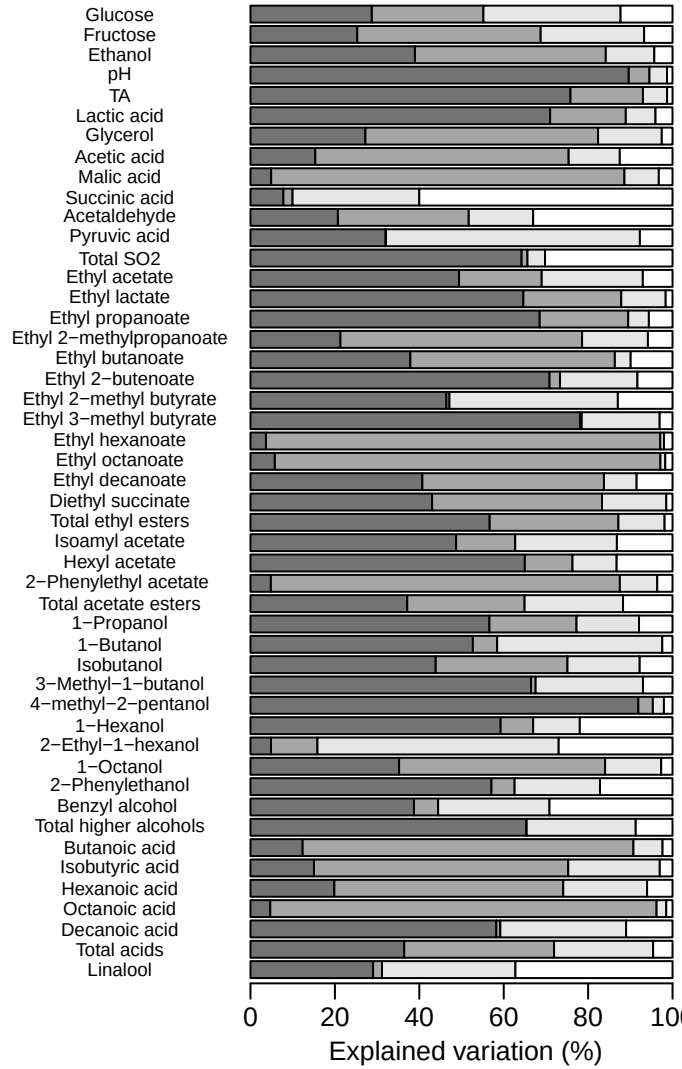
Figure 3. Sum of ethyl esters, acetate esters, higher alcohols and acids (μg/L) in experimental Merlot wines with contributions of individual compounds. The values represent means of triplicates and letters denote significance groups (ANOVA; Tukey's post-hoc $\alpha = 5\%$)

Figure 4. Principal component analysis of the chemical parameters in the experimental Merlot wines: yeast treatments (left) and correlation circle (right).

