Managing ethanol and sensory compounds by non-*Saccharomyces* yeasts

Ana Hranilovic

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School of Agriculture, Food and Wine

Faculty of Sciences

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SUMMARY

The pursuit of flavour and phenolic ripeness, augmented by climate warming and extreme weather events, often leads to excessive sugar accumulation in grapes translating to undesirably high ethanol content in wines. Other common characteristics of such grapes and wines are inadequate acidity and aroma profiles, which all together compromise the quality and marketability of the final product. To tackle these issues, research has devised a number of methods implemented across the entire grape and wine production chain. Among these, partial fermentations with non-*Saccharomyces* yeasts is of particular interest, as it represents an undemanding approach that can also impart 'complexity' and distinctness. However, the full potential of non-*Saccharomyces* yeasts in wine ethanol and flavour management remains elusive, and this work aimed to further explore it.

Research has shown that mixed fermentations with non-*Saccharomyces* yeasts can lead to enhanced wine aroma and sensory properties, and albeit limited, the current range of non-*Saccharomyces* is indeed marketed for this purpose. The potential of commercially available (and thus readily implementable) non-*Saccharomyces* co-inocula was assessed in Shiraz fermentations at two maturity levels; earlier harvest (24 °Brix) and technical ripeness (29 °Brix). Eight yeast treatments trialled in pilot scale fermentations included sequential inoculations using three *Torulaspora delbureckii* strains, one strain each of *Lachancea thermotolerans* and *Metschnikowia pulcherrima,* a commercial blend of non-*Saccharomyces* and *S. cerevisiae,* and appropriate *S. cerevisiae* controls. Fermentation monitoring and comprehensive chemical and sensory analysis allowed for the comparison of the treatments. The results showed a pronounced matrix-derived modulation of wine profiles which was reflective of grape maturity levels. Within each harvest date, however, the yeast treatments had

a significant impact on a range of compositional and wine sensory characters. At earlier harvest stage, certain non-*Saccharomyces* treatments, in particular *T. delbrueckii*, led to an increased wine sensory appeal (i.e. 'floral', 'red fruit', 'aroma intensity', 'spice') compared to the *S. cerevisiae* control ('vegetal, 'acidic' and 'bitter'). These treatments, however, were related to incomplete fermentations in higher ripeness conditions. Thus, some non-*Saccharomyces* yeast showed promise in enhancing the quality of wines produced from earlier harvested grapes and as such represent a complementary approach in managing wine ethanol concentrations.

The following study addressed the selection of lower-ethanol producing non-*Saccharomyces* strain(s) for use in sequential cultures with *Saccharomyces cerevisiae*. Oenological performances of six *M. pulcherrima* strains were evaluated in fermentations with *S. cerevisiae* inoculated after seven days. The best-performing *M. pulcherrima* MP2 strain was further characterised in six sequential fermentations with different *S. cerevisiae* inoculation delays in both synthetic and white grape juice. The analysis of main metabolites, undertaken prior to sequential inoculations and upon fermentation completion, highlighted metabolic interactions and carbon sinks other than ethanol in mixed culture fermentations. Depending on the inoculation delay, MP2 white wines contained between 0.6% and 1.2% (v/v) less ethanol than the *S. cerevisiae* control, with even larger decreases achieved in the synthetic juice. The MP2 wines also had higher concentrations of glycerol and lower concentrations of acetic acid. The analysis of volatile compounds revealed compositional alterations arising from the *S. cerevisiae* inoculation delay, with increased acetate esters and higher alcohols detected in all analysed MP2 treatments.

The concept of intra-specific variability was studied using *L. thermotolerans* as a model. This species harbours several metabolic traits that are of value in oenology, including lactic acid production, potential to decrease ethanol content and modulate flavour in wines. The

relationships between 172 *L. thermotolerans* isolates, sourced from natural and anthropic habitats worldwide, were studied using a 14-microsatellite genotyping method. The resultant clustering revealed that the evolution of *L. thermotolerans* has been driven by the geography and ecological niche of the isolation sources. Isolates originating from anthropic, in particular oenological environments, were genetically close, thus suggesting domestication events within the species. The phenotypic performance of the strains, assessed using a number of agar plate-based growth assays with different carbon sources and physicochemical conditions, provided further support for the observed clustering.

To determine whether, and to what extent, *L. thermotolerans* strains differ in the traits of oenological importance, and harbour signatures of domestication and/or local divergence, 94 previously genotyped strains were trialled in Chardonnay fermentations. The strains and the genetic groups were compared for their fermentation performance, production of primary and secondary metabolites and pH modulation. The common traits of *L. thermotolerans* strains were their glucophilic character, relatively extensive fermentation ability, low production of acetic acid and formation of lactic acid, which significantly affected the pH of the wines. An untargeted analysis of volatile compounds revealed that 58 out of 90 volatiles were affected at an *L. thermotolerans* strain level. Besides the remarkable extent of intra-specific diversity, results confirmed the distinct phenotypic performance of *L. thermotolerans* genetic groups. These observations provide further support for the occurrence of domestication events and allopatric differentiation in *L. thermotolerans* population.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time. I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Ana Hranilovic

23rd February 2018

Date

LIST OF OUTPUTS

Publications

Published manuscripts included in this thesis:

Chapter 2: Hranilovic, A., Li, S., Boss, P.K., Bindon, K., Ristic, R., Grbin, P.R., Van der Westhuizen, T. and Jiranek, V., 2018. Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-*Saccharomyces* inocula. Australian Journal of Grape and Wine Research, 24(2):166-180

Chapter 4: Hranilovic, A., Bely, M., Masneuf-Pomarede, I., Jiranek, V. and Albertin, W., 2017. The evolution of *Lachancea thermotolerans* is driven by geographical determination, anthropisation and flux between different ecosystems. PLOS ONE, e0184652, doi:10.1371/journal.pone.0184652

Manuscripts that are submitted/prepared for submission to peer-reviewed journals:

Chapter 3: Hranilovic, A., Gambetta, J., Jeffery, D., Grbin, P.R. and Jiranek, V. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing; prepared for submission to the International Journal of Food Microbiology

Chapter 5: **Hranilovic, A.**, Gambetta, J., Schmidtke, L., Boss, P.K., Grbin, P.R., Masneuf-Pomarede, I., Bely, M., Albertin, W. and Jiranek, V. Oenological phenomes of *Lachancea thermotolerans* reflect patterns of domestication and allopatric differentiation; submitted to Scientific Reports

Scientific symposia

Hranilovic, A., Albertin, W., Bely, M., Masneuf-Pomarede I., Boss P.K., Grbin, P.R. and Jiranek V., 2017. All yeasts are equal, but some yeasts are more equal than others, ComBio, Adelaide, South Australia (invited speaker)

Hranilovic, A., Albertin, W., Bely, M., Masneuf-Pomarede I., Boss P.K., Grbin, P.R. and Jiranek V., 2017. The other yeast – *Lachancea thermotolerans* diversity study, Crush Grape and Wine Symposium, South Adelaide, Australia (oral presentation)

Hranilovic, A, Bely, M., Masneuf-Pomarede I., Jiranek, V. and Albertin, W., 2017. Microsatellite genotyping reveals grouping of *Lachancea thermotolerans* isolates based on their geographic origin and isolation habitat, ISSY 32, Cork, Ireland (poster presentation)

Tondini, F., **Hranilovic, A.**, Grbin, P.R., Herderich, M., van der Westhuizen, T. and Jiranek, V. 2015. Dealing with spontaneous fermentation: Wild yeast behaviour. 27th International Conference on Yeast Genetics and Molecular Biology, Levico Terme, Italy (poster presentation)

Industry communications

Hranilovic, A., Li, S., Grbin, P.R. and Jiranek, V. 2015. Yeasts for high (and low) alcohol. Australian Society of Viticulture and Oenology Seminar 'Earlier, hotter, shorter – Managing compressed vintages', Adelaide, South Australia (invited speaker)

Ristic, R., **Hranilovic, A**., Li, S., Longo, R., Pham, D., Qesja, B., Schelezki, O. and Jiranek, V., 2016. Integrated strategies to moderate the alcohol content of wines. Wine & Viticulture Journal, 31(5):33-38 (industry article)

Tondini, F., **Hranilovic, A.**, Grbin, P.R., Herderich, M., van der Westhuizen, T. and Jiranek, V. 2016. Dealing with spontaneous fermentation: Wild yeast behaviour. 16th Australian Wine Industry Technical Conference, Adelaide, South Australia (poster presentation)

Hranilovic, A., Grbin, P.R. and Jiranek, V. 2016. Managing ethanol and flavour compounds by non-*Saccharomyces* yeasts. ARC Training Centre Workshop, Charles Sturt University, Wagga Wagga, New South Wales (oral presentation)

Hranilovic, A., Grbin, P.R. and Jiranek, V. 2015. Managing ethanol and flavour compounds by non-*Saccharomyces* yeasts. ARC Training Centre Workshop, Launceston, Tasmania (oral presentation)

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This thesis is dedicated to my brother Tomislav. I think of you when I visit the places you cannot visit, taste the wines you cannot taste and seek the light you cannot seek.

CHAPTER 1

Literature Review and Research Aims

This Literature Review was prepared within the first six months of PhD candidature (December 2014) to provide the background material to the project and define the research aims. Thus, it contains only references published to that point. The relevant literature beyond this review is included in the following Chapters.

1.1. Literature review

1.1.1. Increasing ethanol concentrations in wines

1.1.1.1. Causes and consequences

Wine alcohol content has been progressively rising. Since domestication in Neolithic times, human selection has driven the evolution of grapevine (*Vitis vinifera* L.) towards elevated sugar accumulation, one of the key traits differentiating cultivated grape varieties (*Vitis vinifera* subsp. *sativa*) from their wild ancestors (*Vitis vinifera* subsp. *sylvestris*) (This et al., 2006). Concomitantly to grapevine domestication, subpopulations of *Saccharomyces cerevisiae*, the main fermenting agent, were differentiated and co-evolved to persist in the newly-created oenological environments (Legras et al., 2007, Sicard and Legras, 2011, Camarasa et al., 2011). After the development of starter cultures for fermentations in the early 20th century, efficiency in sugar fermentation imposed itself as a major selection criterion for *S. cerevisiae* yeasts used in the wine industry (González et al., 2011, Pretorius, 2000). Further technological advances in viticulture and oenology, allowing for the cultivation and vinification of longer-ripening fruit, as well as consumer preferences for riper wine flavours, have been other driving forces for increased ethanol levels in wines (Alston et al., 2012, Godden and Muhlack, 2010). A worrisome prediction is the exacerbation of this trend through occurring/projected climate change scenarios, and an increased spate of extreme weather events (Schultz and Jones, 2010).

Excess ethanol content in wines has various negative implications, ranging from health related concerns of consumers and policy makers, to additional trade costs and limitations (de Barros Lopes et al., 2003). High potential alcoholic content often exerts difficulties during both alcoholic and malolactic fermentations, jeopardising their timely completion and likelihood of

success (Sumby et al., 2014, Malacrino et al., 2005, Bisson, 1999). The quality of the obtained wines is also compromised, due to potential off-flavour production (e.g. volatile acidity), and inferior sensory properties related to increased perceptions of 'hotness' and decreased aroma 'complexity' (King et al., 2013, Meillon et al., 2010, Escudero et al., 2007, Goldner et al., 2009). There is therefore a strong demand to develop strategies to manage ethanol content in wines.

1.1.1.2. Mitigation strategies

Over the last decades, various methods for wine ethanol reduction were developed across the whole grape and wine production chain (Pickering, 2000, Schmidtke et al., 2012). These include altered viticultural management leading to lower sugar accumulation during ripening (Stoll et al., 2010, Novello and de Palma, 2013), earlier harvest dates (Bindon et al., 2013), establishment of winemaking practices for unripe grapes (Kontoudakis et al., 2011), stripping the juice of fermentable sugars (Pickering et al., 1999, García-Martín et al., 2010), and, most commonly, physical dealcoholisation of wines post-fermentation via spinning cone columns and membrane technologies (Schmidtke et al., 2012, Saha et al., 2013). Albeit effective, these methods have considerable limitations associated with their cost, additional labour and/or equipment, and detrimental effects on wine quality (Schmidtke et al., 2012). In comparison, the use of lower-ethanol yielding yeast in fermentation represents an inexpensive and readily-implementable alternative, and microbiological methods for ethanol reduction are therefore of great interest.

1.1.1.2.1. Microbiological strategies in ethanol reduction

Two distinct approaches have been pursued in microbiological ethanol reduction: genetic modification (GM) and non-GM strategies (Kutyna et al., 2010). Regardless of the approach, decreased ethanol yields in completed fermentations imply partial diversion of carbon from ethanol formation to alternate metabolic pathways and sinks. This is, in fact, challenging, as the carbon, redox and energy balance in fermenting yeast cells need to be fully maintained (Figure 1). It is also of critical importance that the resulting carbon sinks remain quantitatively and qualitatively compatible with wine sensory quality.

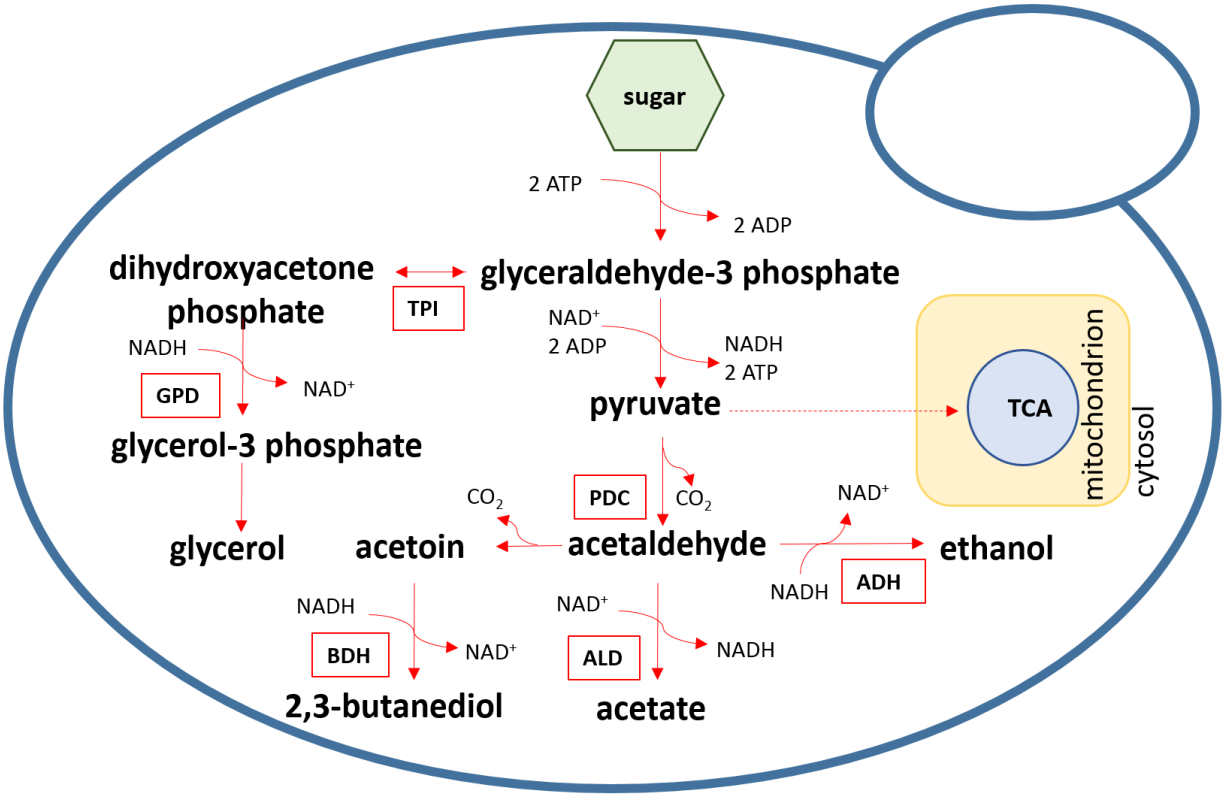

Figure 1. Overview of sugar catabolism in yeast cells. Grape hexoses are metabolised to pyruvate during glycolysis (multiple enzymatic steps). Pyruvate is converted to acetaldehyde (pyruvate decarboxylase, PDC) which is further reduced to ethanol (alcohol dehydrogenase, ADH) in alcoholic fermentation. Acetaldehyde can also be oxidised to acetate (aldehyde dehydrogenase,

ALD) and decarboxylated to acetoin which is further reduced to 2,3-butanediol (butanediol dehydrogenase, BDH). Pyruvate originating is partially shuttled to the mitochondrion and incorporated into the tricarboxylic acid (TCA) cycle. Glycerol is produced via dihydroxyacetone phosphate (glycerol-3-phosphate dehydrogenase, GPD; triosephosphate isomerase, TPI).

A number of *S. cerevisiae* strains were genetically engineered to yield less ethanol via modifications targeting different points of carbohydrate metabolism by exploring either inherent *S. cerevisiae* pathways (Nevoigt and Stahl, 1996, de Barros Lopes et al., 2000, Cambon et al., 2006, Ehsani et al., 2009, Varela et al., 2012, Rossouw et al., 2013) or heterologous gene expression (Dequin and Barre, 1994, Malherbe et al., 2003, Heux et al., 2006, Varela et al., 2012). Among these, the most efficient approaches involve enhanced glycerol production by overexpression of the *GPD1* or *GPD2* genes (Varela et al., 2012, Kutyna et al., 2010, de Barros Lopes et al., 2000). However, even though increased glycerol concentrations *per se* are non-detrimental or even positive for wine quality (Ribéreau-Gayon et al., 2006), glycerol increase leads to excessive acetic acid formation required to maintain a balanced $NADH/NAD^+$ ratio (Figure 1; Eglinton et al., 2002, de Barros Lopes et al., 2003, Cambon et al., 2006, Kutyna et al., 2010). The *GPD1/2* overexpressing *S. cerevisiae* strains were also reported to increase the concentrations of other unfavourable metabolites, i.e. acetaldehyde and acetoin (Eglinton et al., 2002, Cambon et al., 2006, Kutyna et al., 2010). Accordingly, Varela et al. (2012) observed up to a 3.6% (v/v) ethanol decrease in wines produced upon *GPD1* overexpression, however, with side-effects of increased acetate, acetaldehyde and acetoin concentrations in reduced-alcohol wines. Upon *ALD6* deletion, acetate concentrations were acceptable, whereas high acetaldehyde and acetoin levels were only to a degree ameliorated by increasing *BDH1* expression (Varela et al. 2012).

Accumulation of undesired metabolites is, in fact, common not only for glycerol overproducers, but also strains engineered differently to reduce alcohol levels in wines (Dequin and Barre, 1994, Malherbe et al., 2003, Heux et al., 2006, Kutyna et al., 2010). Albeit further modifications can alleviate such anomalies (Varela et al., 2012, Ehsani et al., 2009), maintaining fermentation performance and wine quality in low-alcohol GM yeasts remains a major challenge.

Another barrier ruling out implementation of GM yeasts in the wine industry are the legislative regulations, prompted by perceptions of public opinion. Consequently, microbiological strategies for ethanol reduction that do not involve genetic engineering, and instead rely on natural variation, breeding and/or non-GM mutagenesis, are in high demand. Selection of lower-ethanol producing *S. cerevisiae* strains represents one explored possibility (Loira et al., 2011, Palacios et al., 2007). However, the evolution of *S. cerevisiae* has been thoroughly driven towards quick and efficient conversion of sugars to ethanol as part of the 'make-accumulate-consume' strategy (Piškur et al., 2006). The backbone of such a lifestyle is the Crabtree-effect, i.e. preferential fermentative metabolism in high sugar substrates even in presence of oxygen, contributing to out-competition of other microorganisms (Pronk et al., 1996). Given the genetic differentiation in *S. cerevisiae* populations related to oenological environments (Legras et al., 2007), and its phenotypic manifestation (Camarasa et al., 2011), human selection (albeit initially inadvertent) has further accentuated traits related to fermentation efficiency. Consequently, ethanol yield is rather invariant among *S. cerevisiae* wine strains (Palacios et al., 2007, Varela et al., 2008). Other members of the *Saccharomyces* genus, as well as their inter- and intra-specific hybrids, originating either from natural habitats or breeding programs, were screened for reduced ethanol yields (Bely et al., 2013, Arroyo-López et al., 2010), but their benefits in ethanol reduction appear to be similarly modest. For

example, Bely et al. (2013) observed ethanol decreases of merely 0.34% (v/v) when comparing interspecific *S. uvarum* and *S. cerevisiae* hybrids to *S. cerevisiae* wine strains.

More recently, the concept of evolutionary engineering has been applied to more pro-actively redirect *S. cerevisiae* metabolism away from ethanol production. It involves microbial cultivation in a selective, increasingly challenging environment in order to induce spontaneous mutations and allow for the selection of superior target phenotypes (Portnoy et al., 2011). Osmotic stress treatment with KCl to enhance glycerol production, followed by breeding (Tilloy et al., 2014), resulted in a higher diversion of carbon away from ethanol compared to attempts to stimulate the pentose phosphate pathway using gluconate (Cadière et al., 2011) or glycerol synthesis using sulfites (Kutyna et al., 2012). An ethanol reduction of 1.3% (v/v) relative to the ancestral strain (EC-1118) in Syrah pilot vinifications, accompanied with enhanced glycerol and 2,3-butanediol production without an increase in acetate, have laid ground to claim a research breakthrough for generating the first non-GM *S. cerevisiae* wine strain capable of prominently lowering wine ethanol by Tilloy et al. (2014).

Selection of wine strains has also extended beyond the conventional wine yeast, *S. cerevisiae*, to so-called non-*Saccharomyces* yeasts that offer remarkable variability not only in ethanol yield, but also other oenologically-relevant traits (Jolly et al., 2014). Despite an increasing number of studies focusing on the characterisation of different non-*Saccharomyces* species and strains, their potential in ethanol and wine quality management remains largely underexplored.

1.1.2. Non-*Saccharomyces* yeasts in oenology

1.1.2.1. Ecology of non-*Saccharomyces* yeasts

In oenology, the term 'non-*Saccharomyces* yeasts' refers to a number of yeast species, excluding *S. cerevisiae*, that have been described in the wine-related environments (Jolly et al., 2014). About 40 yeast species been cultured from grape and must substrates (Jolly et al., 2014). Comparatively, the use of culture-independent high-throughput sequencing methods revealed much higher diversity of the microbiota on grapes, whilst also unravelling their regional, site and grape variety-specific dispersal (Bokulich et al., 2014, Taylor et al., 2014, David et al., 2014). While some are arguably rather inconsequential for wine quality, primarily due to their aerobic metabolism (Jolly et al., 2014), a number of yeasts that are found on grapes are subsequently encountered in fermenting grape juices/musts, including *Hansenisapora* spp., *Candida* spp., *Metschnikowia* spp., *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Starmella bacillaris* (Cocolin et al., 2000, Fleet, 2003, Jolly et al., 2003, Combina et al., 2005, Zott et al., 2008, Jolly et al., 2014, David et al., 2014). Grapes are in fact the primary source of these yeasts, whereas the occurrence of *S. cerevisiae* in vineyards is conspicuously rare (Mortimer and Polsinelli, 1999, Taylor et al., 2014). Upon their transfer into the winery, the persistence of non-*Saccharomyces* highly depends on various oenological practices, in particular additions of starter cultures and sulfur dioxide, temperature, clarification, and overall cellar hygiene (Jolly et al., 2003, Zott et al., 2008, Jolly et al., 2014, Albertin et al., 2014). In general, their prevalence is limited, and shortly upon fermentation commencement *S. cerevisiae* becomes a predominant species due to quick ethanol formation and tolerance as its competitive evolutionary edge (Pretorius, 2000, Piškur et al., 2006). While accumulating ethanol concentrations largely determine the survival and succession of species and strains in fermentation, this is not the sole cause for the population decline of non-*Saccharomyces* yeasts

(Holm Hansen et al., 2001, Perez-Nevado et al., 2006, Renault et al., 2013). Research has highlighted the importance of higher oxygen availability for prolonged survival of *L. thermotolerans* and *T. delbreuckii* (Holm Hansen et al., 2001), as well as the mechanisms of quorum sensing and/or cell-cell contact with *S. cerevisiae* as a cause of non-*Saccharomyces* cell death (Nissen and Arneborg, 2003, Nissen et al., 2003, Renault et al., 2013).

1.1.2.2. Mixed culture fermentations

Non-*Saccharomyces* yeasts display sensitivity to a range of (a)biotic stressors and, unlike *S. cerevisiae*, cannot deplete all sugars from the grape juice/must, i.e. 'complete' wine fermentation (Jolly et al., 2014). Moreover, due to the isolation from incomplete or protracted fermentations and/or analytically anomalous wines, these yeasts were long regarded solely as spoilage organisms (Loureiro, 2003, Ciani et al., 2010). Winemakers would therefore generally seek to unselectively inhibit their growth, which is in most cases achieved by the addition of commercially acquired *S. cerevisiae* as a high density inoculum, commonly coupled with SO_2 addition in concentrations toxic for most other microorganisms (Pretorius, 2000). Inoculating a fermentation in such a manner has become a ubiquitous oenological practice, as it ensures reliable and timely processing with a consistent outcome (Pretorius, 2000, González et al., 2011). Nowadays, however, the large inter- and intra-species diversity amongst non-*Saccharomyces* has become more apparent; while some strains cause wine spoilage, others can improve its overall quality. Moreover, co-existence and progression of multiple species results in a more diverse metabolic matrix compared to a *S. cerevisiae* monoculture, which can lead to increased aroma/flavour complexity and palate structure (Lambrechts and Pretorius, 2000, Varela et al., 2009, Ciani et al., 2010). Promoting the proliferation of native microflora by omitting additions of *S. cerevisiae* starter cultures can therefore be beneficial for wine quality.

However, the lack of predictability and reproducibility hinders wider industrial applicability of the un-inoculated fermentation modality.

In an attempt to address the sensory uniformity and decreased complexity of inoculated wines, while avoiding the risks of un-inoculated fermentation, mixed culture inoculation has been proposed as an innovative fermentation management approach in winemaking (Ciani et al., 2010). It implies simultaneous or sequential inoculation of selected non-*Saccharomyces* and *Saccharomyces* strains, where non-*Saccharomyces* proliferate in the early stages of the fermentation, contributing to the chemical and sensory properties of the wine, whereas the later stages are dominated by more competitive *Saccharomyces* yeasts, ensuring fermentation completion. At an industrial level, however, this concept is still in its infancy; as of now only a few non-*Saccharomyces* starters representing four yeast species (i.e. *T. delbrueckii, L. thermotolerans, M. pulcherrima and Pichia kluyveri*) are available on a market saturated with hundreds of *S. cerevisiae* products (Jolly et al., 2014). However, multiple non-*Saccharomyces* selection and characterisation efforts delivering promising results in terms of chemical and sensorial modulation of wine suggest a likely emergence of novel non-*Saccharomyces* starters for the industry in the near future.

1.1.2.3. Contribution of non-*Saccharomyces* yeasts in winemaking

In mixed culture fermentations, the oenological contribution of non-*Saccharomyces* strains is largely defined by the fermentation management. The initial absence of *S. cerevisiae* in sequential inoculations allows for a greater impact of non-*Saccharomyces* yeasts compared to the co-inoculated modality (Ciani et al., 2010). The time interval between the two inoculations and/or inoculation density further affects the fermentation outcome (Kapsopoulou et al., 2007, Ciani et al., 2010, Comitini et al., 2011, Bely et al., 2013). Major alterations in

wine analytical parameters obtained in mixed culture fermentations compared to *S. cerevisiae* controls were reported for concentrations of glycerol (Gobbi et al., 2013, Contreras et al., 2014, Comitini et al., 2011), different organic acids (Gobbi et al., 2013, Bely et al., 2008, Comitini et al., 2011, Kapsopoulou et al., 2005, Kapsopoulou et al., 2007, Contreras et al., 2014), yeast-derived polysaccharides (Comitini et al., 2011, Domizio et al., 2014, Domizio et al., 2011b, Domizio et al., 2011a), phenolic compounds (Benito et al., 2011, Morata et al., 2012, Benito et al., 2012) and a range of flavour-active compounds (Gobbi et al., 2013, Comitini et al., 2011, Zott et al., 2011, Sadoudi et al., 2012, Anfang et al., 2009). Not surprisingly, the main fermentation metabolite is also affected.

1.1.2.3.1. Non-*Saccharomyces* yeasts in ethanol management

The potential of non-*Saccharomyces* diversity in ethanol reduction has initially been overlooked. At the time a number of *S. cerevisiae* strains had been genetically modified to yield less ethanol in wine, and lengthy evolutionary engineering programs had already commenced, this phenotype was not a selection criterion for non-*Saccharomyces* yeasts. There is, accordingly, no mention of yeasts other than *S. cerevisiae* in a review on microbiological methods in ethanol reduction by Kutyna et al. (2010). Nonetheless, lower ethanol yields of some non-*Saccharomyces* yeasts were noticed in several studies that evaluated their oenological potential with another primary focus (Ciani and Maccarelli, 1997, Ciani et al., 2006, Magyar and Tóth, 2011). Likewise, ethanol decreases ranging between 0.2% - 0.9% (v/v) compared to *S. cerevisiae* controls were haphazardly observed in ‘dry’ mix-fermented wines in multiple studies (Ferraro et al., 2000, Soden et al., 2000, Comitini et al., 2011, Di Maio et al., 2011, Sadoudi et al., 2012, Gobbi et al., 2013). Recently, two lines of research on lower-

alcohol non-*Saccharomyces* yeasts emerged concurrently: selection under anaerobic, i.e. typical winemaking conditions, and under aerobic conditions.

1.1.2.3.1.1. Selection of lower-alcohol non-*Saccharomyces* yeasts under anaerobic conditions

The first systematic attempt to select lower-alcohol non-*Saccharomyces* (and non-*cerevisiae*) yeasts under winemaking conditions was reported by Bely et al. (2013), highlighting a *St. bacillaris* strain capable of decreasing wine ethanol in sequential fermentation with *S. cerevisiae*. Upon initial screening of 59 *St. bacillaris* strains, five strains showing acceptable aroma profiles were selected for co- and sequential inoculation trials. While the former modality did not elicit significant ethanol decreases, sequential cultures led to a 0.4 – 0.9% (v/v) ethanol reduction. However, the organoleptic properties of the obtained wines were unsatisfactory. The same authors did not find significant differences between the ethanol yields of *S. cerevisiae* and 30 *T. delbureckii* strains (Bely et al., 2013).

Shortly after, Gobbi et al. (2014) conducted non-*Saccharomyces* pure culture grape juice fermentation trials using 33 strains belonging to nine different yeast species. Comparing pooled strains of single species to the *S. cerevisiae* control, the authors observed significantly lower ethanol yields in *H. uvarum*, *Zygosaccharomyces bailii*, *Z. sapae* and *Z. bisporus* fermentations, and an increased formation of other fermentation by-products. However, these were not evaluated in co-cultures with *S. cerevisiae*, and thus wines contained high levels of residual sugar (31-116 g/L).

Broader characterisation of non-*Saccharomyces* yeasts was undertaken by Contreras et al. (2014), who screened 50 strains belonging to 40 species of 24 genera with the aim of identifying candidates to be used for ethanol reduction in sequential inoculation with *S.*

cerevisiae. Out of the initial number, only fifteen yeasts with lower ethanol yield than *S. cerevisiae* metabolised more than 20% of sugar in a monoculture and were chosen for further trials. Sequential inoculation with two strains of *M. pulcherrima* and one strain each of *Schizosaccharomyces malidevorans* and *C. stellata* reached completion and yielded significantly lower ethanol concentrations than the *S. cerevisiae* control. The most prominent ethanol decrease of 1.3% (v/v) was observed with the use of *M. pulcherrima* AWRI1149 and the consistency of results was confirmed in Chardonnay and Shiraz fermentations, with ethanol reductions of 0.9 and 1.3% (v/v), respectively. As stated by the authors, increased production of glycerol (observed in all mixed fermentations) and some organic acids could partially explain the ethanol reduction, however additional carbon sinks are likely to exist. Chemical composition of obtained wines was mainly analytically satisfactory, apart from the concentration of ethyl acetate in white wine (207.6 mg/L) being above the level (150 mg/L) considered detrimental for quality.

1.1.2.3.1.2. Respiration-based approach in ethanol reduction

An alternative approach in using non-*Saccharomyces* for wine ethanol reduction, as outlined by González et al. (2013), is based on the different regulatory mechanisms of the respiro-fermentative metabolism among yeasts. Unlike *S. cerevisiae*, the archetypical Crabtree-positive species which favours fermentation when sugar concentrations exceed 10 g/L, many non-*Saccharomyces* yeasts do not display such respiration repression (de Deken, 1966). Therefore, aeration during the initial stage of fermentation conducted by a suitable non-*Saccharomyces* strain, followed by the arrest of the aeration coinciding with *S. cerevisiae* sequential inoculation, was proposed to achieve ethanol reduction due to respiration (González et al., 2013). Experimental work exploring this concept is scarce, as is the information

regarding exact metabolic characteristics of different yeasts; nonetheless several early reports (Smith, 1995, Erten and Campbell, 2001) suggested its feasibility.

Use of non-*Saccharomyces* for lowering ethanol levels in wine via respiration was extensively evaluated by Quiros et al. (2014). The screening of 59 non-*Saccharomyces* yeasts under aerated conditions involved the estimation of the respiration quotient (RQ), i.e. ratio of CO_2 produced per O_2 consumed, and concentrations and yields of other key metabolites. Several strains were suitable for lowering ethanol levels by respiration, in particular *M. pulcherrima* IFI1244. Interestingly, the comparison of *M. pulcherrima* and *S. cerevisiae* performance in aerated fermentations revealed that ethanol reduction could be achieved using either of the two species. However, in the case of *S. cerevisiae*, it was accompanied with an increase in acetic acid, whereas in *M. pulcherrima* fermentation acetate concentrations remained undetectable. This novel research avenue will have to carefully assess and fine-tune oxygenation regimes so as to avoid the negative impact of oxygen on wine phenolic and flavour profile.

1.1.2.3.2. Acidity modulation by non-*Saccharomyces* yeasts

Non-*Saccharomyces* yeasts were reported to modulate both volatile and non-volatile acidity in wines. Acetic acid is the main contributor to wine volatile acidity (90-95%), and concentrations above 1 g/L are unacceptable in most wine styles (Ribéreau-Gayon et al., 2006). In *S. cerevisiae* increased production of acetate in high sugar musts is linked to glycerol synthesis in response to hyperosmotic stress due to redox balance restoration (Figure 1; Blomberg and Alder, 1989). However, glycerol and acetate production are not positively correlated in some non-*Saccharomyces* species, in particular *T. delbrueckii*, which was proposed for use in botrytised musts (Bely et al., 2008). Other species reported to produce low

acetate are *M. pulcherrima* and *L. thermotolerans* (Comitini et al., 2011, Gobbi et al., 2013, Contreras et al., 2014). Conversely, high-acetate producers commonly belong to species of *Hanseniaspora, Zygosaccharomyces* and *Schizosaccharomyces,* albeit this phenotype, much like others, is largely strain-dependent (Ciani et al., 2006, Benito et al., 2014, Jolly et al., 2014).

Extensively ripened grapes from hot climates often contain low concentrations of organic acids, which may cause imbalance in wines and facilitate microbial spoilage due to pH dependent SO_2 fractionation (Ribéreau-Gayon et al., 2006). Low acidity is most commonly corrected by addition of tartaric acid, and to a lesser extent other organic acids (Ribéreau-Gayon et al., 2006). Other methods, such as ion exchange (Minguez, 2003), electrodialysis (Ochoa et al., 1999) and blending regimes with grapes/cultivars with retained acidity (Ribéreau-Gayon et al., 2006, Kontoudakis et al., 2011) are also in practice. Alternatively, acidification can be achieved via metabolic activity of some species, in particular *L. thermotolerans,* which is characterised by production of lactic acid in orders of magnitude that by far surpass any other non-GM yeast (Kapsopoulou et al., 2005, Mora et al., 1990, Su et al., 2014). In contrast, some species can have a de-acidifying character, mainly related to, either partial or complete, malic acid degradation (Su et al., 2014). This trait is of particular interest in cooler vine-growing regions where grapes at ripeness contain high levels of malic acid, which in wine imparts 'harsh' acidity, and can support growth of spoilage organisms (Sumby et al., 2014). Among the yeasts reported to (partially) degrade extracellular malate, e.g. *Issatchenkia orientalis, L. thermotolerans, Z. bailii* (Kapsopoulou et al., 2005, Su et al., 2014, Jolly et al., 2014,), *Sc. pombe* has received special attention as it can also lead to wine colour improvement (Benito et al., 2012) and increased release of autolytic polysaccharides (Palomero et al., 2009).

1.1.2.3.3. Impact of non-*Saccharomyces* yeasts on wine aroma and flavour

Wine aroma is perhaps the most important intrinsic characteristic of wine. It is often subdivided into three categories: primary or varietal aroma, derived from the grapes; secondary aroma, formed during fermentation; and tertiary aroma or bouquet, shaped during wine aging (Lambrechts and Pretorius, 2000). Non-*Saccharomyces* yeast can directly affect both primary aroma and secondary aroma by specific enzymatic activities and production of metabolites, respectively.

Within the constraints of a variety, the extent of ripeness was shown to affect the main classes of compounds that shape the primary aroma of wine (Bindon et al., 2013), i.e. methoxypyrazines, C13-norisoprenoids, volatile sulfur compounds and terpenes (Ebeler and Thorngate, 2009). Of these, terpenes are important primarily, but not exclusively, for so-called aromatic varieties, as they impart floral notes at low sensory thresholds (Strauss et al., 1986, Kalua and Boss, 2009). In grapes, terpenes are partially found in the form of odourless glycosylated precursors, which are hydrolysed via yeast activity during fermentation, although *de novo* synthesis has also been reported in yeasts (Carrau et al., 2005). The release of terpenes occurs in two steps; depending on the substrate, α-L-arabinofuranosidase, α-L-rhamnosidase or β-D-apiosidase hydrolyse terpene glycosides to monoterpenyl-β-D-glucosides, which are further cleaved by β-glucoside activity (Gunata et al., 1988). The β-D-glucoside activity is often assessed during selection and characterisation studies of non-*Saccharomyces* yeasts, and has been described in a number of species, including *T. delbrueckii, St. bacillaris, L. thermotolerans, M. pulcherrima, H. uvarum* and *Debaryomyces hansenii* (Rosi et al., 1994, Comitini et al., 2011), potentially driving the increases in terpenes. For instance, Sauvignon Blanc wines co-fermented with *S. cerevisiae* and either *T. delbrueckii* or *St. bacillaris* contained more terpenols than the *S. cerevisiae* monoculture (Sadoudi et al., 2012). Another

group of grape-derived compounds that can be modulated by yeast activity are the volatile thiols. Volatile thiols impart the specific varietal character of Sauvignon Blanc, and strains of some non-*Saccharomyces* species, such as *P. kluyveri, St. bacillaris, L. thermotolerans, T. delbrueckii* and *M. pulcherrima*, were reported to produce an increased amount of volatile thiols (Anfang et al., 2009, Zott et al., 2011).

Major constituent wine secondary aromas are esters and higher alcohols. In *S. cerevisiae*, the biosynthesis of these flavour-active compounds has been well-documented, and information is available on both genetic regulation and physicochemical conditions affecting their production (Hazelwood et al., 2008, Sumby et al., 2010, Cordente et al., 2012). Much less is known about the non-*Saccharomyces* yeasts. Results on secondary aroma alterations in wines produced by non-*Saccharomyces* yeasts are often rather descriptive, and highlight alterations of aroma compounds relative to the *S. cerevisiae* controls. Regardless, valuable, species-specific patterns, as well as extensive intra-specific diversity were revealed in such studies. For example, *L. thermotolerans* is a high producer of ethyl lactate (Comitini et al., 2011, Gobbi et al., 2013), in line with lactate production, and the importance of precursor availability in ethyl ester production (Saerens et al., 2008). Another example is an increased production of ethyl acetate by the apiculate yeasts (Ciani and Maccarelli, 1997, Viana et al., 2008), the main wine ester that becomes faulty in excess of 150 mg/L (Sumby et al., 2010). Ethyl acetate concentrations above this limit were also found in some wines produced with the recently selected lower-ethanol *M. pulcherrima* strain in sequential cultures with *S. cerevisiae* (Contreras et al., 2014). On the other hand, *T. delbrueckii* strains produced very little ethyl acetate and 2-phenylethyl acetate (rose aroma) in Viana et al. (2008). In contrast, 2-phenylethyl acetate concentrations were dramatically higher in *Pichia* and *Hanseniaspora* fermentations (Viana et al., 2008), leading to a remarkable 3- to 9-fold increase in 2-phenylethyl acetate in

Hanseniaspora osmophila - *Saccharomyces* mixed cultures (Viana et al., 2009). The precursor of this ester, 2-phenyethanol, was also increased in co-cultures of *S. cerevisiae* and *M. pulcherrima, St. bacillaris, T. delbrueckii* or *L. thermotolerans*, but this increase was not necessarily observed in their pure cultures (Comitini et al., 2011, Sadoudi et al., 2012, Gobbi et al., 2013). Higher alcohols other than 2-phenylethanol were analysed in multiple other studies (Renault et al., 2009, Comitini et al., 2011, Sadoudi et al., 2012, Gobbi et al., 2013, Contreras et al., 2014) and their concentrations largely depended on the studied species, strain, fermentation conditions and modalities.

Altogether, modulations in concentration and composition of volatiles highlight the immense impact of non-*Saccharomyces* yeasts in shaping wine aroma and flavour, and selection can provide phenotypes suited for specific wine styles. In parallel, more knowledge on fundamental metabolic features (e.g. studies of gene expression and enzymatic activity involved in amino acid uptake, esterification, fatty acid synthesis and other) will help to successfully steer these novel co-starters towards the targeted aroma and flavour profiles.

1.1.3. Concluding remarks

Non-*Saccharomyces* yeasts represent a large pool of inter- and intra-specific diversity that has yet to be fully explored and exploited in oenology. Their different metabolic characteristics to that of *S. cerevisiae*, alongside optimisation of oenological practices such as inoculation and oxygenation modalities, can be used to fine-tune ethanol levels in wine whilst enhancing rather than lessening its overall quality. Thorough selection and characterisation generating the basic understanding of non-*Saccharomyces* growth, metabolic activity and the extent of intra-specific diversity is, however, a pre-requirement for their targeted use in the wine industry.

1.2. Research aims

This research aims to explore the oenological potential and diversity of non-*Saccharomyces* yeasts to modulate the main compositional parameters of wines, i.e. ethanol content, acidity profile and volatile composition, which are often out of balance in the era of excessively ripe grapes. This project's aims will be realised through individual studies comprising the following objectives.

1.2.1. Objective 1: Evaluation of currently available non-*Saccharomyces* starter cultures in the production of Shiraz wines at different grape maturity levels

Perhaps the simplest and the most intuitive way to decrease ethanol in wines is to harvest grapes earlier. However, intense fruit flavours and fuller body are generally preferred by consumers and winemakers alike, and wines made from earlier harvested grapes can be deficient in such sensory characteristics. Based on the comprehensive literature review, mixed culture fermentations with non-*Saccharomyces* can lead to enhanced wine aroma and sensory properties, and albeit limited, the current non-*Saccharomyces* assortment is indeed marketed for this purpose. The potential of commercially available (and thus readily implementable) non-*Saccharomyces* co-inocula was therefore assessed in Shiraz fermentations at two maturity levels. Grapes were harvested at 24 °Brix, i.e. earlier harvest representing sub-optimally ripe fruit, and 29 °Brix, i.e. concomitant with the commercial harvest in the vineyard. The aim was to examine whether the non-*Saccharomyces* treatments could boost overall quality in the earlier harvested fruit, and test their performance at commercial harvest. The fermentations were conducted with eight yeast treatments, including non-*Saccharomyces* starters sequentially inoculated with *S. cerevisiae*, and appropriate *S. cerevisiae* controls, and managed identically. The wines were subjected to an extensive chemical analysis and sensory profiling, allowing for the comparison of these treatments, as reported in Chapter 2.

1.2.2. Objective 2: Selection and characterisation of non-*Saccharomyces* strain(s) for production of lower-alcohol wines in sequential culture with *S. cerevisiae*

Non-*Saccharomyces* yeasts have shown great potential in managing wine ethanol content, however, lower-alcohol starters are still in demand. A study was therefore designed to test and select a non-*Saccharomyces* strain(s) that would decrease wine ethanol in co-culture with *S. cerevisiae*. Strains of *M. pulcherrima*, pre-selected based on their lower ethanol yields, were trialled in a synthetic grape juice medium in sequential inoculations with *S. cerevisiae*. The *M. pulcherrima* strain leading to the greatest ethanol decrease was further tested in a series of sequential inoculations with different time intervals between the two inoculations in both synthetic and real grape juice. The analysis of the main metabolites was undertaken prior to *S. cerevisiae* addition and upon fermentation completion, so as to study the compositional alterations in terms of ethanol, key metabolites and, for a subset of samples, volatile compounds, arising from the delays in sequential inoculation, while highlighting the potential carbon sinks other than ethanol. This study is reported in Chapter 3.

1.2.3. Objective 3: Exploring the intra-specific diversity of *L. thermotolerans*

The following studies focused on the concept of intra-specific diversity among non-*Saccharomyces* yeast, using *L. thermotolerans* as a model, a species of remarkable yet under-explored oenological potential. *L. thermotolerans* is a unique species among oenologically-relevant yeasts, as it abundantly produces lactic acid. The resultant acidification is of value in warmer vintages and regions, where harvested grapes commonly lack acidity. Moreover, lower ethanol yields and aroma enhancement were also reported in wines co-fermented with *L. thermotolerans*. Like many other (non-*Saccharomyces*) yeasts, *L. thermotolerans* inhabits both

wine-related and natural habitats. A collection of *L. thermotolerans* isolates sourced from different ecological niches worldwide was therefore acquired to be studied at both the genetic and phenotypic level. The genetic diversity and population structure in *L. thermotolerans* was studied using a 14-microsatellite set, complemented with plate-based growth assays, as reported in Chapter 4. To understand whether and to what extent the observed genetic diversity was echoed at a phenotypic level, a subset of strains was further tested for their oenological performance in Chardonnay fermentations. Chapter 5 reports the comprehensive oenological phenomes of 94 strains, compared for their fermentation kinetics, yields of main metabolites and volatile composition, and highlights traits of value to the wine industry.

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CHAPTER 2

Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-*Saccharomyces* inocula

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Principal Author

Name of Principal Author (Candidate)	Ana Hranilovic		
Contribution to the Paper	Conceptualisation of the work/experimental design Experimental work/data collection Data collation, interpretation and analysis Manuscript preparation and editing		
Overall percentage (%)	55%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14.2.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Sijing Li		
Contribution to the Paper	Sensory composition - data collection Manuscript preparation and editing		
Signature		Date	13/2/18

Name of Co-Author	Paul Boss		
Contribution to the Paper	Volatile composition - data collection Manuscript preparation and editing		
Signature		Date	14/2/18

Name of Co-Author	Keren Bindon		
Contribution to the Paper	Phenolic composition - data collection Manuscript preparation and editing		
Signature		Date	

Name of Co-Author	Renata Ristic		
Contribution to the Paper	Statistical analysis Manuscript preparation and editing		
Signature		Date	12.02.18

Name of Co-Author	Paul Grbin		
Contribution to the Paper	Conceptualisation of the work/experimental design Manuscript preparation and editing		
Signature		Date	22/02/2018

Name of Co-Author	Tertius van der Westhuizen		
Contribution to the Paper	Conceptualisation of the work/experimental design Manuscript preparation and editing		
Signature		Date	20.2.18

Name of Co-Author	Vladimir Jiranek		
Contribution to the Paper	Conceptualisation of the work/experimental design Manuscript preparation and editing		
Signature		Date	12.2.18

Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-*Saccharomyces* inocula

A. HRANILOVIC[1,2], S. LI[1,2], P.K. BOSS[3], K. BINDON[4], R. RISTIC[1], P. R. GRBIN[1], T. VAN DER WESTHUIZEN[5] and V. JIRANEK[1,2]

[1] The Australian Research Council Training Centre for Innovative Production, The University of Adelaide, Glen Osmond, SA, 5064, Australia; [2] Department of Wine and Food Science, The University of Adelaide, Glen Osmond, SA, 5064, Australia; [3] CSIRO Agriculture and Food, Glen Osmond, SA, 5064, Australia; [4] The Australian Wine Research Institute, Glen Osmond, SA, 5064, Australia; [5] Laffort Australia, Woodville Norh, SA, 5012, Australia
Corresponding author: Professor Vladimir Jiranek, email vladimir.jiranek@adelaide.edu.au

Abstract

Background and Aims: The choice of yeast strain(s) to conduct the fermentation can greatly affect wine chemical and sensory profile. Even though the use of non-*Saccharomyces* co-inocula to build complexity and diversify styles is increasingly in vogue, a limited number of such products are available to date, and more research is required to guide their use in the wine industry. This study evaluates the potential of commercial yeast inocula to modulate the quality of Shiraz wines at two maturity levels.

Methods and Results: Vinification outcomes of eight yeast treatments were compared in earlier (24°Brix) and later (29°Brix) harvested Shiraz fruit. Yeast treatments included five non-*Saccharomyces* products with sequentially inoculated *Saccharomyces cerevisiae*, a commercial blend of non-*Saccharomyces* and *S. cerevisiae* strains, and a *S. cerevisiae* inoculum. Fermentation monitoring, and comprehensive analytical profiling in terms of basic chemistry, volatile composition, phenolic measurements and descriptive sensory analysis, allowed for the comparison of the resulting wines. Both harvest date and yeast inoculation treatments had a significant impact on a range of compositional and, in turn, sensory parameters of the wines.

Conclusions: Certain non-*Saccharomyces* sequential inoculation treatments led to increased appeal of earlier harvest wines compared to the *S. cerevisiae* Control. These treatments, however, were related to an increased risk of arrested fermentation in higher ripeness conditions.

Significance of the Study: This study contributes to a better understanding of yeast inoculum-derived modulation of Shiraz wine quality parameters at different maturity levels.

Keywords: *mixed culture inoculation, non-*Saccharomyces *yeasts, sensory evaluation, Shiraz, volatile compounds, yeast inoculum*

Introduction

Alcoholic fermentation is a step of critical importance in oenology, involving the bioconversion of grape sugars, primarily glucose and fructose, to ethanol and CO_2 with a concomitant release of secondary by-products that affect the chemical and sensory properties of the obtained wine. It is generally conducted by *Saccharomyces cerevisiae* – the 'conventional' wine yeast; however, other species, so-called non-*Saccharomyces* yeasts, are also involved in the fermentation process. Originally regarded as wine spoilage organisms, these yeasts are now being re-evaluated as positive contributors to wine quality (Jolly et al. 2014, Padilla et al. 2016).

Non-*Saccharomyces* yeasts play an important role in uninoculated fermentation, which is characterised by the co-existence and succession of multiple yeast species and strains. Such a complex metabolic matrix can lead to an increased aroma and flavour diversity and superior wine quality (Domizio et al. 2007, Varela et al. 2009, Jolly et al. 2014, Padilla et al. 2016). Due to variable population composition and dynamics, however, and an increased risk of protracted and/or incomplete sugar catabolism and spoilage, this fermentation modality lacks predictability and reproducibility, and has limited industrial applicability (Ciani et al. 2016a). Consequently, the use of a selected *S. cerevisiae* starter culture in the so-called inoculated fermentation has become a common oenological practice. In inoculated fermentations, a high density inoculum contributes to the suppression of native microflora, enabling a reliable and timely fermentation with a consistent outcome (Jolly et al. 2014, Ciani et al. 2016a,). This is, however, often seen to result in overall decreased complexity and sensory uniformity of inoculated wines compared to their spontaneously fermented counterparts (Domizio et al. 2007, Varela et al. 2009, Jolly et al. 2014, Padilla et al. 2016). In attempts to overcome the shortcomings of inoculation while avoiding the risks of its omission, an innovative alternative has been proposed, referred to as the mixed culture fermentation (Ciani et al. 2011, 2016a, Comitini et al. 2011, Jolly et al. 2014). It involves an inoculation of a selected non-*Saccharomyces* strain, with a simultaneously or sequentially added *Saccharomyces* culture. In this a way, non-*Saccharomyces* yeasts, characterised by a limited sugar consumption and sensitivity to various (a)biotic stressors (Renault et al. 2013, Jolly et al. 2014, Kemsawasd et al. 2015, Albergaria and Arneborg 2016), contribute to the chemical and sensory properties of the wine, while the more competitive *Saccharomyces* yeasts

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ensure fermentation completion (Ciani et al. 2011, 2016a, Jolly et al. 2014, Albergaria and Arneborg 2016).

Observations that the inclusion of non-*Saccharomyces* yeasts in the fermentative medium can positively affect wine chemical and, in turn, sensory profile have led to characterisation of a large inter- and intra-species diversity pool in order to select phenotypes to be used in producing certain wine styles. Indeed, a body of research elucidates the contribution of numerous 'wild' non-*Saccharomyces* isolates to the production of primary and secondary fermentation metabolites, as well as their modulation of grape-derived aroma and phenolic substances. Accordingly, major alterations in wine analytical parameters obtained in fermentations (partially) conducted by non-*Saccharomyces* yeasts were reported for ethanol yield (Comitini et al. 2011, Contreras et al. 2014, 2015, Ciani et al. 2016b), glycerol concentration (Comitini et al. 2011, Contreras et al. 2014, 2015), major grape/wine organic acids including acetic acid (Kapsopoulou et al. 2007, Bely et al. 2008, Comitini et al. 2011, Su et al. 2014), a wide range of wine volatiles (Comitini et al. 2011, Sadoudi et al. 2012, Padilla et al. 2016, Varela et al. 2016), yeast-derived polysaccharides (Comitini et al. 2011, Giovani et al. 2012, Domizio et al. 2014), and colour compounds (Benito et al. 2011, Morata et al. 2012). It is therefore likely the industry will see an emergence of novel non-*Saccharomyces* in the near future; however, only a few non-*Saccharomyces* products are currently commercialised and available for wine production, as opposed to hundreds of *Saccharomyces* starters. Likewise, compared to extensive research focusing on the evaluation of *Saccharomyces* yeasts available for winemaking, information on the effect of non-*Saccharomyces* on the composition of wines from different grape cultivars or wine styles is still scarce.

Shiraz is one of the most widely planted red grape cultivars globally (Anderson and Aryal 2013), and a choice of yeast strain to conduct the fermentation markedly affects its vinification outcome (Holt et al. 2013, Whitener et al. 2017). An impact of *Saccharomyces* inocula on a range of Shiraz compositional parameters, including tannins, other phenolic substances and fermentation (by)products, is well recorded (Holt et al. 2013). Conversely, for non-*Saccharomyces* products, information is thus far limited to the wine volatile profile (Whitener et al. 2017). Our work therefore extends the knowledge on the inoculum-derived modulation of Shiraz volatile and non-volatile chemical composition, and resultant sensory perception, using the non-*Saccharomyces* active dry wine yeast products commercially available during the 2015 southern hemisphere vintage. One vinification was undertaken using earlier harvested grapes, generally deemed as suboptimally ripe in terms of their flavour and phenolic profile, yet with a sugar level that translates into a moderate alcohol in wine, increasingly in demand among consumers (Ristic et al. 2016). We hypothesise that the non-*Saccharomyces* co-starters tested would boost aroma, flavour and mouthfeel of wines produced from the earlier harvested fruit. The subsequent harvest was made at a maturity stage required for the production of full-bodied, intensively flavoured wines better fitting a conventional quality benchmark. High sugar and, consequently, ethanol concentration occurring in such fermentations impose stress on yeasts, thereby compromising fermentation progress and final wine composition (Ribéreau-Gayon et al. 2006, Ristic et al. 2016). Non-*Saccharomyces* inocula in such scenarios, again, potentially represent a way to achieve improved composition, for example due to a decrease in volatile acidity and increase in total acidity (Kapsopoulou et al. 2007, Bely et al. 2008, Comitini et al. 2011, Jolly et al. 2014, Su et al. 2014). This study therefore contributes to the understanding of yeast inoculum-derived modulation of Shiraz wine composition at different maturity levels, allowing for informed strain choice to lead to product diversification, stylistic distinctness and, in turn, positioning in an increasingly competitive global wine market.

Materials and methods

Vinification methodology

Shiraz grapes were sourced from a vineyard in McLaren Vale, South Australia (35°17′S, 138°55′E). The regional climate in McLaren Vale is temperate-warm; the average mean January temperature (MJT) for the site is 22.9°C, based on climate data from 2000 to 2016, as obtained from the Australian Bureau of Meteorology (2017). In 2015 the MJT was 22.1°C, and mean maximum temperature exceeded 30°C on 8 days (data not shown). Fruit was harvested at two time points: (i) earlier harvest (H1) when TSS reached 24°Brix, pH 3.4, yeast assimilable nitrogen (YAN) 169 mg/L; and (ii) later harvest (H2); TSS 29°Brix, pH 3.7, YAN 178 mg/L. The two harvest dates (HDs) were 6 days apart (5/02/2015 and 11/02/2015) suggesting rapid sugar accumulation and a compressed vintage effect. Parcels of randomly distributed fruit containing 12 kg fruit were separately destemmed, crushed and distributed into 20 L plastic fermenters. Eight yeast inoculation treatments, three *Torulaspora delbrueckii* strains (AL, BI, PR), *Lachancea thermotolerans* (CO), *Metschnikowia pulcherrima* (FL), an initially uninoculated treatment (PI), a commercial blend of *Saccharomyces cerevisiae, T. delbrueckii and L. thermotolerans* (ME) and a *S. cerevisiae* strain (SC), were established in triplicate at H1 and H2 (Table 1). Following the addition of 100 mg/L of diammonium phosphate (DAP), must was inoculated with 0.2 g/L active dry wine yeast (ADWY) products rehydrated according to the suppliers' instructions. During fermentation at 24°C, the cap was plunged twice per day, and the ferment was sampled frequently for sugar consumption kinetics. If applicable, *S. cerevisiae* PDM (Table 1) was sequentially inoculated into the fermentation after 60 h, with additional 100 mg/L DAP supplementation. After 7 days, the must was pressed off from the skins and transferred into 5 L demijohns. Upon fermentation completion/arrest, 50 mg/L of SO_2 (as potassium metabisulfite) was added to the wines. The wines were then racked from gross lees and cold stabilised at 0°C for approximately 3 months. Prior to bottling, free SO_2 was adjusted to 30 mg/L and wines made at later harvest were acid-adjusted with the addition of 1 g/L of tartaric acid. Bottles were stored at 10°C until chemical and sensory analysis.

Chemical analysis

During the course of the fermentation, the rate of sugar consumption was monitored spectrophotometrically using a commercial enzymatic kit (Megazyme, Bray, Ireland) as described in Walker et al. (2014). Glucose and fructose, ethanol, glycerol, malic, tartaric and acetic acid were quantified by HPLC in centrifuged and filtered (0.45 μm) samples, according to Li et al. (2017). Calibration curves ($R^2 > 0.9999$) relating the concentration of analytes and refractive index or optical density measurements were fitted by least squares regression using Chemstation software (Agilent Technologies, Santa Clara, CA, USA). Titratable acidity (TA) and pH were measured with a Radiometer Titralab 90 model (Radiometer, Brønshøj, Denmark). Wine colour and phenolic substances were determined with the modified Somers method

Table 1. Experimental details of the fermentations with eight yeast treatments and grapes from two harvest dates.

Treatment	Full commercial name/name	Supplier	Species	First† inoculation	Second† inoculation
AL	ZYMAFLORE Alpha	Laffort Oenologie, Bordeaux, France	*Torulaspora delbrueckii*	×	×
BI	Biodiva	Lallemand, Montréal, QC, Canada	*T. delbrueckii*	×	×
CO	CONCERTO	CHR Hansen, Horsholm, Denmark	*Lachancea thermotolerans*	×	×
FL	Flavia	Lallemand	*Metschnikowia pulcherrima*	×	×
PR	PRELUDE	CHR Hansen	*T. delbrueckii*	×	×
PI	Postponed inoculation	NA	Indigenous microflora	–	×
ME	MELODY	CHR Hansen	*T. delbrueckii*, *L. thermotolerans*, *Saccharomyces cerevisiae*	×	–
SC	PDM	Maurivin, Australia	*S. cerevisiae*	×	–

Treatments were in triplicate. †Inoculation regimes, comprising the first inoculation of a respective yeast and/or a second inoculation of PDM after 60 h following the supplier's protocol, are indicated with an ×. AL, BI, PR, *Torulaspora delbrueckii* strains; CO, *Lachancea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae*, *T. delbrueckii* and *L. thermotolerans*; SC, a *S. cerevisiae* strain; NA, not applicable.

(Mercurio et al. 2007). A high-throughput version of the methyl cellulose precipitable (MCP) tannin assay (Mercurio et al. 2007) was applied to spectrally quantify wine tannin concentration. The volatile composition of the wines was analysed by solid phase microextraction (SPME)-GC/MS. Samples were diluted with water (1 in 2 and 1 in 100) to a final volume of 10 mL, with the addition of 3 g of sodium chloride to each 20 mL vial. Samples were spiked with a mixture of five standards: d13-hexanol (9.2 μg for 1:2 dilution, 0.92 μg for 1:100 dilution; C/D/N Isotopes, Pointe-Claire, QC, Canada); d11-hexanoic acid (9.3 μg for 1:2 dilution, 0.93 μg for 1:100 dilution; C/D/N Isotopes); d16-octanal (0.821 μg for 1:2 dilution, 82.1 ng for 1:100 dilution; C/D/N Isotopes); d9-ethyl nonanoate (0.92 μg for 1:2 dilution, 92 ng for 1:100 dilution); d3-linalool (17.3 ng for 1:2 dilution and 1.7 ng for 1:100 dilution, C/D/N Isotopes). The extraction and chromatographic conditions were identical to those reported in Boss et al. (2015). The volatile compounds were identified by comparing mass spectra with the US National Institute of Standards and Technology-11 (NIST-11) and the Wiley Registry 9th edition mass spectral libraries. Volatiles were quantified with ChemStation relative to internal standards belonging to the same chemical group using the peak area of an extracted ion. Calibration curves of respective analytes were used to determine concentration of all volatiles except diethyl succinate, which was quantified relative to d9-ethyl nonanoate.

Sensory analysis

Descriptive sensory analysis (Stone and Sidel 2004) of wines was recorded in a purpose-built sensory laboratory approximately 3 months after bottling. The tasting panel consisted of nine female and three male staff members and students from The University of Adelaide with extensive wine sensory descriptive analysis experience. Six training sessions were conducted to generate applicable attributes, to gain familiarity in recognising and scoring them, and to reach consensus scale use. The panel was presented with standards for aroma attributes, hotness, palate fullness, astringency and palate coarseness. Wine appearance terms (colour and depth) were removed when the panel reached a consensus that differences in these attributes were not perceived. A practice evaluation session was held to verify judge performance (using PanelCheck software, Nofima, Tromsø, Norway), to provide feedback and, based on the consensus, to remove non-discriminating attributes. The wines were then formally evaluated over three sessions, in isolated booths at 22–23°C with data acquisition with FIZZ software (Version 2.2, Biosystèmes, Couternon, France). All wines were presented as 50 mL samples in covered ISO standard glasses with randomly assigned three-digit codes. Panellists rated 28 attributes (Table S1) on a 15 cm unstructured line scale from 0 to 10 marked with anchor points 'low' (10% of the scale), 'medium' (50% of the scale) and 'high' (90% of the scale). To avoid sensory fatigue, panellists were instructed to rinse thoroughly with pectin solution (1 g/L) and rest for at least 1 min between evaluating samples.

Statistical analysis

Basic data processing was undertaken with EXCEL 2010 (Microsoft, Richmond, WA, USA). Data are presented as mean values with standard deviation (SD) from replicate determinations. Two-way ANOVA was performed to assess the effect of harvest date (HD), yeast treatment (YT) and their interaction (IN) on basic chemistry parameters, volatiles and wine colour and phenolics measurements with GraphPad Prism (v6.03v, GraphPad Software, La Jolla, CA, USA). The significance level between measurements was determined separately for each HD using Fisher's least significant difference post-hoc test with significance threshold set at 95%. The concentration of volatiles was visualised in R (v3.3.2, R Development Core Team, Vienna, Austria) with heatmap function, upon data normalisation to set mean as 0 and SD as 1, with a default Euclidean distance and Ward clustering. Panel performance during DA was evaluated with PanelCheck (v1.4.2, Nofima); principal component analysis (PCA) of sensory data was performed using SENPAQ (v6.03, Qi Statistics, Reading, England). Chemical data and sensory data were subjected to PCA in XLSTAT (v2015.4.1, Addinsoft, Paris, France).

Results

Fermentation kinetics and basic wine chemistry parameters

Differences, due to the yeast inoculum, were observed in the rate and the extent of sugar consumption in Shiraz fermentations (Figure 1), and in the composition of final Shiraz wines produced form the two harvests (Table 2). To better understand the effect of the respective inoculation regime on wine composition, differences were separately

considered for each HD, as the HD significantly affected all measurements (Tables 2,S2).

In earlier harvested fruit (H1), all treatments led to complete sugar depletion (Figure 1a), with the duration of fermentation varying from 8 (ME, SC) to 12 days (AL, BI, PR). Even though CO, FL and PI contained a significantly lower sugar concentration at the time of the *S. cerevisiae* sequential inoculation, fermentation was completed 1 day earlier compared to that of AL, BI and PR. Similarly, the lowest extent of sugar consumption preceding *S. cerevisiae* inoculation in H2 fermentations was measured for CO and PI, followed by FL. Nonetheless, PI and FL fermentations were completed on day 11, whereas CO fermented to dryness (i.e. sugar concentration < 4 g/L) on day 16 (Figure 1b). The AL, BI and PR fermentations displayed protracted fermentation, and were terminated on day 20 without reaching completion. The AL and BI H2 wines therefore yielded the lowest ethanol concentration with the highest residual sugar content (Table 2). In H1 wines, ME resulted in a significant ethanol decrease of 0.6% (v/v) compared to that of SC, with no further difference seen among the treatments. Glycerol concentration was significantly affected by the yeast inoculation management ($P < 0.0001$), with FL and PI resulting in the highest glycerol concentration at both HDs (Table 2). In H1, the lowest glycerol concentration was found in BI, AL and PR wines, followed by the SC wine. The same *Torulaspora delbrueckii* inocula (AL, BI and PR) led to lower glycerol concentration than that of other YTs in H2 wines. Conversely, SC wines showed the highest increase in glycerol concentration between the two consecutive HDs; that of 40%. Acetic acid concentration in H1 wines ranged from 0.28 to 0.47 g/L in the FL and CO treatments, respectively (Table 2). *Lachancea thermotolerans* also resulted in the highest acetic acid concentration in H2, statistically equivalent to that of the SC treatment. In higher sugar fermentations, the lowest acetic acid formation was observed in BI and PR wines. The other two treatments involving *T. delbrueckii* inoculation, that is AL and ME, had comparably low acetic acid concentration. The H1 wines showed a large variation in malic acid concentration; CO inoculation regime resulted in 48% lower malic acid concentration compared to that of SC. The latter contained significantly higher malic acid concentration than that of all the other treatments. In H2, the malic acid concentration of SC did not differ from that of AL, CO and PI, and was higher than that of BI, ME and FL. Despite a lower concentration of malic acid, and a similar concentration of tartaric acid, the pH of CO was 0.1 unit lower than that of SC H1 wine.

Volatile profile of sequentially inoculated wines

Thirty-nine volatile compounds were analysed in Shiraz wines (Table 3). A PCA plot and heatmap (Figure 2) provide an overview of the volatile profiles associated with the activity of yeasts inoculated into each treatment at both HDs.

Total acetate esters were significantly affected by the HD, showing an increase with advanced maturity ($P < 0.0001$; Table S3). Conversely, HD did not significantly influence the total ethyl esters; its interaction with YT was, however, significant ($P = 0.0063$; Table S3). Increase in the concentration of total higher alcohols and total terpenes was observed in H2 wines compared to that of H1 wines (Tables 3,S3), except for ME and SC H2, containing comparable total higher alcohols to that of H1 wines fermented with the same treatment (*t*-test *P* values 0.2075 and 0.2362, respectively).

Irrespective of HD, YT showed substantial impact on the production of ethyl esters and the acetates, explaining 76 and 57% of the observed variation, respectively ($P < 0.0001$, Table S3). The main acetates found in the wines were ethyl acetate and isoamyl acetate, accounting for 96% or higher of the total acetate concentration (Table 3). The PI wines had a significantly higher concentration of ethyl acetate than all the treatments except CO H2; SC wines had the lowest concentration. In contrast, ME and PR treatments resulted in the highest concentration of isoamyl acetate, while AL and BI strains produced the lowest concentration at both HDs. Harvest date appeared to have a greater impact on PR, the remaining *T. delbrueckii* treatment, that is, when applied to the H2 grapes, it significantly increased isoamyl acetate production. Other acetates, albeit constituting a small proportion of the total acetates, showed certain strain specific patterns. For example, PR consistently produced the highest concentration of 2-phenylethyl acetate and hexyl acetate while CO and ME produced a higher concentration of isobutyl acetate than the other treatments.

The major ethyl esters found in the wines were ethyl isobutyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and diethyl succinate, differently represented among treatments (Table 3). In AL, BI, PR and CO treatments ethyl isobutyrate was the most abundant ethyl ester; followed by ethyl hexanote, ethyl octanoate and diethyl succinate. Ethyl isobutyrate was the ethyl ester found in the highest concentration in ME H1 wine, while in ME H2 its concentration was exceeded by ethyl hexanoate and ethyl octanoate. The latter was also the case for FL wines. The PI and SC treatments had the most ethyl octanoate followed by ethyl hexanoate. Conversely, ethyl heptanoate, ethyl *Z*-3-hexenoate, ethyl 2-methyl butanoate and ethyl laurate were consistently low in concentration in all treatments. Again, yeast-specific patterns were found in ethyl ester production. For example, ME treatment was particularly linked to an increase in ethyl 2-methyl butanoate, ethyl decanoate and ethyl isovalerate at both HDs. Ethyl heptanoate, albeit present at low concentration, was the only ester that was found to be higher in FL wines than in all the remaining treatments. The AL wines were characterised by the highest ethyl isobutyrate concentration, with a dramatic difference of up to 89% compared to that of the other treatments, and regardless of the maturity level. The remaining wines produced with the sequential inoculation of *T. delbrueckii*, BI and PR, also contained an increased concentration of ethyl isobutyrate.

Yeast treatment was a variable explaining the most variance in total higher alcohols analysed, that is 67% ($P < 0.0001$), significantly affecting 13 out of 15 analysed higher alcohols (Tables 3,S3). In both harvests FL led to the lowest total higher alcohol concentration, comparable to that of PI and SC in H1 and H2, respectively. Furthermore, FL wines contained a concentration of 1-hexanol lower than that of all remaining treatments, with a decrease ranging up to 22% in H1, and 29% in H2. Total higher alcohol concentration in H1 was the highest in ME wine, consistently related with high formation of 1-heptanol, 1-nonanol, 1-octanol, 2-methyl-1-propanol, 3-methyl-1-butanol. In H2, the highest value of total higher alcohols was observed in BI and AL, equivalent to those found in PR, CO and ME. In all treatments except ME and SC, an elevated concentration of phenylethyl alcohol was largely responsible for the increased total higher alcohol concentration in H2 wines. In fact, an increase in phenylethyl alcohol was observed in wines produced from H2 grapes for all YTs except SC, ME and FL.

The *T. delbrueckii* treatments (AL, BI and PR) were associated with an elevated concentration of total terpenes in both

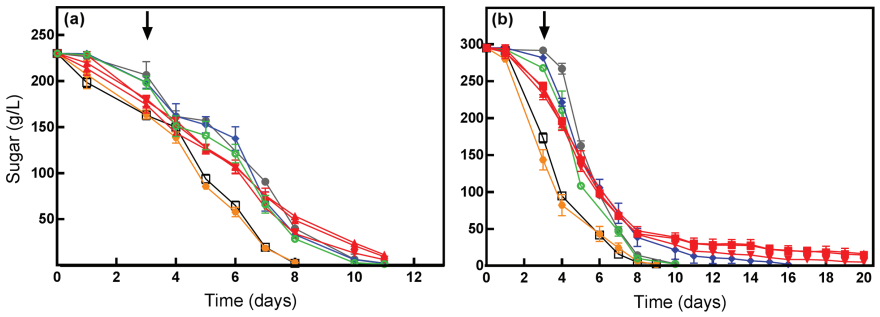

Figure 1. Effect of the (a) earlier and (b) later harvest dates and yeast treatment on the sugar (glucose + fructose) consumption kinetics in Shiraz must fermented with *Torulaspora delbrueckii* strains AL (▲), BI (■), PR (▼); *Lachancea thermotolerans* CO (◆); *Metschnikowia pulcherrima* FL (○); an initially uninoculated treatment PI (●); a commercial blend of *Saccharomyces cerevisiae*, *T. delbrueckii* and *L. thermotolerans* ME (□); and a *S. cerevisiae* strain SC (●). Sequential inoculation of *S. cerevisiae* to treatments AL, BI, CO, FL and PI (day 3) is indicated with an arrow.

harvests, while FL, SC and ME had a comparatively lower concentration of total terpenes (Table 3). The concentration of nerolidol, the most abundant terpene, was elevated in all three treatments initially inoculated with *T. delbrueckii.* This was not the case with linalool, which was present at a concentration in AL and BI higher than that of all the other treatments including PR. The AL wines showed the lowest concentration of hexanoic acid, followed by the BI treatment. The SC, ME and FL wines, in contrast, had a concentration of hexanoic acid relatively higher than that of the other treatments; this being the case with H1 and H2 grapes.

Certain YTs resulted in wines with a more consistent volatile profile irrespective of the HD (Figure 2). For instance, FL wines made at different maturity levels were more similar to one another than to any other YT (Figure 2a). Conversely, treatments PR and CO gave distinct profiles when comparing H1 to H2 for the same treatment. Along PC1 (Figure 2b), a co-localisation of *T. delbrueckii* co-inocula (AL, BI, PR) is apparent, driven primarily by the increase in ethyl isobutyrate, linalool, nerolidol, phenylethyl alcohol and 2-heptanol. The remaining treatments, except CO H2, are separated on the opposite side of the plot, as are all the major ethyl esters except ethyl isobutyrate, that is ethyl hexanoate, ethyl nonate, ethyl decanoate and diethyl succinate. Positioning of H2 treatments above H1 treatments indicates the variability related to HD described by PC2.

Wine colour and phenolic substances

Harvest date had a significant effect on all phenolic substances and colour parameters, except for wine hue (Table 4). Wines produced from H2 had a higher concentration of tannin, anthocyanin and phenolic substances and a higher colour density.

Significant influence of the YT was observed for all the tested parameters, including the wine tannin concentration ($P = 0.0008$, Table 4). At H1, the SC Control resulted in a tannin concentration higher than that of the other YTs, with the increase ranging from 16 (AL) to 24% (BI). Tannin concentration in H2 SC wines was higher than that in PR, CO, PI and FL wines, and comparable to that in BI, AL and ME wines.

Wine colour density was also significantly affected by the yeast inoculation regime ($P < 0.0001$, Table 4). Interestingly, all three *T. delbrueckii* strains (AL, BI, PR) resulted in the lowest colour density of H1 wines. Only ME, a yeast blend containing the same species, had a comparable low colour density. The PR H2 wines had the lowest colour density as well, which was not the case with remaining two *T. delbrueckii* treatments, whereas FL and PI treatments had the highest colour density for both HDs. The PI wine consistently had the lowest wine hue value, indicating a more pronounced shift towards younger blue–purple hues. Phenolic substances in H1 wines were between 8 and 9% higher in the BI treatment compared to the PR, AL and FL treatments, and no further significant differences were seen among the remaining treatments (Table 4). More variation was observed in the H2 wines, with the SC treatment resulting in a concentration of phenolic substances significantly higher than that of CO, ME and PR. The PR wines were characterised by the lowest concentration of phenolic substances, anthocyanin and non-bleachable pigment (Table 4). Similar trends were observed for ME, and an opposite trend for PI wines. Compared to the SC Control, anthocyanin concentration was 6% higher in CO wines and 7% higher in PI wines in H1 and H2, respectively. Inoculation FL led to a 57% increase in stable colour formation measured as the non-bleachable pigment compared to that of PR, BI and ME wines at H1. The second HD resulted in AL and BI wines with the highest non-bleachable pigment concentration, which was, along with that of FL, significantly higher compared to the SC Control treatment.

Descriptive sensory analysis

A significant difference was found in the intensity rating for 22 out of 28 attributes evaluated in Shiraz wines (Table S4). The sensory data for the significantly different attributes were subjected to PCA, with the first two PCs accounting for 61.8% of the total variation in the samples (Figure 3). Along PC1, which explained 40.9% of the variance, wines showing increased astringency, hotness, surface coarseness, bitterness, acidity, vegetal flavour, savoury and earthy aroma/flavour were separated from those characterised by higher sweetness, palate fullness and aroma and flavour intensity, with floral, fruity, confectionery, jammy, spice and licorice aroma/flavour. A clear separation of wines based on their HD can be observed along PC2, accounting for 20.9% of variance. Among H1 wines, the SC wines were perceived as the most acidic and vegetal, whereas the AL, BI, ME and FL wines were seen as more floral, confectionery, fruity and spicy. A differentiation can also be noticed in H2 wines; FL, PI, SC and ME being related to surface coarseness, hotness and astringency, and AL, BI and PR, showing palate fullness, sweetness, and jammy and confectionery flavour. The relationship between the sensory and chemical profile of wines was visualised on a PCA biplot (Figure 4), incorporating sensory data (active variables) and chemical data (supplementary variables), with a correlation matrix provided as supporting data (Table S5). Although mixed classes of volatiles were represented along PC1, certain patterns could, again, be observed, for example co-grouping of the majority of the ethyl esters on the left side of the plot, separately from ethyl isobutyrate on the right. The PC2 appeared to be highly related to the measurement of phenolic substances and colour, and negatively correlated to *Z*-3-hexenol.

Table 2. Effect of harvest date and yeast treatment on the composition of Shiraz wines.

Harvest	Yeast treatment	Final residual sugar (g/L)†	Residual sugar before sequential inoculation (g/L)†	Ethanol (% v/v)	Glycerol (g/L)	Acetic acid (g/L)	Malic acid (g/L)	Tartaric acid (g/L)	pH	TA (g/L as tartaric)
H1	AL	0.25 ± 0.10	174 ± 9 a	13.6 ± 0.3 ab	8.57 ± 0.15 ab	0.35 ± 0.02 abcd	2.79 ± 0.06 d	2.25 ± 0.08	3.71 ± 0.02 ab	8.2 ± 0.2
	BI	0.14 ± 0.06	181 ± 3 a	13.6 ± 0.4 ab	8.28 ± 0.15 a	0.31 ± 0.02 abc	2.77 ± 0.13 cd	2.19 ± 0.06	3.72 ± 0.01 b	8.0 ± 0.3
	CO	0.23 ± 0.07	199 ± 7 b	13.7 ± 0.2 ab	9.52 ± 0.27 c	0.47 ± 0.02 d	1.49 ± 0.05 a	2.08 ± 0.12	3.69 ± 0.01 a	8.3 ± 0.2
	FL	0 ± 0	198 ± 7 b	13.8 ± 0.1 ab	10.51 ± 0.04 d	0.28 ± 0.11 a	1.59 ± 0.22 ab	2.01 ± 0.21	3.72 ± 0.01 b	8.0 ± 0.2
	PR	0.15 ± 0.19	178 ± 6 a	13.8 ± 0.3 ab	8.60 ± 0.15 ab	0.29 ± 0.07 ab	2.63 ± 0.12 c	2.09 ± 0.04	3.75 ± 0.01 c	8.2 ± 0.1
	PI	0.31 ± 0.02	207 ± 15 b	13.9 ± 0.1 ab	10.37 ± 0.28 d	0.43 ± 0.05 cd	1.73 ± 0.02 b	2.15 ± 0.08	3.70 ± 0.01 ab	8.2 ± 0.2
	ME	0 ± 0	NA	13.5 ± 0.2 a	9.20 ± 0.07 c	0.42 ± 0.13 bcd	2.57 ± 0.08 c	1.98 ± 0.13	3.77 ± 0.01 cd	8.0 ± 0.1
	SC	0 ± 0	NA	14.1 ± 0.1 b	8.67 ± 0.25 b	0.35 ± 0.19 abcd	3.09 ± 0.05 e	2.14 ± 0.04	3.79 ± 0.02 d	8.0 ± 0.1
H2	AL	16.46 ± 3.01 d	233 ± 8 a	15.5 ± 0.6 a	9.93 ± 0.43 b	0.40 ± 0.12 ab	2.70 ± 0.04 bc	1.99 ± 0.08	3.81 ± 0.01 a	8.4 ± 0.1
	BI	14.78 ± 2.09 d	243 ± 4 a	15.5 ± 0.4 a	8.83 ± 0.08 a	0.28 ± 0.01 a	2.55 ± 0.02 b	2.24 ± 0.20	3.81 ± 0.01 a	8.5 ± 0.1
	CO	2.19 ± 2.00 b	282 ± 4 c	17.0 ± 0.1 c	11.64 ± 0.02 c	0.66 ± 0.05 d	2.82 ± 0.06 cd	2.15 ± 0.08	3.82 ± 0.02 a	8.4 ± 0.2
	FL	0.38 ± 0.01 a	265 ± 0 b	16.8 ± 0.4 bc	12.59 ± 0.03 e	0.45 ± 0.01 bc	2.57 ± 0.08 b	2.14 ± 0.04	3.83 ± 0.01 a	8.4 ± 0.3
	PR	5.18 ± 1.15 c	240 ± 9 a	16.4 ± 0.4 b	9.71 ± 0.25 b	0.27 ± 0.04 a	2.25 ± 0.11 a	1.97 ± 0.31	3.87 ± 0.03 b	8.5 ± 0.2
	PI	0.28 ± 0.02 a	292 ± 1 c	16.9 ± 0.1 c	12.56 ± 0.27 de	0.42 ± 0.04 b	2.92 ± 0.07 d	2.10 ± 0.07	3.83 ± 0.01 a	8.5 ± 0.1
	ME	0.23 ± 0.01 a	NA	16.8 ± 0.3 bc	11.53 ± 0.13 c	0.36 ± 0.06 ab	2.26 ± 0.06 a	2.11 ± 0.32	3.88 ± 0.01 b	8.3 ± 0.1
	SC	0.24 ± 0.01 a	NA	17.0 ± 0.2 c	12.21 ± 0.14 d	0.56 ± 0.07 cd	2.82 ± 0.08 cd	1.99 ± 0.30	3.83 ± 0.03 a	8.6 ± 0.2
Percentage of variance and level of significance	IN	37.15****	1.80**	3.21***	8.34****	17.60*	54.18****	18.57 ns	5.05***	10.07 ns
	HD	21.75****	77.54****	88.65****	42.17****	6.44*	8.65****	1.40 ns	72.64****	45.73****
	YT	38.16****	18.53****	5.49****	48.23****	47.78****	34.69****	11.19 ns	17.70***	7.85 ns

Values are means of three replicates ± SD; lower case letters denote significant difference among yeast treatments at specified harvest date; significance levels of percentage of variation explained by the harvest date, yeast treatment, and the interaction of these variables are the following: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ (Fisher's least significant difference). †Glucose + fructose concentration. AL, BI, PR, *Torulaspora delbrueckii* strains; CO, *Lachanea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae*, *T. delbrueckii* and *L. thermotolerans*; SC, a *S. cerevisiae* strain. H1, first harvest on 11 February 2015; H2, second harvest on 11 February 2015; NA, not applicable.

Discussion

The use of selected non-*Saccharomyces* yeast to partially conduct the fermentation has become increasingly popular, as a mean to build wine complexity and diversify styles (Jolly et al. 2014). To date a limited number of non-*Saccharomyces* products is available on the market, and more research is required to guide their use by the wine industry. In this study, we applied a sequential inoculation modality with three commercial strains of *T. delbrueckii*, and one strain each of *M. pulcherrima* and *L. thermotolerans*. In order to ascertain that observed differences can be attributed to the respective yeast inoculation, rather than to any other strain(s) found on the grapes, we included a postponed inoculation with the fermentation, initiated by the naturally present microflora. We opted for a time-point as a criterion for a second inoculation, as a critical factor in cellar management during, and post, fermentation (Ribéreau-Gayon et al. 2006). Another tested non-*Saccharomyces* treatment was the commercial blend of *T. delbrueckii*, *L. thermotolerans* and *S. cerevisiae*. These were all compared to a *S. cerevisiae* monoculture of PDM, a strain widely used in the industry. The resultant wines differed in their composition and sensory attributes, indicating the effectiveness of the yeast inoculation treatment. We hereby present a comprehensive dataset generated to determine the effect of non-*Saccharomyces* inocula on the chemical and sensory profile of Shiraz wines produced at two grape maturity levels.

Yeast interactions and alteration of wine composition

Timely and reliable completion of fermentation is of critical importance in winemaking. Novel inoculation regimes that include the use of non-*Saccharomyces* yeasts generally display slower fermentation; alteration of wine composition and an increase in quality are seen to compensate for the delay. Lengthier fermentation observed for sequential inoculation treatments was therefore not surprising. Intriguing was the fact that *T. delbrueckii* treatments (AL, BI and PI) showed delayed completion of fermentation compared to other non-*Saccharomyces* treatments in H1, and its arrest in the H2 wines. As the nitrogen source was supplemented concomitant to *S. cerevisiae* addition, it is unlikely that nitrogen deficiency was the cause for the displayed kinetics. The acclimation of sequentially inoculated *S. cerevisiae* to physicochemical conditions in the commenced ferment, for example the concentration of ethanol already formed or anaerobiosis, might partially explain this discrepancy. It can be assumed, however, that the effect of additional interaction between the yeasts and/or produced metabolites (other than ethanol) were the cause for the displayed kinetics. This is also supported by the fact that H2 *L. thermotolerans* treatment (CO) finished fermentation 5 days later than two other treatments, that is FL and PI, with comparable sugar content consumed prior to *S. cerevisiae* addition. Indeed, rather than co-existing passively, yeasts in fermentation display various interactions (Renault et al. 2013, Jolly et al. 2014, Kemsawasd et al. 2015, Albergaria and Arneborg 2016, Ciani et al. 2016a). *Saccharomyces cerevisiae* is known to negatively affect cell proliferation and survival of non-*Saccharomyces* species via mechanisms including cell–cell contact and microbial peptide secretion, as described for *T. delbrueckii* (Renault et al. 2013) and *L. thermotolerans* (Kemsawasd et al. 2015). The potential effect of non-*Saccharomyces* on *S. cerevisiae* also cannot be excluded. Renault et al. (2013), however, observed a positive effect of *T. delbrueckii* on

Table 3. Effect of harvest date and yeast treatment on the concentration of volatiles in Shiraz wines.