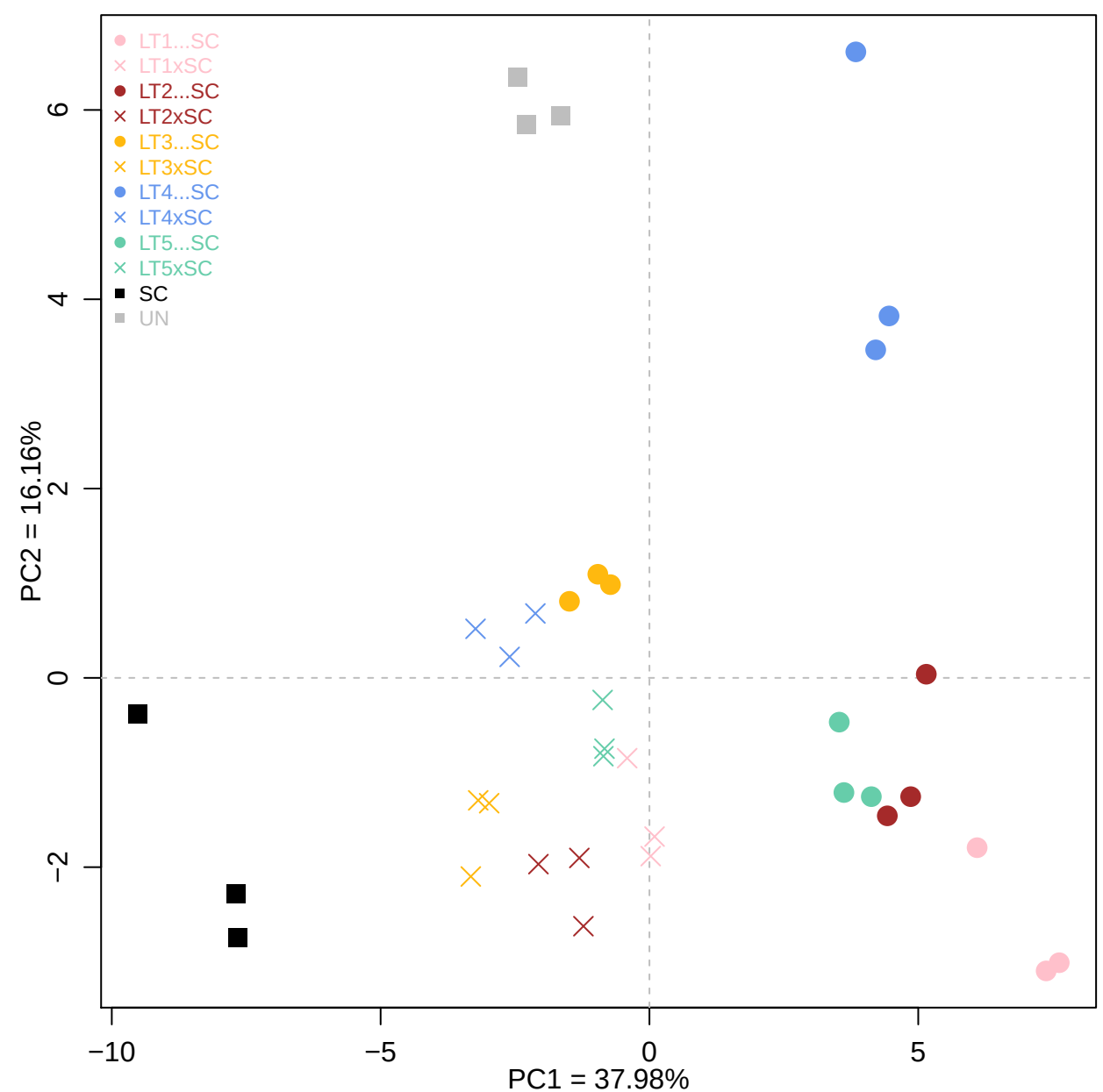
Figure 5. PLS-Regression analysis of RATA sensory profiles of wines: A) yeast treatments; B) configuration of sensory (in black) and chemical (colour-coded as per Figure 4) parameters of wines; C) acidity profiles of wines built with frequencies of four acidity descriptors (Table S5) and significance groups (median test).