		Concentration (μg/L)							
Compound	**Harvest**	**AL**	**BI**	**CO**	**FL**	**PR**	**PI**	**ME**	**SC**
Ethyl acetate	H1	26 638 ± 2005 c	22 662 ± 8238 abc	25 848 ± 3450 bc	24 201 ± 782 abc	21 324 ± 2325 abc	44 833 ± 3229 d	20 046 ± 2195 ab	18 797 ± 825 a
	H2	31 660 ± 2515 bc	30 442 ± 3638 abc	37 821 ± 5823 de	31 195 ± 3374 bc	34 944 ± 2010 cd	42 367 ± 6514 e	27 305 ± 2085 ab	24 519 ± 3611 a
2-Phenylethyl acetate	H1	46 ± 5 d	45 ± 13 d	35 ± 1 abc	41 ± 4 bcd	59 ± 4 e	43 ± 4 cd	41 ± 1 bcd	30 ± 3 a
	H2	52 ± 8 c	45 ± 3 bc	37 ± 2ab	35 ± 3 a	84 ± 5 e	75 ± 3 d	38 ± 6 ab	30 ± 5 a
Ethyl phenyl acetate	H1	0.36 ± 0.02 b	0.26 ± 0.04 a	0.47 ± 0.03 c	0.49 ± 0.03 c	0.38 ± 0.02 b	0.34 ± 0.03 b	0.50 ± 0.05 c	0.60 ± 0.09 d
	H2	0.44 ± 0.03 cd	0.39 ± 0.03 bc	0.47 ± 0.01 d	0.47 ± 0.03 d	0.47 ± 0.06 d	0.33 ± 0.03 ab	0.40 ± 0.01 c	0.30 ± 0.04 a
Hexyl acetate	H1	19 ± 2 a	19 ± 6 a	29 ± 0 b	37 ± 2 c	44 ± 4 c	45 ± 5 c	40 ± 4 c	39 ± 0 c
	H2	16 ± 2 a	15 ± 0 a	25 ± 3 b	27 ± 2 b	62 ± 6 cd	68 ± 13 d	61 ± 1 cd	55 ± 8 c
Isoamyl acetate	H1	637 ± 67 a	662 ± 272 a	1306 ± 99 cd	1060 ± 112 bc	1350 ± 85 d	948 ± 24 b	1530 ± 67 d	859 ± 61 ab
	H2	590 ± 19 a	545 ± 28 a	1219 ± 36 bc	977 ± 147 b	2283 ± 110 e	1864 ± 285 d	2106 ± 341 de	1368 ± 201 c
Isobutyl acetate	H1	48 ± 5 b	30 ± 3 a	64 ± 9 c	50 ± 3 b	57 ± 3 bc	34 ± 4 a	64 ± 3 c	24 ± 1 a
	H2	54 ± 2 c	37 ± 4 ab	68 ± 6 d	43 ± 4 b	81 ± 8 e	63 ± 19 cd	64 ± 3 cd	31 ± 2 a
Total acetate esters	H1	27 773 ± 2085 b	23 849 ± 8531 ab	27 847 ± 3561 b	25 887 ± 902 ab	23 399 ± 2421 ab	46 321 ± 3265 c	22 164 ± 2270 ab	20 334 ± 890 a
	H2	32 665 ± 2545 ab	31 372 ± 3672 a	39 521 ± 5870 cd	32 767 ± 3530 ab	38 083 ± 2137 bcd	44 810 ± 6834 d	30 211 ± 2435 a	26 426 ± 3826 a
Diethyl succinate	H1	118 ± 9 bc	55 ± 6 a	124 ± 5 bc	110 ± 1 b	126 ± 5 bc	205 ± 19 e	160 ± 26 d	141 ± 14 cd
	H2	170 ± 16 d	111 ± 7 ab	106 ± 3 a	156 ± 10 cd	229 ± 37 f	196 ± 10 e	199 ± 14 e	135 ± 10 bc
Ethyl 2-methyl butanoate	H1	4.3 ± 0.3 de	2.7 ± 0.4 b	3.9 ± 0.5 cd	1.7 ± 0.2 a	4.6 ± 0.4 e	1.6 ± 0.2 a	4.4 ± 0.4 de	3.3 ± 0.2 c
	H2	3.6 ± 0.1 c	3.3 ± 0.3 bc	4.4 ± 0.4 d	2.1 ± 0.1 a	5.3 ± 0.4 e	2.3 ± 0.1 a	4.8 ± 0.6 de	3.0 ± 0.5 b
Ethyl butyrate	H1	48 ± 3 ab	49 ± 1 ab	56 ± 6 b	57 ± 3 b	81 ± 5 c	56 ± 3 b	45 ± 3 a	45 ± 2 a
	H2	51 ± 3 ab	60 ± 9 b	43 ± 1 a	59 ± 8 b	107 ± 12 d	60 ± 6 b	74 ± 9 c	59 ± 8 b
Ethyl decanoate	H1	31 ± 4 a	46 ± 7 a	87 ± 8 b	95 ± 15 b	99 ± 14 b	122 ± 14 c	162 ± 6 d	167 ± 25 d
	H2	42 ± 4 a	65 ± 7 b	72 ± 4 b	100 ± 2 c	136 ± 15 d	134 ± 1 d	168 ± 25 e	141 ± 6 d
Ethyl heptanoate	H1	0.79 ± 0.12 b	0.81 ± 0.01 bc	0.92 ± 0.13 bc	1.08 ± 0.16 d	0.94 ± 0.11 c	0.53 ± 0.06 a	0.60 ± 0.04 a	0.63 ± 0.11 a
	H2	0.71 ± 0.03 cd	0.62 ± 0.09 c	0.81 ± 0.06 de	1.16 ± 0.10 f	0.06 ± 0 a	0.47 ± 0.01 b	0.86 ± 0.03 e	0.61 ± 0.06 c
Ethyl hexanoate	H1	404 ± 61 a	450 ± 44 ab	594 ± 34 de	535 ± 49 cd	609 ± 12 e	462 ± 23 ab	482 ± 53 bc	624 ± 37 e
	H2	308 ± 6 a	303 ± 11 a	375 ± 27 ab	516 ± 64 d	689 ± 94 e	441 ± 15 bc	699 ± 3 e	478 ± 50 cd
Ethyl isobutyrate	H1	1676 ± 179 e	843 ± 102 c	657 ± 103 b	214 ± 16 a	1114 ± 108 d	187 ± 18 a	560 ± 58 b	235 ± 8 a
	H2	1545 ± 123 e	1131 ± 34 d	872 ± 133 c	221 ± 7 a	1436 ± 238 e	244 ± 86 a	597 ± 82 b	173 ± 6 a
Ethyl isovalerate	H1	15 ± 1 c	11 ± 1 b	19 ± 3 d	10 ± 1 ab	18 ± 1 d	7 ± 0 a	22 ± 2 e	17 ± 1 cd
	H2	17 ± 0 b	16 ± 1 b	18 ± 2 b	12 ± 1 a	28 ± 2 c	11 ± 1 a	26 ± 3 c	17 ± 2 b
Ethyl laurate	H1	3 ± 0 a	3 ± 3 a	10 ± 0 b	10 ± 1 b	10 ± 0 b	14 ± 1 c	13 ± 1 bc	18 ± 6 d
	H2	6 ± 1 ab	5 ± 0 a	6 ± 1 ab	11 ± 0 cd	14 ± 1 d	10 ± 2 bc	21 ± 0 e	13 ± 2 d
Ethyl octanoate	H1	136 ± 6 a	168 ± 13 a	380 ± 13 b	411 ± 50 b	363 ± 46 b	512 ± 11 c	560 ± 100 c	693 ± 65 d
	H2	144 ± 13 a	139 ± 0 a	207 ± 23 a	432 ± 32 c	327 ± 20 b	542 ± 4 d	610 ± 94 d	598 ± 17 d
Ethyl Z-3-hexenoate	H1	3.8 ± 0.4 e	2.1 ± 0.1 c	2.8 ± 0.3 d	1.8 ± 0.2 bc	4.9 ± 0.4 f	1.2 ± 0.2 a	1.6 ± 0.3 ab	1.8 ± 0.2 bc
	H2	2.1 ± 0.1 d	1.2 ± 0.1 bc	1.3 ± 0.2 bc	1.1 ± 0.1 ab	4.0 ± 0.2 e	0.7 ± 0 a	1.6 ± 0.2 c	1.0 ± 0.1 ab
Total ethyl esters	H1	2441 ± 263 d	1631 ± 177 ab	1934 ± 173 bc	1447 ± 136 a	2430 ± 193 d	1568 ± 90 a	2009 ± 247 c	1945 ± 159 bc
	H2	2290 ± 167 b	1834 ± 69 a	1706 ± 194 a	1511 ± 124 a	2975 ± 420 c	1641 ± 124 a	2400 ± 231 b	1618 ± 101 a
Isoamyl butanoate	H1	1.2 ± 0.1 cd	0.8 ± 0.1 ab	1.0 ± 0.1 b	0.6 ± 0.1 a	1.6 ± 0.2 d	0.5 ± 0 a	1.00 ± 0.1 b	0.7 ± 0.1 ab
	H2	1.2 ± 0.1 bc	1.2 ± 0.1 bc	0.8 ± 0.1 ab	0.5 ± 0 a	3.0 ± 0.4 d	0.7 ± 0.1 a	1.6 ± 0.2 c	1.1 ± 0.8 b
Octanoic acid methyl ester	H1	0.6 ± 0 a	0.8 ± 0.1 a	2.2 ± 0.1 c	2.9 ± 0.2 d	1.8 ± 0.2 b	3.4 ± 0.1 e	2.4 ± 0.2 c	4.8 ± 0.4 f
	H2	0.7 ± 0.1 a	0.5 ± 0.3 a	0.9 ± 0.1 a	2.8 ± 0.2 c	1.5 ± 0.1 b	3.2 ± 0.1 d	2.5 ± 0.4 c	3.9 ± 0.2 e
1-Butanol	H1	41 ± 2 d	26 ± 2 bc	25 ± 3 b	30 ± 1 c	36 ± 2 d	40 ± 1 d	18 ± 1 a	26 ± 4 bc
	H2	49 ± 4 f	26 ± 2 a	41 ± 5 cd	43 ± 1 de	37 ± 4 c	65 ± 3 g	31 ± 3 b	48 ± 6 ef
1-Heptanol	H1	62 ± 4 a	66 ± 9 a	82 ± 7 b	65 ± 4 a	65 ± 3 a	1057 ± 4 c	103 ± 4 c	108 ± 3 c
	H2	66 ± 2 a	71 ± 3 a	83 ± 4 b	64 ± 5 a	65 ± 3 a	82 ± 8 b	118 ± 8 d	105 ± 3 c
1-Hexanol	H1	4580 ± 78 bc	4863 ± 106 cd	4703 ± 183 bc	3929 ± 104 a	4739 ± 112 bc	5048 ± 277 d	4142 ± 146 a	4467 ± 74 b
	H2	5351 ± 119 d	4973 ± 274 bc	5215 ± 194 cd	3779 ± 88 a	4938 ± 322 bc	4847 ± 42 b	5010 ± 177 bc	4983 ± 241 bc
1-Nonanol	H1	8.5 ± 0.6 ab	9.0 ± 0.8 b	9.6 ± 1.0 b	7.5 ± 0.2 a	7.0 ± 0.6 a	13.5 ± 0.5 c	13.6 ± 0.07 c	12.7 ± 1.7 c

(Continues)

Table 3. Continued

Compound	Harvest	Concentration (µg/L) AL	BI	CO	FL	PR	PI	ME	SC
	H2	9.7 ± 0.8 bc	10.8 ± 0.3 cd	8.9 ± 0.9 ab	10.0 ± 1.3 bc	8.0 ± 0.3 a	13.6 ± 0.9 e	11.9 ± 1.2 d	13.5 ± 1.7 e
1-Octanol	H1	22 ± 12 a	34 ± 3 cd	31 ± 3 bc	23 ± 1 a	29 ± 2 b	36 ± 2 d	52 ± 1 e	50 ± 3 e
	H2	19 ± 1 a	22 ± 2 ab	21 ± 0 ab	23 ± 2 b	22 ± 2 ab	33 ± 4 c	40 ± 3 d	48 ± 3 e
1-Octen-3-ol	H1	0.84 ± 0.09 ab	0.94 ± 0.03 b	0.78 ± 0.03 a	0.75 ± 0.07 a	0.82 ± 0.09 ab	0.85 ± 0.03 ab	0.88 ± 0.13 ab	0.93 ± 0.14 b
	H2	1.15 ± 0.05 bcd	1.25 ± 0.1 d	1.02 ± 0.07 ab	0.99 ± 0.04 a	1.27 ± 0.06 d	1.02 ± 0.08 abc	1.16 ± 0.02 cd	1.25 ± 0.18 d
2-Ethyl-1-hexanol	H1	4.1 ± 0.1 a	4.3 ± 0.4 a	4.4 ± 0.7 ab	4.8 ± 0.2 ab	4.8 ± 0.7 ab	4.4 ± 0.4 ab	4.9 ± 0.2 ab	5.4 ± 0.8 b
	H2	4.8 ± 0.5 ab	4.8 ± 0.2 ab	4.1 ± 0.3 a	4.9 ± 0.3 ab	4.5 ± 0.4 ab	4.5 ± 0.3 ab	5.5 ± 1.9 b	4.9 ± 0.7 ab
2-Heptanol	H1	14.7 ± 1.1 d	17.0 ± 1.0 e	12.7 ± 0.3 bc	13.1 ± 0.5 bc	13.5 ± 0.3 cd	13.3 ± 0.6 c	11.4 ± 0.2 a	12.0 ± 0.7 ab
	H2	17.2 ± 0.3 cd	17.3 ± 0.2 d	15.8 ± 1 ab	15.7 ± 0.4 a	16.1 ± 0.7 abc	17.0 ± 1.5 bcd	15.0 ± 0.1 a	16.1 ± 0.7 abc
2-Methyl-1-propanol	H1	124 885 ± 15 183 c	118 860 ± 1049 bc	148 807 ± 12 814 d	104 896 ± 15 368 b	114 219 ± 11 674 bc	60 727 ± 753 a	171 107 ± 21 013 e	73 821 ± 4957 a
	H2	148 269 ± 5462 cd	139 504 ± 8498 c	149 735 ± 2288 cd	109 260 ± 11 223 b	153 174 ± 4380 cd	106 187 ± 12 961 b	158 554 ± 13 932 d	73 039 ± 6186 a
3-Methyl-1-butanol	H1	439 334 ± 24 418 bc	429 811 ± 29 424 bc	501 379 ± 46 035 de	308 418 ± 17 043 a	456 182 ± 20 194 cd	306 594 ± 8635 a	539 682 ± 28 490 e	389 569 ± 26 651 b
	H2	490 717 ± 18 684 cd	487 334 ± 13 457 cd	512 201 ± 57 916 d	383 069 ± 15 669 a	504 959 ± 17 828 cd	452 324 ± 40 607 bc	525 037 ± 42678d	401 826 ± 57 630 ab
3-Methylthio-1-propanol	H1	1473 ± 190 a	1309 ± 146 a	2510 ± 238 b	1612 ± 243 a	1537 ± 201 a	2355 ± 151 b	2314 ± 97 b	3135 ± 445 c
	H2	1781 ± 88	1613 ± 237	1480 ± 186	1647 ± 102	1328 ± 72	3532 ± 769	2122 ± 127	1675 ± 205
Benzyl alcohol	H1	587 ± 38	587 ± 28	560 ± 38	570 ± 29	591 ± 54	585 ± 8	618 ± 72	611 ± 56
	H2	813 ± 41 abc	761 ± 22 a	745 ± 23 a	826 ± 67 abc	827 ± 89 b	872 ± 55 c	851 ± 61 c	793 ± 39 abc
Phenylethyl alcohol	H1	255 652 ± 30 727 bc	275 224 ± 16 415 c	227 529 ± 23 691 ab	208 395 ± 8016 a	247 516 ± 25 345 bc	202 457 ± 5736 a	281 357 ± 11 820 c	248 348 ± 21 660 bc
	H2	305 353 ± 10 281 ef	322 694 ± 28 667 f	246 394 ± 18 317 cd	200 201 ± 11 603 b	274 884 ± 39 849 de	240 924 ± 35 207 cd	231 227 ± 6037 bc	155 807 ± 22 283 a
E-3-Hexen-1-ol	H1	47 ± 2 a	50 ± 2 ab	50 ± 2 ab	54 ± 1 b	52 ± 3 ab	55 ± 3 b	47 ± 3 a	50 ± 3 ab
	H2	54 ± 2 abc	49 ± 3 a	51 ± 5 ab	53 ± 2 abc	56 ± 7 bc	52 ± 0 ab	58 ± 8 c	54 ± 2 abc
Z-3-Hexen-1-ol	H1	1111 ± 31 d	1077 ± 28 d	962 ± 69 b	949 ± 13 b	1218 ± 39 e	852 ± 61 a	1053 ± 87 cd	984 ± 42 bc
	H2	830 ± 38 cd	803 ± 40 cd	707 ± 34 b	604 ± 24 a	828 ± 76 cd	579 ± 19 a	867 ± 85 d	757 ± 21 bc
Total higher alcohols	H1	827 820 ± 70 676 c	831 936 ± 47 213 c	886 664 ± 83 085 c	628 965 ± 40 824 ab	826 219 ± 57 630 c	578 886 ± 15 630 a	1 000 524 ± 61 733 d	721 199 ± 53 899 b
	H2	953 336 ± 34 723 c	957 883 ± 51 207 c	916 702 ± 78 975 c	699 599 ± 38 788 a	941 148 ± 62 633 c	809 532.23 ± 89 659.39 ab	923 947 ± 63 122 c	639 169 ± 86 624 a
ß-Citronellol	H1	1.7 ± 0.1 b	1.2 ± 0.2 a	1.5 ± 0.1 ab	1.6 ± 0.2 b	1.5 ± 0.1 ab	1.4 ± 0 ab	1.7 ± 0.1 b	2.2 ± 0.2 c
	H2	2.3 ± 0.3 d	1.8 ± 0.1 b	2.0 ± 0.2 bc	2.0 ± 0.2 bc	1.9 ± 0.3 bc	2.2 ± 0 cd	1.4 ± 0.2 a	1.9 ± 0.3 bc
Linalool	H1	3.1 ± 0.1 ef	3.3 ± 0.25 f	2.6 ± 0.1 bc	2.8 ± 0.2 de	2.3 ± 0.1 a	2.4 ± 0 ab	2.6 ± 0.1 bc	2.7 ± 0.1 cd
	H2	3.3 ± 0.1 de	3.5 ± 0.3 e	2.9 ± 0.1 c	3.1 ± 0.1 cd	2.5 ± 0.2 ab	2.6 ± 0.1 b	2.2 ± 0.1 a	2.4 ± 0.3 ab
Nerolidol	H1	12.9 ± 1.8 c	9.8 ± 0.4 bc	4.0 ± 0.6 ab	3.0 ± 0.4 a	9.6 ± 0.8 bc	3.6 ± 0.3 a	6.9 ± 0.8 ab	6.8 ± 0.7 ab
	H2	24.6 ± 12.6 d	16.8 ± 2.5 c	15.4 ± 1.0 bc	8.3 ± 0.9 a	32.6 ± 4.6 e	12.0 ± 1.0 abc	11.2 ± 1.6 abc	10.4 ± 1.4 ab
Total terpenes	H1	17.7 ± 2.1 b	14.3 ± 0.9 b	8.1 ± 0.8 a	7.5 ± 0.8 a	13.4 ± 1.0 b	7.4 ± 0.4 a	11.2 ± 1.03 a	11.7 ± 1 ab
	H2	30.2 ± 13.0 d	22.0 ± 2.9 c	20.2 ± 1.3 bc	13.3 ± 1.1 a	36.9 ± 5.0 e	16.8 ± 1.01 abc	14.9 ± 1.9 ab	14.6 ± 2 ab
ß-Damascenone	H1	1.00 ± 0.13 ab	0.95 ± 0.11 a	1.16 ± 0.18 bc	1.07 ± 0.06 ab	1.10 ± 0.04 abc	1.17 ± 0.04 bc	1.28 ± 0.11 c	1.14 ± 0.14 bc
	H2	1.16 ± 0.12	1.15 ± 0.09	1.27 ± 0.15	1.20 ± 0.15	1.16 ± 0.12	1.21 ± 0.09	1.20 ± 0.09	1.21 ± 0.15
Hexanoic acid	H1	2950 ± 331 a	3149 ± 621 a	4518 ± 113 bc	4849 ± 373 cd	5104 ± 308 cde	4212 ± 140 b	5164 ± 644 de	5694 ± 464 e
	H2	2021 ± 242 a	2869 ± 228 b	3114 ± 320 bc	4492 ± 464 ef	4097 ± 266 de	3536 ± 253 cd	6045 ± 225 g	5090 ± 428 f

Values are means of three replicates ± SD. Lower case letters denote significant difference. $P < 0.05$ (Fisher's least significant difference) among yeast treatments at specified harvest date. AL, BI, PR, *Torulaspora delbrueckii* strains; CO, *Lachancea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae*, *T. delbrueckii* and *L. thermotolerans*; and SC, a *S. cerevisiae* strain. H1, first harvest on 11 February 2015; H2, second harvest on 11 February 2015.

S. cerevisiae when separated, and no effect in their co-culture. Stuck and sluggish multi-starter fermentations were, in contrast, previously reported (Ciani et al. 2006). It is therefore plausible that, in our study, observed fermentation kinetics imply a negative effect of tested *T. delbrueckii* and *L. thermotolerans* strains on *S. cerevisiae*, in particular for H2 wines. Such negative interactions were absent in the case of *M. pulcherrima* (FL) and microflora present on grapes (PI). Interaction mechanisms are undoubtedly extremely complex, and strain- and condition-dependent, and potentially exacerbated by higher sugar-related H2 conditions (Blomberg and Adler 1989, Ribéreau-Gayon et al. 2006). This phenomenon may offer an explanation for the increase in glycerol concentration between the two consecutive harvests, in particular the glycerol excess in SC H2 treatment (Table 2). High glycerol concentration in PI and FL treatments agrees with reports that uninoculated fermentations and co-fermentations with some non-*Saccharomyces*, including *M. pulcherrima*, generally produce more glycerol than *S. cerevisiae* monoculture (Comitini et al. 2011, Contreras et al. 2014, 2015, Jolly et al. 2014, Varela et al. 2016). While glycerol formation in *S. cerevisiae* is linked to acetic acid production, this is not the case for some non-*Saccharomyces* (Ribéreau-Gayon et al. 2006, Bely et al. 2008, Comitini et al. 2011). Low volatile acidity production is, in particular, considered a general trait of *T. delbrueckii* (Bely et al. 2008, Comitini et al. 2011, Jolly et al. 2014). Accordingly, H2 *S. cerevisiae* wine had an acetic acid concentration higher than that of all the other wines, except CO, whereas *T. delbrueckii* treatments yielded the lowest acetic acid concentration. Acids other than acetic are affected by yeasts during the fermentation, of which malic acid is in general the most abundant in grape juice/must (Ribéreau-Gayon et al. 2006, Su et al. 2014). Malic acid is related to 'harsh' wine sensory descriptors, and is known to decline during grape ripening (Ribéreau-Gayon et al. 2006, Su et al. 2014). *Schizosaccharomyces pombe* is the only yeast species capable of fully metabolising malic acid during fermentation, while other yeasts can be involved in either its partial degradation or an increase in concentration, depending on the strain and conditions (Kapsopoulou et al. 2007, Jolly et al. 2014, Su et al. 2014, Benito et al. 2015). The lowest malic acid concentration was measured in CO H1 wine, followed by FL and PI (Table 1). A potential contribution of other microorganisms on grapes cannot be excluded as the fermentation was non-sterile. The fact, however, that CO contained a significantly lower concentration of malic acid than that of the initially uninoculated treatment PI (theoretically allowing for the most prolific native microbial activity) suggests that differences in malic acid concentration were attributable to the yeast inoculation regime. *Lachancea thermotolerans* was, in fact, previously reported to be capable of reducing the initial malic acid concentration by up to 0.42 g/L (Kapsopoulou et al. 2007), and the same strain as used in our study previously led to the lowest malic acid concentration compared to other inocula in Sauvignon Blanc (Beckner Whitener et al. 2016) and Shiraz wines (Whitener et al. 2017). The lower pH value of the CO H1 wine could have been influenced by the formation of other organic acids that were not quantified in the study, primarily lactic acid, production of which is a trait of *L. thermotolerans* (Kapsopoulou et al. 2007, Jolly et al. 2014, Su et al. 2014, Benito et al. 2015). The same effects, however, were not observed in H2.

Our findings, related to fermentation performance and basic chemical profile of the wines, highlight the necessity for further fundamental studies focusing on performance and interactions of oenological yeasts, along with a validation under commercial winemaking conditions, in order to optimise inoculation regimes and select mutually compatible strains to guarantee the target wine style.

Discernible volatile profiles of wines produced

Wine volatile flavour and aroma profile is shaped by several viticultural and oenological inputs. Of these, both grape-derived precursors, altered in composition and concentration throughout ripening, and the yeast strains used for fermentation, significantly define final wine aromatic makeup (Ribéreau-Gayon et al. 2006, Bindon et al. 2013, Holt et al. 2013, Padilla et al. 2016, Whitener et al. 2017). As seen elsewhere (Bindon et al. 2013), in the current study greater ripeness generally favoured the production of yeast-derived volatiles (Table 3). Depending on the YT, however, volatile profiles of the wines were either similar or quite different between the two HDs (Figure 2a).

The majority of the 39 compounds analysed (Table 3) were esters. Production mechanisms of these yeast-derived metabolites imparting 'fruity' aromas are well documented for *S. cerevisiae* (Sumby et al. 2010), but less so for the non-*Saccharomyces* species and strains. Overall, wines co-fermented with non-*Saccharomyces* showed altered composition and/or an increase in ester concentration, which was particularly evident in *T. delbrueckii* treatments. Such findings are in contrast with those of Whitener et al. (2017), who observed that *T. delbrueckii* wine volatile composition lacked a distinct pattern when compared to that of other treatments. Of the *T. delbrueckii* treatments, AL and BI showed highly similar volatile profiles within each HD, different from PR, which appeared to be more affected by HD (Figure 2a). Strain-dependent ester production has previously been described for *T. delbrueckii* (Renault et al. 2009). Nonetheless, ethyl isobutyrate was consistently overproduced in all three *T. delbrueckii* sequential inoculations at both HDs. Ethyl isobutyrate has recently been identified as an activity/growth marker for *T. delbrueckii*, and typically increases in concentration in pure and sequential cultures (Renault et al. 2015). Furthermore, an increase of ethyl heptanoate in both FL fermentations is suggestive of its role as a *M. pulcherrima* metabolic marker. Whilst ethyl heptanoate has been omitted from some studies featuring volatile profiling of *M. pulcherrima* co-fermented wines (Padilla et al. 2016, Varela et al. 2016), an overproduction of this ester has been observed in wines sequentially fermented with two other *Metschnikowia* species, that is *M. chrysoperlae* and *M. fruticola* (Liu et al. 2017).

The second most abundant group of volatile analytes in the current study was the higher alcohols. These wine (off) flavour compounds, primarily derived either from amino acids (Ehrlich pathway) or grape hexoses (anabolic synthesis), are complexing at low concentration, and at high concentration are a fault (Hazelwood et al. 2008). 3-Methyl-1-butanol and 2-phenyl alcohol were the most important and abundant wine aromatic alcohols, imparting fusel and rose aroma, respectively (Table 3). Trends in 3-methyl-1-butanol concentration comparing the different YTs were consistent: FL, PI and SC resulted in comparatively lower, and CO and ME in higher, concentration than that of most treatments at both HDs. The consistent trend was not the case for phenylethyl alcohol, which decreased with increasing ripeness in SC, ME and FL wines, as opposed to an increase in the

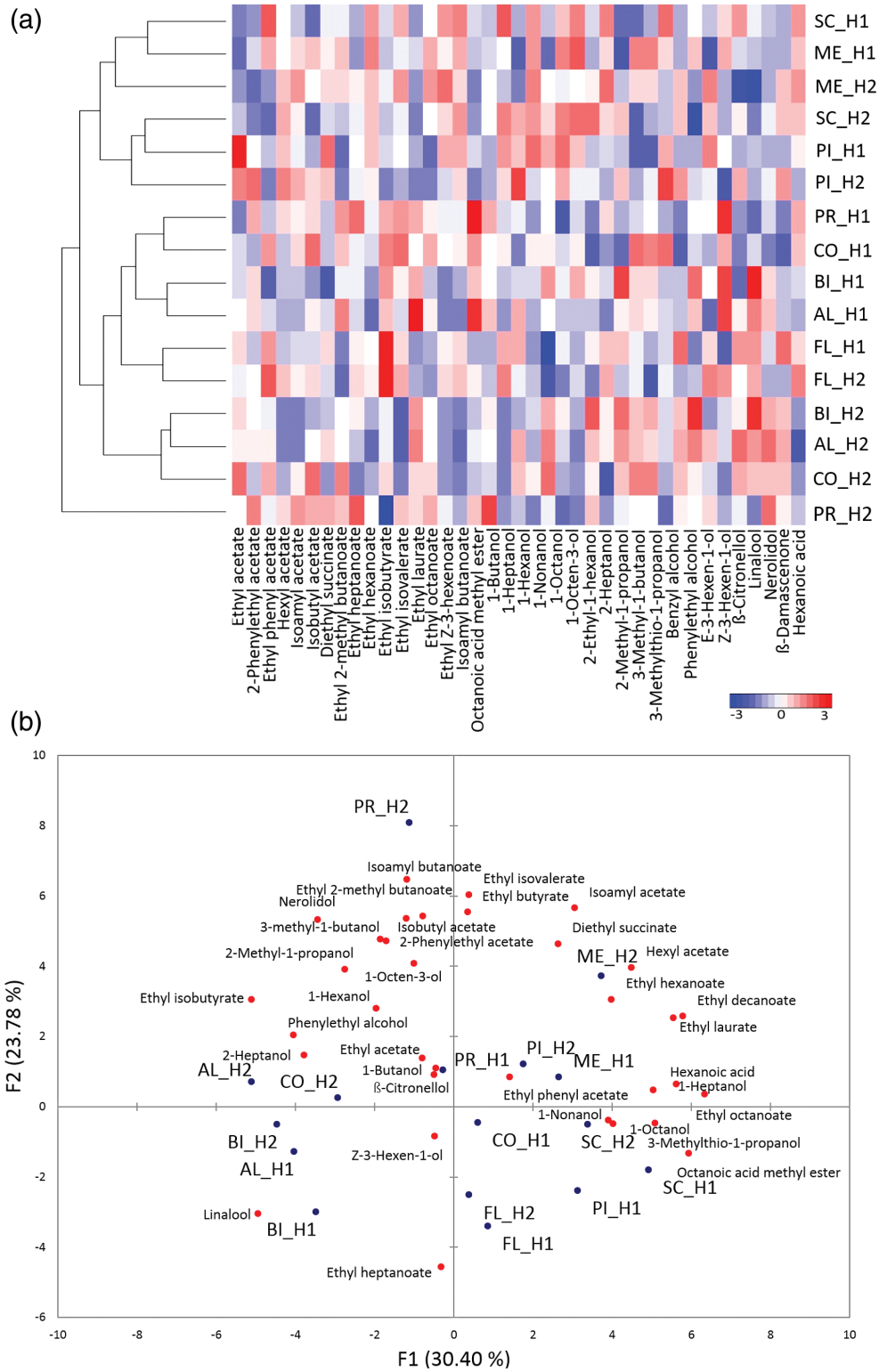

Figure 2. (a) Clustering of yeast treatments based on their volatile profile in wines produced at earlier (H1) and later (H2) harvest dates. (b) The discrimination of yeast inoculation treatments based on the quantification of 34 out of 39 significantly differing volatiles accounted for 54% of variance for first and second principal components. AL, BI, PR, *Torulaspora delbrueckii* strains; CO, *Lachancea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae, T. delbrueckii* and *L. thermotolerans*; and SC, a *S. cerevisiae* strain.

remaining wines. Moreover, phenylethyl alcohol was particularly high in wines with arrested fermentation, that is AL and BI, followed by PR, H2 wines. Given the demonstrated role of phenylethyl alcohol in yeast quorum sensing (Hazelwood et al. 2008, Zupan et al. 2013, Avbelj et al. 2016), a phenomenon through which individual microbial cells regulate their phenotype and adapt to environmental changes (Avbelj et al. 2016), it is worth further exploring whether the phenylethyl alcohol overproduction was related to stressful conditions leading to fermentation arrest. Previously, an increase in phenylethyl alcohol was generally attributed to mixed non-*Saccharomyces* and *Saccharomyces* fermentations (Comitini et al. 2011, Sadoudi et al. 2012, Jolly et al. 2014, Padilla et al. 2016) rather than their respective monocultures (Sadoudi et al. 2012). Furthermore, major differences were observed in the terpene concentration.

Terpenes, predominantly originating from grapes, are released and modulated by microbial activity, although modest de novo synthesis by yeasts has been reported (Carrau et al. 2005). The AL, BI, PR treatments possessed an elevated concentration of terpenes, potentially due to strong β-glucosidase activity as a general trait of *T. delbrueckii* strains (Renault et al. 2009, Comitini et al. 2011, Padilla et al. 2016).

Altogether, observed differences appear to be reflective of different metabolic activities and regulation between the evaluated yeast species and strains, of a *S. cerevisiae* strain inoculated in a differently initiated fermentation, and of resulting microbial interactions. Fundamental research, as conducted to date for *S. cerevisiae* (Hazelwood et al. 2008, Sumby et al. 2010), is required to characterise the known and/or possibly novel pathways of aromatic compound synthesis in non-*Saccharomyces* yeasts.

Phenolic substances and wine colour

Given their major role in red wine aesthetics, flavour, mouthfeel and wine grade (Ribéreau-Gayon et al. 2006, Mercurio et al. 2010), a range of measurements assessing the concentration of phenolic substances and colour was determined in Shiraz wines. An increase in the concentration of phenolic substances and in wine colour was observed in H2 wines, congruent with well-established patterns of their development and accumulation in grape skins during berry ripening (Ribéreau-Gayon et al. 2006, Bindon et al. 2013, Li et al. 2017). Less is known about yeast-derived effects and underlying mechanisms of mediation of the aforementioned wine chemical parameters. Arguably, yeast effects are comparatively less pertinent than grape-derived determinants, but as strongly suggested by our results, these are considerable. Different yeast inoculation regimes resulted in wines of significantly discernible phenolic and chromatic profile when identical cap management and duration of skin contact were applied.

Tannin concentration in wines was significantly affected by YT at both HDs. The extraction and retention mechanisms of these macromolecules under winemaking conditions are still not fully understood. The ethanol-mediated extraction is emphasised in the literature (Canals et al. 2005), much as is the physical breakdown of grape solids (Busse-Valverde et al. 2010). Fitting the yeast influence into these models is far from accomplished. As mentioned, SC treatment resulted in higher tannin concentration compared to that of the remaining treatments in H1 wines (Table 4). Given the fermentation kinetics and ethanol concentration formed by the time-point when the fermentations were pressed off skins (Figure 1a), tannin concentration in H1 wines was accordant with the ethanol-assisted extraction model in all treatments, except ME. Despite a similarity in the fermentation kinetics, the ME treatment resulted in a lower final tannin concentration than that of SC. A deviation from the ethanol-assisted tannin extraction hypothesis was even more evident in H2 wines, where the tannin concentration of the SC wine was similar to that of ME, AL and BI wines (Table 4). The SC and ME wines had similar sugar consumption/ethanol formation kinetics for H2, different to that of AL and BI (Figure 1b) resulting in incomplete fermentation and thereby lower final ethanol concentration (Table 2). This indicates a more complex mechanism of tannin extraction and retention during and post-fermentation on skins than that explained by the ethanol-mediated extraction hypothesis. Such findings agree with Carew et al. (2013), who observed a discrepancy between final tannin concentration in Pinot Noir wines and the respective yeast fermentation kinetics. Among alternative explanations for the difference in tannin concentration, Carew et al. (2013) discussed breakdown of grape solids, differential fining of phenolic compounds by yeasts, and expression levels of enzymatic activity. With regards to the latter, potential differential secretion of other enzymes contributing to tannin release (e.g. β-glucosidase, proteolytic enzymes, pectinase) by different yeast strains (Comitini et al. 2011, Jolly et al. 2014, Padilla et al. 2016), and by the same strain under different conditions, might have also contributed to the differences observed in the current study.

Different *S. cerevisiae* strains were reported to vary based on their tannin binding affinity (Mazauric and Salmon 2006, Sidari et al. 2007). Besides interacting with yeast cell walls, primarily via mannoproteins, glucans and chitins (Salmon 2006), tannins were reported to interact with the plasma membrane and cytoplasmic components upon diffusion through non-viable yeast cell walls (Mekoue Nguela et al. 2015). Furthermore, the tannin binding capacity of yeast can be strongly affected by the composition of the fermentation medium (Rinaldi et al. 2016). For instance, medium supplements overrode differences attributable to yeast strain, with the mean absorbed tannin concentration almost doubled upon vitamin and peptone enrichment (Rinaldi et al. 2016). Differences in yeast cell morphology, especially cell wall and the plasma membrane composition/structure, and response to differences in medium composition arising from HD might have also played a role in yeast-derived mediation of wine tannin concentration. Our findings highlighted the need for further research on such factors among and within different species of wine-related yeasts.

In addition to tannin concentration, YT significantly influenced the colour profile and other phenolic substances measured in the wines. Yeasts are known to affect wine colour in several ways, one of which, is the adhesion/adsorption of pigmented compounds to yeast cells (Mazauric and Salmon 2006, Sidari et al. 2007). A differential binding affinity to the cells of tested strains was likely to contribute to differences in wine colour and concentration of phenolic substances. Indeed, a variation in lees colour intensity was observed visually, albeit not measured instrumentally. While some insight is available for phenolic fining by *S. cerevisiae* wine strains (Mazauric and Salmon 2006, Sidari et al. 2007), less is known about the extent of that diversity among species and strains of non-*Saccharomyces* yeasts. Furthermore, extracellular enzymatic activity, primarily glycosidase and pectinase, differentially expressed in wine yeast species and strains (Comitini et al. 2011, Jolly et al. 2014, Padilla et al. 2016), might have impacted parameters defining phenolic substances and chromatic wine profile. Finally, as phenolic substances bind to yeast metabolic by-products, primarily pyruvic acid and acetaldehyde, certain non-*Saccharomyces* strains have been proposed to be used for colour stabilisation due to increased stable pigment formation (Benito et al. 2011, Morata et al. 2012). Metabolic activity clearly varies between species, strains and their co-existence, and is dependent on medium composition. Different production rates and final concentration of metabolites that were not measured (e.g. pyruvic acid and acetaldehyde) might therefore account for some variation between YT within the same HD, as well as differences when the same YT was applied to another HD. Interestingly, PR treatment was consistently related to lower concentration of phenolic substances and to lower colour measurements at both HDs, whereas the remaining two *T. delbrueckii* treatments, AL and BI, showed more variability. Our results thus suggest an intraspecific diversity among *T. delbrueckii* strains with regards to

Table 4. Effect of harvest date and yeast treatment on the tannin concentration and Somers measurements in Shiraz wines.

Harvest	Yeast treatment	Tannin concentration (mg/L epicatechin)	Wine colour density (AU)	Wine hue	Phenolic substances (AU)	Anthocyanin (mg/L)	Non-bleachable pigment (AU)
H1	AL	826 ± 39 a	10.7 ± 0.4 a	0.64 ± 0.05 abc	36.2 ± 0.8 a	474 ± 11 abc	1.96 ± 0.07 abc
	BI	907 ± 72 a	10.8 ± 0.3 a	0.62 ± 0.01 a	39.4 ± 0.8 b	503 ± 15 bcd	1.84 ± 0.01 ab
	CO	899 ± 127 a	11.8 ± 0.9 bc	0.62 ± 0.04 a	38.6 ± 4.0 ab	511 ± 40 d	1.97 ± 0.14 abc
	FL	906 ± 15 a	12.0 ± 0.2 c	0.67 ± 0.05 bc	36.3 ± 1.4 a	473 ± 13 ab	2.20 ± 0.07 c
	PR	889 ± 102 a	10.2 ± 0.5 a	0.63 ± 0 a	35.9 ± 1.3 a	471 ± 16 ab	1.77 ± 0.08 a
	PI	830 ± 82 a	12.1 ± 0.4 c	0.60 ± 0.01 a	38.7 ± 0.2 ab	506 ± 4 cd	2.07 ± 0.09 bc
	ME	902 ± 87 a	11.0 ± 0.9 ab	0.68 ± 0.03 c	38.0 ± 0.8 ab	464 ± 11 a	1.87 ± 0.16 ab
	SC	1083 ± 62 b	11.7 ± 0.5 bc	0.63 ± 0 a	38.7 ± 0.5 ab	479 ± 2 abc	2.01 ± 0.08 abc
H2	AL	1378 ± 40 cd	15.2 ± 0.1 cd	0.63 ± 0.02 abc	49.0 ± 0.9 abc	592 ± 4 ab	3.94 ± 0.04 d
	BI	1398 ± 27 cd	15.6 ± 0.8 d	0.63 ± 0.01 abc	50.3 ± 1.8 bc	589 ± 17 ab	4.00 ± 0.35 d
	CO	1178 ± 94 ab	14.1 ± 0.3 ab	0.61 ± 0.03 ab	46.5 ± 2.3 a	629 ± 22 cd	2.83 ± 0.07 a
	FL	1299 ± 130 bc	15.6 ± 0.6 d	0.64 ± 0.04 bc	49.9 ± 3.7 bc	644 ± 44 de	3.44 ± 0.19 c
	PR	1087 ± 53 a	13.2 ± 0.3 a	0.64 ± 0 bc	46.5 ± 1.0 a	598 ± 9 abc	2.77 ± 0.13 a
	PI	1275 ± 32 bc	16.1 ± 0.6 d	0.59 ± 0.01 a	51.4 ± 1.1 c	664 ± 6 e	3.24 ± 0.02 bc
	ME	1342 ± 44 cd	14.7 ± 0.7 bc	0.66 ± 0.02 c	47.7 ± 0.9 ab	575 ± 19 a	2.98 ± 0.38 ab
	SC	1439 ± 230 d	14.7 ± 1.0 bc	0.62 ± 0.01 ab	51.5 ± 2.7 c	615 ± 18 bcd	3.13 ± 0.20 b
Percentage of variance and level of significance	IN	5.41*	3.94**	4.14 ns	2.23 ns	3.28*	8.66****
	HD	72.84****	80.14****	1.45 ns	86.88****	83.59****	78.01****
	YT	11.18***	10.70****	48.87***	4.77**	8.07****	10.09****

Values are means of three replicates ± SD; lower case letters denote a significant difference among yeast treatments at specified harvest date; significance levels of percentage of variation explained by the harvest date, yeast treatments, and the interaction of these variables are the following: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ (Fisher's least significant difference). AL, BI, PR, *Torulaspora delbrueckii* strains; CO, *Lachancea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae*, *T. delbrueckii* and *L. thermotolerans*; and SC, a *S. cerevisiae* strain. H1, first harvest on 11 February 2015; H2, second harvest on 11 February 2015.

their effect on wine phenolic and colour profile, as observed for the profile of wine volatiles. Such observations warrant further investigation, with a controlled experimental and instrumental set-up allowing for the quantification of phenolic substances absorbed by yeasts after monitoring their population dynamics and estimating cell surface, in conjunction with metabolite production and enzymatic activity determination, and the assessment of the interrelation of the variables in a given medium.

Effects of non-volatile and volatile compounds on wine sensory perception

Substantial literature highlights compositional particularities of wines obtained with non-*Saccharomyces* co-inocula under different fermentative conditions (Ciani et al. 2006, Kapsopoulou et al. 2007, Bely et al. 2008, Comitini et al. 2011, Sadoudi et al. 2012, Contreras et al. 2014, 2015, Varela et al. 2016). Sensory implications, however, are not always clarified. In some studies the sensory aspect is lacking, whilst in others the methodology employed does not allow for detailed wine profiling and comparison. To broaden such understanding, we hereby present an extensive descriptive analysis dataset obtained by a well-trained panel.

A noticeable differentiation of wines from the two HDs was congruent with overall compositional analysis. As expected, taste attributes were relatively simple to define and explain in relation to certain aspects of wine composition. For example, residual sugar concentration was positively correlated with 'sweetness' perception ($R = 0.95$; Table S5), and negatively correlated with 'acidity' ($R = -0.90$) and 'bitterness' ($R = -0.74$). Lack of clear relationship between 'acidity' and pH/TA is potentially linked to fluctuations due to acid adjustment and alterations during stabilisation, whereas further analysis of other aspects of wine composition, for example polysaccharides, might give more insight into the 'bitterness' perception (Mercurio and Smith 2008). Regarding palate sensation attributes, a clear relationship between 'hotness' and ethanol concentration was observed ($R = 0.84$), as documented in studies incorporating progressive grape ripening (Heymann et al. 2013, Bindon et al. 2014, Li et al. 2017). Increasing residual sugar, ethanol and tannin concentration were positively associated with 'palate fullness', whereas both 'astringency' and 'palate coarseness' were positively correlated with tannin concentration. 'Astringency' also positively correlated with colour density, phenolic substances, anthocyanin and polymeric pigment, as well as ethanol and glycerol concentration, reflective of increased grape ripeness. Higher perception of astringency with increasing tannin concentration is well established (Mercurio and Smith 2008), and its link to measurement of some other phenolic substances such as polymeric pigment has also been reported (Bindon et al. 2013). Further quantification and characterisation of wine macromolecules, primarily tannins and polysaccharides (Mercurio and Smith 2008), could explain subtly perceived differences, particularly within the same HD.

Interactions between volatile and non-volatile compounds are complex and matrix-dependent, and the effect of volatile profile on aroma/flavour perception was thus less conclusive. Nonetheless, certain correlative, although not necessarily causative, relationships between variables warrant highlighting. First, the AL H1 wine was scored the highest in the attributes aroma intensity, red fruit and confectionery, while the SC H1 wine was perceived as the most vegetal. A compound likely to contribute to such perception is ethyl isobutyrate, most pronounced in the AL H1 wine. Ethyl isobutyrate was recently reported as a *T. delbrueckii* activity marker, conferring strawberry and red fruit sensory notes (Renault et al. 2015). Fruity esters are known to mask vegetative, generally undesired, notes in wines (Escudero et al. 2004), and variations in their composition elicit significant aroma alterations (Pineau et al. 2009).

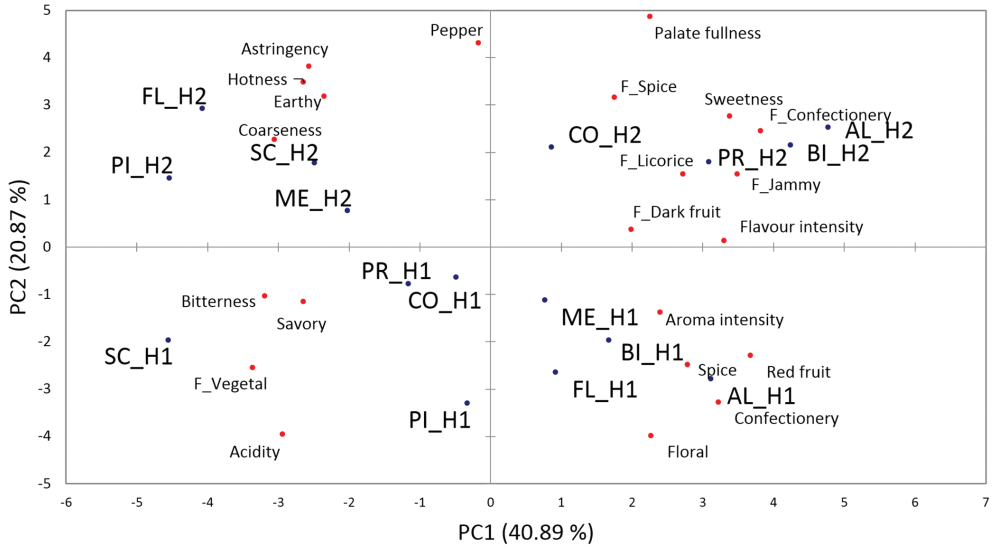

Figure 3. Principal component (PC) analysis biplot of sensory data (●) for Shiraz wines produced with eight yeast treatments (●) using earlier (H1) and later (H2) harvested fruit. AL, BI, PR *Torulaspora delbrueckii* strains; CO, *Lachancea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae, T. delbrueckii* and *L. thermotolerans*; and SC, a *S. cerevisiae* strain.

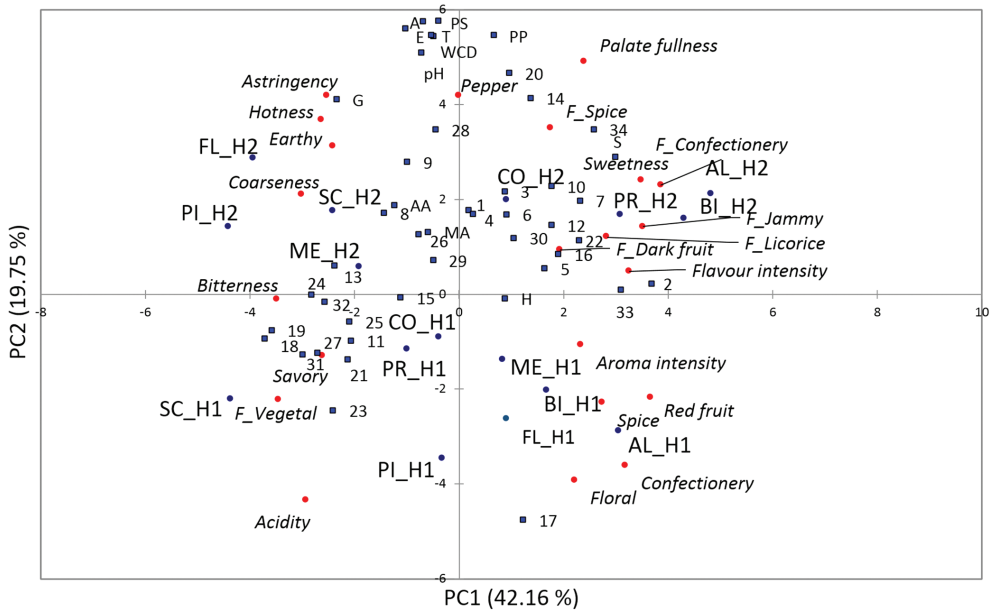

Figure 4. Principal component (PC) analysis biplot of sensory data [active variables (●)] and chemical data [supplementary variables (■)] for Shiraz wines produced with eight different yeast treatments (●) (Table 1) using earlier (H1) and later (H2) harvested grapes. AL, BI, PR *Torulaspora delbrueckii* strains; CO, *Lachancea thermotolerans*; FL, *Metschnikowia pulcherrima*; PI, an initially uninoculated treatment; ME, a commercial blend of *Saccharomyces cerevisiae, T. delbrueckii* and *L. thermotolerans*; and SC, a *S. cerevisiae* strain. The concentration/measurement of respective analytes are coded as: 1, ethyl acetate; 2, ethyl isobutyrate; 3, isobutyl acetate; 4, ethyl butyrate; 5, ethyl 2-methyl butanoate; 6, ethyl isovalerate; 7, 2-methyl-1-propanol; 8, isoamyl acetate; 9, 1-butanol; 10, 3-methyl-1-butanol; 11, ethyl hexanoate; 12, isoamyl butanoate; 13, hexyl acetate; 14, 2-heptanol; 15, ethyl heptanoate; 16, 1-hexanol; 17, *Z*-3-hexen-1-ol; 18, octanoic acid methyl ester; 19, ethyl octanoate; 20, 1-octen-3-ol; 21, 1-heptanol; 22, linalool; 23, 1-octanol; 24, ethyl decanoate; 25, 1-nonanol; 26, diethyl succinate; 27, 3-methylthio-1-propanol; 28, beta-citronellol; 29, ethyl phenyl acetate; 30, 2-phenylethyl acetate; 31, hexanoic acid; 32, ethyl laurate; 33, phenylethyl alcohol; 34, nerolidol. A, anthocyanin; AA, acetic acid; E, ethanol; G, glycerol; H, hue; MA, malic acid; PP, non-bleachable pigment; PS, phenolic substances; S, residual sugar; T, tannin; WCD, wine colour density.

As hypothesised, modulation of volatile profile seen in certain non-*Saccharomyces* treatments thus potentially led to aroma/flavour enhancement of earlier harvested wines. In contrast, an impacting factor in the aroma perception of AL, BI and PR H2 wines was, certainly, residual sugar, known to cause an increased concentration of volatiles in the headspace (Robinson et al. 2009). When comparing the grouping of wines based on their volatile profile compared to grouping based on sensory perception, a correspondence can be observed if the wines from different YTs were produced within the same HD. For example, AL and BI H1 wines were characterised by both similar volatile and sensory profile, and thus closely clustered on two PCA biplots (Figures 3–4), separately from, for example, the SC H1 wine. Certain wines, however, produced with the same YT, but from a different HD were seen as different despite their close GC-MS volatile profile. In this case, changes in ethanol concentration are likely to have largely influenced the behaviour of volatiles in the wine liquid phase and the headspace. Increasing ethanol concentration generally leads to aroma/flavour dampening

due to decreased volatility of compounds (Robinson et al. 2009), potentially explaining the observed discrepancies. For example, FL H1 and H2 wines showed the closest volatile profile, yet were perceived differently, with significantly lower scores in aroma intensity, red fruit, spice, floral, and pepper for H2 compared to H1 wines.

Conclusion

Our results show marked matrix-based modulation of wine sensory perception that is reflective of grape HD, but yeast inoculum-derived differences observed in the chemical composition of wines are also apparent. Of particular interest is the increase in intensity of descriptors generally regarded as more appealing in earlier harvest wine profiles obtained by certain non-*Saccharomyces* co-inocula compared to that of a *S. cerevisiae* Control, and the chemical basis for such perception in terms of increased production of aromatic compounds. Further validation across a range of conditions is required to confirm such claims, but at the moment non-*Saccharomyces* yeast appear to be a valuable tool to optimise the quality of wines made from earlier harvests, as might occur with efforts to modulate wine ethanol concentration.

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Table S1. Definitions and standards of taste, palate sensation, aroma and flavour attributes assessed in descriptive analysis of Shiraz wines.

Table S2. Summary of two-way ANOVA of basic chemical parameters, Somers measurements and tannin concentrations in Shiraz wines.

Table S3. Qualitative and quantitative information and two-way ANOVA summary of volatile compounds analysed in Shiraz wines.

Table S4. Mean intensity ratings for significantly different attributes in Shiraz wines made with different yeast treatments at earlier (H1) and later (H2) harvest dates.

Table S5. Correlation matrix of significantly different chemical and sensory parameters in Shiraz wines (correlation efficiencies > 0.5 and <−0.5 are in bold).

Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-*Saccharomyces* inocula

Ana Hranilovic[1,2], Sijing Li[1,2], Paul Boss[3], Keren Bindon[4], Renata Ristic[1], Paul Grbin[1], Tertius van der Westhuizen[5], Vladimir Jiranek[1,2]

[1] The Australian Research Council Training Centre for Innovative Wine Production

[2] The University of Adelaide, Department of Wine and Food Science, PMB 1, Glen Osmond, SA 5064, Australia

[3] CSIRO Agriculture and Food, PMB2, Glen Osmond, SA 5064, Australia

[4] The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064

[5] Laffort Australia, Woodville North SA 5012

CORRESPONDING AUTHOR Prof Vladimir Jiranek, The University of Adelaide, Department of Wine and Food Science, PMB 1, Glen Osmond, SA 5064, Australia, telephone +61 (0)8 8313 7415, email vladimir.jiranek@adelaide.edu.au

Table S1. Definitions and standards of taste, palate sensation, aroma and flavour attributes assessed in descriptive analysis of Shiraz wines.

Attribute	Type	Definition	Standard †
Acidity	Taste	Intensity of acid taste perceived in the mouth or after expectorating	-
Sweetness	Taste	Intensity of sweet taste perceived in the mouth or after expectorating	-
Bitterness	Taste	Intensity of bitter taste perceived in the mouth or after expectorating	-
Palate fullness	Palate sensation	Overall impression of weight or substantiveness of the wine in the mouth	0.3 g/L Xanthan gum
Astringency	Palate sensation	Overall level of astringency sensation	0.3 g/L Tarac seed tannin
Surface coarseness	Palate sensation	Texture felt on mouth surfaces	'Touch standards' comprising of a range of fabrics and powders
Hotness	Palate sensation	Intensity of warmth or heat perceived in the mouth or after expectorating	14% Ethanol in filtered water
Flavour intensity	Flavour	Perception of overall flavour intensity	-
F_Red fruit	Flavour	Perception of fresh raspberry, strawberry, sour cherry and red plum	Half a strawberry, 2 canned cherries, 1 slice of plum and 2 raspberries
F_Dark fruit	Flavour	Perception of fresh and strewed dark fruit blackberry, blueberry and plum aroma	Half a tea spoon of forest berry jam, 1 blackberry and 2 blue berries
F_Jammy	Flavour	Perception of jam and prune	1 Tea spoon of prune jam and 1 chopped prune
F_Vegetal	Flavour	Perception of cut grass, leaf and fresh herb	Half a eucalyptus leaf and two blades of grass
F_Confectionery	Flavour	Perception of strawberry cream and bubble gum	1 cm of Bubble gum, half of a raspberry cream lolly, quarter of a marshmallow and 2.5 cm of red snake lolly
Spice	Flavour	Perception of mixed spice, e.g. cinnamon, clove and nutmeg	Half a clove, a pinch of mixed spice powder and nut meg powder
Pepper	Flavour	Perception of black and white pepper	Half a crack of black pepper and a pinch of white pepper

Aroma intensity	Aroma	Perception of overall aroma intensity	-
Red fruit	Aroma	Perception of fresh raspberry, strawberry, sour cherry and red plum	Same as red fruit flavour
Dark fruit	Aroma	Perception of fresh and strewed dark fruit blackberry, blueberry and plum aroma	Same as dark fruit flavour
Jammy	Aroma	Perception of jam and prune	Same as jammy flavour
Vegetal	Aroma	perception of cut grass, leaf and fresh herb	Same vegetal flavour
Confectionery	Aroma	Perception of strawberry cream and bubble gum	Same as confectionery flavour
Spice	Aroma	Perception of mixed spice, e.g. cinnamon, clove and nutmeg	Same as spice flavour
Savoury	Aroma	Perception of soy sauce, oyster sauce and bacon	2 Drops of soy sauce, 1 drop of oyster sauce, 3 g of bacon
Earthy	Aroma	Perception of musty and damp soil and mushroom	2 cm x 1 cm Unwashed potato peel, a pinch of earth and 1 slice of mushroom in water
Floral	Aroma	Perception of floral fragrance and perfume	-
Pepper	Aroma	Perception of black and white pepper	Same as pepper flavour
Other	Aroma/flavour	Any aroma/flavour not encompassed in the attribute list	-

† Unless otherwise specified, standards were prepared in 30 mL 2014 Shiraz cask wine (Yalumba, South Australia).

Table S2: Summary of two-way ANOVA of basic chemical parameters, Somers measurements and tannin concentrations in Shiraz wines.

Parameter	Source of variation	% of variation	*P* value	Signifi-cance	SS	DF	MS	F
Residual sugar (g/L)	IN	37.15	< 0.0001	****	479.40	7	68.48	58.01
	HD	21.75	< 0.0001	****	280.70	1	280.70	237.80
	YT	38.16	< 0.0001	****	492.40	7	70.34	59.59
	R				37.77	32	1.18	
Residual sugar at sequential inoculation (g/L)	IN	1.80	0.0084	**	1009	5	201.80	4.04
	HD	77.54	< 0.0001	****	43595	1	43595	873.10
	YT	18.53	< 0.0001	****	10419	5	2084	41.73
	R				1198	24	49.93	
Ethanol (% v/v)	IN	3.21	0.0003	***	3.24	7	0.4627	5.55
	HD	88.65	< 0.0001	****	89.43	1	89.43	1073
	YT	5.49	< 0.0001	****	5.54	7	0.7918	9.50
	R				2.67	32	0.0833	
Glycerol (g/L)	IN	8.35	< 0.0001	****	8.67	7	1.24	30.36
	HD	42.17	< 0.0001	****	43.78	1	43.78	1074
	YT	48.23	< 0.0001	****	50.07	7	7.15	175.50
	R				1.30	32	0.04	
Acetic acid (g/L)	IN	17.60	0.0197	*	0.13	7	0.0183	2.86
	HD	6.44	0.0109	*	0.05	1	0.0469	7.32
	YT	47.78	< 0.0001	****	0.35	7	0.0497	7.75
	R				0.21	32	0.0064	
Malic acid (g/L)	IN	54.18	< 0.0001	****	5.84	7	0.8344	99.55
	HD	8.65	< 0.0001	****	0.93	1	0.9324	111.30
	YT	34.69	< 0.0001	****	3.74	7	0.5342	63.73
	R				0.27	32	0.0083	
Tartaric acid (g/L)	IN	27.86	< 0.0001	****	0.67	7	0.0960	12.32
	HD	0.25	0.3839	ns	0	1	0.0060	0.78
	YT	61.55	< 0.0001	****	1.49	7	0.2122	27.23
	R				0.25	32	0.0078	
pH	IN	5.05	0.0007	***	0.01	7	0.0012	5.00
	HD	72.64	< 0.0001	****	0.13	1	0.1292	504.10
	YT	17.7	< 0.0001	****	0.03	7	0.0044	17.55
	R				0.01	32	0.0002	
TA (g/L as tartaric)	IN	10.07	0.2982	ns	0.28	7	0.0399	1.27
	HD	45.73	< 0.0001	****	1.27	1	1.2680	40.25
	YT	7.85	0.4583	ns	0.22	7	0.0311	0.99
	R				1.01	32	0.0315	
Tannin (mg/L epicatechin)	IN	5.41	0.0475	*	138731	7	19819	2.34
	HD	72.84	< 0.0001	****	1867000	1	1867000	220.60
	YT	11.18	0.0008	***	286620	7	40946	4.84
	R				270751	32	8461	
Wine colour (AU)	IN	3.94	0.0072	**	7.72	7	1.10	3.46
	HD	80.14	< 0.0001	****	156.90	1	156.90	492.10
	YT	10.70	< 0.0001	****	20.95	7	2.99	9.39

	R				10.20	32	0.32	
Hue	IN	4.14	0.8853	ns	0.0018	7	0.0002	0.41
	HD	1.45	0.3206	ns	0.0006	1	0.0006	1.01
	YT	48.87	0.0008	***	0.0219	7	0.0031	4.90
	R				0.0203	32	0.0006	
Phenolic substances (AU)	IN	2.23	0.1546	ns	39.92	7	5.70	1.66
	HD	86.88	< 0.0001	****	1559	1	1559	453.80
	YT	4.77	0.0061	**	85.63	7	12.23	3.56
	R				109.90	32	3.47	
Anthocyanins (mg/L)	IN	3.28	0.0166	*	7697	7	1100	2.96
	HD	83.59	< 0.0001	****	196193	1	196193	527.50
	YT	8.07	< 0.0001	****	18931	7	2704	7.27
	R				11901	32	371.90	
Non-bleachable pigment (AU)	IN	8.66	< 0.0001	****	2.35	7	0.34	12.18
	HD	78.01	< 0.0001	****	21.14	1	21.14	768.20
	YT	10.09	< 0.0001	****	2.73	7	0.40	14.19
	R				0.88	32	0.03	

Significance levels of percentage of variation explained by the harvest date (HD), yeast treatments (YT), and the interaction (IN) of these variables are the following: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. SS, sum of squares; DF, degrees of freedom; MS, mean of squares; R, residual.

Table S3. Qualitative and quantitative information and two-way ANOVA summary of volatile compounds analysed in Shiraz wines.

Compound	CAS No.	RT	Ion	Aroma quality[†]	Aroma detection threshold (µg/L) [‡]		Determined range (µg/L)	Source of variation	% of variation	*P* value	Significance
Ethyl acetate	141-78-6	3.413	61	Fruity (low levels); solventy , balsamic (high levels)	15000 [§]	H1	18797.18 - 44833.27	IN	7.77	0.0427	*
						H2	24519.09 - 42366.85	HD	18.47	< 0.0001	****
								YT	58.98	< 0.0001	****
2-Phenylethyl acetate	103-45-7	18.846	104	Rose, floral	250 [¶]	H1	29.56 - 59.21	IN	17.83	< 0.0001	****
						H2	29.68 - 83.90	HD	5.20	< 0.0001	****
								YT	69.84	< 0.0001	****
Ethyl phenyl acetate	101-97-3	18.388	91	Rose, floral	650 [††]	H1	0.26 - 0.60	IN	48.22	< 0.0001	****
						H2	0.30 - 0.47	HD	0.58	0.2232	ns
								YT	39.13	< 0.0001	****
Hexyl acetate	142-92-7	9.444	84	Fruity, herbal	670 [‡‡]	H1	19.00 - 45.40	IN	13.34	< 0.0001	****
						H2	14.98 - 68.05	HD	4.03	< 0.0001	****
								YT	77.36	< 0.0001	****
Isoamyl acetate	123-92-2	6.561	87	Banana	30 [¶]	H1	637.23 - 1529.85	IN	16.43	< 0.0001	****
						H2	544.62 - 2283.38	HD	9.34	< 0.0001	****
								YT	68.47	< 0.0001	****
Isobutyl acetate	110-19-0	4.852	73	Solvent, fruity, apple	1600 [§§]	H1	24.38 - 64.32	IN	11.64	0.0004	***
						H2	31.38 - 81.35	HD	6.66	< 0.0001	****
								YT	71.63	< 0.0001	****
Total acetate esters	na	na	na	na	na	H1	20334.28 - 46320.79	IN	7.31	0.0641	ns
						H2	26425.86 - 44809.83	HD	19.47	<0.0001	****
								YT	57.83	<0.0001	****
Diethyl succinate	123-25-1	16.63	129	Fruity, fermented, floral	1250000 [§]	H1	55.31 - 205.15	IN	17.87	< 0.0001	****
						H2	105.64 - 228.86	HD	13.01	< 0.0001	****
								YT	61.84	< 0.0001	****

Ethyl 2-methyl butanoate	7452-79-1	5.414	102	Fruity, strawberry, anise	1 ¶	H1	1.56 - 4.57	IN	4.72	0.0056	**
						H2	2.12 - 5.26	HD	1.70	0.0049	**
								YT	87.62	< 0.0001	****
Ethyl butyrate	105-54-4	5.189	88	Floral, fruity, strawberry	20 ¶	H1	45.06 - 80.94	IN	15.12	< 0.0001	****
						H2	42.88 - 107.14	HD	7.78	< 0.0001	****
								YT	68.17	< 0.0001	****
Ethyl decanoate	110-38-3	16.053	101	Soapy, waxy, floral	200 ¶¶	H1	98.93 - 86.76	IN	4.15	0.0036	**
						H2	135.98 - 72.11	HD	0.45	0.0964	ns
								YT	90.52	< 0.0001	****
Ethyl heptanoate	106-30-9	10.559	113	Fruity, grape	2 [a]	H1	0.53 - 1.08	IN	35.23	< 0.0001	****
						H2	0.06 - 1.16	HD	5.57	< 0.0001	****
								YT	52.40	< 0.0001	****
Ethyl hexanoate	123-66-0	8.556	88	Green apple peel, fruity	14 ¶¶	H1	404.07 - 623.99	IN	28.76	< 0.0001	****
						H2	302.55 - 699.38	HD	3.15	0.0016	**
								YT	59.65	< 0.0001	****
Ethyl isobutyrate	97-62-1	4.264	71	Strawberry, fruity	15 §§	H1	186.74 - 1675.75	IN	2.29	0.0043	**
						H2	172.78 - 1544.93	HD	0.80	0.0046	**
								YT	94.14	< 0.0001	****
Ethyl isovalerate	108-64-5	5.654	88	Pineapple, apple, fruity	3 ¶	H1	7.36 - 22.02	IN	7.68	< 0.0001	****
						H2	10.95 - 27.76	HD	8.04	< 0.0001	****
								YT	79.35	< 0.0001	****
Ethyl laurate	106-33-2	19.311	101	Fruity, floral	500 §	H1	3.01 - 17.57	IN	19.76	< 0.0001	****
						H2	5.42 - 21.15	HD	0.09	0.546	ns
								YT	72.10	< 0.0001	****
Ethyl octanoate	106-32-1	12.484	127	Sour apple, soap	2 ¶	H1	135.78 - 692.83	IN	3.54	0.0017	**
						H2	138.74 - 609.15	HD	0.58	0.0317	*
								YT	92.18	< 0.0001	****
Ethyl Z-3-hexenoate	64187-83-3	10.797	97	Tropical	nf	H1	1.23 - 4.85	IN	4.66	< 0.0001	****
						H2	0.73 - 3.95	HD	14.23	< 0.0001	****

								YT	78.94	< 0.0001	****
Total ethyl esters	na	na	na	na	na	H1	1446.95 - 2440.49	IN	7.61	0.0708	ns
						H2	1510.67 - 2974.53	HD	19.74	<0.0001	****
								YT	56.18	<0.0001	****
Isoamyl butanoate	106-27-4	9.32	71	Fruity	nf	H1	0.48 - 1.56	IN	14.84	<0.0001	****
						H2	0.54 - 3.03	HD	7.78	<0.0001	****
								YT	67.51	<0.0001	****
Octanoic acid methyl ester	111-11-5	11.63	127	Orange	200 [a]	H1	0.62 - 4.82	IN	3.52	<0.0001	****
						H2	0.54 - 3.92	HD	2.10	<0.0001	****
								YT	92.59	<0.0001	****
1-Butanol	71-36-3	7.202	56	Medicinal	150000 [a]	H1	18.06 - 40.60	IN	12.59	< 0.0001	****
						H2	25.70 - 64.48	HD	27.7	< 0.0001	****
								YT	54.87	< 0.0001	****
1-Heptanol	111-70-6	12.765	83	Grape, sweet	1000 [b]	H1	61.52 - 108.23	IN	6.80	< 0.0001	****
						H2	64.31 - 117.66	HD	0.00	0.9016	ns
								YT	88.86	< 0.0001	****
1-Hexanol	111-27-3	10.891	69	Green, grassy	8000 [¶]	H1	3928.64 - 5047.63	IN	17.00	< 0.0001	****
						H2	3778.83 - 5351.42	HD	12.97	< 0.0001	****
								YT	59.85	< 0.0001	****
1-Nonanol	143-08-8	16.312	70	Fatty, green	1000 [c]	H1	7.04 - 13.64	IN	7.05	0.0109	*
						H2	8.03 - 8.85	HD	1.55	0.0334	*
								YT	81.34	< 0.0001	****
1-Octanol	111-87-5	14.58	84	Moss, nut, mushroom	1 [d]	H1	28.84 - 31.26	IN	3.90	0.0003	***
						H2	22.31 - 20.98	HD	7.85	< 0.0001	****
								YT	84.99	< 0.0001	****
1-Octen-3-ol	3391-86-4	12.705	72	Earthy, mushroomy, mouldy	40 [e]	H1	0.75 - 0.94	IN	4.04	0.3027	ns
						H2	0.98 - 1.27	HD	62.07	< 0.0001	****
								YT	19.20	0.0002	***
2-Ethyl-1-hexanol	104-76-7	13.384	83	Citrus, green	8000 [f]	H1	4.11 - 5.40	IN	10.78	0.6117	ns
						H2	4.05 - 5.52	HD	0.54	0.6062	ns

								YT	25.21	0.1185	ns
2-Heptanol	543-49-7	10.37	83	Fruity, mouldy, musty	70 [c]	H1	11.43 - 16.95	IN	7.44	0.0032	**
						H2	15.03 - 17.32	HD	50.72	< 0.0001	****
								YT	33.27	< 0.0001	****
2-Methyl-1-propanol	78-83-1	6.235	74	Wine, solvent	40000 [¶]	H1	114219.41 - 148807.33	IN	8.25	0.0004	***
						H2	73038.81 - 158553.88	HD	5.11	< 0.0001	****
								YT	79.53	< 0.0001	****
3-Methyl-1-butanol	123-51-3	8.322	55	Whiskey, malt, burnt	30000 [¶]	H1	306593.86 - 539681.65	IN	9.52	0.008	**
						H2	383069.31 - 525036.71	HD	10.49	< 0.0001	****
								YT	67.16	< 0.0001	****
3-Methylthio-1-propanol	505-10-2	17.249	106	Boiled potato, rubber	500 [¶]	H1	1308.80 - 3134.59	IN	32.73	< 0.0001	****
						H2	1327.47 - 3531.50	HD	0.99	0.1026	ns
								YT	55.08	< 0.0001	****
Benzyl alcohol	100-51-6	19.749	107	Flower	200000 [g]	H1	559.61 - 617.89	IN	2.30	0.4674	ns
						H2	745.33 - 872.10	HD	82.67	< 0.0001	****
								YT	4.22	0.1249	ns
Phenylethyl alcohol	60-12-8	20.177	91	Honey, spice, rose, lilac	14000 [¶]	H1	202456.82 - 281356.72	IN	28.81	< 0.0001	****
						H2	155807.15 - 322693.83	HD	0.19	0.5495	ns
								YT	54.49	< 0.0001	****
E-3-Hexen-1-ol	928-97-2	11.079	82	Moss, fresh	400 [¶]	H1	46.78 - 55.20	IN	28.47	0.0237	*
						H2	48.80 - 57.99	HD	10.67	0.0114	*
								YT	13.45	0.2835	ns
Z-3-Hexen-1-ol	928-96-1	11.461	82	Grass	400 [¶]	H1	852.22 - 1218.17	IN	2.82	0.0352	*
						H2	578.50 - 867.03	HD	61.23	< 0.0001	****
								YT	30.81	< 0.0001	****
Total higher alcohols	na	na	na	na	na	H1	578885.65 - 1000523.79	IN	13.31	0.0014	**
						H2	639169.23 - 957882.61	HD	6.03	0.0007	***
								YT	67.15	<0.0001	****
ß-Citronellol	106-22-9	18.029	123	Rose	100 [¶]	H1	1.24 - 2.18	IN	31.76	< 0.0001	****
						H2	1.38 - 2.31	HD	22.16	< 0.0001	****

								YT	28.83	< 0.0001	****
Linalool	78-70-6	14.426	121	Flower, lavender	15 ¶	H1	2.31 - 2.60	IN	9.67	0.0017	**
						H2	2.45 - 2.91	HD	0.95	0.0927	ns
								YT	79.21	< 0.0001	****
Nerolidol	7212-44-4	22.092	93	Wood, flower, wax	1000 [a]	H1	3.01 - 12.90	IN	13.21	0.001	***
						H2	8.31 - 32.59	HD	33.37	< 0.0001	****
								YT	40.71	< 0.0001	****
Total terpenes	na	na	na	na	na	H1	7.37 - 17.65	IN	13.70	0.001	***
						H2	13.31 - 30.16	HD	33.92	<0.0001	****
								YT	39.10	<0.0001	****
ß-Damascenone	23726-93-4	18.917	121	Apple, rose, honey	0.05 ¶	H1	9.45 - 12.78	IN	9.87	0.5771	ns
						H2	11.46 - 12.68	HD	11.43	0.0147	*
								YT	23.76	0.0895	ns
Hexanoic acid	142-62-1	10.989	99	Leafy, wood, varnish	420 ¶¶	H1	2950.35 - 5693.61	IN	7.74	0.0006	***
						H2	2020.84 - 6045.27	HD	5.68	< 0.0001	****
								YT	79.65	< 0.0001	****

Significance levels of percentage of variation explained by the harvest date (HD), yeast treatments (YT), and the interaction (IN) of these variables are the following: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. RT, retention time; na, not applicable; nf, not found. † Obtained from flavornet (http://www.flavornet.org) by Terry Acree and Heinrich Arn; ‡ thresholds are reported for matrices with > 10% (ABV) ethanol, except Du et al. (2010), which was in water; § Moyano et al. (2002); ¶ Guth (1997); †† Burdock (2009); ‡‡ Peinado et al. (2004); §§ Sumby et al. (2010); ¶¶ Ferreira et al. (2000); [a] Welke et al. (2014); [b] Jiang and Zhang (2010); [c] Du et al. (2010); [d] Clarke and Bakker (2011); [e] Callejón et al. (2016); [f] Buttery et al. (1988); [g] Gómez-Míguez et al. (2007).

Table S4. Mean intensity ratings for significantly different attributes in Shiraz wines made with different yeast treatments at earlier (H1) and later (H2) harvest date.

Attribute/ Treatment	AL_H1	AL_H2	BI_H1	BI_H2	CO_H1	CO_H2	FL_H1	FL_H2	PR_H1	PR_H2	PI_H1	PI_H2	ME_H1	ME_H2	SC_H1	SC_H2	*P* value
Aroma intensity	10.1 d	9.8 d	9.8 d	9.0 abc	9.4 bcd	9.5 bcd	9.7 cd	8.9 ab	9.4 bcd	9.6 bcd	9.6 bcd	9.4 bcd	9.8 d	9.3 bcd	8.4 a	9.7 cd	0.0081
Red fruit	6.8 e	6.1 bcde	5.9 abcde	5.9 abcde	5.9 abcde	5.8 abcde	6.3 cde	4.7 a	5.3 abcd	6.3 cde	6.0 abcde	5.0 abc	6.6 de	5 abc	4.8 ab	5 abc	0.0414
Confectionery	5.0 c	4 abc	4.9 c	4.1 abc	4.1 abc	3.8 abc	4.0 abc	3.0 a	4.0 abc	3.9 abc	4.0 abc	3.1 abc	4.6 bc	3.6 ab	3.2 a	3.3 a	0.0161
Spice	4.5 cd	4 abcd	4.6 d	4 abcd	3.9 abc	3.8 abc	4.2 bcd	3.6 ab	4.0 dcba	4.1 abcd	4.0 abcd	3.4 abc	3.9 abc	3.9 abc	3.6 ab	4.2 bcd	0.0931
Earthy	4.2 abcd	4.1 abc	4.0 ab	3.6 ab	3.9 ab	3.9 ab	3.2 a	5.2 d	3.6 ab	4.1 abc	3.9 ab	5.1 cd	3.9 ab	4.4 bcd	3.9 ab	5.2 d	0.0064
Floral	3.4 cd	2.6 abc	4.0 d	2.7 abc	2.7 abc	2.5 abc	3.3 bcd	2.1 a	2.3 ab	2.8 abc	3.4 cd	2.3 ab	3.2 bcd	2.4 ab	2.2 a	2.6 abc	0.0032
Pepper	3.6 abc	4.4 de	4.1 cde	3.7 abcd	3.9 abcde	4.1 bcde	3.3 ab	4.5 e	4.1 bcde	4.2 cde	3.1 a	4.2 cde	4.0 bcde	3.8 abcde	3.7 abcd	3.8 abcde	0.0508
Flavour intensity	10.4 cde	10.5 e	10.4 de	10.1 abcde	9.7 abcd	10.1 abcde	10.2 bcde	9.7 abcd	9.4 a	10.4 cde	9.8 abcde	10.1 abcde	10.3 bcde	9.6 ab	9.4 a	9.7 abc	0.0213
F_Dark fruit	9.5 bc	8.6 abc	9.1 abc	8.3 ab	8.5 abc	9.1 abc	8.9 abc	8.7 abc	8.4 ab	9.6 c	8.2 a	8.2 a	8.4 abc	8.9 abc	8.1 a	8.8 abc	0.3267
F_Jammy	6.4 bcd	6.8 cd	6.5 bcd	6.6 cd	5.7 abc	6.4 bcd	5.9 abc	6.1 bcd	5.7 abc	7.2 d	6 abc	4.9 a	5.6 abc	6 abc	5.4 ab	5.7 abc	0.0254
F_Vegetal	3.2 bc	2.8 ab	4.0 cd	2.3 a	3.4 bc	3.5 bcd	3.9 cd	3.7 bcd	3.9 cd	3.3 bc	3.7 cd	4.0 cd	3.5 bcd	3.9 cd	4.4 d	3.5 bcd	0.0025
F_Confectionery	4.5 abc	6.4 d	4.5 abc	6.5 d	4.2 ab	4.7 bc	4.3 abc	3.9 ab	4.0 ab	5.5 cd	4.1 ab	3.9 ab	4.6 abc	3.7 ab	3.4 a	3.9 ab	< 0.0001
F_Spice	4.0 abc	4.2 abcd	4.6 bcd	4.7 cd	4.4 abcd	4.6 bcd	4.3 abcd	4.4 abcd	3.9 ab	4.8 d	3.7 a	3.9 abc	4.5 bcd	4.4 abcd	3.7 a	4.5 bcd	0.0456
F_Licorice	3.7 cd	3.4 abcd	3.3 abcd	3.6 abcd	3.3 abcd	3.7 d	3.1 abcd	2.9 ab	3.7 cd	3.6 bcd	2.8 a	3.1 abcd	3.3 abcd	3.3 abcd	2.9 abc	3.3 abcd	0.3745
Savoury	3.4 cde	2.6 ab	3.6 e	2.2 a	2.9 abcde	2.8 abcd	2.9 abcde	3.5 cde	3.5 cde	3.0 abcde	2.7 abc	3.5 de	3.1 bcde	3.1 bcde	3.3 bcde	3.4 cde	0.022
Acidity	7 cde	4.6 a	7.2 ef	5.1 ab	7.2 ef	6.0 bc	6.5 cde	6.6 cde	7.2 cdef	6.0 bcd	7.2 def	7 cde	7.4 ef	6.8 cde	8.2 f	6.8 cde	< 0.0001
Sweetness	5.5 cd	9.2 e	5.0 abc	8.8 e	4.4 ab	5.5 cd	4.7 abc	4.5 abc	5.3 bc	6.5 d	4.9 abc	4.4 ab	4.7 abc	5.2 bc	4.1 a	4.4 ab	< 0.0001
Bitterness	6.1 abcd	5.0 a	5.8 abcd	5.0 a	5.8 abcd	6.5 bcd	6.7 cd	6.7 cd	5.8 abcd	5.7 abc	6.3 bcd	6.9 d	5.4 ab	5.8 abcd	6.5 bcd	6.4 bcd	0.0227
Palate fullness	8.8 ab	9.9 d	8.9 abc	9.4 bcd	8.7 ab	9.8 cd	8.7 ab	9 abcd	9.0 abc	9.4 bcd	8.5 ab	9 abc	8.8 ab	8.9 abc	8.3 a	9.3 bcd	0.0485
Astringency	7.8 abc	7.8 abc	8.2 abc	8.3 abc	8.4 abc	8.9 cde	8.2 abc	9.9 de	7.7 a	8.7 abcd	7.7 ab	9.8 de	8.6 abc	8.9 bcde	8.3 abc	10.1 e	0
Surface coarseness	6.1 a	6.2 a	7.0 abc	6.7 abc	7.1 abc	7.1 abc	6.4 ab	7.6 bc	7.3 abc	6.4 ab	6.3 ab	7.6 bc	7.6 bc	7.8 c	6.9 abc	7.7 c	0.0889
Hotness	8.3 a	8.2 a	9.0 abcd	8.3 a	9.4 bcde	10.0 def	8.5 ab	10.5 ef	8.9 abcd	9.8 cdef	8.8 abc	10.6 f	8.7 ab	10.1 ef	8.5 ab	10.7 f	<0.0001

Values are means of three replicates; lower case letters denote significant differences (Fisher's LSD).

Table S5. Correlation matrix of significantly different chemical and sensory parameters in Shiraz wines (correlation efficiencies > 0.5 and < -0.5 are in bold)