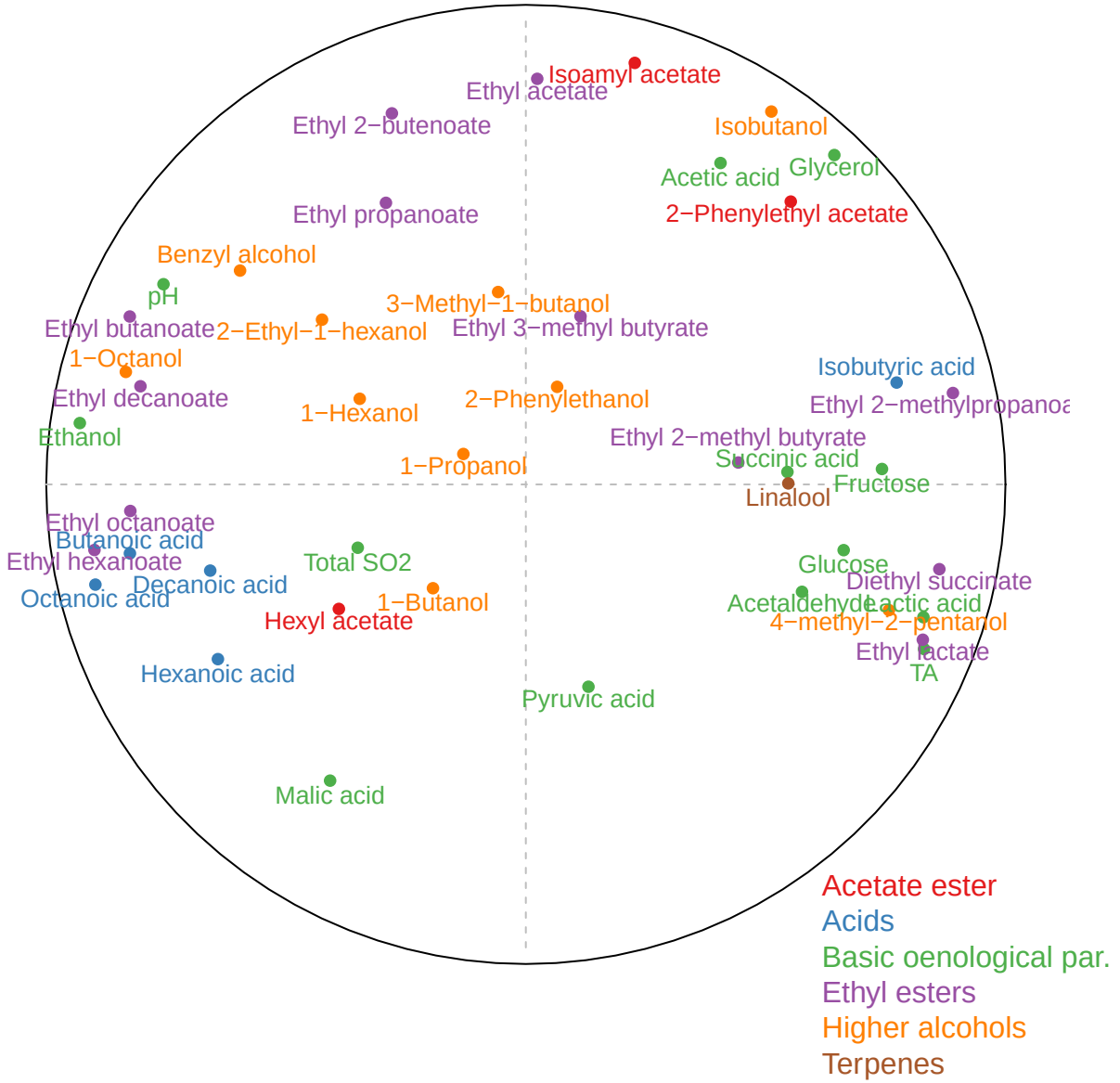

pH
Time (days)
Profile 1
Profile 2
Profile 3
Profile 4
Profile 5
Profile 6
TSS (Baumé)
Profile 1'
Profile 2'
Profile 3'
Profile 4'
Profile 5'
LT3...SC (3)
LT3xSC (3)
LT4...SC (3)
LT1xSC (3)
LT5xSC (3)
UN (3)
SC (3)
LT2xSC (3)
LT4xSC (3)
LT1...SC (3)
LT2...SC (3)
LT1...SC (3)
LT3...SC (3)
LT3xSC (3)
LT4...SC (3)
LT4xSC (3)
LT5...SC (3)
LT5xSC (3)
SC (3)
UN (3)