	AI	RF	CON	SP	EA	FL	PE	FI	F_DF	F_JA	F_VE	F_CON	F_SP	F_LI	SA	AC	SW	BI	PF	AS	SC	HO	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	T	WCD	H	TP	A	PP	S	E	G	AA	MA	pH
AI	1.00																																																																		
RF	**0.68**	1.00																																																																	
CON	**0.64**	**0.88**	1.00																																																																
SP	**0.65**	**0.59**	**0.78**	1.00																																																															
EA	0.01	**-0.53**	**-0.59**	-0.27	1.00																																																														
FL	**0.63**	**0.70**	**0.85**	**0.78**	-0.31	1.00																																																													
PE	-0.02	-0.19	-0.23	-0.18	0.33	-0.34	1.00																																																												
FI	**0.63**	**0.76**	**0.61**	0.46	-0.11	**0.58**	0.24	1.00																																																											
F_DF	0.51	0.42	0.37	**0.61**	0.08	0.31	0.20	0.50	1.00																																																										
F_JA	0.30	**0.53**	0.41	**0.55**	-0.30	0.31	0.14	**0.56**	**0.68**	1.00																																																									
F_VE	-0.27	**-0.55**	-0.33	-0.26	0.24	-0.11	-0.03	-0.49	-0.15	**-0.58**	1.00																																																								
F_CON	0.25	**0.57**	0.37	0.28	-0.36	0.18	0.21	**0.68**	0.20	**0.72**	**-0.88**	1.00																																																							
F_SP	0.17	0.24	0.17	0.31	0.04	0.11	0.38	0.38	**0.54**	**0.53**	-0.43	0.44	1.00																																																						
F_LI	0.42	0.49	0.46	0.47	-0.41	0.08	0.14	0.36	**0.56**	0.49	-0.49	0.49	0.48	1.00																																																					
SA	0.03	-0.39	-0.13	0.07	0.48	0.02	0.26	-0.22	0.17	-0.44	**0.74**	**-0.72**	-0.21	-0.21	1.00																																																				
AC	-0.19	-0.25	-0.02	-0.10	0.04	0.13	-0.35	-0.48	-0.23	**-0.67**	**0.80**	**-0.87**	-0.44	-0.44	**0.66**	1.00																																																			
SW	0.13	0.38	0.22	0.21	-0.35	0.01	0.18	0.49	0.11	**0.67**	**-0.82**	**0.94**	0.26	0.46	**-0.71**	**-0.90**	1.00																																																		
BI	-0.25	**-0.58**	**-0.56**	-0.40	**0.60**	-0.28	-0.06	-0.42	-0.07	-0.51	**0.70**	**-0.76**	-0.36	-0.47	**0.61**	**0.54**	**-0.74**	1.00																																																	
PF	0.29	0.19	-0.03	0.09	0.06	-0.19	**0.53**	0.48	0.36	**0.56**	**-0.62**	**0.72**	**0.55**	**0.58**	-0.43	**-0.90**	**0.71**	-0.36	1.00																																																
AS	-0.21	**-0.57**	**-0.67**	-0.43	**0.85**	-0.45	0.37	-0.18	0.03	-0.33	0.21	-0.32	0.35	-0.26	0.33	0.03	-0.37	**0.55**	0.15	1.00																																															
SC	-0.28	**-0.64**	-0.49	-0.41	0.48	-0.41	0.28	-0.48	-0.27	**-0.61**	0.45	**-0.55**	0.21	-0.23	0.42	0.33	**-0.54**	0.34	-0.13	**0.71**	1.00																																														
HO	-0.09	**-0.58**	**-0.65**	-0.36	**0.78**	-0.43	0.30	-0.29	0.15	-0.27	0.35	-0.44	0.30	-0.18	0.38	0.08	-0.47	**0.62**	0.13	**0.89**	**0.68**	1.00																																													
1	0.13	0.04	-0.24	-0.30	0.23	-0.07	-0.12	0.22	-0.01	0.15	-0.15	0.19	-0.11	-0.15	-0.36	-0.37	0.18	0.29	0.30	0.17	-0.24	0.31	1.00																																												
2	0.45	**0.67**	**0.60**	**0.54**	-0.47	0.24	0.19	**0.59**	**0.57**	**0.71**	**-0.59**	**0.69**	0.24	**0.79**	-0.28	**-0.55**	**0.69**	**-0.63**	0.50	**-0.56**	**-0.65**	**-0.52**	-0.08	1.00																																											
3	0.30	0.30	0.06	-0.19	-0.07	-0.22	0.35	0.27	0.35	0.17	-0.14	0.18	0.34	0.47	-0.14	-0.24	0.10	-0.18	0.37	0.09	0.08	0.25	0.25	0.36	1.00																																										
4	0.01	-0.06	-0.17	0.02	0.02	-0.23	0.13	-0.10	0.31	0.31	-0.03	0.10	0.26	0.26	0.01	-0.16	0.13	-0.13	0.12	0.06	0.00	0.28	0.14	0.25	0.48	1.00																																									
5	0.19	0.36	0.29	0.21	-0.42	-0.12	0.15	0.13	0.50	0.32	-0.17	0.21	0.38	**0.76**	-0.10	-0.14	0.20	-0.44	0.26	-0.23	-0.05	-0.14	-0.37	**0.61**	**0.66**	0.42	1.00																																								
6	0.02	0.17	0.03	0.01	-0.22	-0.28	0.17	0.02	0.39	0.25	-0.14	0.16	0.48	**0.52**	-0.17	-0.15	0.15	-0.41	0.23	0.02	0.13	0.06	-0.33	0.35	**0.65**	**0.52**	**0.90**	1.00																																							
7	0.25	0.48	0.38	0.10	-0.30	0.02	0.41	0.48	0.38	0.39	-0.43	0.49	**0.59**	**0.58**	-0.33	-0.43	0.40	**-0.62**	0.46	-0.11	0.03	-0.11	-0.13	**0.55**	**0.74**	0.16	**0.70**	**0.66**	1.00																																						
8	0.05	-0.18	-0.32	-0.31	0.26	-0.33	0.12	-0.15	0.18	-0.19	0.32	-0.30	0.24	0.07	0.19	0.17	-0.33	0.18	-0.02	0.44	0.47	**0.61**	0.16	-0.18	**0.70**	**0.66**	0.43	**0.62**	0.28	1.00																																					
9	0.21	-0.30	-0.48	-0.27	**0.65**	-0.33	0.24	0.08	0.03	-0.14	0.04	-0.04	-0.27	-0.08	0.19	-0.27	0.05	0.49	0.37	0.42	0.00	0.48	**0.63**	-0.06	0.11	0.09	-0.33	-0.34	-0.32	0.14	1.00																																				
10	0.19	0.34	0.25	0.02	-0.28	-0.12	0.39	0.35	0.28	0.20	-0.35	0.40	**0.57**	**0.65**	-0.26	-0.36	0.33	**-0.56**	0.47	-0.01	0.17	-0.02	-0.22	0.48	**0.74**	0.18	**0.78**	**0.75**	**0.93**	0.40	-0.26	1.00																																			
11	-0.29	-0.34	-0.27	-0.17	0.02	-0.28	-0.04	**-0.54**	0.10	-0.19	**0.58**	**-0.57**	-0.03	-0.15	0.38	**0.54**	**-0.52**	0.20	-0.50	0.10	0.32	0.30	-0.29	-0.23	0.28	**0.60**	0.38	**0.53**	0.00	**0.68**	-0.28	0.06	1.00																																		
12	0.22	0.29	0.14	0.27	-0.19	-0.07	0.16	0.21	**0.58**	**0.53**	-0.24	0.34	0.44	**0.59**	-0.11	-0.29	0.32	-0.40	0.31	-0.11	-0.21	0.03	-0.07	**0.58**	**0.58**	**0.85**	**0.75**	**0.78**	0.44	**0.56**	-0.08	0.48	0.46	1.00																																	
13	-0.04	-0.38	-0.48	-0.36	0.39	-0.32	-0.11	-0.33	-0.08	-0.43	0.49	-0.48	-0.12	-0.22	0.31	0.34	-0.44	0.39	-0.24	0.44	0.44	**0.58**	0.22	-0.42	0.33	**0.57**	0.09	0.30	-0.19	**0.87**	0.33	-0.02	**0.62**	0.34	1.00																																
14	0.17	-0.07	-0.12	0.20	0.35	-0.02	0.40	0.43	0.35	0.46	-0.39	0.51	0.42	0.27	-0.12	**-0.70**	**0.54**	-0.07	**0.70**	0.31	-0.09	0.29	0.41	0.30	0.00	0.18	-0.16	-0.16	0.09	-0.09	**0.53**	0.08	-0.46	0.16	-0.12	1.00																															
15	-0.18	-0.32	-0.04	-0.03	0.08	-0.16	0.20	-0.39	-0.14	-0.19	0.13	-0.31	-0.13	-0.10	0.24	0.12	-0.20	0.12	-0.11	0.00	0.33	0.04	-0.33	-0.13	-0.24	-0.42	-0.15	-0.27	0.02	-0.40	-0.14	-0.08	-0.07	**-0.53**	-0.48	-0.12	1.00																														
16	0.34	0.18	0.09	0.26	-0.26	0.11	-0.22	0.22	0.14	0.32	-0.28	0.41	0.07	0.42	-0.47	-0.50	0.50	-0.27	0.50	-0.25	-0.34	-0.08	0.39	0.33	0.12	0.13	0.14	0.09	0.05	0.03	0.29	0.17	-0.26	0.24	0.06	0.46	-0.36	1.00																													
17	0.25	0.42	**0.69**	**0.56**	**-0.66**	0.48	-0.25	-0.01	0.08	0.03	0.11	-0.08	-0.20	0.31	0.19	0.39	-0.08	-0.40	-0.41	**-0.74**	-0.25	**-0.66**	**-0.68**	0.39	-0.10	-0.02	0.41	0.17	0.11	-0.21	**-0.60**	0.10	0.23	0.17	-0.24	**-0.52**	0.14	-0.09	1.00																												
18	-0.47	**-0.64**	**-0.65**	**-0.55**	0.41	-0.36	-0.26	**-0.66**	**-0.52**	**-0.72**	**0.64**	**-0.73**	-0.45	**-0.74**	0.35	**0.64**	**-0.67**	**0.60**	**-0.61**	0.41	0.44	0.36	-0.06	**-0.84**	-0.33	-0.05	-0.39	-0.13	**-0.65**	0.27	0.10	-0.51	0.46	-0.30	0.59	-0.47	-0.10	-0.32	-0.21	1.00																											
19	-0.40	**-0.59**	**-0.59**	**-0.55**	0.37	-0.38	-0.23	**-0.64**	-0.46	**-0.72**	**0.65**	**-0.73**	-0.34	**-0.63**	0.33	**0.63**	**-0.67**	0.48	**-0.59**	0.42	**0.59**	0.40	-0.12	**-0.80**	-0.11	0.06	-0.17	0.11	-0.40	0.50	0.00	-0.25	**0.59**	-0.16	**0.72**	**-0.53**	-0.10	-0.32	-0.15	**0.94**	1.00																										
20	-0.07	-0.15	-0.34	0.01	0.30	-0.28	0.22	0.20	0.27	0.41	-0.39	0.48	**0.55**	0.23	-0.32	**-0.64**	**0.52**	-0.22	**0.62**	0.42	0.06	0.31	0.19	0.14	0.08	0.41	0.15	0.37	0.15	0.26	0.25	0.24	-0.09	0.47	0.23	**0.68**	-0.44	0.41	**-0.53**	-0.10	-0.06	1.00																									
21	-0.22	-0.31	-0.31	-0.30	0.09	-0.13	**-0.52**	-0.45	-0.38	**-0.52**	0.42	-0.50	-0.21	-0.44	-0.04	0.44	-0.43	0.21	-0.43	0.20	0.42	0.18	-0.08	**-0.66**	-0.16	-0.19	-0.07	0.16	-0.24	0.32	-0.19	-0.10	0.32	-0.23	0.49	-0.48	-0.12	0.04	-0.07	**0.70**	**0.81**	0.03	1.00																								
22	-0.03	0.28	0.31	0.25	-0.15	0.25	0.28	**0.54**	0.10	0.46	**-0.54**	**0.63**	0.22	0.16	-0.28	**-0.51**	**0.58**	-0.33	0.40	-0.19	-0.41	-0.42	-0.01	0.41	-0.32	-0.45	-0.22	-0.36	0.18	**-0.76**	-0.07	0.04	**-0.73**	-0.27	**-0.85**	0.50	0.21	0.00	-0.08	**-0.60**	**-0.74**	0.12	**-0.59**	1.00																							
23	-0.17	-0.28	-0.15	-0.14	0.11	0.05	-0.34	-0.39	-0.39	**-0.66**	**0.56**	**-0.60**	-0.20	-0.46	0.33	**0.69**	**-0.61**	0.21	**-0.61**	0.20	0.51	0.09	-0.45	**-0.67**	-0.31	-0.22	-0.11	0.08	-0.32	0.24	-0.32	-0.16	0.37	-0.24	0.45	**-0.58**	-0.11	-0.26	0.21	**0.75**	**0.82**	-0.14	**0.82**	**-0.53**	1.00																						
24	-0.38	-0.45	**-0.52**	**-0.52**	0.24	-0.39	-0.18	-0.50	-0.32	**-0.53**	**0.53**	**-0.54**	-0.12	-0.45	0.19	0.49	-0.51	0.30	-0.43	0.40	**0.55**	0.39	-0.09	**-0.64**	0.11	0.28	0.07	0.37	-0.17	**0.68**	-0.10	-0.03	**0.65**	0.12	**0.80**	-0.46	-0.32	-0.24	-0.15	**0.79**	**0.93**	0.14	**0.77**	**-0.76**	**0.75**	1.00																					
25	-0.18	-0.29	-0.35	-0.40	0.36	-0.05	-0.38	-0.19	**-0.56**	**-0.61**	0.24	-0.32	-0.27	**-0.66**	-0.02	0.31	-0.32	0.27	-0.36	0.38	0.37	0.19	0.13	**-0.74**	-0.32	-0.34	-0.49	-0.22	-0.38	0.13	0.09	-0.28	-0.05	-0.46	0.42	-0.23	-0.24	-0.10	-0.35	**0.71**	**0.72**	0.09	**0.79**	-0.30	**0.73**	**0.64**	1.00																				
26	-0.02	-0.10	-0.41	-0.46	0.28	-0.34	-0.06	-0.05	-0.07	-0.04	0.07	-0.04	-0.20	-0.31	-0.17	-0.09	0.02	0.09	-0.03	0.18	-0.03	0.26	0.50	-0.14	0.44	**0.54**	0.06	0.30	0.00	**0.64**	0.36	-0.01	0.39	0.37	0.70	-0.08	**-0.52**	0.06	-0.38	0.37	0.47	0.28	0.31	**-0.55**	0.07	**0.59**	0.34	1.00																			
27	-0.40	-0.38	-0.43	**-0.70**	0.17	-0.31	-0.20	-0.33	**-0.69**	**-0.80**	0.44	-0.45	**-0.57**	**-0.60**	0.10	0.47	-0.41	0.38	-0.51	0.18	0.26	0.10	0.14	**-0.59**	-0.05	-0.25	-0.34	-0.19	-0.28	0.21	0.16	-0.15	0.17	-0.41	0.43	-0.38	-0.15	-0.14	-0.21	**0.68**	**0.66**	-0.26	**0.52**	-0.33	0.51	**0.54**	**0.68**	0.35	1.00																		
28	-0.26	-0.23	**-0.53**	**-0.53**	0.34	**-0.56**	0.43	0.13	-0.13	-0.06	-0.14	0.23	-0.11	-0.08	-0.09	-0.35	0.27	0.18	0.42	0.34	-0.10	0.10	0.21	0.01	0.05	-0.13	-0.08	0.01	-0.08	-0.05	**0.53**	0.02	-0.29	-0.03	0.03	0.25	-0.26	0.01	**-0.54**	0.18	0.06	0.37	-0.10	0.23	-0.15	0.06	0.16	0.27	0.29	1.00																	
29	**-0.54**	-0.06	-0.26	**-0.53**	-0.25	-0.50	0.18	-0.20	-0.14	-0.01	0.05	0.00	-0.03	-0.08	-0.22	0.06	0.00	-0.06	-0.06	-0.06	-0.03	-0.17	-0.23	-0.01	0.25	-0.04	0.37	0.47	0.27	0.09	-0.35	0.26	0.32	0.11	-0.08	-0.51	0.01	-0.36	-0.01	0.22	0.24	-0.08	0.15	-0.04	0.08	0.32	-0.04	0.21	0.24	0.44	1.00																
30	0.26	0.22	0.05	-0.03	-0.01	-0.01	0.29	0.41	0.21	0.21	-0.11	0.32	0.05	0.28	0.02	-0.25	0.27	-0.11	0.27	-0.05	-0.25	0.06	0.41	0.42	**0.56**	**0.67**	0.22	0.17	0.21	0.46	0.41	0.23	0.10	**0.60**	0.41	0.35	**-0.61**	0.23	-0.09	-0.27	-0.22	0.22	-0.46	-0.12	-0.40	-0.02	-0.28	0.47	0.00	0.18	-0.17	1.00															
31	-0.40	-0.50	-0.38	-0.31	0.12	-0.33	-0.22	**-0.74**	-0.25	**-0.53**	**0.61**	**-0.73**	-0.15	-0.35	0.36	**0.67**	**-0.65**	0.28	**-0.62**	0.24	**0.61**	0.30	-0.42	**-0.58**	-0.02	0.24	0.16	0.36	-0.19	**0.52**	-0.34	-0.08	**0.81**	0.07	**0.59**	**-0.64**	0.10	-0.41	0.18	**0.72**	**0.85**	-0.14	**0.68**	**-0.77**	**0.74**	**0.84**	0.37	0.27	0.33	-0.26	0.29	-0.30	1.00														
32	-0.42	-0.49	**-0.54**	-0.47	0.17	-0.45	-0.25	**-0.61**	-0.24	-0.37	0.50	**-0.53**	-0.20	-0.45	0.09	0.40	-0.41	0.24	-0.44	0.25	0.41	0.32	-0.07	**-0.54**	0.09	0.34	0.12	0.42	-0.17	**0.61**	-0.13	-0.08	**0.75**	0.17	**0.71**	-0.46	-0.17	-0.15	-0.11	**0.74**	**0.87**	0.13	**0.77**	**-0.76**	**0.61**	**0.91**	0.49	**0.64**	0.44	-0.02	0.38	-0.13	**0.86**	1.00													
33	0.04	**0.53**	0.49	0.17	**-0.62**	0.21	0.22	**0.61**	0.06	0.44	-0.50	**0.75**	0.21	0.44	-0.45	-0.47	**0.71**	**-0.73**	0.35	**-0.52**	-0.45	**-0.66**	-0.11	**0.67**	0.21	0.00	0.36	0.22	**0.60**	-0.24	-0.32	**0.54**	-0.34	0.28	-0.44	0.21	-0.23	0.18	0.27	**-0.66**	**-0.59**	0.14	-0.42	**0.58**	-0.38	-0.39	-0.34	-0.15	-0.16	0.12	0.19	0.37	**-0.52**	-0.43	1.00												
34	0.22	0.31	0.04	0.17	-0.05	-0.11	0.41	**0.56**	**0.56**	**0.71**	-0.48	**0.70**	0.45	**0.55**	-0.31	**-0.72**	**0.69**	-0.39	**0.73**	-0.02	-0.42	-0.02	0.25	**0.68**	0.46	**0.54**	0.47	0.47	0.43	0.22	0.27	0.43	-0.09	**0.75**	0.04	**0.59**	**-0.55**	0.43	-0.22	-0.51	-0.47	**0.69**	-0.46	0.23	**-0.56**	-0.21	-0.43	0.30	-0.43	0.42	0.09	**0.64**	-0.47	-0.19	0.50	1.00											
T	-0.30	-0.50	**-0.65**	-0.33	0.51	**-0.59**	0.36	-0.07	-0.07	0.05	-0.27	0.26	0.32	-0.01	-0.22	**-0.58**	0.39	0.00	**0.57**	**0.61**	0.33	0.45	0.17	-0.14	-0.04	0.08	-0.10	0.14	0.04	0.10	0.41	0.16	-0.21	0.04	0.12	**0.63**	-0.02	0.24	**-0.72**	0.08	0.08	**0.82**	0.11	0.18	-0.10	0.12	0.24	0.18	0.00	**0.55**	0.00	-0.06	-0.08	0.14	-0.04	0.37	1.00										
WCD	-0.26	-0.43	**-0.65**	-0.47	**0.57**	**-0.55**	0.32	0.02	-0.10	0.05	-0.31	0.28	0.25	-0.13	-0.31	**-0.61**	0.36	0.11	**0.55**	**0.64**	0.24	**0.52**	0.51	-0.18	0.09	0.08	-0.26	-0.03	0.05	0.15	**0.56**	0.08	-0.29	-0.05	0.15	**0.66**	-0.07	0.22	**-0.90**	0.05	0.03	**0.71**	0.02	0.20	-0.27	0.07	0.27	0.34	0.12	**0.55**	-0.03	0.09	-0.23	0.08	-0.06	0.35	**0.91**	1.00									
H	0.06	0.23	0.27	0.19	-0.12	0.05	0.16	0.13	0.26	0.18	-0.11	0.10	0.30	0.16	0.03	0.00	0.10	-0.48	-0.04	-0.09	0.16	-0.23	**-0.60**	0.24	0.20	0.08	**0.56**	**0.62**	**0.56**	0.16	**-0.55**	0.49	0.27	0.33	-0.06	-0.29	0.12	-0.39	0.37	-0.13	0.10	0.12	0.17	-0.03	0.25	0.24	-0.02	0.08	-0.25	-0.14	0.35	-0.20	0.36	0.28	0.28	0.10	0.01	-0.19	1.00								
TP	-0.17	-0.41	**-0.62**	-0.34	**0.62**	-0.49	0.39	0.08	0.04	0.11	-0.29	0.29	0.40	-0.04	-0.22	**-0.61**	0.34	0.10	**0.62**	**0.71**	0.30	**0.59**	0.43	-0.15	0.09	0.17	-0.19	0.05	0.06	0.22	**0.54**	0.12	-0.24	0.07	0.21	**0.73**	-0.15	0.25	**-0.87**	0.02	0.02	**0.82**	0.00	0.16	-0.22	0.09	0.23	0.29	-0.01	**0.52**	-0.12	0.14	-0.20	0.06	-0.09	0.44	**0.94**	**0.97**	-0.14	1.00							
A	-0.14	-0.41	**-0.64**	-0.40	**0.65**	-0.51	0.44	0.08	0.11	0.11	-0.18	0.19	0.37	-0.03	-0.13	**-0.55**	0.21	0.28	**0.62**	**0.74**	0.28	**0.71**	**0.58**	-0.16	0.22	0.19	-0.21	-0.02	0.04	0.28	**0.66**	0.08	-0.21	0.04	0.23	**0.73**	-0.11	0.22	**-0.93**	-0.01	-0.03	**0.67**	-0.12	0.12	-0.36	0.02	0.10	0.29	-0.01	0.51	-0.10	0.23	-0.26	-0.01	-0.17	0.42	**0.81**	**0.92**	-0.32	**0.94**	1.00						
PP	-0.18	-0.25	-0.48	-0.29	0.40	-0.47	0.37	0.16	-0.04	0.27	**-0.54**	**0.54**	0.31	0.04	-0.44	**-0.80**	**0.62**	-0.16	**0.70**	0.43	0.05	0.28	0.40	0.08	0.04	0.09	-0.16	0.02	0.14	-0.04	0.47	0.15	-0.42	0.05	-0.06	**0.73**	-0.07	0.27	**-0.75**	-0.17	-0.20	**0.78**	-0.15	0.39	-0.41	-0.13	0.10	0.24	-0.09	**0.56**	-0.03	0.12	-0.39	-0.09	0.16	0.51	**0.91**	**0.94**	-0.07	**0.92**	**0.83**	1.00					
S	0.01	0.27	0.07	0.06	-0.27	-0.09	0.22	0.43	-0.03	**0.56**	**-0.80**	**0.92**	0.24	0.32	**-0.74**	**-0.89**	**0.97**	**-0.69**	**0.69**	-0.26	-0.47	-0.41	0.18	**0.55**	0.03	0.05	0.08	0.10	0.32	-0.35	0.08	0.29	**-0.54**	0.22	**-0.43**	**0.53**	-0.21	0.45	-0.20	**-0.53**	**-0.56**	**0.56**	-0.36	**0.60**	**-0.53**	-0.43	-0.19	0.04	-0.26	0.40	0.08	0.21	**-0.63**	-0.36	**0.67**	**0.64**	0.49	0.47	0.04	0.43	0.29	**0.71**	1.00				
E	-0.16	-0.48	**-0.69**	-0.38	**0.64**	**-0.59**	0.37	-0.06	0.20	0.09	-0.07	0.06	0.38	0.04	-0.06	-0.46	0.13	0.31	**0.57**	**0.76**	0.38	**0.76**	0.45	-0.18	0.26	0.28	-0.01	0.19	0.06	0.43	**0.59**	0.15	-0.02	0.18	0.38	**0.62**	-0.12	0.23	**-0.84**	0.09	0.12	**0.75**	0.07	-0.08	-0.21	0.21	0.12	0.36	-0.05	0.47	-0.03	0.15	-0.03	0.21	-0.26	0.43	**0.84**	**0.87**	-0.13	**0.92**	**0.94**	**0.76**	0.18	1.00			
G	-0.09	**-0.56**	**-0.73**	**-0.52**	**0.82**	**-0.52**	0.24	-0.22	-0.04	-0.27	0.18	-0.29	0.09	-0.32	0.12	-0.13	-0.25	**0.58**	0.25	**0.83**	**0.54**	**0.87**	**0.52**	**-0.53**	0.18	0.04	-0.32	-0.10	-0.15	0.41	**0.65**	-0.11	0.00	-0.20	0.45	0.32	0.09	0.01	**-0.85**	0.36	0.38	0.34	0.27	-0.28	0.01	0.32	0.38	0.41	0.21	0.34	-0.10	-0.04	0.13	0.30	**-0.61**	-0.03	**0.61**	**0.74**	-0.25	**0.73**	**0.81**	**0.52**	-0.17	**0.83**	1.00		
AA	0.11	-0.16	-0.30	-0.27	0.37	-0.20	0.03	-0.13	-0.02	-0.24	0.09	-0.24	0.10	-0.08	-0.03	-0.01	-0.31	0.45	0.25	0.48	0.33	0.49	0.27	-0.41	0.07	-0.48	-0.19	-0.14	-0.09	0.02	0.31	-0.04	-0.26	-0.45	-0.01	-0.03	0.24	0.09	-0.47	0.21	0.16	-0.06	0.31	-0.12	0.10	0.04	0.26	-0.10	0.09	0.23	0.07	-0.46	-0.01	-0.01	**-0.54**	-0.26	0.21	0.28	-0.29	0.27	0.39	0.12	-0.23	0.39	**0.63**	1.00	
MA	-0.13	-0.25	-0.16	-0.02	0.24	-0.17	0.37	0.14	0.06	-0.14	0.18	-0.02	-0.06	0.13	0.42	-0.01	0.05	0.23	0.21	0.24	0.14	0.00	-0.26	0.06	-0.27	-0.28	0.00	-0.11	-0.15	-0.21	0.28	0.00	-0.30	-0.13	-0.08	0.30	0.00	-0.08	-0.06	0.01	-0.05	0.23	-0.13	0.32	0.08	-0.06	0.03	-0.31	-0.02	**0.53**	-0.01	0.01	-0.16	-0.23	0.17	0.18	0.33	0.14	0.06	0.22	0.17	0.19	0.06	0.22	0.04	0.01	1.00
pH	-0.29	-0.42	**-0.62**	-0.38	0.44	**-0.62**	0.42	-0.03	0.18	0.12	-0.02	0.13	0.39	0.09	-0.06	-0.40	0.22	0.07	0.48	**0.61**	0.37	**0.53**	0.17	-0.06	0.32	0.42	0.26	0.49	0.23	**0.53**	0.32	0.33	0.19	0.41	0.45	0.45	-0.26	0.12	**-0.61**	0.11	0.23	**0.82**	0.15	-0.13	-0.04	0.42	0.13	0.46	-0.03	0.49	0.21	0.24	0.16	0.41	0.01	**0.56**	**0.80**	**0.72**	0.24	**0.79**	**0.72**	**0.68**	0.26	**0.86**	**0.57**	0.06	0.34	1.00

Measurements are coded as follows: AI, aroma intensity; RF, red fruit; CON, confectionery; SP, spice; EA, earthy; FL, floral; PE, pepper; FI, flavour intensity; F_DF, dark fruit flavour; F_JA, jammy flavour; F_CON, confectionery flavour; F_SP, spice flavour; F_LI, licorice flavour; SA, savoury; AC, acidity; SW, sweetness; BI, bitterness; PF, palate fullness; AS, astringency; SC, surface coarseness; HO, hotness; 1, ethyl acetate; 2, ethyl isobutyrate; 3,isobutyl acetate; 4, ethyl butyrate; 5, ethyl 2-methyl butanoate; 6, ethyl isovalerate;7, 2-methyl-1-propanol; 8, isoamyl acetate; 9, 1-butanol; 10, 3-methyl-1-butanol; 11, ethyl hexanoate; 12, isoamyl butanoate; 13, hexyl acetate; 14, 2-heptanol; 15, ethyl heptanoate; 16, 1-hexanol; 17, Z-3-hexen-1-ol; 18, octanoic acid methyl ester; 19, ethyl octanoate; 20, 1-octen-3-ol; 21, 1-heptanol; 22, linalool; 23, 1-octanol; 24, ethyl decanoate; 25, 1-nonanol; 26, diethyl succinate; 27, 3-methylthio-1-propanol; 28, beta-citronellol; 29, ethyl phenyl acetate; 30, 2-phenylethyl acetate; 31, hexanoic acid; 32, ethyl laurate; 33, phenylethyl alcohol; 34, nerolidol; T, tannins; WCD, wine colour density; H, hue; TP, phenolic substances; A, anthocyanins; PP, non-bleachable pigment; S, residual sugar; E, ethanol; G, glycerol; AA, acetic acid; MA, malic acid.

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CHAPTER 3

Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing

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Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 20/2/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Joanna Gambetta
Contribution to the Paper	Analysis of the volatile compounds Manuscript preparation and editing
Signature	Date 20/02/2018

Name of Co-Author	David Jeffery
Contribution to the Paper	HPLC-MS analysis Manuscript preparation and editing
Signature	Date 20/2/2018

Name of Co-Author	Paul Grbin		
Contribution to the Paper	Conceptualisation of the work/experimental design Manuscript preparation and editing		
Signature		Date	22/01/2018

Name of Co-Author	Vladimir Jiranek		
Contribution to the Paper	Conceptualisation of the work/experimental design Manuscript preparation and editing		
Signature		Date	20.1.18

Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: the effect of sequential inoculation timing

Ana Hranilovic[a,b], Joanna M. Gambetta[a,1], David W. Jeffery[a,b], Paul R. Grbin[b], Vladimir Jiranek[a,b*]

[a] The Australian Research Council Training Centre for Innovative Wine Production, 5064 Urbrrae, South Australia, Australia

[b] Department of Wine and Food Science, The University of Adelaide, 5064 Urbrrae, South Australia, Australia

[*] Corresponding author: vladimir.jiranek@adelaide.edu.au

[1] Present address: National Wine and Grape Industry Centre, School of Agricultural and Wine Science, Charles Sturt University, Wagga Wagga, New South Wales, 2678, Australia

Abstract

In Latin, *'pulcherrima'* is a superlative form of an adjective that translates as beautiful. Apart from being 'the most beautiful' yeast, *Metshnikowia pulcherrima* has a great potential in production of lower-alcohol wines. In this work, we evaluated the oenological performance of six *M. pulcherrima* strains used in sequential cultures with *Saccharomyces cerevisiae.* The *M. pulcherrima* MP2 strain was further characterised in six sequential fermentations with different *S. cerevisiae* inoculation delays. The analysis of main metabolites, undertaken prior to sequential inoculations and upon fermentation completion in both Chemically Defined Grape Juice Medium (CDGJM) and white grape juice, highlighted metabolic interactions and carbon sinks other than ethanol in mixed culture fermentations. Depending on the inoculation delay, MP2 white wines contained between 0.6% and 1.2% (v/v) less ethanol than the *S. cerevisiae* control, with even larger decreases achieved in the CDGJM. The lower-alcohol wines also had higher concentrations of glycerol and the TCA cycle by-products (i.e. succinate and fumarate), and lower concentrations of acetic acid. The analysis of volatile compounds revealed compositional alterations arising from the *S. cerevisiae* inoculation delay, with increased acetate esters and higher alcohols detected in all analysed MP2 treatments.

Highlights

- *Metschnikowia pulcherrima* MP2 was selected for production of lower-alcohol wines
- Alcohol decrease in white wines ranged between 0.6 and 1.2% (v/v)
- Volatile profiles of lower-alcohol wines depended on sequential inoculation timing

Keywords

Metschnikowia pulcherrima, non-*Saccharomyces* yeasts, mixed culture fermentations, lower-alcohol wines, volatile compounds

1. Introduction

Recent trends in seeking riper fruit (Godden et al., 2015), exacerbated by climate warming (Schultz and Jones, 2010) and the phenomenon of compressed vintages (Schelezki et al., 2018), drive excessive sugar accumulation in grapes translating to undesirably high ethanol levels in wines. The implied oenological repercussions are severe; high sugars/alcohol exert difficulties on each of the alcoholic and malolactic fermentations, jeopardising their timely completion and likelihood of success (Bisson, 1999; Malacrino et al., 2005; Sumby et al., 2014). Chemical and sensory profiles of resultant wines also are compromised through increased perceptions of 'hotness' and decreased aroma 'complexity' (Heymann et al., 2013; King et al., 2013), and, given the rising demand for wines with moderate alcohol levels and the presence of higher taxation rates above certain alcohol % (v/v), so is their consumer acceptance and marketability (Saliba et al., 2013).

Winemakers seek to mitigate the adverse concentrations of ethanol via external inputs and/or interventions, implemented across the whole grape and wine production chain (Longo et al., 2017; Varela et al., 2015). Among these, microbiological methods are of great interest, as the use of a yeast that yields less moles of ethanol per mole of fermented sugar, i.e., one that partially diverts carbon to other end-products, represents an inexpensive and readily-implementable strategy. However, ethanol yield is a rather invariant trait in wine strains of *Saccharomyces cerevisiae*, the main fermentative species used in wineries (Palacios et al., 2007). Novel *S. cerevisiae* strains have therefore been generated to produce less ethanol using recombinant DNA techniques (Kutyna et al., 2010; Varela et al., 2012) and more recently, evolutionary engineering (Tilloy et al., 2014; 2015). Moreover, the selection has expanded to species other than *S. cerevisiae*, as these so-called non-*Saccharomyces* yeasts offer largely untapped diversity for oenologically-relevant traits, ethanol yield included (Jolly et al. 2014). Indeed, even though metabolic pathways involved in central carbon metabolism are largely

conserved among different species (Fig. 1), their regulation, and thus ethanol production efficiency, vary greatly. A number of non-*Saccharomyces* strains have been selected based on their ability to divert carbon away from ethanol production and trialled in co-culture with *S. cerevisiae*, which is required to complete the fermentation (Ciani et al., 2016; Contreras et al., 2014; Jolly et al., 2014; Rossouw and Bauer, 2016). Among these, strains of *Metschnikowia pulcherrima* were the best performers in several studies.

One such study focused on selection of non-*Saccharomyces* yeasts capable of lowering ethanol levels via respiration in aerated conditions (Quiros et al., 2014). The selected *M. pulcherrima* CECT12841 was subsequently tested in co-culture with *S. cerevisiae* with controlled oxygenation at fermentation onset, conferring an ethanol decrease of 2.2% (v/v) with acceptable acetic acid content (Morales et al., 2015). Significant ethanol decrease was also achieved in aerated *M. pulcherrima* co-cultures by Tronchoni et al. (2018), however, these were linked to inferior wine quality (oxidised/solventy character, lacking in fruitiness) compared to anaerobically fermented *S. cerevisiae.* To avoid quality loss and comply with standard reductive winemaking practices, the screening for lower-alcohol non-*Saccahromyces* has also been conducted under anaerobic conditions. Under such conditions, Contreras et al. (2014) have selected another *M. pulcherrima* strain, AWRI1149. When sequentially inoculated with *S. cerevisiae* upon 50% sugar depletion, strain AWRI1149 resulted in an ethanol decrease of 0.9 and 1.6% (v/v) in Chardonnay and Shiraz, respectively (Contreras et al., 2014), with a further decrease achieved in *S. uvarum* co-culture (Contreras et al., 2015). However, several *M. pulcherrima* fermentations were relatively lengthy and/or resulted in concentrations of ethyl acetate that are potentially detrimental for wine quality (Contreras et al. 2014, Varela et al. 2016). Cell immobilisation was used in another study to increase non-*Saccharomyces* inoculation rates and accelerate sugar consumption prior to *S. cerevisiae* inoculation (Canonico et al., 2016). The *M. pulcherrima* strain DiSVA269, tested alongside three other non-

Saccharomyces species, resulted in up to 1.4% (v/v) less ethanol production than the *S. cerevisiae* monoculture. Nonetheless, yeast immobilisation is still an experimental practice in winemaking rather than an industrial one (Genisheva et al., 2014).

Despite their potential, fit-for-purpose *M. pulcherrima* strains are still lacking and their use in the wine industry requires further guidance. This study addressed the selection and characterisation of a lower-ethanol producing *M. pulcherrima* strain for use in sequential culture with *S. cerevisiae* in a defined medium and grape juice, focusing not only on the production of ethanol but also assessing compositional alterations, i.e., main metabolites and volatile compounds, arising from the delays in sequential inoculation.

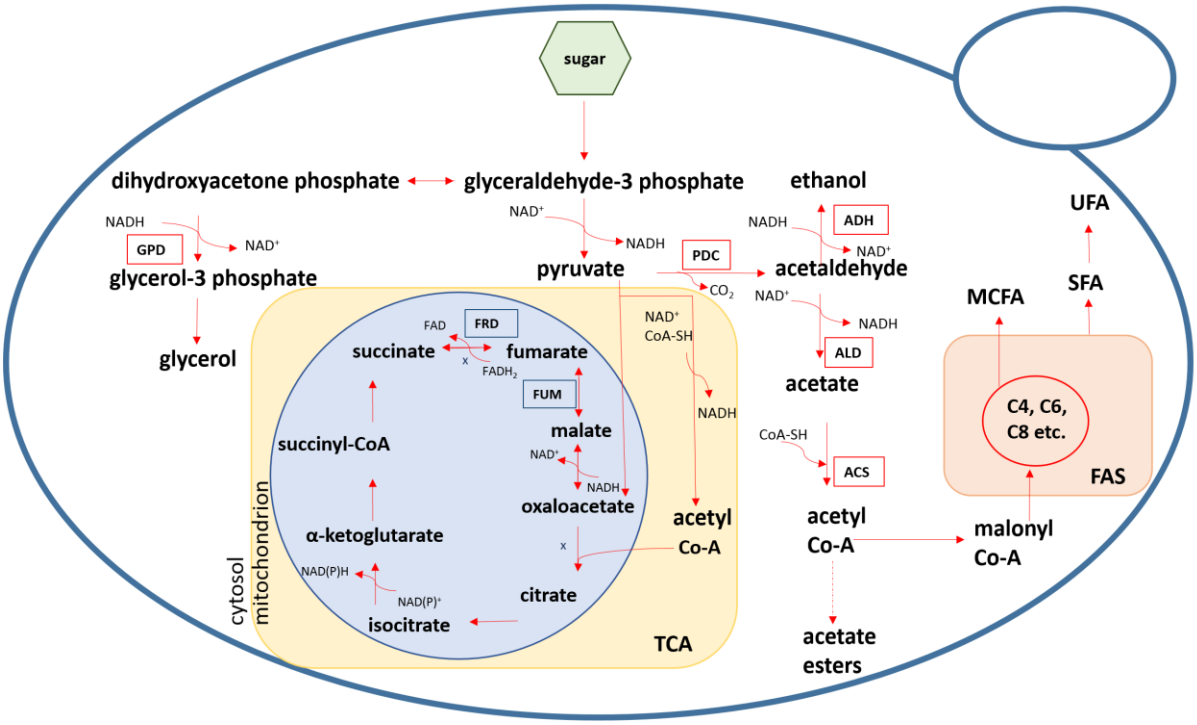

Fig. 1. Overview of central carbon metabolism in yeasts. Grape hexoses (glucose and fructose) are metabolised to pyruvate during glycolysis via multiple enzymatic steps. In alcoholic fermentation, via acetaldehyde (pyruvate decarboxylase, PDC) pyruvate is converted to ethanol (alcohol dehydrogenase, ADH). Glycerol-phosphatase-decarboxylase (GPD) and

aldehyde dehydrogenase (ALD) are involved in the formation of glycerol and acetate, respectively. Acetate can be converted to acetyl-CoA (acetyl-CoA synthase, ACS), contributing to the acetate esters and fatty acids synthesis. In the FAS (fatty acids synthase) complex, malonyl-CoA undergoes sequential 2-carbon elongation to palmitate (C16:0), that can be further elongated to saturated fatty acids (SFA) and desaturated to unsaturated fatty acids (UFA). Medium chain fatty acids (MCFA) can be released from the FAS complex and partake in ethyl ester formation. Pyruvate originating from glycolysis is partially shuttled to the mitochondrion and incorporated into the tricarboxylic acid (TCA) cycle. The normal TCA cycle is indicated with arrows in a clockwise direction. Under anaerobic conditions, some TCA reactions are inhibited (indicated with a symbol 'x'), resulting in branching of the cycle. Fumarase (FUM) catalyses interconversion of malate and fumarate, and fumarate reductase (FRD) causes reduction of fumarate to succinate.

2. Materials and methods

2.1. Strains and culture conditions. *S. cerevisiae* and *M. pulcherrima* strains were isolated from a commercial starter preparation (EC-1118, Lalvin, Montreal, Canada) and an un-inoculated Shiraz fermentation (Yalumba, Angaston, South Australia), respectively. The latter was previously identified via sequencing and blasting of ITS1/ITS4 PCR products, as described elsewhere (Contreras et al., 2015). Cryogenically stored cultures (−80 °C in 25% glycerol) were grown for 2 days at 28 °C on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Yeast starter cultures were established by inoculating approximately 10^7 cells into 100 mL of a 1:1 mixture of YPD broth and the medium to be used for fermentations in 250 mL Erlenmeyer flasks. After 24 h incubation at 28 °C with agitation (120 rpm), cell density and viability were determined by flow cytometry coupled with propidium iodide (PI) DNA staining

(Accuri C6, BD, New Jersey, USA). Cultures (45 mL) were transferred into 50 mL tubes, centrifuged (10 minutes; 3214 rcf) and re-suspended in buffered phosphate saline to 10^9 viable cells/mL. Standardised volumes (0.5 mL) of the obtained cultures were then inoculated into 100 mL fermentations at a rate of 5×10^6 viable cells/mL.

2.2. Fermentation media. Three filter-sterilised (0.2 µm) media were used for fermentation trials: (1) Chemically Defined Grape Juice Medium (CDGJM; McBryde et al., 2006) with 230 g/L sugar (equimolar glucose and fructose) and 350 mg/L yeast assimilable nitrogen (YAN) as a mixture of amino acids and ammonium chloride (McBryde et al., 2006); (2) CDGJM with 257 g/L sugar and 350 mg/L YAN, and (3) grape juice (GJ; a Chardonnay and Semillon blend sourced from Treasury Wine Estates, South Australia) with 253 g/L sugar and 350 mg/L YAN. The initial GJ sugar (190 g/L) and YAN (160 mg/L) concentrations were adjusted by addition of glucose and fructose, and an amino acid and ammonium chloride mixture (McBryde et al., 2006), respectively.

2.3. Fermentation set up. All fermentations were conducted in a custom-made 'Tee-bot' fermentation platform. Built on an EVO Freedom workdeck (Tecan, Männedorf, Switzerland), 'Tee-bot' allowed for the automatic sampling of up to 96 simultaneously conducted fermentations in 150 mL fermentation vessels. The fermentations were mixed with a magnetic stirrer and sealed with an airlock. Upon inoculation, the fermenters were incubated with agitation (300 rpm) at controlled temperature under self-induced anaerobic conditions.

2.4. Fermentation modalities and monitoring. Six pre-selected *M. pulcherrima* (MP1-MP6) strains were tested in CDGJM containing 230 g/L sugar with *S. cerevisiae* inoculated after 7

days (Table 1) at 24 °C. The MP2 strain was subsequently trialled in higher sugar CDGJM and GJ with consecutive sequential inoculations with *S. cerevisiae* (Table 1) at 22.5 °C. All treatments consisted of 100 mL triplicates with *S. cerevisiae* (SC) EC-1118 as a control. During fermentation, aliquots (200 µL) were automatically sampled into 96-well plates and used to spectrophotometrically monitor (Infinite 200 PRO, Tecan, Männedorf, Switzerland) fermentation progress via total sugar consumption (K-FRUGL enzymatic kit, Megazyme, Ireland) and microbial growth. In fermentations with multiple MP strains, growth was measured by absorbance at 600 nm (OD_{600}). In the follow-up fermentations, growth of the SC monoculture and MP2 treatments prior to the secondary inoculation was monitored via PI-flow cytometry. After *S. cerevisiae* inoculations, the two yeasts were quantified at four time-points (24 h, 48 h and 72 h after secondary inoculation, and at fermentation completion) via YPD and lysine medium (CM0191, Thermo Fisher Scientific, Waltham, MA, USA) plate counts after a 2-day incubation at 28 °C. Being selective against SC growth, lysine medium allowed for MP quantification. Prior to *S. cerevisiae* inoculation, 1 mL samples were collected for high performance liquid chromatography (HPLC) analysis. The sequential inoculation and sampling were both carried out via a septum-sealed port thereby maintaining anaerobiosis. The final sample was centrifuged (10 min, 3214 rcf) in 50 mL tubes to separate the yeast from the supernatant used for downstream analysis.

Table 1. Yeast inoculation treatments involving six *M. pulcherrima* strains (MP1–MP6) tested in sequentially inoculated fermentations with *S. cerevisiae*, and an *S. cerevisiae* (SC) control. The inoculation timing (with 5×10^6 viable cells/mL) is indicated with an 'x'.

Yeast treatment	**Day of *M. pulcherrima* inoculation**	**Day of *S. cerevisiae* inoculation**						
	0	0	3	4	5	6	7	~50% sugar
MP1	x						x	
MP2	x						x	
MP3	x						x	
MP4	x						x	
MP5	x						x	
MP6	x						x	
MP2 + SC3	x		x					
MP2 + SC4	x			x				
MP2 + SC5	x				x			
MP2 + SC6	x					x		
MP2 + SC7	x						x	
MP2 + SC50%	x							x
SC		x						

2.5. Chemical analyses. HPLC was used to quantify glucose, fructose, ethanol, glycerol and organic acids (acetic, malic and succinic) as described by Li et al. (2017). Briefly, upon centrifugation and filtration (0.45 µm), samples were transferred into crimp-cap vials containing 300 µL inserts and capped. An Agilent 1100 (Santa Clara, CA, USA) instrument was fitted with an HPX-87H column (300 mm × 7.8 mm, BioRad, Hercules, CA, USA). The eluent was 2.5 mM H_2SO_4 with a flow rate of 0.5 mL/min and the column was maintained at 60 °C for a 35-minute run time. The injection volume was 20 µL and signals were detected using an Agilent G1315B diode array detector (DAD, organic acids; 210 nm) and G1362A refractive index detector (RID, hexoses and alcohols) from injections. ChemStation software (version B.01.03) was used for instrument control and data analysis, and analytes were

quantified using external calibration curves ($R^2 > 0.99$). The final ethanol concentrations in MP2 follow-up fermentations were determined with an alcolyser (Anton Paar, Graz, Austria).

Fumarate identification was undertaken with a ThermoFinnigan Surveyor HPLC connected to a ThermoFinnigan LCQ Deca XP Plus mass spectrometer using electrospray ionisation in negative ion mode. Separation was performed with a 250 × 2.1 mm i.d., 5 µm, 100 Å Alltima C18 column operated at 25 ºC and protected by a 7.5 × 2.1 mm i.d. guard cartridge of the same material. The solvents were 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in acetonitrile (solvent B), with a flow rate of 0.200 mL/min. The linear gradient for solvent B was: 0 min, 1%; 10 min, 10%; 20 min, 35%; 30min, 90%; 35 min, 90%; 36 min, 1%; 46 min, 1%. An injection volume of 10 µL was used. The mass spectrometer had the following conditions: nitrogen was used for sheath gas, 30 arbitrary units and auxiliary gas, 20 arbitrary units; the ion spray voltage, capillary voltage, tube lens offset voltage and capillary temperature were set at -3500 V, 20 V, 35 V and 250 ºC, respectively; helium was used as the collision gas, and normalised collision energy, activation Q, activation time and isolation width were 35%, 0.250, 30 ms and *m*/*z* 2, respectively. Data acquisition and processing were performed using Xcalibur software (version 1.3). The identification of fumarate by mass spectrometry was verified via the HPLC method described above (which was also used to collect the fraction of unknown metabolite), via matching retention times with the pure compound, spiking of fumarate into samples to confirm its increase in peak height and area (i.e., concentration) and separation of fumaric acid from its isomer maleic acid.

Headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS) was carried out in duplicate to determine the volatile composition of three mixed culture wines and the SC control. The samples (0.5 mL) were diluted with deionised water (4.5 mL) in 20 mL vials supplemented with 2 g NaCl and 10 µL of ethanolic internal standard mixture (Gambetta et al., 2016; d_4-3-methyl-1-butanol, 2.4 µg/L; d_3-hexyl

acetate,0.025 µg/L; d_{13}-1-hexanol, 0.05 µg/L; d_5-2-phenylethanol, 0.5 µg/L; d_{19}-ecanoic acid, 0.05 µg/L and d_5-ethyl dodecanoate, 0.001 µg/L). The instrument settings and extraction conditions were identical to those reported in Gambetta et al. (2016).

2.6. Data analysis. Basic data processing was undertaken with EXCEL 2010 (Microsoft, Richmond, WA, USA). Data are presented as mean values with standard deviation (SD) from replicate fermentations. Sugar consumption and yeast population dynamics were plotted in GraphPad Prism (v6.03v, GraphPad Software). Metabolite yields were derived from the HPLC data for two fermentation stages. Partial fermentation (PF) yields were calculated as a ratio of measured metabolite and sugar (glucose + fructose) consumed prior to the *S. cerevisiae* inoculation, while final fermentation (FF) yields represent a ratio of final metabolites and consumed sugar. Measured and derived parameters were submitted to one-way analysis of variance (ANOVA) and Tukey's Honestly Significantly Different (HSD) post-hoc test in XLSTAT (version 2015.4.1, Addinsoft, Paris, France) with significance threshold set at 0.05. Significantly different volatile compounds were normalised and subjected to Principal Component Analysis (PCA) analysis in XLSTAT.

3. Results

3.1. *Metschnikowia pulcherrima* candidate strain selection. Six MP strains were pre-selected from an in-house collection of non-*Saccharomyces* isolates based on their lower ethanol yield per consumed sugar (data not shown) and trialled in sequential inoculations with *S. cerevisiae* added after 7 days. The SC control displayed the highest rate and extent of growth and sugar consumption (Fig. A.1). An increase in OD_{600} and sugar consumption rate upon *S. cerevisiae* inoculation was apparent in all MP treatments, and suggestive of successful *S. cerevisiae*

implementation (Fig. A.1). Some variation was observed in MP1-MP5 fermentation duration but each went to completion, whereas MP6 still contained 12 g/L of residual sugar after 15 days of fermentation (Fig. A.1; Table 2). Prior to *S. cerevisiae* inoculation, MP1 and MP2 consumed more sugar than other MP treatments (Table A.1). The SC control achieved the highest ethanol concentration (12.8%; Table 2), whereas the final sequential fermentations ranged between 10.3% and 11.5% ethanol (Table 2). Notably, MP2 yielded the largest ethanol decrease, in the order of 20% (or 2.5% v/v), compared to the SC control (Table 2). All MP fermentations had significantly higher ($P < 0.0001$) glycerol and succinic acid concentrations than the SC control but were up to 80% lower in acetic acid (Table 2).

Table 2. Analytical profiles of final wines produced using six *M. pulcherrima* strains that were sequentially inoculated with *S. cerevisiae* strain on day 7 (MP1-6), and an *S. cerevisiae* control (SC).

Parameter	MP1	MP2	MP3	MP4	MP5	MP6	SC	P values
Residual sugar[1] (g/L)	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	12 ± 0^{A}	0 ± 0^{B}	< 0.0001
Ethanol (% v/v)	11.5 ± 0.1^{AB}	10.3 ± 0.6^{B}	11.5 ± 1.1^{AB}	11.1 ± 0.5^{B}	11.5 ± 0.6^{AB}	11.1 ± 0.2^{B}	12.8 ± 0.1^{A}	0.0054
Ethanol yield (g/g)	0.39 ± 0^{AB}	0.35 ± 0.02^{B}	0.40 ± 0.04^{AB}	0.38 ± 0.02^{B}	0.39 ± 0.02^{AB}	0.40 ± 0.01^{AB}	0.44 ± 0^{A}	0.0050
Glycerol (g/L)	8.9 ± 0.2^{AB}	9.7 ± 0.4^{A}	8.9 ± 0.4^{AB}	8.8 ± 0.5^{AB}	8.4 ± 0.4^{B}	9.0 ± 0^{AB}	5.5 ± 0^{C}	< 0.0001
Acetic acid (g/L)	0.20 ± 0.02^{B}	0.16 ± 0.03^{B}	0.18 ± 0.02^{B}	0.17 ± 0.02^{B}	0.21 ± 0.02^{B}	0.13 ± 0.02^{B}	0.66 ± 0.08^{A}	< 0.0001
Succinic acid (g/L)	1.7 ± 0.1^{BC}	1.9 ± 0.2^{B}	1.6 ± 0.1^{BC}	1.5 ± 0.2^{C}	1.6 ± 0^{BC}	2.9 ± 0.1^{A}	0.9 ± 0.1^{D}	< 0.0001

[1] Glucose + fructose; letters denote significant differences within a row (ANOVA; $P < 0.05$).

3.2. MP2 consecutive sequential inoculations in a higher sugar environment

3.2.1. Fermentation kinetics. The MP2 strain led to the lowest ethanol concentration/yield and was further tested in a suite of sequential fermentations with different *S. cerevisiae*

inoculation timings. The tested strains were able to grow (Fig. A.2) and catabolise sugars (Fig. 2a and b) despite the higher initial sugar content (~255 g/L) in the CDGJM and GJ. Irrespective of the sequential inoculation timing, the presence of MP2 at fermentation onset did not inhibit the growth of sequentially added *S. cerevisiae*. Conversely, rapid MP2 population decline upon *S. cerevisiae* inoculation was seen in all fermentation modalities, as indicated by the absence of growth on all lysine plates from three days after secondary inoculation (Fig. A.2.). Despite comparable initial sugar levels and incubation conditions, all treatments completed fermentation on average three days faster in GJ than in CDGJM. The sugar consumption kinetics were faster in the SC controls than in the co-cultures. In the latter, the delay of *S. cerevisiae* inoculation prolonged the fermentation accordingly. The MP2 treatment sequentially inoculated after consuming approximately 50% sugar was the slowest, reaching completion after 15 and 18 days in GJ and CDGJM, respectively.

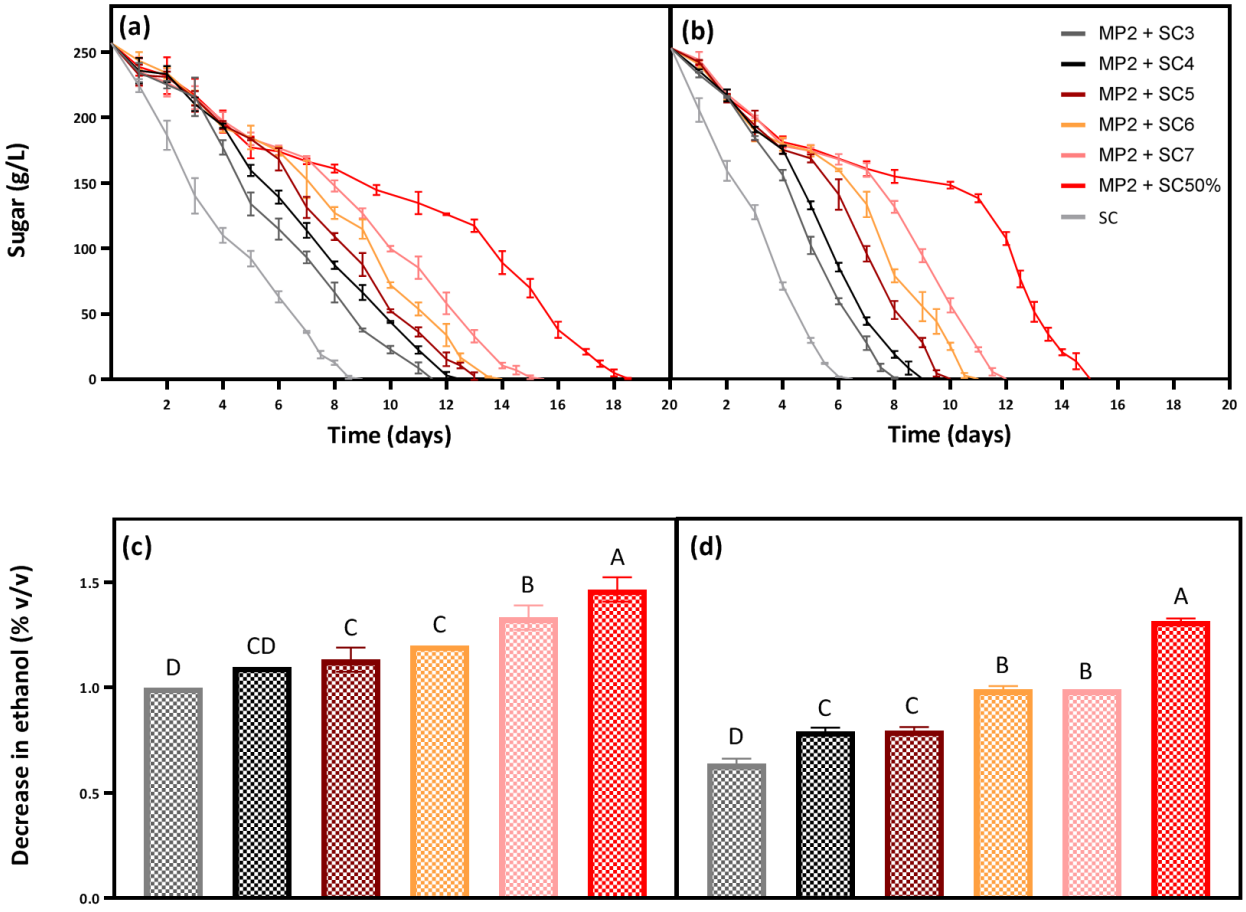

Fig. 2. Sugar (glucose + fructose) consumption kinetics in (a) CDGJM and (b) GJ for six MP2 sequential inoculation treatments and *S. cerevisiae* (SC) control, and the final ethanol concentration decrease in (c) CDGJM and (d) GJ. Error bars represent standard deviations (SD) of analysed triplicates. Different letters denote significant differences among treatments (ANOVA; $P < 0.05$).

3.2.2. Concentrations and yields of produced metabolites. Even though a matrix-derived effect on metabolite production was seen between CDGJM (Table 3) and GJ (Table 4), some trends were consistent. MP2 showed glucophilic character, with residual glucose/fructose ratios decreasing from day three onwards. Final ethanol concentrations in SC controls were higher than in any of the final MP2 wines (Fig. 2c and d). In fact, the extent of sugar consumed by MP2 alone prior to co-inoculation showed a significant ($P < 0.0001$) negative correlation with final ethanol content ($r = -0.94$ and -0.98 in CDGJM and GJ, respectively; Table A.3). In CDGJM, SC reached 15.5% ethanol, whereas MP2 final wines contained from 1% (v/v) (MP2 + SC3) to 1.5% (v/v) (MP2 + SC50%) less ethanol ($P < 0.0001$; Fig. 2c). Similarly in GJ, MP2 + SC3 treatment had 0.6% (v/v) less ethanol and MP2 + SC50% was 1.2% (v/v) less than SC ($P < 0.0001$; Fig. 2d). In sequential fermentations, final glycerol concentrations were up to 45% and 32% higher in CDGJM (MP2 + SC3) and GJ (MP2 + SC6), respectively, than in SC, with the MP2 strain alone contributing up to ~50% of final glycerol concentrations (Tables 3 and 4). Irrespective of the media composition, SC had higher acetic acid concentrations than all sequential fermentations except MP2 + SC3 in GJ (Tables 3 and 4). As seen in the screening fermentations with six MP strains (Table A.1), acetic acid concentrations in CDGJM were higher in PFs than in FFs (Table 3), whereas the opposite was true for GJ (Table 4). Succinic and malic acid concentrations were seemingly more dependent on the medium; SC values were the lowest in CDGJM (Table 3), and intermediary in GJ (Table 4). In sequential treatments in

both media, further progression of fermentation with MP2 alone resulted in accumulation of succinic acid (Tables 3 and 4). Malic acid concentrations in all MP2 partial fermentations (PF) were higher than those of SC wines (Tables 3 and 4) and decreased from PF to FF.

Similarly to concentrations, yields of metabolites were affected to a degree by the fermentation matrix (Tables 3 and 4). The SC ethanol yields (0.48 g in CDGJM, 0.47 g in GJ) were significantly higher than any MP2 ethanol yields ($P < 0.0001$). In CDGJM, MP2 PF ethanol yields peaked on day 3, and remained either stable or dropped thereafter; in GJ, they increased with fermentation progression. Irrespective of the medium, ethanol yields increased after *S. cerevisiae* inoculations. Glycerol yields for the SC control were lower than for any MP2 treatment ($P < 0.0001$), regardless of the fermentation stage and the medium (Tables 3 and 4). In MP2 alone, glycerol yields were halved between day 3 (83 mg/g sugar) and day 13 (42 mg/g sugar) in CDGJM. Even though this drop was less apparent in GJ, glycerol yield of MP2 + SC50% PF was lower than the remaining ones. The timing of *S. cerevisiae* inoculation did not appear to greatly affect glycerol production in mixed cultures. In CDGJM, the steady drop in MP2 PF acetic acid yields with fermentation progression followed the trend in glycerol production (Table 3). A decrease in acetic acid yields from PFs to FFs corresponded to the decline in concentration of this metabolite upon *S. cerevisiae* inoculation (Table 3), although such a trend was not seen in GJ (Table 4). Nonetheless, the decline in acetic acid yield with postponed *S. cerevisiae* inoculation in FFs, and overall higher acetic acid yield in SC, were consistent between the two media. MP2 alone produced more succinic acid per gram of sugar early on during the fermentation in either medium, with higher yields in GJ than in CDGJM. In final MP2 fermentations, succinic acid yields increased with the *S. cerevisiae* delay, reaching higher values than SC in CDGJM (Table 3), and intermediary in GJ (Table 4).

3.2.3. Fumarate accumulation. HPLC analysis revealed that sugar consumption in MP2 was related to an increase of an unknown UV-detectable metabolite that was fractionally collected from the instrument and subjected to HPLC-MS analysis. The unknown analyte was identified as fumaric acid based on the matching mass spectra and retention times when compared to the pure compound (Fig. A.3). After confirming the separation of fumaric and maleic acids (isomers), the identity was further validated via spiking experiments with authentic fumaric acid and HPLC analysis. Fumaric acid standards were then prepared and analysed by HPLC, allowing for its quantification in samples via UV detection at 210 nm (Fig. 3; Table A.2). All MP2 treatments displayed a consistent trend of fumarate accumulation prior to *S. cerevisiae* inoculation, followed by a dramatic decrease in the final samples. Despite this decrease, the final MP2 wines contained more fumarate than the SC controls (Fig. 3; Table A.2). Highly significant ($P < 0.0001$) positive correlations were established between extent of sugar consumption by MP2 and fumarate production ($r > 0.93$ depending on the fermentation stage and the medium; Table A.3). Moreover, negative correlations ($P < 0.0001$) between final ethanol concentrations and fumarate production in partial ($r = -0.75$ (CDGJM), $r = -0.85$ (GJ); Table A.3) and final fermentations ($r = -0.94$ (CDGJM), $r = -0.93$ (GJ); Table A.3) were also detected.

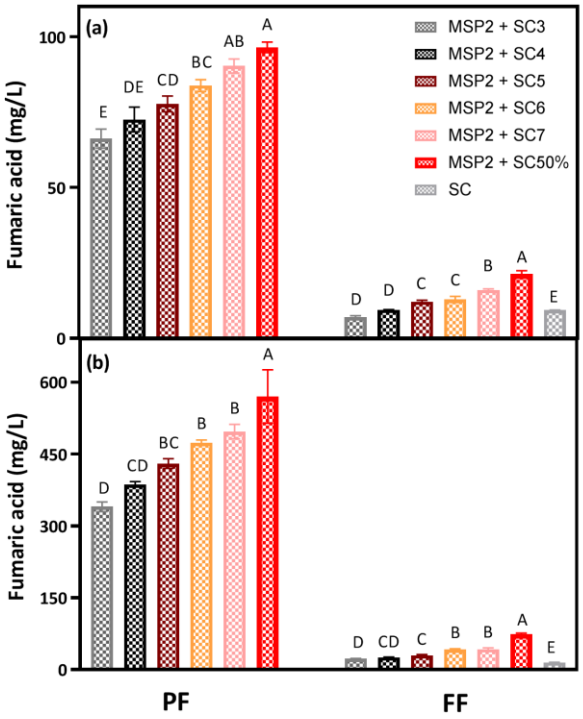

Fig. 3. Fumaric acid concentrations (mg/L) with six MP2 sequential inoculation modalities (Table 1) and SC controls in (a) CDGJM and (b) GJ. Error bars represent standard deviations (SD) of triplicate fermentations. Letters denote significant differences (ANOVA; $P < 0.05$) between treatments at the partial fermentation (PF) and final fermentation (FF) stage.

Table 3. Concentrations of metabolites at different fermentation stages (partial fermentation, PF; final fermentation, FF) produced with six MP2 sequential inoculation treatments and an *S. cerevisiae* control (SC) in Chemically Defined Grape Juice Medium (CDGJM).

Parameter		Stage	Yeast treatment							P values
			MP2 + SC3	MP2 + SC4	MP2 + SC5	MP2 + SC6	MP2 + SC7	MP2 + SC50%	SC	
Sugars	Glucose (g/L)	PF	97 ± 0^{A}	89 ± 1^{B}	84 ± 1^{C}	78 ± 2^{D}	74 ± 1^{E}	49 ± 0^{F}		< 0.0001
		FF	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	ns
	Fructose (g/L)	PF	110 ± 0^{A}	105 ± 1^{B}	103 ± 3^{B}	96 ± 2^{C}	95 ± 1^{C}	77 ± 0^{D}		< 0.0001
		FF	0.3 ± 0.2	0.2 ± 0	0.2 ± 0.1	0.1 ± 0	0 ± 0.2	0 ± 0	0.4 ± 0.3	ns
Alcohols	Ethanol (% v/v)	PF	2.4 ± 0.1^{D}	2.9 ± 0.2^{CD}	3.1 ± 0.1^{BC}	3.3 ± 0.1^{BC}	3.5 ± 0.4^{B}	5.7 ± 0.1^{A}		< 0.0001
		FF	14.5 ± 0^{B}	14.4 ± 0^{BC}	14.4 ± 0.1^{C}	14.3 ± 0^{C}	14.2 ± 0.1^{D}	14.0 ± 0.1^{E}	15.5 ± 0^{A}	< 0.0001
	Glycerol (g/L)	PF	4.1 ± 0.2^{B}	4.9 ± 0.8^{AB}	4.9 ± 0.1^{AB}	5.1 ± 0.3^{A}	5.3 ± 0.1^{A}	5.4 ± 0.1^{A}		0.0057
		FF	11.0 ± 0.1^{A}	10.9 ± 0.1^{AB}	10.9 ± 0.1^{AB}	10.6 ± 0.1^{B}	10.7 ± 0.1^{B}	10.7 ± 0.1^{B}	6.0 ± 0^{C}	< 0.0001
Acids	Acetic (g/L)	PF	0.31 ± 0.05^{A}	0.27 ± 0.02^{AB}	0.28 ± 0.02^{A}	0.29 ± 0.02^{A}	0.29 ± 0.01^{A}	0.21 ± 0.03^{B}		0.0072
		FF	0.17 ± 0.02^{BC}	0.22 ± 0.05^{B}	0.14 ± 0.01^{C}	0.16 ± 0.01^{C}	0.13 ± 0.01^{C}	0.14 ± 0.01^{C}	0.67 ± 0.01^{A}	< 0.0001
	Succinic (g/L)	PF	0.5 ± 0^{C}	0.6 ± 0^{BC}	0.6 ± 0^{BC}	0.7 ± 0.1^{AB}	0.7 ± 0.1^{AB}	0.9 ± 0.1^{A}		0.0002
		FF	1.7 ± 0^{BC}	1.6 ± 0^{C}	1.8 ± 0^{B}	1.7 ± 0.1^{BC}	1.7 ± 0^{BC}	1.9 ± 0.1^{A}	0.9 ± 0^{D}	< 0.0001
	Malic (g/L)	PF	3.1 ± 0.1^{A}	3.0 ± 0.1^{A}	3.0 ± 0.1^{A}	2.9 ± 0^{A}	3.0 ± 0^{A}	2.6 ± 0.1^{B}		< 0.0001
		FF	2.89 ± 0.01^{AB}	2.85 ± 0.02^{BC}	2.90 ± 0.02^{AB}	2.90 ± 0.04^{AB}	2.93 ± 0.02^{A}	2.80 ± 0.03^{C}	2.55 ± 0.02^{D}	< 0.0001
Metabolite yields	Ethanol (g/g)	PF	0.39 ± 0.02^{A}	0.37 ± 0.03^{AB}	0.35 ± 0.02^{AB}	0.32 ± 0.02^{B}	0.32 ± 0.04^{B}	0.34 ± 0.01^{AB}		0.0252
		FF	0.45 ± 0^{B}	0.44 ± 0^{C}	0.44 ± 0^{C}	0.44 ± 0^{C}	0.44 ± 0.01^{C}	0.43 ± 0^{D}	0.48 ± 0^{A}	< 0.0001
	Glycerol (mg/g)	PF	83 ± 4^{A}	77 ± 10^{A}	69 ± 5^{AB}	62 ± 2^{B}	61 ± 0^{B}	42 ± 1^{C}		< 0.0001
		FF	43 ± 0^{A}	42 ± 0^{A}	42 ± 0^{A}	41 ± 0^{A}	42 ± 1^{A}	42 ± 0^{A}	23 ± 0^{B}	< 0.0001
	Acetic acid (mg/g)	PF	6.34 ± 0.97^{A}	4.22 ± 0.22^{B}	4 ± 0.06^{B}	3.49 ± 0.33^{B}	3.3 ± 0.12^{B}	1.59 ± 0.26^{C}		< 0.0001
		FF	0.67 ± 0.08^{BC}	0.85 ± 0.19^{B}	0.56 ± 0.05^{C}	0.61 ± 0.05^{C}	0.49 ± 0.03^{C}	0.55 ± 0.04^{C}	2.59 ± 0.02^{A}	< 0.0001
	Succinic acid (mg/g)	PF	9.08 ± 0.34^{A}	9.47 ± 0.67^{A}	9.04 ± 0.06^{A}	8.81 ± 1.35^{A}	7.95 ± 0.7^{AB}	6.69 ± 0.44^{B}		0.0048
		FF	6.7 ± 0.2^{BC}	6.4 ± 0.2^{C}	6.9 ± 0.1^{B}	6.8 ± 0.3^{BC}	6.8 ± 0.1^{BC}	7.4 ± 0.2^{A}	3.4 ± 0^{D}	< 0.0001

Letters denote significant differences within a row (ANOVA; $P < 0.05$).

Table 4. Concentrations of metabolites at different fermentation stages (partial fermentation, PF; final fermentation, FF) produced with six MP2 sequential inoculation treatments and an *S. cerevisiae* control (SC) in Chardonnay grape juice (GJ).

Parameter		Stage	Yeast treatment							P values
			MP2 + SC3	MP2 + SC4	MP2 + SC5	MP2 + SC6	MP2 + SC7	MP2 + SC50%	SC	
Sugars	Glucose (g/L)	PF	86 ± 1^{A}	81 ± 2^{B}	77 ± 2^{C}	71 ± 0^{D}	69 ± 2^{D}	57 ± 1^{E}		< 0.0001
		FF	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	ns
	Fructose (g/L)	PF	98 ± 1^{A}	95 ± 2^{B}	92 ± 1^{BC}	89 ± 1^{C}	88 ± 2^{C}	82 ± 2^{D}		< 0.0001
		FF	0.3 ± 0.1	0.2 ± 0	0.2 ± 0	0.3 ± 0.1	0.2 ± 0	0.3 ± 0.2	0.4 ± 0.1	ns
Alcohols	Ethanol (% v/v)	PF	1.8 ± 0.1^{D}	2.2 ± 0.1^{C}	2.4 ± 0.2^{C}	3.1 ± 0.2^{B}	3.3 ± 0.1^{B}	4.1 ± 0.2^{A}		< 0.0001
		FF	14.5 ± 0^{B}	14.4 ± 0^{C}	14.4 ± 0^{C}	14.2 ± 0^{D}	14.2 ± 0^{D}	13.9 ± 0^{E}	15.1 ± 0^{A}	< 0.0001
	Glycerol (g/L)	PF	3.9 ± 0.1^{D}	4.6 ± 0.2^{C}	4.7 ± 0.2^{C}	5.3 ± 0.1^{B}	5.4 ± 0^{B}	6 ± 0.2^{A}		< 0.0001
		FF	10.9 ± 0.1^{B}	11 ± 0^{AB}	11.1 ± 0^{AB}	11.4 ± 0.3^{A}	11.2 ± 0.2^{AB}	11.3 ± 0.1^{AB}	7.8 ± 0.2^{C}	< 0.0001
Acids	Acetic (g/L)	PF	0.17 ± 0.01^{B}	0.21 ± 0.01^{A}	0.22 ± 0.01^{A}	0.21 ± 0.01^{A}	0.22 ± 0.01^{A}	0.15 ± 0.03^{B}		0.0003
		FF	0.46 ± 0.01^{A}	0.38 ± 0.02^{B}	0.35 ± 0.01^{BC}	0.33 ± 0.03^{C}	0.32 ± 0.01^{C}	0.26 ± 0.02^{D}	0.45 ± 0^{A}	< 0.0001
	Succinic (g/L)	PF	1.6 ± 0^{B}	1.6 ± 0.1^{B}	1.7 ± 0^{B}	1.8 ± 0.1^{A}	1.9 ± 0.1^{A}	1.9 ± 0.1^{A}		< 0.0001
		FF	2.30 ± 0^{C}	2.37 ± 0.06^{BC}	2.4 ± 0^{ABC}	2.43 ± 0.06^{AB}	2.47 ± 0.06^{AB}	2.5 ± 0^{A}	2.43 ± 0.06^{AB}	0.0003
	Malic (g/L)	PF	2.3 ± 0.1	2.3 ± 0	2.3 ± 0	2.3 ± 0	2.3 ± 0.1	2.3 ± 0		ns
		FF	1.89 ± 0.06^{BC}	1.92 ± 0.09^{ABC}	1.98 ± 0.05^{ABC}	2.05 ± 0.06^{A}	2.02 ± 0.03^{AB}	1.85 ± 0.01^{C}	1.98 ± 0.03^{ABC}	0.0049
Metabolite yields	Ethanol (g/g)	PF	0.20 ± 0.01^{C}	0.22 ± 0.01^{C}	0.23 ± 0.02^{BC}	0.26 ± 0.02^{AB}	0.27 ± 0.01^{A}	0.28 ± 0.01^{A}		< 0.0001
		FF	0.453 ± 0^{B}	0.449 ± 0^{C}	0.450 ± 0^{C}	0.443 ± 0^{D}	0.443 ± 0^{D}	0.434 ± 0^{E}	0.470 ± 0^{A}	< 0.0001
	Glycerol (mg/g)	PF	57 ± 2^{AB}	59 ± 4^{A}	56 ± 2^{AB}	57 ± 1^{AB}	57 ± 2^{AB}	52 ± 0^{B}		0.0388
		FF	43 ± 0^{B}	44 ± 0^{AB}	44 ± 0^{AB}	45 ± 1^{A}	44 ± 1^{AB}	45 ± 0^{AB}	31 ± 1^{C}	< 0.0001
	Acetic acid (mg/g)	PF	0.9 ± 0^{C}	1.2 ± 0.1^{AB}	1.3 ± 0^{AB}	1.4 ± 0.1^{AB}	1.4 ± 0^{AB}	1.1 ± 0.2^{BC}		0.0005
		FF	1.8 ± 0^{A}	1.5 ± 0.1^{B}	1.4 ± 0^{BC}	1.3 ± 0.1^{C}	1.3 ± 0^{C}	1.0 ± 0.1^{D}	1.8 ± 0^{A}	< 0.0001
	Succinic acid (mg/g)	PF	23 ± 1^{A}	21 ± 2^{AB}	20 ± 1^{B}	20 ± 1^{B}	20 ± 0^{B}	16 ± 0^{C}		< 0.0001
		FF	9.1 ± 0^{C}	9.4 ± 0.2^{BC}	9.5 ± 0^{ABC}	9.6 ± 0.2^{AB}	9.8 ± 0.2^{AB}	9.9 ± 0^{A}	9.6 ± 0.2^{AB}	0.0003

Letters denote significant differences within a row (ANOVA; $P < 0.05$).

3.2.4. Analysis of volatile compounds in the wines. Alongside the SC control, three sequentially inoculated MP2 wines (MP2 + SC3, MP2 + SC6 and MP2 + SC50%) with large differences in their gross metabolite composition in grape juice were subjected to analysis of volatile compounds by HS-SPME-GC-MS (Table 5). From 31 quantified compounds, 20 volatiles were significantly different between the treatments (ANOVA; $P < 0.05$; Table 5). In MP2 treatments, a delay in sequential inoculation caused an increase in total acetate esters, mainly driven by ethyl acetate. In fact, ethyl acetate was 2.5-fold and 5-fold higher in MP2 × SC3 and MP2 × SC50%, respectively, than in SC. Isoamyl acetate and phenylethyl acetate were also dramatically increased in sequential wines, whereas hexyl acetate was the only measured acetate found to be higher in SC than in any MP2 treatments. The concentrations of total ethyl esters did not significantly differ among the treatments. However, MP2 + SC50% had the lowest concentrations of several ethyl esters, including ethyl esters of medium chain fatty acids (MCFA), i.e., hexanoate, octanoate, decanoate, and diethyl succinate. Conversely, ethyl propanoate was higher in MPS2 + SC50% and MP2 + SC6 than in the remaining two treatments. The concentrations of major higher alcohols (3-methyl-1-butanol, 2-methyl-1-propanol and 2-phenylethanol) were higher in mixed culture fermentations than in the SC control, which was between 66 and 71% lower in total higher alcohols than MP2 wines. In contrast, 1-hexanol was higher in SC wine. The prolonged persistence of MP2 promoted the increase in monoterpenoids, in particular linalool, and α-terpineol was also found to be higher in MP2 wines than in the SC one. The wines differed significantly in concentrations of hexanoic and 3-methyl butanoic acids. In sequential fermentations, the measured acids were generally lower than in the SC control, showing a decreasing trend with *S. cerevisiae* inoculation delay. Accordingly, total measured acids were 18% lower in MP2 + SC3 than in SC, and 30% and 52% lower in MP2 + SC4 and MP2 + SC50%, respectively (Table 5).

Table 5. Concentrations (µg/L) of volatiles in final wines produced with three MP2 sequential inoculation treatments and an *S. cerevisiae* control (SC).

Compound	SC	MP2 × SC3	MP2 × SC6	MP2 × SC50%	P values
ethyl acetate	27599 ± 434^{D}	69580 ± 739^{C}	110139 ± 5987^{B}	140359 ± 12888^{A}	< 0.0001
isoamyl acetate	2269 ± 358^{B}	10655 ± 1173^{A}	13756 ± 2577^{A}	11938 ± 543^{A}	< 0.0001
phenylethyl acetate	446 ± 57^{B}	3372 ± 642^{A}	3843 ± 746^{A}	3066 ± 7481^{A}	0.0006
hexyl acetate	66 ± 1^{A}	39 ± 3^{B}	46 ± 3^{B}	45 ± 7^{B}	0.0003
ethyl phenylacetate	4.5 ± 0.3^{C}	5.4 ± 0.2^{B}	5.7 ± 0.3^{AB}	6.1 ± 0.2^{A}	0.0006
total acetates	30384 ± 3782^{D}	83651 ± 6389^{C}	127791 ± 6908^{B}	155414 ± 10418^{A}	< 0.0001
ethyl hexanoate	308 ± 14^{A}	306 ± 25^{A}	294 ± 38^{A}	216 ± 31^{B}	0.0124
ethyl butanoate	185 ± 25	218 ± 33	218 ± 33	174 ± 15	ns
2-ethyl-1-hexanol	82 ± 45	67 ± 38	79 ± 44	82 ± 45	ns
ethyl decanoate	47 ± 11^{AB}	58 ± 1^{A}	64 ± 14^{A}	30 ± 9^{B}	0.0160
ethyl octanoate	32 ± 5^{A}	34 ± 1^{A}	31 ± 7^{A}	17 ± 3^{B}	0.0081
ethyl propanoate	22 ± 3^{B}	23 ± 2^{B}	32 ± 1^{A}	36 ± 4^{A}	0.0005
diethyl succinate	10.4 ± 0.5^{B}	13.9 ± 1.5^{A}	11.5 ± 1.8^{AB}	6.1 ± 0.8^{C}	0.0004
ethyl 3-methyl butanoate	1.4 ± 1.3	nd	nd	nd	ns
ethyl lactate	0.02 ± 0	0.01 ± 0	0.02 ± 0	0.02 ± 0	ns
total ethyl esters	687 ± 88	719 ± 96	729 ± 129	560 ± 37	ns
3-methyl-1-butanol	111731 ± 5709^{B}	189216 ± 10820^{A}	198329 ± 16193^{A}	182921 ± 7594^{A}	< 0.0001
2-phenylethanol	23950 ± 3740^{B}	91727 ± 17735^{A}	100415 ± 21106^{A}	92659 ± 14214^{A}	0.0010
2-methyl-1-propanol	16858 ± 3272^{B}	169369 ± 28206^{A}	223093 ± 33727^{A}	189178 ± 32663^{A}	< 0.0001
1-butanol	485 ± 66^{B}	782 ± 36^{A}	812 ± 85^{A}	623 ± 33^{B}	0.0005
1-hexanol	233 ± 23^{A}	92 ± 8^{B}	91 ± 14^{B}	91 ± 6^{B}	< 0.0001
benzyl alcohol	29.7 ± 2.9	31.2 ± 2.9	33.0 ± 1.0	30.9 ± 3.8	ns
1-octanol	3.2 ± 0	2.2 ± 0	2.6 ± 0.3	2.6 ± 0.5	ns
total higher alcohols	153288 ± 12813^{B}	451218 ± 56808^{A}	522776 ± 42335^{A}	465504 ± 35084^{A}	< 0.0001
linalool	2.0 ± 0.1^{C}	2.3 ± 0.1^{C}	2.9 ± 0.2^{B}	3.3 ± 0.1^{A}	< 0.0001
limonene	2.0 ± 0.5	1.6 ± 0.5	1.7 ± 0.6	2.1 ± 0.7	ns
α-terpineol	1.6 ± 0.3^{B}	2.0 ± 0.2^{A}	2.3 ± 0.1^{A}	2.2 ± 0.1^{A}	0.0031
total monoterpenoids	5.5 ± 0.6^{B}	5.9 ± 0.6^{B}	6.9 ± 0.3^{AB}	7.6 ± 0.6^{A}	0.0053
octanoic acid	10250 ± 3412	8534 ± 2373	6997 ± 1010	4845 ± 1375	ns
decanoic acid	2550 ± 763	1850 ± 395	1839 ± 347	1192 ± 328	ns
hexanoic acid	1697 ± 188^{A}	1543 ± 164^{A}	1407 ± 166^{A}	912 ± 127^{B}	0.0019
3-methyl butanoic acid	223 ± 57^{A}	101 ± 32^{B}	89 ± 11^{B}	89 ± 13^{B}	0.0031
total acids	14720 ± 4411	12026 ± 2958	10331 ± 1528	7039 ± 1815	ns
nonanal	14 ± 1	10 ± 2	10 ± 2	14 ± 7	ns
benzaldehyde	0.01 ± 0^{B}	0.02 ± 0^{B}	0.02 ± 0^{B}	0.03 ± 0.01^{A}	0.0092
total aldehydes	14.01 ± 0.1	10.02 ± 2.0	10.02 ± 2.0	14.03 ± 7.01	ns

Letters denote significant differences within a column (ANOVA; $P < 0.05$).

The 20 volatile compounds that were significantly different in wines (ANOVA, $P < 0.05$) were normalised and subjected to PCA (Fig. 4). A separation of the SC monoculture from mixed inoculation treatments was seen along PC1, accounting for 69% of the total explained variance. The SC treatment was characterised by higher amounts of 1-hexanol, hexanoic acid, and their respective esters, alongside other major ethyl esters and 3-methyl-butanoic acid. The MP2 treatments were associated with production of acetate esters, higher alcohols (other than 1-hexanol), monoterpenoids, ethyl propanoate and benzaldehyde. The MS2 × SC50% was separated along PC2 (lower right quadrant, Fig. 4) from the remaining two MP2 wines, which showed more resembling volatile profiles.

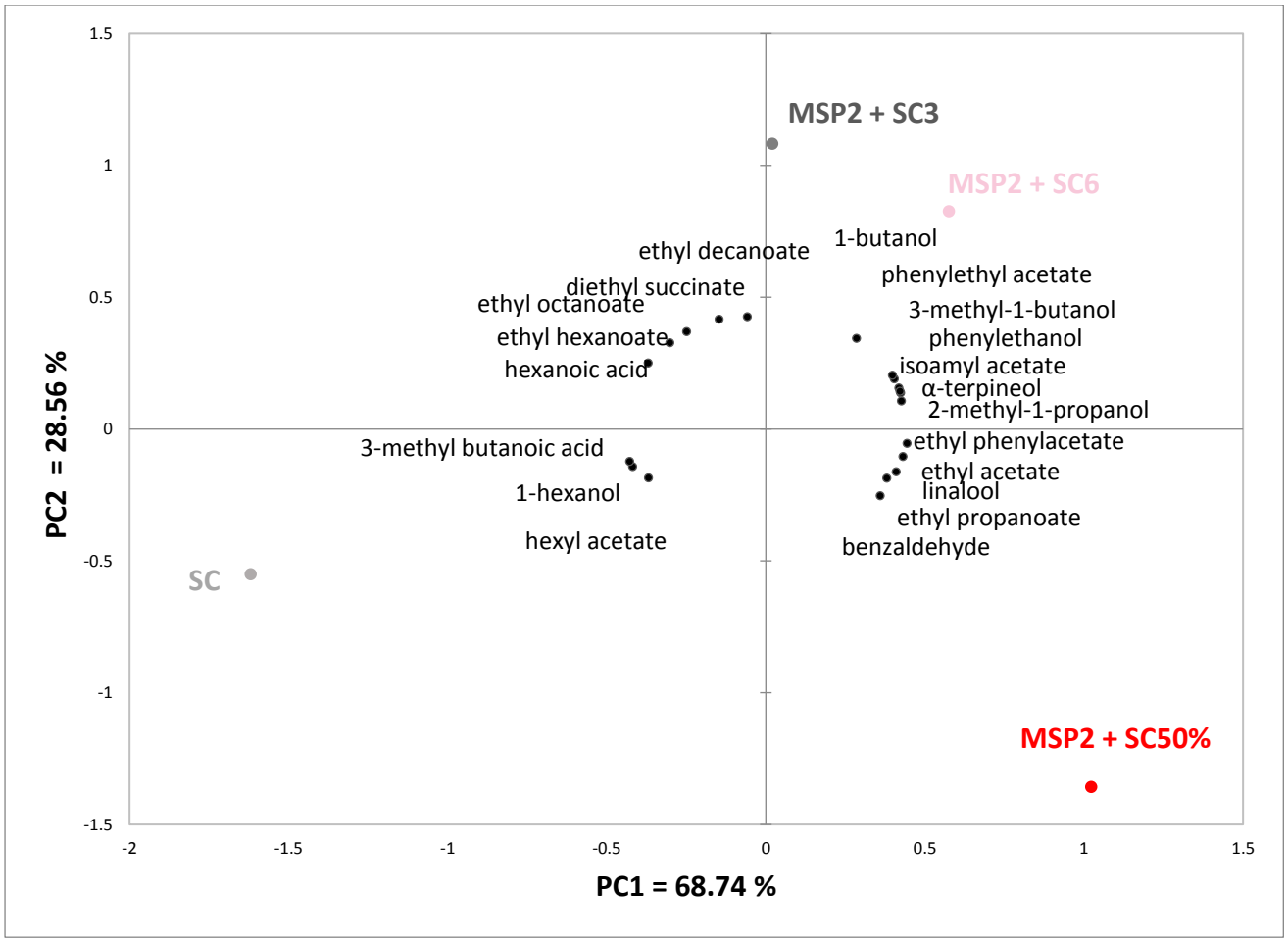

Fig. 4. PCA bi-plot of significantly different volatile compounds for wines produced with three MP2 sequential inoculation treatments and an *S. cerevisiae* control (SC).

4. Discussion

Wine microbiologists are on a quest for a yeast capable of lowering wine ethanol content while enhancing overall quality. Besides techniques employed to expand intrinsically low diversity in ethanol yields among *S. cerevisiae* wine strains (Tilloy et al., 2015), efforts have been extended to encompass the diversity of non-*Saccharomyces* yeasts (Jolly et al. 2014). Sequential inoculations are the most explored modalities, as the initial absence of *S. cerevisiae* allows for the maximised proliferation and metabolic contribution of the non-*Saccharomyces* strains (Ciani et al. 2016). In this study we initially selected and characterised a lower-ethanol *M. pulcherrima* strain to then focus on evaluation of timing of *S. cerevisiae* sequential inoculation. As seen previously (Contreras et al., 2014; Tronchoni et al., 2018), differences in sugar catabolism and metabolite production were seen among multiple tested *M. pulcherrima* strains. Nonetheless, the *S. cerevisiae* control consistently produced more ethanol than any MP sequential treatment, which is in accord with some studies (Contreras et al., 2015; Contreras et al., 2014; Varela et al., 2016) but disagrees with others (Sadoudi et al., 2017), and further emphasises the intra-specific diversity among yeasts. Besides lower ethanol, MP treatments generally showed an increase in glycerol and a decrease in acetate. This was also the case for the strain MP2, selected for further trials due to timely fermentation completion and the lowest final ethanol concentrations and yields (Table 2).

The lower ethanol phenotype of MP2 was then validated in a series of sequential inoculations in two media with higher initial sugar concentrations, where ethanol modulation becomes further relevant (Table 1). Fermentations inoculated with *S. cerevisiae* alone finished first, whereas fermentation completion was delayed in co-cultures to an extent depending on the timing of *S. cerevisiae* inoculation (Fig. A.1). In agreement with previous studies (Contreras et al., 2014; Sadoudi et al., 2017; Sadoudi et al., 2012; Varela et al., 2016), *S. cerevisiae* addition induced rapid population decline in *M. pulcherrima* (Fig. A.2). The maintained

viability of MP2 in treatments with postponed sequential inoculation indicated that an antagonistic activity of *S. cerevisiae*, rather than alcohol stress, anoxia and/or other abiotic stressors, caused the observed cell death. Mechanisms like cell-cell contact and quorum-sensing were reported to elicit non-*Saccharomyces* cell death in *S. cerevisiae* co-cultures with *Torulaspora delbrueckii* (Renault et al., 2013) and *Lachancea thermotolerans* (Kemsawasd, Branco, et al., 2015), but information about *M. pulcherrima* is lacking. Nonetheless, consecutive sequential inoculations allowed for differential MP2 proliferation and sugar consumption among the treatments, in turn affecting the extent of carbon diverted away from ethanol.

The fate of carbon diverted during fermentations by lower-ethanol (*M. pulcherrima*) yeast strains remains largely elusive. The role of respiratory metabolism in *M. pulcherrima* has been highlighted in aerobic cultures (Quiros et al., 2014). In anaerobiosis, a decrease in ethanol was either partially explained by an increase in the production of some organic acids and glycerol (Contreras et al., 2015), or sinks that remained undetected (Varela et al., 2016). In the current study, we observed an accumulation of a metabolite that was positively correlated to MP2 sugar consumption (Table A.3). It was identified as fumarate; a transient metabolite that does not qualify as a major acid in wine (Ribereau-Gayon et al., 2006). Besides several early reports on the inhibitory effects of fumaric acid on malolactic fermentation (Cofran and Meyer, 1970; Pilone et al., 1973), fumarate has not received much attention in a winemaking context. In a study by Magyar et al. (2014) fumarate concentrations released by *S. cerevisiae* (i.e. 17-21 mg/L) were higher than those in *S. uvarum*, *S. bacillaris* and *C. stellata* treatments, and comparable to the ones in our SC controls (Fig. 3; Table A.2). The fumarate increases detected in MP2 treatments were dramatic, and potentially suggest an important role of the tricarboxylic acid (TCA) cycle in *M. pulcherrima* during fermentation. Maintained activity of the TCA cycle during fermentation is well documented, as is its importance in winemaking due to the

excretion of acids affecting wine sensory balance (Camarasa et al., 2003). In a branched TCA cycle, fumarate is produced on the reductive TCA branch from malate via fumarase activity (Fig. 1). Fumarate reductase then converts fumarate to succinate. This reductive branch 'dead-end' is, in fact, the main succinate production pathway in fermenting *S. cerevisiae* (Camarasa et al., 2003). One possible explanation for increased fumarate accumulation in MP2 is therefore a comparable TCA cycle activity in both yeasts, with different MP2 regulation at the point of fumarate production/consumption. However, in MP2 partial fermentations, succinate yields were, depending on the medium and time-point, at least 1.7 times higher than in the SC controls, and malate concentrations were also higher (Tables 3 and 4). In *M. pulcherrima*, the contribution of the oxidative and reductive branch in succinate formation remains to be studied, as well as the response to different nitrogen sources. Nonetheless, given the increase in concentrations/yields of both fumarate and succinate, we hypothesise that the proportion of carbon shuttled towards the TCA cycle, and thus rerouted away from ethanol production, is higher in MP2 than in *S. cerevisiae* fermentations. Different compositions of the media used may explain the altered concentrations of TCA cycle by-products and other metabolites observed in GJ and CDGJM, and this warrants further investigation.

Another interesting observation was the depletion of fumarate in the final stages of sequential fermentations (Fig. 3), implying metabolite exchange between the two species. More specifically, given the analytical data prior to and following sequential inoculation (Tables 3 and 4), and branching of the TCA cycle in *S. cerevisiae* (Fig. 1), it is likely that fumarate released by MP2 was assimilated by *S. cerevisiae* to be converted to succinate. Similar interactions have previously been reported for acetaldehyde in *S. cerevisiae* co-cultures with *Starmella bombicola* (Ciani and Ferraro, 1988), *T. delbrueckii* (Ciani et al 2006, Bely et al. 2008) and *L. thermotolerans* (Ciani et al. 2006), and acetoin with *St. bombicola* (Ciani and Ferraro, 1988). The underlying molecular mechanisms of this phenomenon remain to be

elucidated. Until recently, interactions between *M. pulcherrima* and *S. cerevisiae* have only been inferred from modulated production of metabolites (Sadoudi et al., 2012). The first attempt to study interactions on a molecular level has revealed changed expression levels of genes involved in glyceropyruvic fermentation and the pyruvate dehydrogenase bypass in *S. cerevisiae* sequentially inoculated into an *M. pulcherrima* fermentation (Sadoudi et al., 2017). Our study implies that other constituents of central carbon metabolism, i.e. the TCA cycle, are also affected.

Significant increases in glycerol and decreases in acetic acid in sequential *M. pulcherrima* fermentations agree with a number of previous studies (Contreras et al., 2015; Contreras et al., 2014; Sadoudi et al., 2017). In general, glycerol is formed at the beginning of fermentation as a response to hyperosmotic stress. Decreasing glycerol yields with the progression of sugar consumption in MP2 alone, especially in CDGJM, agree with this model (Table 3). In addition to osmo-adaptation, glycerol formation in *S. cerevisiae* by glycerol 3-phosphate dehydrogenases (GPD) serves as a redox valve to eliminate excess cytosolic NADH in anaerobic conditions (Ansell et al., 1997). Expression of homologous genes *GPD1* and *GPD2* is induced by osmotic stress and anoxia, respectively (Ansell et al. 1997). The levels of *GPD1* transcript showed an initial overexpression followed by a drop in *S. cerevisiae* sequentially inoculated into *M. pulcherrima* fermentation in a study by Sadoudi et al. (2017), from which *GDP2* was omitted. We observed that about 50% of glycerol in all final sequential fermentations was formed after *S. cerevisiae* inoculation with maintained anaerobiosis, regardless of the residual sugar level, highlighting the need for further research on *GPD2* expression by *S. cerevisiae* in co-cultures. Irrespective of the mechanism, glycerol yields in both pure and finalised MP2 fermentations were higher than those of *S. cerevisiae*, offering a partial explanation for ethanol decrease. In fact, glycerol remains the key carbon sink in a range

of lower-ethanol yeasts, and has either negligible or positive sensory contributions (Goold et al., 2017).

Initial glycerol formation by *S. cerevisiae* is coupled with acetic acid production to restore the reduced NADH form (Fig. 1) (Ansell et al., 1997; Kutyna et al., 2010). This is not necessarily the case for some non-*Saccharomyces* yeasts, for example *T. delbrueckii* (Bely et al. 2008). Given our analytical data did not show a link between glycerol and acetic acid production (Table A.3), *M. pulcherrima* may also differ in this way. In agreement with other reports (Comitini et al., 2011; Sadoudi et al., 2017), sequential fermentations consistently contained less acetic acid than *S. cerevisiae* controls. Apart from the drop in acetate production upon *S. cerevisiae* inoculation in CDGJM, partial consumption of acetate also occurred in pure MP2 cultures. This was evident from the lower acetate concentrations in such cultures upon 50% sugar consumption in both media (Table 3 and 4). A plausible explanation is that consumed acetate was converted to acetyl-coenzyme A (Fig. 1), a proportion of which was used for other biochemical processes, for example esterification. An increase in acetate esters in MP2 fermentations (Table 5) supports this hypothesis, and is in line with previous studies (Contreras et al., 2014; Sadoudi et al., 2012; Varela et al., 2016). However, unlike some other studies (Contreras et al., 2014; Varela et al., 2016), ethyl acetate concentrations did not exceed 150 mg/L, i.e., the point where it is generally perceived as a fault rather than fruity/complexing (Sumby et al., 2010). As for ethyl esters, even though their total concentrations remained comparable, different patterns were seen among the treatments. The MP2 treatment inoculated after 50% sugar consumption contained the lowest amounts of ethyl esters of MCFA, including ethyl hexanoate, ethyl octanoate and ethyl decanoate (Table 5). In agreement with the literature (Dennis et al., 2012; Saerens et al., 2010), their respective precursors were either significantly lower (hexanoic acid) or decreased (octanoic and decanoic acid) in the same MP2 treatment. Lower concentrations of MCFA in sequential fermentations, also seen elsewhere (Contreras et

al., 2014; Shekhawat et al., 2017), alongside the progressive drop with a delay in *S. cerevisiae* inoculation (Table 5), suggest that MP2 and *S. cerevisiae* differ in their MCFA production capacity under given fermentative conditions. It remains to be elucidated whether lower MCFA levels perhaps reflect decreased fatty acid biosynthesis in MP2 compared to *S. cerevisiae.* Another possibility is that promoted elongation in MP2 leads to less MCFA intermediates released from the fatty acid synthase complex in comparison to *S. cerevisiae*. Although it remains to be validated, this scenario is plausible because *M. pulcherrima* has recently been characterised as an oleaginous yeast (Maina et al., 2017; Santamauro et al., 2014) and it aligns with the previously discussed availability of acetyl-CoA derived from acetic acid.

A dramatic increase in concentrations of higher alcohols was seen in sequential fermentations (Table 5), in accord with several reports on *M. pulcherrima* co-fermentations (Shekhawat et al., 2017; Tronchoni et al., 2018). The effect of higher alcohols on wine quality is somewhat elusive; the commonly quoted positive contribution below 300 mg/L, and detrimental one above 400 mg/L, is seemingly lacking experimental support, and the research has highlighted their matrix-dependent effects in wine aroma modulation (de-la-Fuente-Blanco et al., 2016). Implications for wine quality of higher alcohol overproduction observed in sequential fermentations (Table 5) therefore need to be evaluated, as do the underlying mechanisms driving their increase. The link between higher alcohols and their respective amino acid precursors has been long recognised, as well as the biotic and abiotic factors affecting their release (Hazelwood et al., 2008). One of these is the well-characterised preferential consumption of different amino acids in *S. cerevisiae* (Jiranek et al., 1995; Kemsawasd, Viana, et al., 2015), with the information recently extended to some non-*Saccharomyces* species (Gobert et al., 2017; Kemsawasd, Viana, et al., 2015). Even though the relationship between amino acid composition and final volatiles is not necessarily straightforward in mixed cultures (Gobert et al., 2017), we can hypothesise that differences in amino acid metabolism between

S. cerevisiae and co-cultures are reflected in the volatile profiles of the wines. Apart from nitrogenous sources, higher alcohols can also be synthesised from glucose via pyruvate (Cordente et al., 2012). Partial sugar diversion towards higher alcohols instead of ethanol remains a possibility in mixed cultures. Another modulating factor is yeast interactions. 2-Phenylethanol has a role in yeast quorum sensing, a phenomenon through which individual microbial cells regulate their phenotype and adapt to environmental changes (Avbelj et al., 2016; Hazelwood et al., 2008). As an increase in 2-phenylethanol is generally attributed to mixed fermentations with non-*Saccharomyces* (Comitini et al., 2011; Padilla et al., 2016; Sadoudi et al., 2012), and not necessarily their respective monocultures (Sadoudi et al., 2012), it is worth further exploring whether its overproduction is related to a sequential culture response.

Terpenes, predominantly originating from grapes (Marais, 1983) are released and modulated by microbial activity, although *de novo* synthesis has been reported for both *S. cerevisiae* and non-*Saccharomyces* yeasts (Carrau et al., 2005; Padilla et al., 2016; Rossouw and Bauer, 2016). Strong β-glucosidase activity associated with *M. pulcherrima* strains (Comitini et al., 2011) could account for the increase in monoterpenoids seen in the sequentially inoculated wines. Altogether, it is likely that yeast treatments affected sensory properties of the wines, however, this effect remains to be investigated in the future studies. Other aspects to be explored include the performance of the MP2 strain in non-sterile grape juice/must at a larger scale, as well as the suitability for production of wines destined for malolactic fermentation.

In conclusion, this study describes the selection and thorough characterisation of an *M. pulcherrima* strain for production of lower-ethanol dry wines in sequential fermentations with *S. cerevisiae*. Depending on the inoculation delay, MP2 white wines contained between 0.6% and 1.2% (v/v) less ethanol than the *S. cerevisiae* monoculture, with even larger decreases

achieved in CDGJM. The experimental set-up with increasing delays in *S. cerevisiae* inoculation allowed for the evaluation of metabolite production dynamics in MP2 alone, and their subsequent modulation in co-cultures, and highlighted several potential carbon sinks accounting for the ethanol loss. Besides increases in glycerol and decreases in acetate, of particular interest are the higher concentrations and yields of the TCA cycle by-products (i.e. fumarate and succinate) in MP2 fermentations, as well as the altered volatile profiles, without detection of any apparent aroma off-flavours. Accordingly, the selected and characterised MP2 strain shows promise when used in conjunction with *S. cerevisiae* as a means of modulating ethanol level and balancing flavour profile upon fermenting grapes with a high sugar content.

Appendix A

Fig. A.1. Microbial growth (a) and sugar (glucose + fructose) consumption kinetics (b) in fermentations conducted with six *M. pulcherrima* strains (MP1–MP6), sequentially inoculated with *S. cerevisiae* on day seven (indicated with an arrow) and an *S. cerevisiae* control (SC) in CDGJM. Error bars are standard deviations (SD) of fermentation triplicates.

Table A.1. Concentrations and yields of metabolites produced with *S. cerevisiae* (SC) and six *M. pulcherrima* (MP) strains, prior to *S. cerevisiae* sequential inoculation (PF) on day 7, and in final samples (FF) in Chemically Defined Grape Juice Medium (CDGJM).

Fig. A.2. Population dynamics in six MP2 sequential inoculation regimes (Table 1) and a S. cerevisiae (SC) control in CDGJM (top panel) and GJ (bottom panel): (a) MP2 + SC3; (b) MP2 + SC4; (c) MP2 + SC5; (d) MP2 + SC6; (e) MP2 + SC7; (f) MP2 + SC50%. The growth of SC monoculture (•) and MP2 prior to sequential inoculation (--) was monitored via flow cytometry. The growth of MP2 (□) and S. cerevisiae (-□-) after sequential inoculation was

quantified via lysine and YPD plate counts at four time-points: 24 h, 48 h and 72 h after secondary inoculation, and at fermentation completion.

Fig. A.3. Results for identification of an unknown peak isolated by HPLC and analysed by HPLC-MS with electrospray ionisation in negative mode, showing (a) HPLC-MS chromatogram and (b) full scan MS of fumaric acid standard (*m*/*z* 115 = $[M-H]^-$, *m*/*z* 161 = fumaric/formic adduct ion, *m*/*z* 231 = fumaric/fumaric cluster ion), and (c) HPLC-MS chromatogram with extracted ion chromatogram (XIC) of *m*/*z* 115 (inset) and (d) full scan MS of the unknown compound with retention time and full scan MS consistent with fumaric acid.