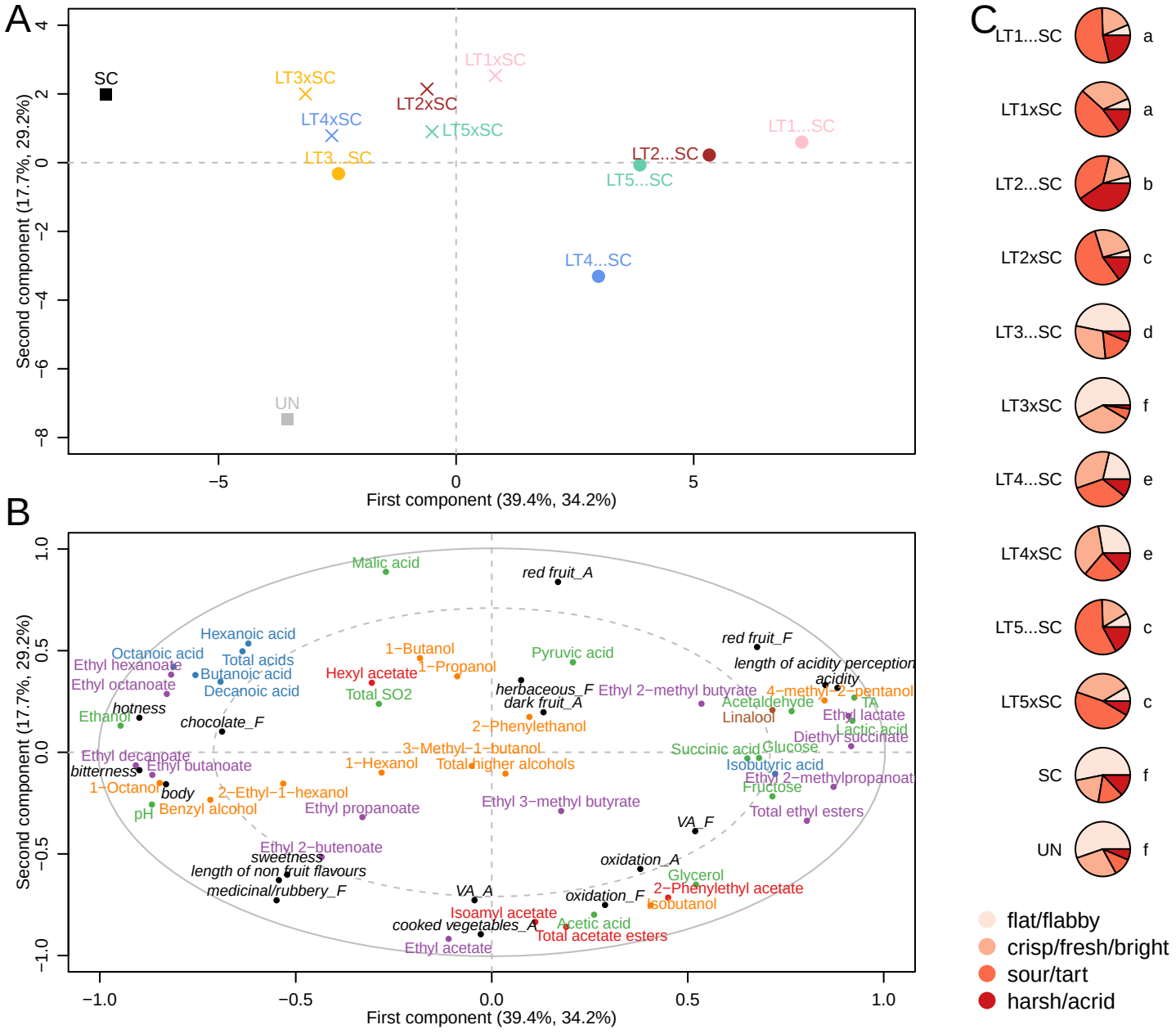

−4
−2
0
2
4
Normalized Z−score
LT1...SC
LT1xSC
LT2...SC
LT2xSC
LT3...SC
LT3xSC
LT4...SC
LT4xSC
LT5...SC
LT5xSC
SC
UN


Glucose
Fructose
Ethanol
pH
TA
Lactic acid
Glycerol
Acetic acid
Malic acid
Succinic acid
Acetaldehyde
Pyruvic acid
Total SO2
Ethyl acetate
Ethyl lactate
Ethyl propanoate
Ethyl 2−methylpropanoate
Ethyl butanoate
Ethyl 2−butenoate
Ethyl 2−methyl butyrate
Ethyl 3−methyl butyrate
Ethyl hexanoate
Ethyl octanoate
Ethyl decanoate
Diethyl succinate
Total ethyl esters
Isoamyl acetate
Hexyl acetate
2−Phenylethyl acetate
Total acetate esters
1−Propanol
1−Butanol
Isobutanol
3−Methyl−1−butanol
4−methyl−2−pentanol
1−Hexanol
2−Ethyl−1−hexanol
1−Octanol
2−Phenylethanol
Benzyl alcohol
Total higher alcohols
Butanoic acid
Isobutyric acid
Hexanoic acid
Octanoic acid
Decanoic acid
Total acids
Linalool
0
20
40
60
80
100
Explained variation (%)
LT
INOC
INTER
RES