Table A.2. Fumaric acid concentrations and yields in partial fermentations (PF) and final fermentations (FF) conducted with six MP2 sequential inoculation regimes (Table 1) and an *S. cerevisiae* control (SC) in CDGJM and GJ.

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Appendix A

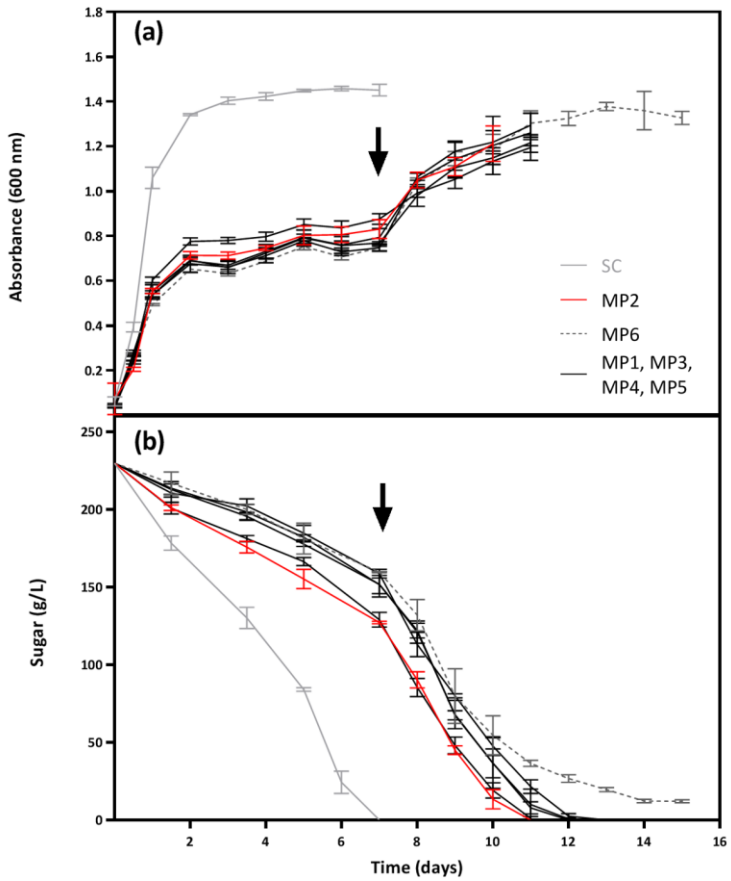

Fig. A.1. Microbial growth (a) and sugar (glucose + fructose) consumption kinetics (b) in fermentations conducted with six *M. pulcherrima* strains (MP1–MP6), sequentially inoculated with *S. cerevisiae* on day seven (indicated with an arrow) and an *S. cerevisiae* control (SC) in CDGJM. Error bars are standard deviations (SD) of fermentation triplicates.

Table A.1. Concentrations and yields of metabolites produced with *S. cerevisiae* (SC) and six *M. pulcherrima* (MP) strains, prior to *S. cerevisiae* sequential inoculation (PF) on day 7, and in final samples (FF) in Chemically Defined Grape Juice Medium (CDGJM).

Parameter		Stage	Yeast treatments							P values
			MP1	MP2	MP3	MP4	MP5	MP6	SC	
Sugars	Glucose (g/L)	PF	55 ± 2^{B}	54 ± 0^{B}	69 ± 3^{A}	71 ± 2^{A}	71 ± 2^{A}	72 ± 0^{A}		< 0.0001
		FF	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	1 ± 0^{A}	0 ± 0^{B}	< 0.0001
	Fructose (g/L)	PF	74 ± 2^{B}	74 ± 0^{B}	86 ± 4^{A}	87 ± 1^{A}	88 ± 1^{A}	88 ± 0^{A}		< 0.0001
		FF	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{B}	11 ± 0^{A}	0 ± 0^{B}	< 0.0001
Alcohols	Ethanol (% v/v)	PF	4.3 ± 0.2^{A}	4.5 ± 0.1^{A}	2.8 ± 0.2^{B}	3.0 ± 0.2^{B}	3.0 ± 0.1^{B}	2.9 ± 0.1^{B}		< 0.0001
		FF	11.5 ± 0.1^{AB}	10.3 ± 0.6^{B}	11.5 ± 1.1^{AB}	11.1 ± 0.5^{B}	11.5 ± 0.6^{AB}	11.1 ± 0.2^{B}	12.8 ± 0.1^{A}	0.0054
	Glycerol (g/L)	PF	4.8 ± 0.2^{AB}	5.1 ± 0.1^{A}	2.9 ± 0.1^{BC}	2.9 ± 0.1^{BC}	2.8 ± 0.1^{C}	2.9 ± 0^{BC}		< 0.0001
		FF	8.9 ± 0.2^{AB}	9.7 ± 0.4^{A}	8.9 ± 0.4^{AB}	8.8 ± 0.5^{AB}	8.4 ± 0.4^{B}	9.0 ± 0^{AB}	5.5 ± 0^{C}	< 0.0001
Acids	Acetic (g/L)	PF	0.24 ± 0.01^{A}	0.22 ± 0.01^{AB}	0.19 ± 0.01^{C}	0.20 ± 0.02^{BC}	0.19 ± 0^{C}	0.22 ± 0.01^{AB}		0.0002
		FF	0.20 ± 0.02^{B}	0.16 ± 0.03^{B}	0.18 ± 0.02^{B}	0.17 ± 0.02^{B}	0.21 ± 0.02^{B}	0.13 ± 0.02^{B}	0.66 ± 0.08^{A}	< 0.0001
	Succinic (g/L)	PF	0.9 ± 0^{A}	0.9 ± 0.1^{A}	0.5 ± 0^{B}	0.5 ± 0^{B}	0.5 ± 0^{B}	0.5 ± 0^{B}		< 0.0001
		FF	1.7 ± 0.1^{BC}	1.9 ± 0.2^{B}	1.6 ± 0.1^{BC}	1.5 ± 0.2^{C}	1.6 ± 0^{BC}	2.9 ± 0.1^{A}	0.9 ± 0.1^{D}	< 0.0001
	Malic (g/L)	PF	2.8 ± 0.1	3.0 ± 0	2.9 ± 0.1	2.9 ± 0	2.9 ± 0	3.0 ± 0		ns
		FF	2.9 ± 0.1^{AB}	3.1 ± 0.2^{A}	2.8 ± 0.1^{AB}	2.8 ± 0.2^{AB}	2.8 ± 0.1^{AB}	2.9 ± 0.1^{AB}	2.6 ± 0^{B}	0.0055
Metabolite yields	Ethanol (g/g)	PF	0.34 ± 0.03	0.35 ± 0.01	0.30 ± 0.05	0.34 ± 0	0.33 ± 0.01	0.33 ± 0.01		0.3283
		FF	0.39 ± 0^{AB}	0.35 ± 0.02 B	0.40 ± 0.04 AB	0.38 ± 0.02 B	0.39 ± 0.02 AB	0.40 ± 0.01 AB	0.44 ± 0 A	0.0050
	Glycerol (mg/g)	PF	47 ± 4^{A}	50 ± 1 AB	39 ± 4 C	41 ± 3 BC	40 ± 1 C	41 ± 1 BC		0.0011
		FF	39 ± 1^{AB}	42 ± 2 A	39 ± 2 AB	38 ± 2 AB	37 ± 2 B	41 ± 0 A	24 ± 0 C	< 0.0001
	Acetic acid (mg/g)	PF	2.4 ± 0.1^{B}	2.2 ± 0.1 B	2.5 ± 0.4 B	2.8 ± 0.3 AB	2.7 ± 0.1 AB	3.1 ± 0.1 A		0.0030
		FF	0.9 ± 0.1^{B}	0.7 ± 0.1 B	0.8 ± 0.1 B	0.7 ± 0.1 B	0.9 ± 0.1 B	0.6 ± 0.1 B	2.9 ± 0.3 A	< 0.0001
	Succinic acid (mg/g)	PF	8 ± 0^{A}	9 ± 1 A	7 ± 1 B	7 ± 0 B	7 ± 0 B	7 ± 0 B		< 0.0001
		FF	7 ± 0^{BC}	8 ± 1 B	7 ± 0 BC	7 ± 1 BC	7 ± 0 BC	13 ± 0 A	4 ± 0 D	< 0.0001

Letters denote significant differences within a row (ANOVA; $P < 0.05$).

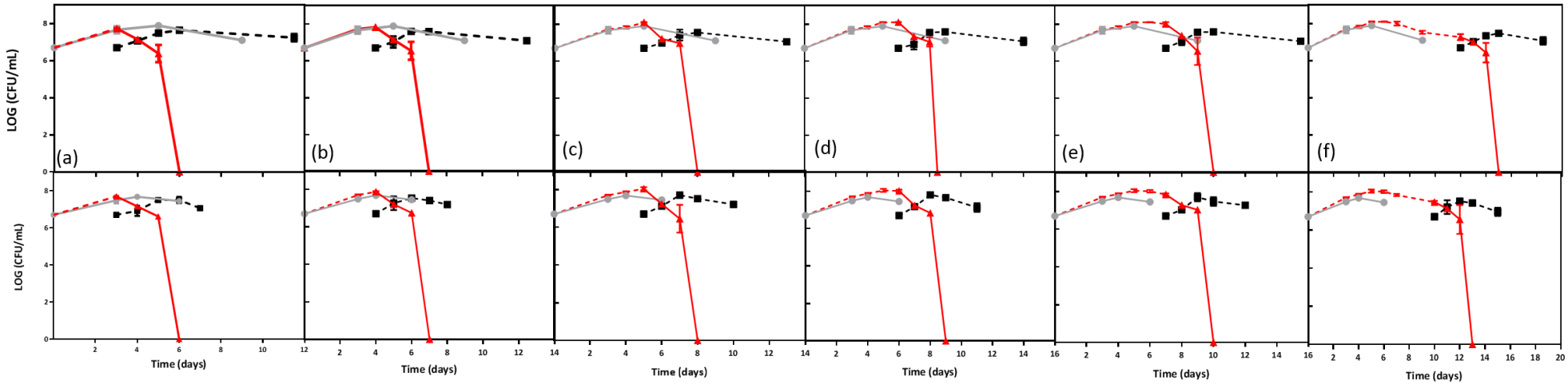

Fig. A.2. Population dynamics in six MP2 sequential inoculation regimes (Table 1) and a *S. cerevisiae* (SC) control in CDGJM (top panel) and GJ (bottom panel): (a) MP2 + SC3; (b) MP2 + SC4; (c) MP2 + SC5; (d) MP2 + SC6; (e) MP2 + SC7; (f) MP2 + SC50%. The growth of SC monoculture (◦) and MP2 prior to sequential inoculation (--) was monitored via flow cytometry. The growth of MP2 (▲) and *S. cerevisiae* (-■-) after sequential inoculation was quantified via lysine and YPD plate counts at four time-points: 24 h, 48 h and 72 h after secondary inoculation, and at fermentation completion.

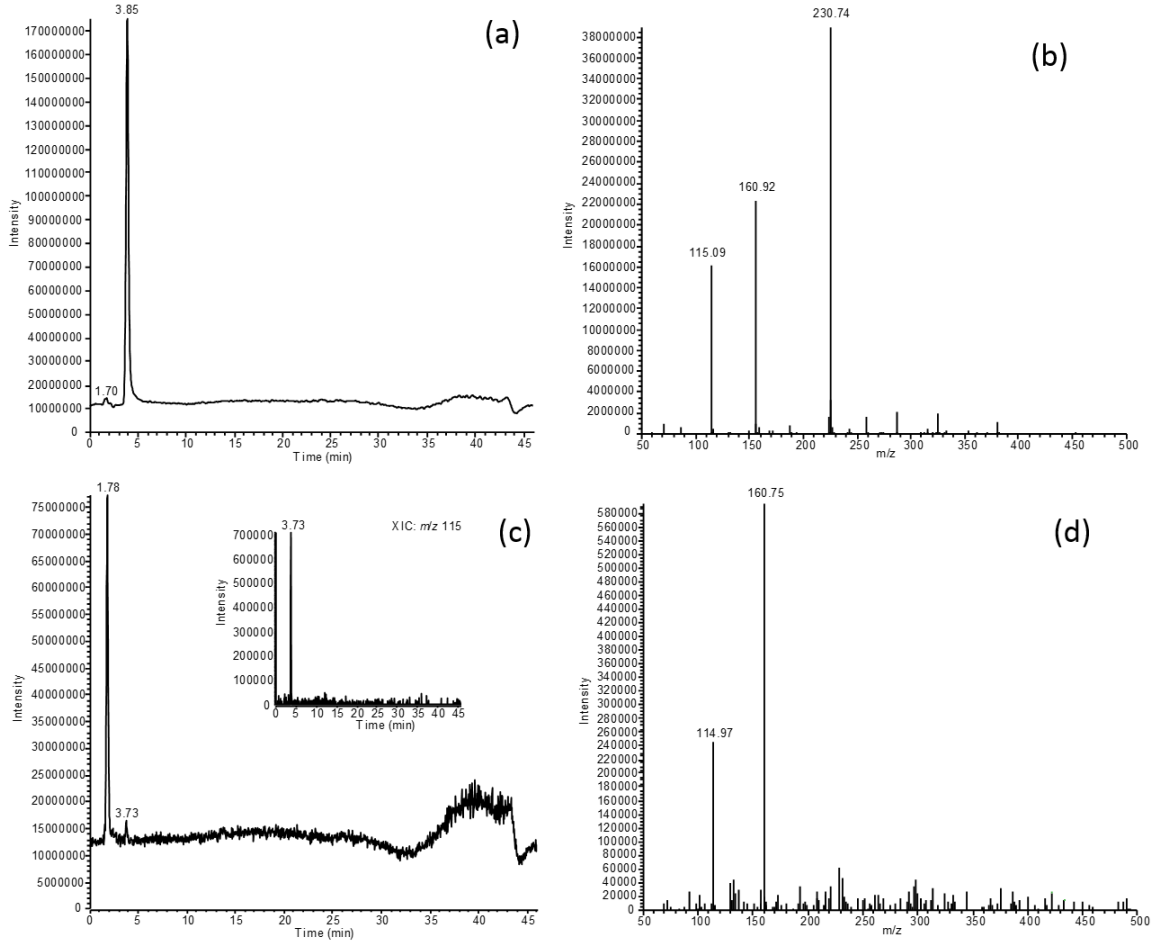

Fig. A.3. Results for identification of an unknown peak isolated by HPLC and analysed by HPLC-MS with electrospray ionisation in negative mode, showing (a) HPLC-MS chromatogram and (b) full scan MS of fumaric acid standard (*m*/*z* 115 = $[M-H]^-$, *m*/*z* 161 = fumaric/formic adduct ion, *m*/*z* 231 = fumaric/fumaric cluster ion), and (c) HPLC-MS chromatogram with extracted ion chromatogram (XIC) of *m*/*z* 115 (inset) and (d) full scan MS of the unknown compound with retention time and full scan MS consistent with fumaric acid.

Table A.2. Fumaric acid concentrations and yields in partial fermentations (PF) and final fermentations (FF) conducted with six MP2 sequential inoculation regimes (Table 1) and an *S. cerevisiae* control (SC) in CDGJM and GJ.

Medium	Yeast treatment	Fumaric acid (mg/L)		Fumaric acid yield (mg/g)	
		PF	FF	PF	FF
CDGJM	MP2 + SC3	66 ± 3^{E}	9 ± 0^{D}	1.34 ± 0.07^{A}	0.036 ± 0^{D}
	MP2 + SC4	73 ± 4^{DE}	9 ± 0^{D}	1.15 ± 0.06^{B}	0.037 ± 0^{D}
	MP2 + SC5	78 ± 2^{CD}	12 ± 1^{C}	1.10 ± 0.05^{BC}	0.047 ± 0.002^{C}
	MP2 + SC6	81 ± 2^{BC}	13 ± 1^{C}	1.01 ± 0.03^{C}	0.050 ± 0.004^{C}
	MP2 + SC7	90 ± 2^{AB}	16 ± 0^{B}	1.03 ± 0.01^{C}	0.062 ± 0.001^{B}
	MP2 + SC50%	97 ± 2^{A}	21 ± 1^{A}	0.74 ± 0.01^{D}	0.083 ± 0.004^{A}
	SC		7 ± 1^{E}		0.027 ± 0.002^{E}
GJ	MP2 + SC3	340 ± 10^{D}	23 ± 1 D	4.97 ± 0.21	0.09 ± 0^{D}
	MP2 + SC4	387 ± 6^{CD}	26 ± 1 CD	4.99 ± 0.26	0.10 ± 0^{CD}
	MP2 + SC5	430 ± 10^{BC}	30 ± 2 C	5.11 ± 0.31	0.12 ± 0.01^{C}
	MP2 + SC6	473 ± 6^{B}	42 ± 2 B	5.09 ± 0.12	0.16 ± 0.01^{B}
	MP2 + SC7	497 ± 15^{B}	42 ± 3 B	5.21 ± 0.04	0.17 ± 0.01^{B}
	MP2 + SC50%	570 ± 56^{A}	74 ± 2 A	4.97 ± 0.41	0.29 ± 0.01^{A}
	SC		15 ± 1 E		0.06 ± 0^{E}

Table A.3. Pearson's correlation coefficients between metabolites in partial fermentations (PF) and final fermentations (FF) in CDGJM (lower matrix, i.e. below the empty diagonal) and GJ (upper matrix, i.e. above the empty diagonal). Significant correlations ($P < 0.05$) are in bold.

Variables	Sugar_PF	Ethanol_PF	Ethanol_FF	Glycerol_PF	Glycerol_FF	Acetic acid_PF	Acetic acid_FF	Succininc acid_PF	Succinic acid_FF	Fumaric acid_PF	Fumaric acid_FF	Malic acid PF	Malic acid FF
Sugar_PF		**0.98**	**-0.98**	**0.96**	**0.62**	-0.27	**-0.94**	**0.84**	**0.87**	**0.96**	**0.95**	-0.21	-0.05
Ethanol PF	**0.97**		**-0.97**	**0.96**	**0.63**	-0.26	**-0.92**	**0.88**	**0.87**	**0.96**	**0.94**	-0.20	-0.03
Ethanol FF	**-0.94**	**-0.91**		**-0.96**	**-0.89**	0.30	**0.85**	**-0.82**	-0.35	**-0.93**	**-0.85**	0.20	0.17
Glycerol PF	**0.72**	**0.59**	**-0.73**		**0.72**	-0.11	**-0.94**	**0.91**	**0.88**	**0.95**	**0.89**	-0.08	0.09
Glycerol FF	**-0.60**	-0.39	**-0.93**	**-0.72**		0.15	**-0.61**	**0.61**	0.04	**0.64**	**0.54**	-0.21	-0.04
Acetic acid PF	**-0.70**	**-0.74**	**0.59**	-0.42	0.31		0.02	-0.02	-0.09	-0.24	-0.44	**0.51**	**0.63**
Acetic acid FF	-0.44	-0.37	**0.96**	-0.23	**-0.97**	-0.08		**-0.80**	**-0.74**	**-0.90**	**-0.90**	0.12	0.01
Succininc acid PF	**0.89**	**0.79**	**-0.84**	**0.83**	**-0.74**	**-0.59**	-0.38		**0.76**	**0.88**	**0.75**	0.00	0.26
Succinic acid FF	**0.78**	**0.79**	**-0.97**	0.32	**0.96**	**-0.51**	**-0.98**	**0.64**		**0.88**	**0.65**	-0.18	0.11
Fumaric acid PF	**0.93**	**0.84**	**-0.94**	**0.83**	**-0.71**	**-0.50**	**-0.50**	**0.87**	**0.65**		**0.89**	-0.13	0.01
Fumaric acid FF	**0.97**	**0.94**	**-0.75**	**0.65**	**0.46**	**-0.65**	**-0.58**	**0.80**	**0.67**	**0.92**		-0.31	-0.26
Malic acid PF	**-0.91**	**-0.93**	**0.79**	**-0.48**	0.40	**0.80**	0.22	**-0.73**	**-0.71**	**-0.72**	**-0.85**		0.23
Malic acid FF	**-0.51**	**-0.59**	**-0.84**	-0.25	**0.93**	**0.66**	**-0.93**	-0.42	**0.86**	-0.24	0.35	**0.71**	

CHAPTER 4

The evolution of *Lachancea thermotolerans* is driven by geographic origin, anthropisation and flux between different ecosystems

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Ana Hranilovic
Contribution to the Paper	Conceptualisation, formal analysis, investigation, methodology, software, visualisation, writing – original draft, writing – review and editing
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 12.2.18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Marina Bely
Contribution to the Paper	Conceptualization, formal analysis, funding acquisition, methodology, project administration, writing – review and editing
Signature	Date 20.2.18

Name of Co-Author	Isabelle Masneuf-Pomarede
Contribution to the Paper	Conceptualization, formal analysis, funding acquisition, methodology, project administration, writing – review and editing
Signature	Date 20.02.2018

Name of Co-Author	Vladimir Jiranek		
Contribution to the Paper	Conceptualization, funding acquisition, project administration, project administration, writing – review and editing		
Signature		Date	12.2.18

Name of Co-Author	Warren Albertin		
Contribution to the Paper	Formal analysis, investigation, software, visualisation, writing – review and editing		
Signature		Date	20.2.18

PLOS ONE

RESEARCH ARTICLE

The evolution of *Lachancea thermotolerans* is driven by geographical determination, anthropisation and flux between different ecosystems

Ana Hranilovic[1,2], Marina Bely[3], Isabelle Masneuf-Pomarede[3,4], Vladimir Jiranek[1,2], Warren Albertin[3,5]*

1 The Australian Research Council Training Centre for Innovative Wine Production, Adelaide, South Australia, Australia, **2** Department of Wine and Food Science, The University of Adelaide, Urbrrae, South Australia, Australia, **3** Unité de recherche Œnologie, Institut de la Science de la Vigne et du Vin, University of Bordeaux, Villenave d'Ornon, France, **4** Bordeaux Sciences Agro, Gradignan, France, **5** ENSCBP, Bordeaux INP, Pessac, France

* warren.albertin@u-bordeaux.fr

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Abstract

The yeast *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) is a species with remarkable, yet underexplored, biotechnological potential. This ubiquist occupies a range of natural and anthropic habitats covering a wide geographic span. To gain an insight into *L. thermotolerans* population diversity and structure, 172 isolates sourced from diverse habitats worldwide were analysed using a set of 14 microsatellite markers. The resultant clustering revealed that the evolution of *L. thermotolerans* has been driven by the geography and ecological niche of the isolation sources. Isolates originating from anthropic environments, in particular grapes and wine, were genetically close, thus suggesting domestication events within the species. The observed clustering was further validated by several means including, population structure analysis, F-statistics, Mantel's test and the analysis of molecular variance (AMOVA). Phenotypic performance of isolates was tested using several growth substrates and physicochemical conditions, providing added support for the clustering. Altogether, this study sheds light on the genotypic and phenotypic diversity of *L. thermotolerans*, contributing to a better understanding of the population structure, ecology and evolution of this non-*Saccharomyces* yeast.

Introduction

The terms 'yeast' and '*Saccharomyces cerevisiae*' are often used interchangeably. Not surprisingly so; this microorganism, accompanying humans' progress since Neolithic times [1], is widely used for the production of food, beverages, biofuel and a variety of biochemicals. It is also the best studied eukaryotic model organism, with genome sequences available for hundreds of strains [2–4], and ongoing projects aimed at determining biological functions and genetic interactions of each and every component of its genome [5, 6]. Less is known about

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other species, commonly referred to as 'non-conventional' or 'non-*Saccharomyces*' yeasts. Scientific interest in them is, however, gaining momentum, as their uncommon physiological, metabolic and cellular functions warrant their further exploration and, ultimately, biotechnological application. One species of remarkable, yet underexplored, biotechnological potential is *Lachancea thermotolerans*.

Formerly known as *Kluyveromyces thermotolerans*, *L. thermotolerans* is the type species of the genus *Lachancea* [7]. This genus was proposed by Kurtzman in 2003 to accommodate a group from several different genera showing similarities at the rRNA level. To date, the genus harbours 11 other species: *L. cidri*, *L. dasiensis*, *L. fantastica*, *L. fermentati*, *L. kluyveri*, *L. lanzarotensis*, *L. meyersi*, *L. mirantina*, *L. nothofagi*, *L. quebecensis* and *L. walti*. From the ecological viewpoint, most *Lachancea* species are ubiquitous [8]. Accordingly, *L. thermotolerans* commonly occupies a range of natural and anthropic habitats, including insects, plants, soil and horticultural crops, in particular grapes and wine [9–12]. As so-called protoploid *Saccharomycetaceae*, the *Lachancea* species have diverged from the *S. cerevisiae* lineage prior to the ancestral whole genome duplication, and as such offer a complementary model to study evolution and speciation in yeast [13].

Apart from the taxonomic re-classification of *L. thermotolerans*, the DNA sequencing era also resulted in extensive genomic information. The nuclear genome of the type strain CBS 6430 is 10.6 Mb and contains 5,350 annotated genes organised in eight chromosomes [13, 14]. Mitochondrial genome sequences are available for 50 strains, and are highly conserved within the species [9]. Despite the ample genomic information, the ploidy of *L. thermotolerans* remains controversial; diploid according to some authors [13, 14], haploid according to the others [9, 15].

Another underexplored trait is the peculiar ability of *L. thermotolerans* to produce lactic acid during alcoholic fermentation [16]. Lactic acid production is an uncommon metabolic activity among yeasts [17] but it is, however, of great biotechnological interest [18, 19]. The maximum reported lactic acid concentration obtained during *L. thermotolerans* alcoholic fermentation is 16.6 g/L [15]. In comparison, wildtype *S. cerevisiae* strains in such conditions normally produce only about 0.2–0.4 g/L [18, 19]. While yields obtained by *L. thermotolerans* remain insufficient for industrial bulk chemical production, they are of interest for processes in which alcoholic fermentation with concomitant acidification is a benefit; notably winemaking.

Indeed, the use of *L. thermotolerans* inocula to partially conduct fermentation is being increasingly explored in winemaking [20–23]. The resultant biological acidification is considered to positively affect the organoleptic quality and microbial stability of the resulting wines [16]. Other positive chemical and sensorial modulations include lower final ethanol content [21], increasingly in demand on the market [24], and improved wine aroma, flavour and mouthfeel [16, 20, 21]. Accordingly, several *L. thermotolerans* co-starters are now commercially available to be used in wine fermentations with either simultaneously or sequentially inoculated *S. cerevisiae* [16].

Population genetics studies in several yeast species, including *L. thermotolerans*, have revealed differentiation of subpopulations according to their geographical and/or ecological origin [25]. In *L. thermotolerans*, grouping based on the geographical origin has been determined by the mitochondrial and nucleic DNA analysis of 50 isolates [9]. Nonetheless, information on population diversity, evolution and structure is lacking. In the current study, we explore the relationships of 172 isolates from diverse ecological niches worldwide. Using a 14-loci microsatellite genotyping method, coupled with phenotyping assays, we demonstrate that both geographic localisation and anthropisation have significantly contributed to the diversity and evolution of *L. thermotolerans*.

Materials and methods

Yeast isolates, culture conditions and DNA preparation

Yeast isolates catalogued as *L. thermotolerans* were obtained from multiple yeast culture collections and generous laboratories worldwide. Excluding any obvious issues of selective enrichment inherent to any culture-based study, the sample set represented diverse ecological niches (e.g. oenological environments, plant material, insects) covering a wide geographic span (S1 Table). The isolates were mapped in Fig 1 using R package maps [26]. In addition, the type strains of 11 other *Lachancea* species (S1 Table), were included in the study. Cryogenically stored isolates (-80˚C in 25% glycerol) were cultured on YPD plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar) for 3 days at 24˚C. DNA template for genotyping was prepared by heating a suspension of approximately 10^7 cells in 100 μL of 20 mM NaOH at 94˚C for 10 minutes, followed by storage at -20˚C. For phenotyping purposes, approximately 10^7 cells were grown for 24 hours at 24˚C in 500 μL YPD agitated on a rotary shaker in deep 96-well plate format.

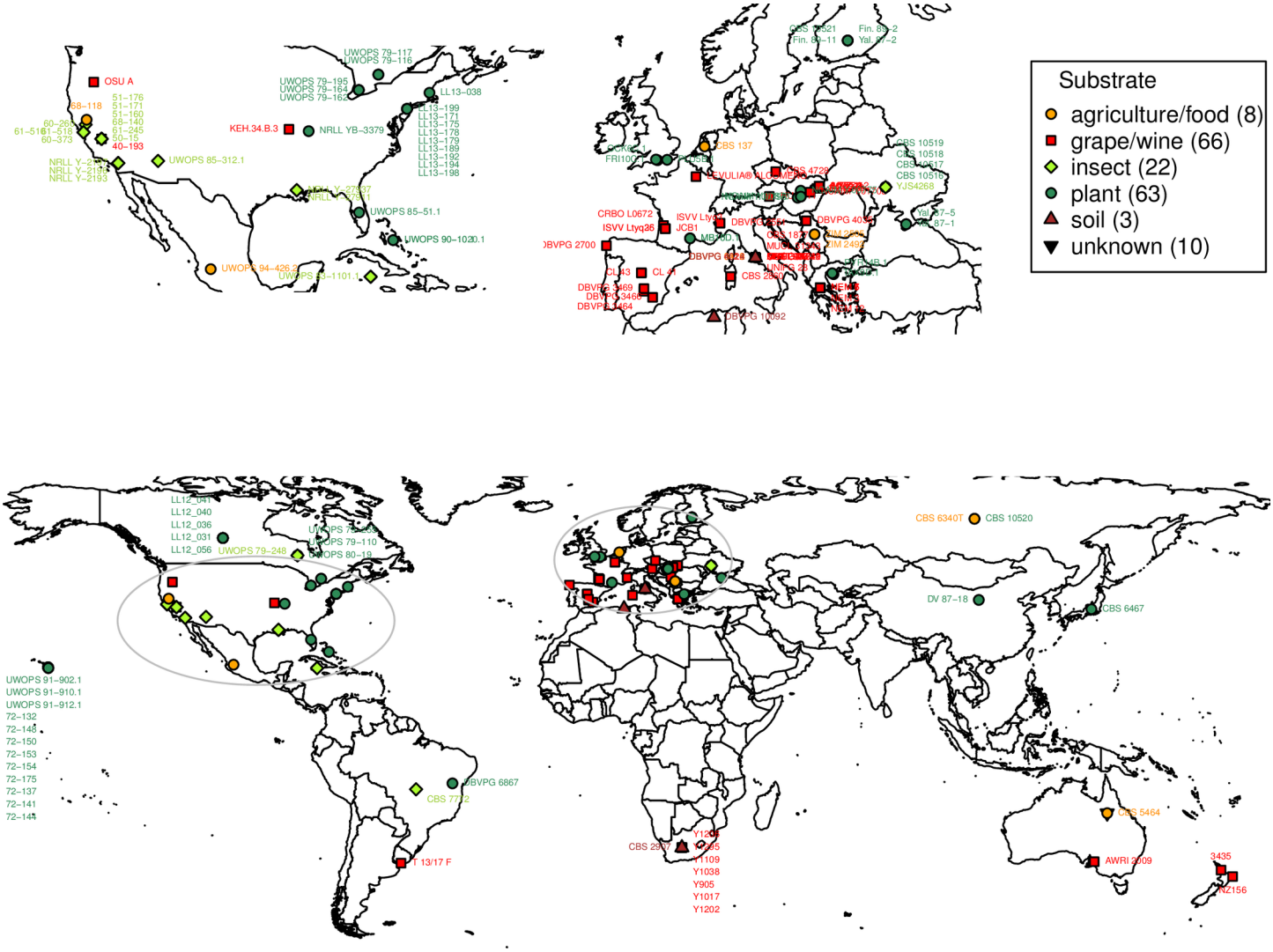

Fig 1. Geographic origin of the genotyped *L. thermotolerans* isolates obtained from different substrates. Isolates with unknown origin (see S1 Table) are not represented on the map.

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Microsatellite loci

Microsatellite markers were detected within the genomic sequence of *L. thermotolerans* CBS 6340 type strain as described previously [27]. Primers were designed using the 'Design primers' tool on the SGD website (http://www.yeastgenome.org/cgi-bin/web-primer). In addition, five microsatellite loci developed by Banilas et al. [15] were included in the study. In order to reduce the cost associated with primer fluorescent labelling, forward primers were tailed on the 5'-end with the M13 sequence as described by Schuelke [28]. Amplification specificity and optimal PCR conditions were assessed for all the loci (Table 1).

Microsatellite amplification

PCRs were carried out in a final volume of 15 μL containing 1 μL of DNA template solution, 1X Taq-&GO (MP Biomedicals, Illkirch, France), 0.05 μM of forward primer, and 0.5 μM of reverse and labelled primer. Universal M13 primers were labelled either with FAM-, HEX-, PET- or NED- fluorescent dyes (Eurofins MWG Operon, Les Ulis, France). Amplifications were performed in an iCycler (Biorad, Hercules, CA, USA) thermal cycler. The program comprised an initial denaturation of 1 minute at 94˚C; 30 annealing cycles with 30 seconds at 94˚C, 35 seconds at Tm, or Tm +10˚C with a 1˚C decrease per cycle until Tm was achieved, 30 seconds at 72˚C; and a final elongation at 72˚C for 10 minutes (Table 1). Upon initial amplification verification by a microchip electrophoresis system (MultiNA, Shimazdu), amplicons were diluted in deionised water (1,200-fold for HEX, 2,400-fold for PET, 3,600-fold for FAM and NED). Amplified fragment sizes varied from 86 to 566 base pairs, allowing for the multiplexing of all the amplicons in formamide. LIZ 600 molecular marker (100-fold dilution) was added to each multiplex, heated for 4 minutes at 94˚C. The sizes were of amplicons were then measured on an ABI3730 DNA analyser (Applied Biosystems), and recorded using GeneMarker Demo software v2.4.0 (SoftGenetics).

Microsatellite data analysis

Microsatellite data, i.e. recorded alleles sizes, were analysed using R software [26]. To examine the genetic relationships between genotyped *L. thermotolerans* isolates, a dendrogram was constructed using Bruvo's distance, particularly well adapted for cases of unknown/multiple ploidy levels [29], and Neighbour Joining (NJ) clustering [30] using poppr [31], ape [32], plotrix [33] and geiger [34] packages. The robustness of the identified clusters was further tested by several means, including node reliability assessment based on the algorithm by Prosperi et al. [35], a dendrogram construction with Bruvo's distance and UPGMA clustering, and principal component analysis (PCA) of the allelic data using ade4 package [36]. Population differentiation among obtained genetic groups was tested with the fixation index (F_{ST}), computed with polysat [37] package. Bootstrapping (n = 100) of the F_{ST} indexes was performed, and confidence intervals were calculated for the obtained values.

Population structure analysis based on the Bayesian approach was performed in R package LEA [38], using non-negative matrix factorization (sNMF) algorithm [39] for estimating individual ancestry coefficients. Models with number of populations (K) ranging from 1 to 40 were tested in 100 repetitions. Two models were selected for graphical representation: (i) K = 12 resulting in the lowest cross-entropy value, and (ii) K = 8 featuring the minimal ancestral population number and statistically equivalent cross-entropy to K = 12 (Kruskal–Wallis (KW) test; alpha = 0.05; package agricolae).

Analysis of molecular variance (AMOVA) was performed to assess whether the genetic distance was significantly explained by the substrate and geographical origin of isolation using the pegas package 0.6 [40] with 1,000 permutations. The relationship between genetic distance

Table 1. Microsatellite loci for *L. thermotolerans* genotyping.

Locus	Chr.	Coordinates	Motif	Primers[b]	Dye	Tm	Number of alleles	Size range	Coding sequence	Function
LT2A	2	610672–610712	ACA	F:TGACAAAAGTTTATCCCCCC	NED	62	24	385–438	XP_002552115	RNA-binding protein
				R:AGCACTGGCGATATCTTGGTT						
LT3A	3	129153–129184	AGC	F:CAGTACCAGCGCCAGTTCTA	PET	60[c]	25	293–352	XP_002552291	peroxin family member
				R:TTCTGTAGCTTGGGGTTGTGT						
LT3B	3	621739–621768	AGC	F:ACAGCAGCAGCAACAGCAA	NED	60[c]	9	86–111	no similarity found	na
				R:TTCGCCAAGCTGCTGATACTA						
LT4A	4	897528–897557	AGA	F:AGAAGGAGGACTCAGCGGATT	NED	60[c]	12	222–260	no similarity found	na
				R:ATGCCTAAGCGAATCAGATGC						
LT5B	5	317191–317225	ATA	F:AACGCTGACGTGCTGAAAGA	FAM	56	10	275–314	no similarity found	na
				R:GAAAGAGGCAGTAACGGATTT						
LT6B	6	134618–134640	ACA	F:TTCCTAGGTCTGGACCTCCAA	PET	60[c]	24	106–161	no similarity found	na
				R:TATTGCTGCTGCTTTTGCTG						
LT7A	7	1417616–1417644	TGT	F:TTTTTTCTTGATGCCCCGGT	FAM	60	10	131–150	XP_002555739	unknown; kinase suppression effect
				R: CGAACTGTGGTTCCTTCACAT						
LT8A	8	638186–638223	TCC	F:TGAAATAGAGTCCCGTGTGAA	PET	62	28	182–240	XP_002556192	vacuolar protein sorting
				R: AAATAACGCAGAAAGCGAGG						
LT8B	8	239222–239256	ATG	F:CAGCATCCGCACAGTAGCTAA	HEX	60[c]	9	261–286	XP_002555998	nuclear DNA helicase
				R:TTATCTCCTTATGCGGGCGTA						
MA2[a]	1	358081–358339	CA	F:AATTTTACGAAGGGAGAGAGGG	NED	60[c]	44	298–358	XP_002551596	bud-site selection nutrient signaling
				R:CTGCTGATGGTTTCTTCTGTGA						
MD3[a]	4	259537–259789	CAA	F:ACAAGAAAGCGAAGGAAAACAG	FAM	62	41	353–485	XP_002552792	unknown; hypothetical ORF
				R:CCCAGTAGAACGTGATTAAGCC						
ME11[a]	5	1381401–1381503	TG	F:CGGTTCTTAGCTTACCAACAGC	HEX	52	30	148–209	XP_002554109	mitotic spindle-associated protein
				R:ACTCGAACAGCCAGAGCTTAAC						
ME4[a]	5	576050–576253	GA	F:TGGCCTCTTCTGTCTTTCCTAA	HEX	60[c]	34	346–421	no similarity found	na
				R:CTCATCAACCAACACACTCCAT						
MH6[a]	8	372940–373089	TGT	F:CTTGCTGTTGTCGTAACCTCTG	PET	62	49	374–566	XP_002556014	ER-associated protein degradation; hypothetical ORF
				R:AATCCCAATAATCTCACACCC						

Chr.—chromosome; Tm—melting temperature.

[a] Banilas et al. (2016)

[b] M13 sequence was attached at the 5' end of the forward primer

c touch-down PCR commenced at Tm + 10˚C with a 1˚C decrease per cycle (see Materials and Methods).

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and geographic localisation was further verified by Mantel's test, allowing for the correlation of two distance matrices [41]. A genetic distance matrix obtained from microsatellite data was

correlated to a kilometric distance matrix obtained from coordinates of isolation using ade4 and sp packages [42], with the number of permutations set at 1,000.

Phenotypic analysis

Plate-based assays were performed to assess the growth rate and extent of 132 *L. thermotolerans* alongside 11 non-*thermotolerans* strains using different carbon sources and physicochemical conditions. Cell density and viability of pre-established yeast cultures was determined by flow cytometry coupled with propidium iodide DNA staining (Quanta SC MPL, Beckman Coulter, France). Cultures were diluted to 10^5 viable cells/mL and 2 μL of the obtained dilution was plated onto the appropriate media. All tests were performed in triplicate and, unless otherwise specified, incubated at 24˚C. Growth on standard YPD was evaluated at 3 temperatures: 24˚C (control), 8˚C (lower temperature) and 30˚C (higher temperature). In media for testing carbon utilisation, 2% glucose in YPD was substituted with 2% of one of the following carbon sources: fructose, xylose, mannose, galactose and glycerol. Osmotolerance was tested on plates containing 25% and 50% (w/v) of equimolar concentrations of glucose and fructose. Plates were imaged after 3, 6 and, for 8˚C condition, 10 days of incubation, and analysed using ImageJ2 software [43]. Upon converting uploaded images into a binary mode (black background, white foreground), colony sizes were determined via pixel density measurements using the ROI (region of interest) function. The colony size from each condition was compared to that on the standard YPD plate incubated at 24˚C for 3 or 6 days. Phenotyping data was analysed using R packages gplots, RColorBrewer, plot3D and agricolae [26]. A heatplot and a dendrogram (Euclidean distance and Ward clustering) were constructed to visualise the performance of individual phenotyped isolates. The differences among the determined *L. thermotolerans* genetic groups were tested with KW tests and post-hoc multiple comparison of modalities to assess levels of significance (alpha = 0.05).

Results

Polymorphic microsatellite markers for *L. thermotolerans*

The genomic sequence of *L. thermotolerans* type strain CBS 6340 was mined to identify tandem iterations of two or more nucleotides, located on positions other than the 5'-end and 3'-end of the chromosomes to exclude possible (sub)telomeric positions. Primer pairs were designed to amplify microsatellites, and their amplification specificity was ascertained using a sub-panel of 15 *L. thermotolerans* isolates using a microchip electrophoresis system MultiNA. Nine loci covering seven of the eight CBS 6340 chromosomes were retained for further analysis, five of these situated within putative coding sequences (Table 1). This set of microsatellites was extended with five markers previously used for *L. thermotolerans* genotyping [15]. All 14 markers were tested on 11 non-*thermotolerans* type strains, resulting in a good amplification of several markers (S2 Table). Some of the microsatellites developed for *L. thermotolerans* were therefore deemed as potentially suitable for diversity studies of other species belonging to the genus *Lachancea*. Eight loci were amplified in *L. quebecensis*, a species very closely related to *L. thermotolerans*. Amplification on all loci was, however, exclusive for *L. thermotolerans* strains, allowing for taxonomic confirmation at a species level, and thus confirming the identity of the 172 *L. thermotolerans* isolates. A comparable number of genotyped isolates originated from anthropic environments and nature: 75 and 88, respectively. Given the importance of the species to oenology, most of the samples from the anthropic milieu were reported as isolated from wine-related environments. Moreover, both anthropic and natural sub-groups comprised representatives from each continent/region of isolation.

All markers were polymorphic, with the number of alleles varying between 9 for loci LT3B and LT8B, and 49 for locus MH6 (Table 1). Interestingly, a single allele per locus was obtained for all tested isolates. Of the 172 isolates, 136 distinct genotypes were observed, confirming the discriminatory power of the microsatellite analysis.

Genetic proximity and divergence between *L. thermotolerans* isolates

Genetic relationships between *L. thermotolerans* isolates were further examined using Bruvo's distance and the NJ clustering method. The resulting dendrogram (Fig 2) enabled the visualisation and delineation of genetic groups. Some groups mainly comprised isolates originating from natural environments, grouped together based on their origin. One such group, 'Americas', consisted of 17 isolates mainly from natural habitats in the Americas (15/17), i.e. southern USA (9/17), Caribbean (4/17) and Brazil (2/17). A second 'wild' group, 'Canada trees', contained 20 North American isolates of which 18 were found to originate from plant material (*Quercus sp*. and *Prunus sp*.) across Canada. The third wild group ('Hawaii/California') harboured 21 isolates from Hawaii (12/21) and California (7/21), sourced from cacti and insects, respectively. Interestingly, identical genotypes could be observed among Hawaiian samples collected from the same habitat with a two-decade temporal span. Isolates 72_148 and 72_175 were collected approximately 20 years prior to the UWOPS 91–902.1, thus indicating the persistence of certain clonal variants. Finally, two separate, albeit small, clusters with tree exudate isolates from Eurasia were differentiated ('Other'). In addition to 'wild' groups, genetic proximity of isolates originating predominantly from anthropic habitats could also be observed. These 'domesticated' isolates were, in fact, grouped in two separate clusters. The larger group ('Domestic 1') consisted of 36 isolates, the majority from grapes and wine. The 23 oenological samples showed diverse geographic origin; two isolates from New Zealand (NZ156, 3435) and one from Australia (AWRI 2009) clustered closely to 20 European isolates, mostly from the Mediterranean region. It also included six isolates from agriculture and food-related environments from more distant geographical origins, i.e. Russia (CBS 6340T), Europe (CBS 137, DBVPG 3418, ZIM 2492) and North America (68_118, UWOPS 94–426.2). The second 'domesticated' group, 'Domestic 2' contained 21 grape/wine representatives from different continents, including Europe (Italy, Spain, Austria), Africa (South Africa) and Americas (USA, Uruguay). The remaining two South African isolates from soil (CBS 2907, DBVPG 10092) also clustered in this group, as well as the two isolates of unknown origin (IMAT 2508, IMAT 2510). The remaining genetic clusters were mixed with regards to the location and/or substrate of isolation of their constituents. Seven isolates from 'Mix Eastern Europe' formed one such branch. Four of these were isolated from grapes, and three from other plant material (*Quercus sp*. and *Betula sp*.). These clustered close to a group with a total of 24 isolates from Europe (16/24) and North America (8/24), with the representatives of oenological (13/24) and natural habitats (9/24) from both continents, i.e. 'Mix Europe/North America'. In addition to 12 European oak isolates, the last mixed group ('Europe oak/France grapes') encompassed four isolates associated with grapes originating from two French wine regions (i.e. Burgundy and Bordeaux), an Australian and an isolate of unknown origin.