a cd a d ef ef cd e bc d f b
Ethyl esters (total)
0 50000 150000
cde ab cde cde cde de a bc e cde bcd cde
Higher alcohols (total)
0e+00 2e+05 4e+05
bcd d bc d bcd cd a bcd b bcd cd a
Acetate ester (total)
0 500 1500 2500
ef b de bc cd bc bc bc f cd a f
Acids (total)
0 4000 8000 12000
LT1...SC LT1xSC LT2...SC LT2xSC LT3...SC LT3xSC LT4...SC LT4xSC LT5...SC LT5xSC SC UN
Total Ethyl esters
Ethyl acetate
Ethyl lactate
Ethyl propanoate
Ethyl 2−methylpropanoate
Ethyl butanoate
Ethyl 2−butenoate
Ethyl 2−methyl butyrate
Ethyl 3−methyl butyrate
Ethyl hexanoate
Ethyl octanoate
Ethyl decanoate
Diethyl succinate
Total Acetate ester
Isoamyl acetate
Hexyl acetate
2−Phenylethyl acetate
Total Higher alcohols
1−Propanol
1−Butanol
Isobutanol
3−Methyl−1−butanol
4−methyl−2−pentanol
1−Hexanol
2−Ethyl−1−hexanol
1−Octanol
2−Phenylethanol
Benzyl alcohol
Total Acids
Butanoic acid
Isobutyric acid
Hexanoic acid
Octanoic acid
Decanoic acid


LT1...SC
LT1xSC
LT2...SC
LT2xSC
LT3...SC
LT3xSC
LT4...SC
LT4xSC
LT5...SC
LT5xSC
SC
UN
PC2 = 16.16%
PC1 = 37.98%
−10
−5
0
5
6
4
2
0
−2


Isoamyl acetate
Ethyl acetate
Ethyl 2−butenoate
Isobutanol
Glycerol
Acetic acid
2−Phenylethyl acetate
Ethyl propanoate
Benzyl alcohol
pH
3−Methyl−1−butanol
Ethyl butanoate
2−Ethyl−1−hexanol
Ethyl 3−methyl butyrate
1−Octanol
Isobutyric acid
Ethyl 2−methylpropanoa
Ethyl decanoate
1−Hexanol
2−Phenylethanol
Ethanol
Ethyl 2−methyl butyrate
Succinic acid
1−Propanol
Fructose
Linalool
Ethyl octanoate
Butanoic acid
Ethyl hexanoate
Total SO2
Glucose
Diethyl succinate
Decanoic acid
Octanoic acid
1−Butanol
Acetaldehyde
Lactic acid
Hexyl acetate
4−methyl−2−pentanol
Ethyl lactate
Hexanoic acid
TA
Pyruvic acid
Malic acid
Acetate ester
Acids
Basic oenological par.
Ethyl esters
Higher alcohols
Terpenes


A
SC
LT3xSC
LT1xSC
LT2xSC
LT4xSC
LT5xSC
LT3...SC
LT1...SC
LT2...SC
LT5...SC
LT4...SC
UN
Second component (17.7%, 29.2%)
First component (39.4%, 34.2%)
B
Malic acid
red fruit_A
Hexanoic acid
Octanoic acid
Ethyl hexanoate
Total acids
Butanoic acid
Ethyl octanoate
Decanoic acid
1−Butanol
1−Propanol
Hexyl acetate
Total SO2
Pyruvic acid
herbaceous_F
dark fruit_A
Ethyl 2−methyl butyrate
2−Phenylethanol
red fruit_F
length of acidity perception
acidity
Acetaldehyde
4−methyl−2−pentanol
TA
Linalool
Ethyl lactate
Lactic acid
Diethyl succinate
hotness
Ethanol
chocolate_F
Ethyl decanoate
bitterness
Ethyl butanoate
1−Octanol
body
2−Ethyl−1−hexanol
pH
Benzyl alcohol
Ethyl propanoate
3−Methyl−1−butanol
1−Hexanol
Total higher alcohols
Succinic acid
Glucose
Isobutyric acid
Ethyl 2−methylpropanoat
Fructose
Ethyl 3−methyl butyrate
Total ethyl esters
VA_F
Ethyl 2−butenoate
sweetness
length of non fruit flavours
medicinal/rubbery_F
oxidation_A
Glycerol
VA_A
oxidation_F
2−Phenylethyl acetate
Isobutanol
Isoamyl acetate
cooked vegetables_A
Acetic acid
Total acetate esters
Ethyl acetate
Second component (17.7%, 29.2%)
First component (39.4%, 34.2%)
C
LT1...SC a
LT1xSC a
LT2...SC b
LT2xSC c
LT3...SC d
LT3xSC f
LT4...SC e
LT4xSC e
LT5...SC c
LT5xSC c
SC f
UN f
flat/flabby
crisp/fresh/bright
sour/tart
harsh/acrid