Validation of observed clustering

Several approaches were used to validate the proposed clustering identified on the Bruvo's NJ dendrogram (Fig 3). As classical bootstrapping is poorly reliable with microsatellite data, the Prosperi et al. [35] algorithm-based reliability assessment was used to test the robustness of the tree nodes. The reliability values of all major tree nodes exceeded 70% (i.e. bootstrap support > 70; Fig 3B), thus strongly supporting the observed clustering. Next, an UPGMA

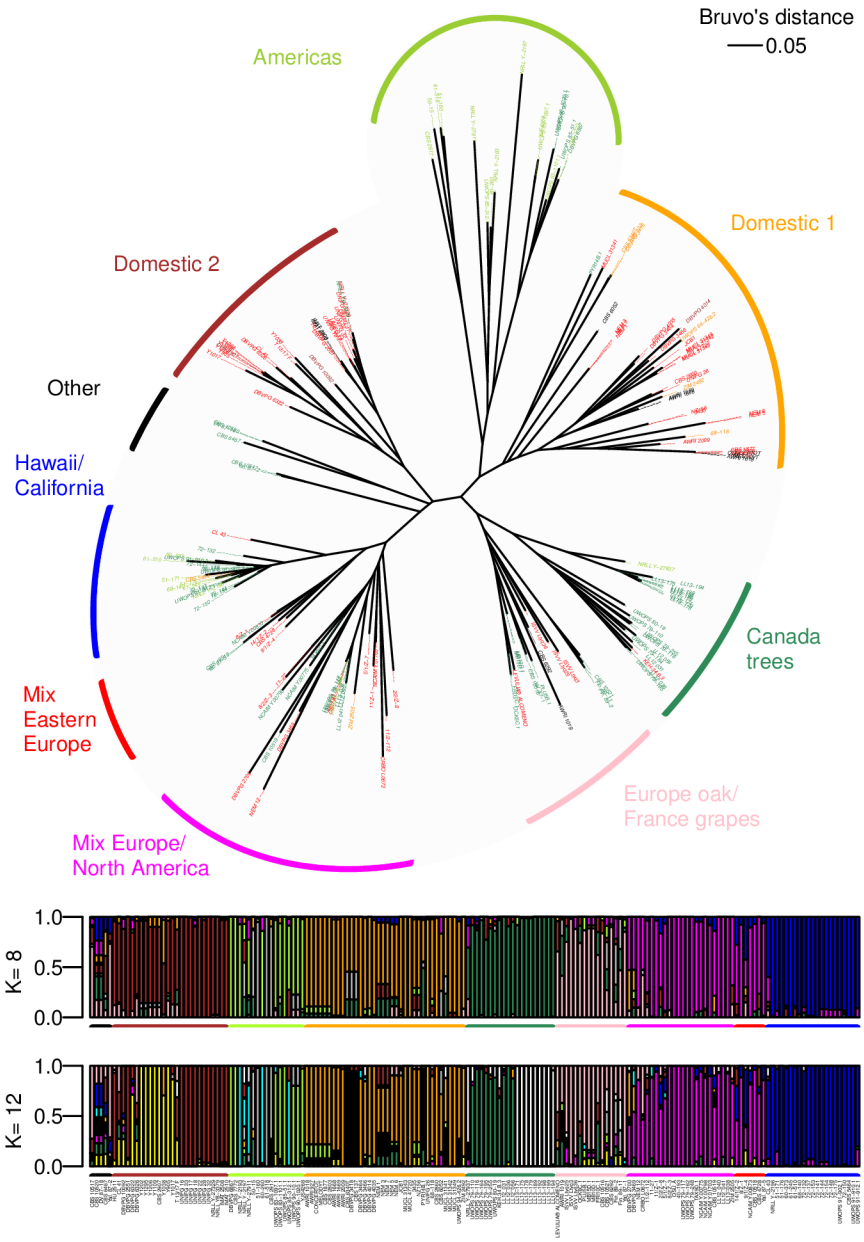

Fig 2. Genetic relationships between 172 *L. thermotolerans* isolates determined using 14 microsatellite makers. Colour-coding of isolates corresponds to isolation substrate, as per Fig 1. (A) Dendrogram constructed using Bruvo's distance and NJ clustering. (B) Barplot representing population structure (K = 8 and K = 12). The posterior probability (y-axis) of assignment of each isolate (vertical bar) to inferred ancestral populations is shown with different colours.

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algorithm was used as an alternative to NJ clustering to plot Bruvo's distance matrix. Both clustering methods resulted in largely consistent genetic grouping (Fig 3C), albeit 'Mix Eastern Europe' clustered among the 'Mix Europe/North America' group on the UPGMA dendrogram. A congruent separation of genetic groups could also be observed on the PCA plot of the genetic polymorphism data (Fig 3D), showing a co-localisation of the 'Mix Eastern Europe' and 'Mix Europe/North America' group, and a suitably resolved partitioning of other groups.

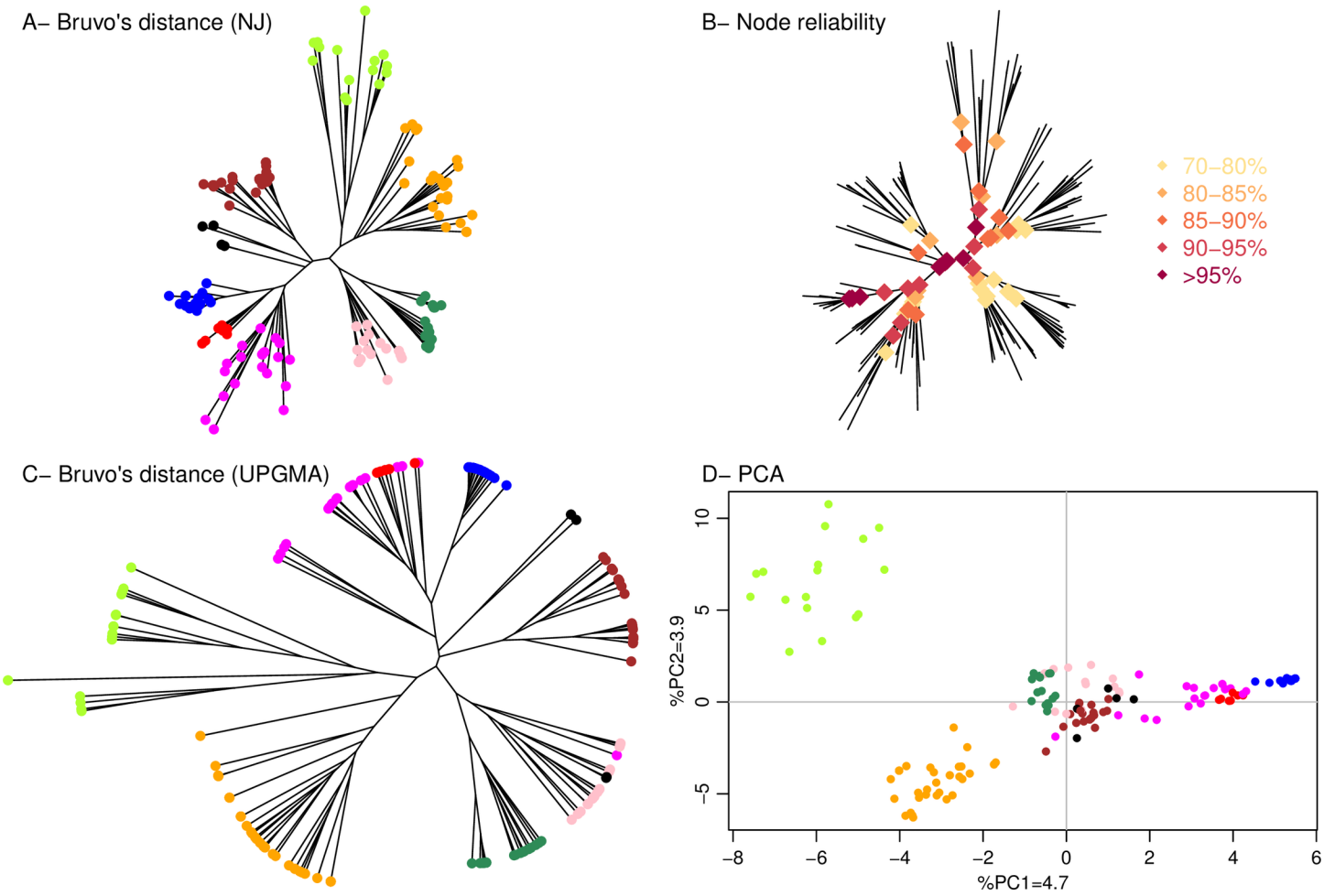

Fig 3. Genetic clustering of 172 *L. thermotolerans* isolates determined using 14 microsatellite makers. Each dot represents a genotype, with colours corresponding to determined genetic groups as per Fig 2. (A) Dendrogram constructed Bruvo's distance and NJ clustering. (B) Reliability assessment of the nodes of the dendrogram constructed using Bruvo's distance and NJ clustering. (C) Dendrogram constructed Bruvo's distance and UPGMA clustering. (D) PCA of the allelic data.

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In order to evaluate the differentiation of these populations, a pairwise fixation index F_{ST} was calculated for eight genetic groups (Table 2), as two minor groups ('Other') were excluded due to insufficient population size. Overall, a significant differentiation between populations was suggested, with the lowest pairwise F_{ST} value between the 'Mix Eastern Europe' and 'Mix Europe/North America' clusters, in accord with previous observations. Conversely, 'Hawaii/California' was the most differentiated population, followed by the 'Canada trees'. Interestingly, a comparably low degree of differentiation was obtained between 'Domestic 2' and 'Mix Eastern Europe' and 'Americas' groups, while 'Domestic 1' had the lowest pairwise F_{ST} with 'Europe oak/France grapes' group.

Population structure analysis was further conducted to infer ancestral populations (Fig 2B). The number of populations (K) ranged from 1 to 40. The absolute lowest cross-entropy values were found for K = 12, but the cross-entropy values were statistically equivalent (KW test) for K = 8 and up to K = 20 (S1 Fig). Among the 'wild' groups, the 'Hawaii/California' isolates were assigned to a distinct single ancestry, regardless of the total number of populations. The group of 'Americas' isolates, conversely, showed less homogeneity with multiple ancestries. A single

Table 2. Pairwise F_{ST} distance matrix. F_{ST} values are given in the upper matrix, whereas the lower matrix indicates bootstrap values and, in brackets, associated confidence intervals.

	Hawaii /California	Domestic 2	Canada trees	Americas	Mix Europe/ North America	Domestic 1	Europe oak/ France grapes	Mix Eastern Europe
Hawaii /California	na	0.404	0.495	0.413	0.322	0.466	0.425	0.348
Domestic 2	0.404 (0.280–0.440)	na	0.28	0.205	0.271	0.28	0.227	0.204
Canada trees	0.495 (0.318–0.495)	0.28 (0.084–0.280)	na	0.26	0.31	0.342	0.218	0.319
Americas	0.413 (0.413–0.579)	0.205 (0.205–0.400)	0.260 (0.212–0.478)	na	0.272	0.273	0.216	0.258
Mix Europe/ North America	0.322 (0.322–0.522)	0.271 (0.248–0.371)	0.310 (0.204–0.420)	0.272 (0.241–0.349)	na	0.291	0.238	0.116
Domestic 1	0.466 (0.429–0.531)	0.280 (0.261–0.37)	0.342 (0.177–0.392)	0.273 (0.273–0.420)	0.291 (0.256–0.347)	na	0.225	0.256
Europe oak/ France grapes	0.425 (0.339–0.482)	0.227 (0.227–0.354)	0.218 (0.127–0.330)	0.216 (0.216–0.346)	0.238 (0.172–0.269)	0.225 (0.225–0.331)	na	0.263
Mix Eastern Europe	0.348 (0.348–0.500)	0.204 (0.204–0.326)	0.3188 (0.185–0.407)	0.258 (0.205–0.315)	0.116 (0.116–0.314)	0.256 (0.203–0.288)	0.263 (0.189–0.294)	na

https://doi.org/10.1371/journal.pone.0184652.t002

and a dual ancestry was indicated for the 'Canada trees' group under the K = 8 and K = 12 scenario, respectively. This also seemed to be the case for the 'Domestic' groups of isolates. The two closely related mixed groups ('Mix Eastern Europe', 'Mix Europe/North America') showed similar population structure and a common ancestry, separate to that of 'Europe oak/France grapes' group. All these groups had a proportion of mixed origin isolates, especially in K = 12 simulation model. Overall, such results were in strong accord with the previous analysis (dendrograms, PCA, etc.).

To determine whether, and to what extent, the isolation substrate and geographic origin have significantly shaped *L. thermotolerans* genetic variation, an AMOVA was performed. The genetic distance was tested in relation to the continent/region of provenance (S1 Table), and habitat types grouped either as 'domestic' or 'wild'. Both geographic location and habitat were found to be significant, explaining 20.85% and 13.58% of variation, respectively ($P < 0.0001$). The relationship between genetic distance and geography was further confirmed by Mantel's test, indicating a significant link between the genetic and kilometric distance matrices of the whole sample set (P = 0.00009), samples from Europe (P = 0.00009) and Americas (P = 0.00019).

Phenotypic variability of the tested sample set

Phenotyping assays testing growth performance of 132 *L. thermotolerans* and 11 non-*thermotolerans* strains showed substantial variability at the species/strain level (Fig 4). Using the phenotypic dataset, a dendrogram was built using Euclidean distance and Ward's clustering. In general, one cluster of isolates (A) displayed a lower degree of growth on all substrates and conditions except glucose, with a subset of isolates growing well at 8˚C. Conversely, the second group (B) showed better growth on all tested substrates. Group C was less prolific at lower and higher temperatures, under osmotic stress and on xylose, compared to fructose, galactose, mannose and glycerol. The largest and the most variable group, D, contained isolates generally exhibiting osmotolerance. It featured a subset with lesser growth at 30˚C and on glycerol, and another with an extensive growth on xylose.

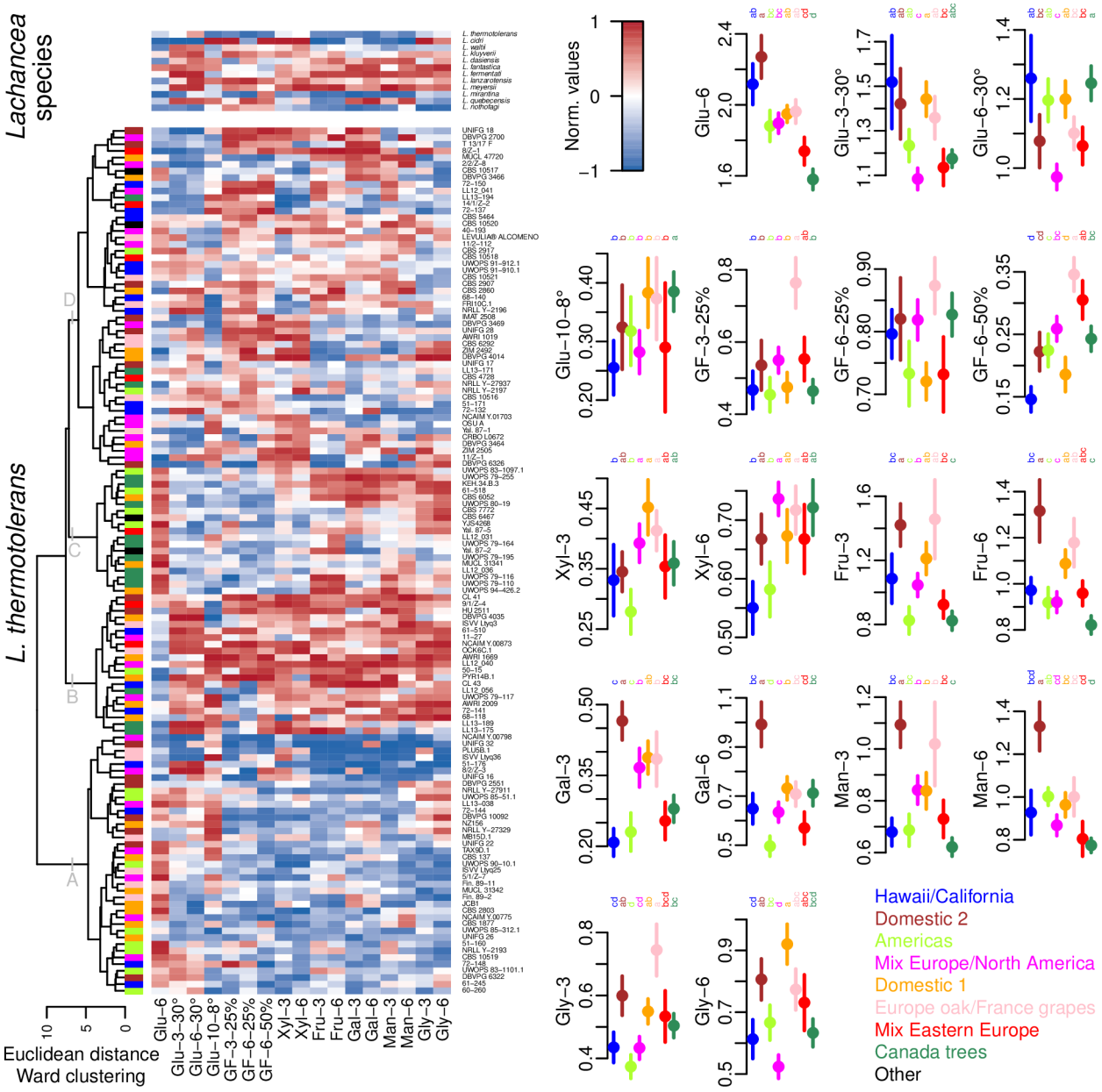

Fig 4. Phenotypic performance tested on plates using different carbon sources and physicochemical conditions. Dendrogram constructed with Euclidean distance and Ward clustering using normalised values of obtained growth of 132 *L. thermotolerans* and 11 non-*thermotolerans* strains in tested conditions, and/or a corresponding heatplot (left). Comparison of phenotypic performance at a genetic group level (right). Glu–glucose, GF–equimolar mixture of glucose and fructose, Xyl–xylose, Fru–fructose, Gal–galactose, Man–mannose, Gly–glycerol; unless otherwise specified, carbon sources were supplemented in concentration of 2%, and incubation temperature was 24˚C; numbers 3, 6 and 10 refer to the incubation duration. No quantifiable growth was observed for 'GF-3-50%', 'G-3-8°' and 'G-6-8°' modalities, thus not included graphical representation. Colours of the represented individuals/genetic groups correspond to Figs 2 and 3. Dots and bars represent normalised growth means and ranges, respectively, and letters denote significance levels between genetic groups (KW tests; alpha = 0.05).

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Several findings regarding the comparison of phenotypic performance at the genetic group level warrant highlighting. Firstly, the two 'domestic' groups ('Domestic 1' and 'Domestic 2') were among groups displaying superior growth aptitude in the majority of tested conditions. Next, the 'Europe oak/France grapes' group, followed by the 'Mix Eastern Europe' group, grew

best on plates testing osmotolerance. Interestingly, among natural isolates, these groups contained representatives sourced from grape musts in Sauternes and mummified grapes in Tokay, i.e. high sugar concentration substrates. Finally, a superior growth of 'Canada trees' isolates was observed at 8˚C compared to all other groups, without being impaired at 30˚C.

Discussion

Despite the rapid progress in DNA sequence analysis, microsatellites, rather than being obsolete, represent an informative, cost-effective tool for genotyping purposes, well adapted to large sample sizes. In fact, few genetic markers, if any, have found such widespread application for population diversity, ecology and evolution studies [44]. In yeasts, they were successfully applied to elucidate population structure of several species, including *S. cerevisiae* [45, 46], *S. uvarum* [47], *Torulaspora delbrueckii* [27], *Starmerella bacillaris* [48], *Hanseniaspora uvarum* [49] and *Brettanomyces bruxellensis* [50]. A set of five microsatellites has thus far been developed for *L. thermotolerans* [15], hereby extended with nine novel loci. This improved multilocus genotyping method was used on 172 isolates of diverse geographic and ecological origin, shedding light on *L. thermotolerans* diversity and population structure.

The resultant clustering revealed that the evolution of *L. thermotolerans* has been driven by the geography and the ecological niche of the isolation sources. This observation was subsequently confirmed with F-statistic, Mantel's test and AMOVA results. A link between phylogeny and geography has previously been reported for this species; a differentiation in relation to habitat has, conversely, not been established [9]. While the overall clustering remains congruent between both studies, the enlarged sample size with a balanced number of natural and anthropic isolates might account for such disparity. Indeed, the current study provides a compelling case for domestication occurrence within *L. thermotolerans* population, implying selection, intended or not, of variants related to anthropic environments. Scientific interest in microbial domestication is on the rise, and has been confirmed for *S. cerevisiae* [46, 51] and, more recently, for *T. delbrueckii* [27]. In each of these species, a separate wine-related lineage was detected, along with groups of individuals associated with other bioprocesses (e.g. baking, dairy, bioethanol etc.). Strikingly, two separate structured (F_{ST} = 0.280) *L. thermotolerans* domestic sub-populations with distant ancestries were hereby resolved, indicating multiple domestication events. Both clades were comprised largely of wine-related samples, with isolates from other anthropic environments (i.e. milk, distilling, fruits) clustering among the oenological ones. This suggests that, while some strains occupy diverse anthropic niches, further differentiation has not been achieved, although a larger sample subset (i.e. more isolates from anthropic environments other than grapes and wine) is required to confirm this hypothesis. Persistence in the grape and wine-related ecosystems involves survival in rather extreme conditions, ranging from the frequent exposure to agrochemicals, especially sulphur and copper, in vineyards, to the particularly harsh conditions during winemaking. Accumulated sugars exert the initial hyperosmotic stress, while fermentation leads to the accumulation of ethanol concentrations toxic for the yeast cells [52]. Several other (a)biotic stressors are also imposed, including oxygen and nutrient depletion, unfavourable physicochemical conditions (low pH, temperature shocks, SO_2 addition, etc.) and inhibitory microbial interactions [16, 52]. It is therefore plausible that such selective environments have led to differentiation of the two domestic clusters. Interestingly, both domestic clusters encompassed representatives from Europe and so-called 'New World' winegrowing countries (Australia and New Zealand for 'Domestic 1'; Americas and South Africa for 'Domestic 2'), hinting at a contributing role of viti-vinicultural expansion towards a wider dispersal of some genotypes. This is in line with

well-established expansion of grape-growing and winemaking practices from the Mediterranean basin to, ultimately, all wine regions across the globe [53].

Groups harbouring isolates from both cultivated and natural ecosystems, on the other hand, suggest the inter-connectivity of different ecological niches. A free flow of individuals can lead to absence of differentiation between cultivated and wild environments within a limited geographic span, as previously reported for *S. cerevisiae* communities in New Zealand [54] and USA [55]. The isolation proximity of certain samples within 'mixed' groups supports this observation, in particular within the 'Mix Eastern Europe' cluster, and among 'Mix Eastern Europe' and some 'Mix Europe/North America' genotypes. Common vectors for the inferred yeast dissemination between different ecological reservoirs are insects like bees, wasps and fruit flies [56, 57], while dispersal over a larger geographical span, also seen among mixed groups, requires other carriers—likely birds [58] and humans. The carryover between ecosystems is also indicated within *L. thermotolerans* 'natural' groups, in particular within the 'Hawaii/California' group. Given the spatial isolation of the Hawaiian islands, and their volcanic origin, migration events are to be presumed. This may also be the case with the seemingly most heterogeneous cluster of American isolates. Altogether, this dataset paints a comprehensive picture of *L. thermotolerans* evolution being shaped by anthropisation and geographic origin, as well as the macroorganism-mediated flux between different ecosystems.

Colonisation of a given niche is known to lead to evolutionary differentiation, harnessing adaptation to specific environmental conditions [25]. A set of plate-based growth assays was therefore carried out to examine whether the genotypic diversity is echoed on a phenotypic level. Interestingly, the overall prolific growth of 'domestic' groups could be observed, that might have contributed to their inter-continental dispersal and persistence in a large range of anthropic-related environments. Evidence for a narrower ecological adaptation was also suggested; e.g. a superior growth of Canadian isolates at 8˚C, possibly reflecting their adaptation to (sub)boreal climate conditions. Overall, a marked intra-specific diversity at a phenotypic level could be observed, to a degree supporting genetic differentiation. Further experimental verification of genotype-phenotype inter-groups relationships, however, is required to support such claims.

Apart from population structure, microsatellites can be used to elucidate life cycle of studied organisms [27, 59]. The ploidy of *L. thermotolerans* is controversial. Due to its sporulation ability, it was originally deemed to be a diploid species [14]. Conversely, Freel et al. [9] have reported most natural isolates to be haploid, in line with the single-allele microsatellite patterns observed in Banilas et al. [15]. As only one allele per locus was recorded on all 14 microsatellite loci for all 172 isolates used in this study, additional support for the haploid status of *L. thermotolerans* is provided. Nonetheless, absence of heterozygosity and/or diploidisation of haploids cannot be excluded. Further elucidation of the species' life cycle particularities is thus still required, as well as establishing sporulation conditions, mating patterns, occurrence and distribution of heterothallic and/or homothallic variants, and their potential implications for the diversity and evolution of the species.

In conclusion, this study provides a valuable insight into the genotypic and phenotypic diversity of *L. thermotolerans*, contributing to a better understanding of population structure, ecology and the evolution of this remarkable yeast species.

Supporting information

S1 Table. List of microorganisms used in the current study. Genotyping was undertaken on all the listed *L. thermotolerans* isolates, and phenotyping on isolates/strains in bold. Italicised isolates were obtained in the isolated DNA format.
(PDF)

S2 Table. Amplification of *L. thermotolerans* microsatellite markers on *Lachancea* species. Numbers are coded as following: 0—no amplification, 1—faint band, 2—medium intensity band, 3—full intensity band as determined using a microchip electrophoresis system.
(PDF)

S1 Fig. Kruskal–Wallis test of cross-entropy values for numbers simulated ancestral populations.
(PDF)

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Author Contributions

Conceptualization: Marina Bely, Isabelle Masneuf-Pomarede, Vladimir Jiranek.

Formal analysis: Ana Hranilovic, Marina Bely, Isabelle Masneuf-Pomarede, Warren Albertin.

Funding acquisition: Marina Bely, Isabelle Masneuf-Pomarede, Vladimir Jiranek.

Investigation: Ana Hranilovic, Warren Albertin.

Methodology: Ana Hranilovic, Marina Bely, Isabelle Masneuf-Pomarede, Warren Albertin.

Project administration: Marina Bely, Isabelle Masneuf-Pomarede, Vladimir Jiranek.

Software: Ana Hranilovic, Warren Albertin.

Visualization: Ana Hranilovic, Warren Albertin.

Writing – original draft: Ana Hranilovic.

Writing – review & editing: Marina Bely, Isabelle Masneuf-Pomarede, Vladimir Jiranek, Warren Albertin.

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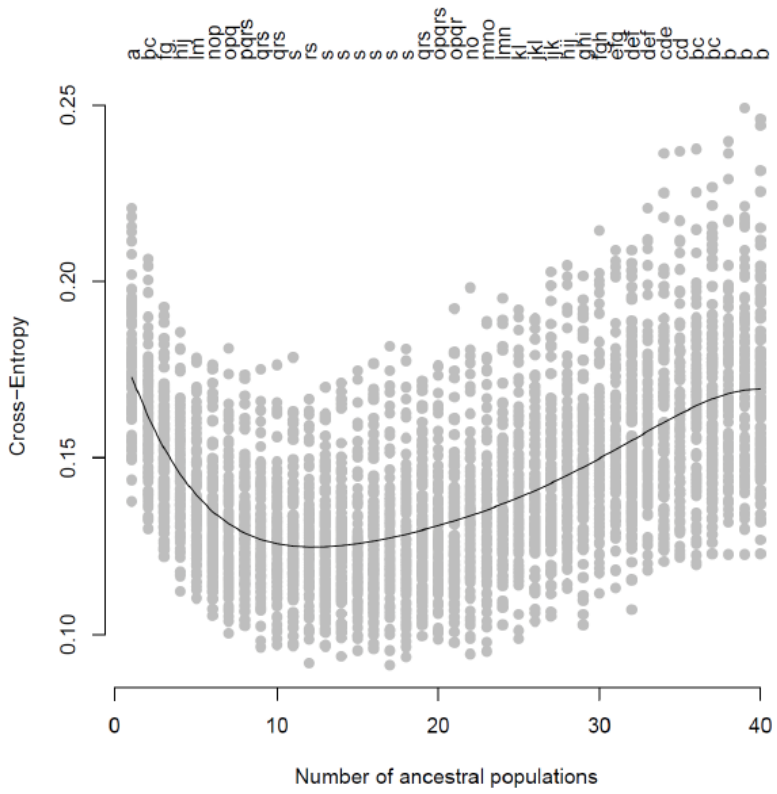

S1 Fig. Kruskal–Wallis test of cross-entropy values for numbers simulated ancestral populations.

S1 Table. List of microorganisms used in the current study. Genotyping was undertaken on all the listed *L. thermotolerans* isolates, and phenotyping on isolates/strains in bold. Italicised isolates were obtained in the isolated DNA format.

Species	Strain	Source	Isolation origin	Continent/region	Isolation habitat	Habitat type	Genetic group	Reference
L. thermotolerans	3435	Auckland University	New Zealand	Australia/Oceania	grapes	grape/wine	Domestic 1	na
L. thermotolerans	**11/2-112**	University of Debrecen	Slovakia, Mala Trna	Europe	grapes, mummified	grape/wine	Mix Europe/N. America	Sipiczki 2016 [a]
L. thermotolerans	**11/Z-1**	University of Debrecen	Slovakia, Mala Trna	Europe	grapes, mummified	grape/wine	Mix Europe/N. America	Sipiczki 2016 [a]
L. thermotolerans	11-27	University of Debrecen	Slovakia, Mala Trna	Europe	grapes, mummified	grape/wine	Mix Europe/N. America	Sipiczki 2016 [a]
L. thermotolerans	**14/1/Z-2**	University of Debrecen	Hungary, Erdöbénye	Europe	grapes, mummified	grape/wine	Mix E. Europe	Sipiczki 2016 [a]
L. thermotolerans	**2/2/Z-8**	University of Debrecen	Hungary, Sárazsadány	Europe	grapes, mummified	grape/wine	Mix Europe/N. America	Sipiczki 2016 [a]
L. thermotolerans	**5/1/Z-7**	University of Debrecen	Hungary, Vinicky	Europe	grapes, mummified	grape/wine	Mix Europe/N. America	Sipiczki 2016 [a]
L. thermotolerans	**8/2/Z-3**	University of Debrecen	Slovakia, Černochov	Europe	grapes, mummified	grape/wine	Mix Europe/N. America	Sipiczki 2016 [a]
L. thermotolerans	**8/Z-1**	University of Debrecen	Slovakia, Černochov	Europe	grapes, mummified	grape/wine	Mix E. Europe	Sipiczki 2016 [a]
L. thermotolerans	9/1/Z-4	University of Debrecen	Slovakia, Mala Trna	Europe	grapes, mummified	grape/wine	Mix E. Europe	Sipiczki 2016 [a]
L. thermotolerans	**40-193**	Phaff YCC	USA, California	North America	grapes, cv. Alicante Bouchet	grape/wine	Mix Europe/N. America	na
L. thermotolerans	**50-15**	Phaff YCC	USA, Southern California, Pinon Flats, San Jacinto Mnts.	North America	*Drosophila pseudoobscura*	insect	Americas	na
L. thermotolerans	**51-160**	Phaff YCC	USA, California, Aspen Valley, Yosemite area	North America	*Drosophila azteca*	insect	Americas	na
L. thermotolerans	**51-171**	Phaff YCC	USA, California, Mather, Yosemite area	North America	*Drosophila pseudoobscura*	insect	Hawaii/California	na
L. thermotolerans	**51-176**	Phaff YCC	USA, California, Mather, Yosemite area	North America	*Aulacigaster sp.*	insect	Hawaii/California	na
L. thermotolerans	**60-260**	Phaff YCC	USA, UCD campus	North America	*Aulacigaster sp.*on *Ulmus carpinifolia* exudate	insect	Americas	na
L. thermotolerans	60-373	Phaff YCC	USA, Lake Beryessa area	North America	*Drosophila pseudoobscura*	insect	Hawaii/California	na
L. thermotolerans	**61-245**	Phaff YCC	USA, North California Pacific Coast, Gualala River area	North America	*Aulacigaster sp.*	insect	Americas	na
L. thermotolerans	**61-510**	Phaff YCC	USA, Lake Beryessa area	North America	*Drosophila pseudoobscura*	insect	Hawaii/California	na
L. thermotolerans	**61-518**	Phaff YCC	USA, Lake Beryessa area	North America	*Drosophila melanogaster*	insect	Americas	na

L. thermotolerans	**68-118**	Phaff YCC	USA, Yuba City	North America	conveyer belt scrapings (Sunsweet Prune)	agriculture/food	Domestic 1	na
L. thermotolerans	**68-140**	Phaff YCC	USA, California	Australia/Oceania	bees	insect	Hawaii/California	na
L. thermotolerans	**72-132**	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	**72-137**	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	**72-141**	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	**72-144**	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	**72-148**	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	**72-150**	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum flux* clear drip	plant	Hawaii/California	na
L. thermotolerans	72-153	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum flux* tacky gum	plant	Hawaii/California	na
L. thermotolerans	72-154	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	72-175	Phaff YCC	Hawaii, Ahumoa, Saddle Rd area	Australia/Oceania	*Myoporum sandwicens*exudate	plant	Hawaii/California	na
L. thermotolerans	AWRI 1018	AWMCC	na	na	na	na	Domestic 1	na
L. thermotolerans	**AWRI 1019**	AWMCC	na	na	na	na	Europe oak/France grapes	na
L. thermotolerans	AWRI 1668	AWMCC	na	na	na	na	Domestic 1	na
L. thermotolerans	**AWRI 1669**	AWMCC	na	na	na	na	Domestic 1	na
L. thermotolerans	**AWRI 2009**	AWMCC	Australia, South Australia	Australia/Oceania	grapes	grape/wine	Domestic 1	na
L. thermotolerans	AWRI 927	AWMCC	Italy	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	**CBS 10516**	CBS-KNAW	Ukraine	Europe	*Quercus sp.* exudate	plant	Europe oak/France grapes	na
L. thermotolerans	**CBS 10517**	CBS-KNAW	Ukraine	Europe	*Quercus sp.* exudate	plant	Other	na
L. thermotolerans	**CBS 10518**	CBS-KNAW	Ukraine	Europe	*Quercus sp.* exudate	plant	Mix E. Europe	na
L. thermotolerans	**CBS 10519**	CBS-KNAW	Ukraine	Europe	*Quercus sp.* exudate	plant	Mix Europe/N. America	Freel et al. 2014 [b]
L. thermotolerans	**CBS 10520**	CBS-KNAW	Russia	Russia/Asia	*Quercus sp.* exudate	plant	Other	Freel et al. 2014 [b]
L. thermotolerans	**CBS 10521**	CBS-KNAW	Finland	Europe	*Quercus sp.* exudate	plant	Europe oak/France grapes	Freel et al. 2014 [b]
L. thermotolerans	**CBS 137**	CBS-KNAW	Netherlands	Europe	date	agriculture/food	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**CBS 1877**	CBS-KNAW	Italy	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	**CBS 2803**	CBS-KNAW	Italy	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	**CBS 2860**	CBS-KNAW	Italy, Sardinia	Europe	grape must	grape/wine	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**CBS 2907**	CBS-KNAW	South Africa	Africa	soil	soil	Domestic 2	Freel et al. 2014 [b]

L. thermotolerans	**CBS 2917**	CBS-KNAW	na	na	*Drosophila sp.*	insect	Hawaii/California	na
L. thermotolerans	**CBS 4728**	CBS-KNAW	former Czechoslovakia	Europe	grapes	grape/wine	Mix E. Europe	Freel et al. 2014 [b]
L. thermotolerans	**CBS 5464**	CBS-KNAW	Australia	Australia/Oceania	cotton seed	agriculture/food	Hawaii/California	na
L. thermotolerans	**CBS 6052**	CBS-KNAW	na	na	na	na	Domestic 1	na
L. thermotolerans	**CBS 6292**	CBS-KNAW	Australia	Australia/Oceania	na	na	Europe oak/France grapes	na
L. thermotolerans	**CBS 6340T**	CBS-KNAW	Russia	Russia/Asia	mirabelle plum conserve	agriculture/food	Domestic 1	Freel et al. 2014 [b], Naumova et al. 2007 [c]
L. thermotolerans	**CBS 6467**	CBS-KNAW	Japan	Russia/Asia	tree exudate	plant	Other	na
L. thermotolerans	**CBS 7772**	CBS-KNAW	Brazil	South/Central America	*Uca sp.*	insect	Americas	Freel et al. 2014 [b]
L. thermotolerans	**CL 41**	University of Leon	Spain	Europe	grapes	grape/wine	Domestic 2	na
L. thermotolerans	**CL 43**	University of Leon	Spain	Europe	grapes	grape/wine	Hawaii/California	na
L. thermotolerans	CONCERTO™	CHR Hansen	'Mediterranean country''	na	na	na	Domestic 1	na
L. thermotolerans	**CRBO L0672**	CRBOeno	France	Europe	grapes, fermentation	grape/wine	Mix Europe/N. America	na
L. thermotolerans	**DBVPG 10092**	DBVPG	Algeria	Africa	soil, apple orchard	soil	Domestic 2	na
L. thermotolerans	**DBVPG 2551**	DBVPG	Italy, Piemonte	Europe	wine cv. Barbera	grape/wine	Domestic 2	Freel et al. 2014 [b]
L. thermotolerans	**DBVPG 2700**	DBVPG	Spain, La Mancha, Campo de Santiago	Europe	grapes cv. Airen	grape/wine	Mix Europe/N. America	Freel et al. 2014 [b]
L. thermotolerans	DBVPG 3418	DBVPG	Italy	Europe	milk	agriculture/food	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	DBVPG 3464	DBVPG	Spain, La Mancha, Valdepenas	Europe	grapes	grape/wine	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**DBVPG 3466**	DBVPG	Spain, La Mancha, La Encomienda	Europe	grapes	grape/wine	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**DBVPG 3469**	DBVPG	Spain, La Mancha, Manzanares	Europe	grapes	grape/wine	Mix Europe/N. America	Freel et al. 2014 [b]
L. thermotolerans	**DBVPG 4014**	DBVPG	Italy	Europe	caverns	soil	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**DBVPG 4035**	DBVPG	ex Yugoslavia	Europe	grapes, must	grape/wine	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**DBVPG 6322**	DBVPG	Italy	Europe	grapes	grape/wine	Domestic 2	na
L. thermotolerans	**DBVPG 6326**	DBVPG	Italy	Europe	grapes, raisins	grape/wine	Domestic 2	na
L. thermotolerans	DBVPG 6867	DBVPG	Brazil	South/Central America	*Pilosocereus arrabida*	plant	Americas	Freel et al. 2014 [b]
L. thermotolerans	DV 87-18	na	"Far East"	Russia/Asia	*Quercus sp.*exudate	plant	Other	Naumova et al. 2007 [c]
L. thermotolerans	**Fin. 89-11**	na	Finland	Europe	*Quercus sp.*exudate	plant	Europe oak/France grapes	Naumova et al. 2007 [c]
L. thermotolerans	**Fin. 89-2**	na	Finland	Europe	*Quercus sp.*exudate	plant	Europe oak/France grapes	Freel et al. 2014 [b], Naumova et al. 2007 [c]

L. thermotolerans	**FRI10C.1**	NCYC	UK, Fritham, New Forest	Europe	*Quercus sp.*	plant	Europe oak/France grapes	Robinson et al. 2016 [d]
L. thermotolerans	**HU 2511**	BOKU	Austria	Europe	grapes	grape/wine	Domestic 2	na
L. thermotolerans	**IMAT 2508**	IMAT	na	na	na	na	Domestic 2	na
L. thermotolerans	IMAT 2510	IMAT	na	na	na	na	Domestic 2	na
L. thermotolerans	**ISVV Ltyq25**	ISVV	France, Sauternes	Europe	grapes, high sugar must	grape/wine	Europe oak/France grapes	na
L. thermotolerans	**ISVV Ltyq3**	ISVV	France, Sauternes	Europe	grapes, high sugar must	grape/wine	Europe oak/France grapes	na
L. thermotolerans	**ISVV Ltyq36**	ISVV	France, Sauternes	Europe	grapes, high sugar must	grape/wine	Europe oak/France grapes	na
L. thermotolerans	**JCB1**	ISVV	France, Sauternes	Europe	grapes, high sugar must	grape/wine	Domestic 1	na
L. thermotolerans	**KEH.34.B.3**	na	USA, Missouri, Ste. Genevieve	North America	grapes, fermentation	grape/wine	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	**LEVULIA® ALCOMENO**	AEB	France, Burgundy	Europe	grapes, fermentation	grape/wine	Europe oak/France grapes	na
L. thermotolerans	**LL12_031**	LL	Canada	North America	*Quercus sp.*tree bark	plant	Canada trees	na
L. thermotolerans	**LL12_036**	LL	Canada	North America	*Acer sp.* bark	plant	Canada trees	na
L. thermotolerans	**LL12_040**	LL	Canada	North America	*Acer sp.* bark	plant	Mix Europe/N. America	na
L. thermotolerans	**LL12_041**	LL	Canada	North America	*Quercus sp.*bark	plant	Mix Europe/N. America	na
L. thermotolerans	**LL12_056**	LL	Canada	North America	planted *Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	**LL13-038**	LL	USA, Massachusetts, Woburn	North America	*Quercus sp.*bark	plant	Mix Europe/N. America	na
L. thermotolerans	**LL13-171**	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	**LL13-175**	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	LL13-178	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	LL13-179	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	**LL13-189**	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	LL13-192	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na

L. thermotolerans	**LL13-194**	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	LL13-198	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	LL13-199	LL	Canada, New-Brunswick, Oak Point Provincial Park	North America	*Quercus sp.*bark	plant	Canada trees	na
L. thermotolerans	MB10D.1	NCYC	France, Montbarri	Europe	*Quercus sp.*	plant	Europe oak/France grapes	Robinson et al. 2016 [d]
L. thermotolerans	MB15D.1	NCYC	France, Montbarri	Europe	*Quercus sp.*	plant	Europe oak/France grapes	Robinson et al. 2016 [d]
L. thermotolerans	MELODY™	CHR Hansen	Mediterranean country''	na	na	na	Domestic 1	na
L. thermotolerans	**MUCL 31341**	MUCL	Italy	Europe	wine	grape/wine	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	**MUCL 31342**	MUCL	Italy	Europe	wine	grape/wine	Domestic 1	na
L. thermotolerans	MUCL 31343	MUCL	Italy	Europe	grapes, fermentation	grape/wine	Domestic 1	na
L. thermotolerans	MUCL 31349	MUCL	Italy	Europe	wine	grape/wine	Domestic 1	na
L. thermotolerans	**MUCL 47720**	MUCL	Italy	Europe	wine	grape/wine	Domestic 1	na
L. thermotolerans	**NCAIM Y.00775**	NCAIM	Hungary, Babat	Europe	*Carpinus betulu* exudate	plant	Mix Europe/N. America	na
L. thermotolerans	**NCAIM Y.00798**	NCAIM	Hungary, Csikóváralja	Europe	brown rotten *Quercus sp.*	plant	Mix Europe/N. America	na
L. thermotolerans	**NCAIM Y.00873**	NCAIM	Hungary, Budapest	Europe	rotten material of a cavity of *Betula pendula*	plant	Mix E. Europe	na
L. thermotolerans	**NCAIM Y.01703**	NCAIM	Hungary, Nagyeged	Europe	grapes	grape/wine	Mix Europe/N. America	na
L. thermotolerans	*NEM 1*	ITAP-DEMETER	Greece	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	*NEM 12*	ITAP-DEMETER	Greece	Europe	grapes	grape/wine	Mix Europe/N. America	na
L. thermotolerans	*NEM 3*	ITAP-DEMETER	Greece	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	*NEM 5*	ITAP-DEMETER	Greece	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	*NEM 6*	ITAP-DEMETER	Greece	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	*NEM 7*	ITAP-DEMETER	Greece	Europe	grapes	grape/wine	Domestic 1	na
L. thermotolerans	**NRLL Y-2193**	NRRL/ARS	USA, San Jacinto, California	North America	*Drosophila pseudoobscura*	insect	Americas	na
L. thermotolerans	**NRLL Y-2196**	NRRL/ARS	USA, San Jacinto, California	North America	*Drosophila pseudoobscura*	insect	Hawaii/California	na
L. thermotolerans	**NRLL Y-2197**	NRRL/ARS	USA, San Jacinto, California	North America	*Drosophila pseudoobscura*	insect	Americas	na
L. thermotolerans	**NRLL Y-27329**	NRRL/ARS	USA, West Virginia	North America	grapes	grape/wine	Domestic 2	na

L. thermotolerans	**NRLL Y-27911**	NRRL/ARS	USA, Louisiana	North America	gut of a fishfly	insect	Americas	Freel et al. 2014 [b]
L. thermotolerans	**NRLL Y-27937**	NRRL/ARS	USA, Louisiana	North America	surface of fishfly	insect	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	NRLL YB-3379	NRRL/ARS	USA, Marion, Illinois	North America	rotten log	plant	Domestic 2	na
L. thermotolerans	**NZ156**	CRPR	New Zealand	Australia/Oceania	grapes cv. Chardonnay	grape/wine	Domestic 1	na
L. thermotolerans	**OCK6C.1**	NCYC	UK, Ocknell, New Forest	Europe	*Quercus sp.*	plant	Europe oak/France grapes	Robinson et al. 2016 [d]
L. thermotolerans	**OSU A**	OSU	USA, Oregon	North America	grapes	grape/wine	Mix Europe/N. America	na
L. thermotolerans	**PLU5B.1**	NCYC	UK, Plumpton vineyard	Europe	*Quercus sp.*	plant	Europe oak/France grapes	Robinson et al. 2016 [d]
L. thermotolerans	**PYR14B.1**	NCYC	Greece, Pyradikia	Europe	*Quercus sp.*	plant	Domestic 1	Robinson et al. 2016 [d]
L. thermotolerans	**T 13/17 F**	University of the Republic	Uruguay	South/Central America	grapes cv. Tannat	grape/wine	Domestic 2	na
L. thermotolerans	**TAX9D.1**	NCYC	Greece, Taxiarchis	Europe	*Quercus sp.*	plant	Mix Europe/N. America	Robinson et al. 2016 [d]
L. thermotolerans	**UNIFG 26**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 1	na
L. thermotolerans	**UNIFG 28**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	**UNIFG 16**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	**UNIFG 17**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	**UNIFG 18**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	**UNIFG 22**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	**UNIFG 32**	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	UNIFG 33	UNIFG	Italy	Europe	wine	grape/wine	Domestic 2	na
L. thermotolerans	**UWOPS 79-110**	UWOPS	Canada, Ontario	North America	black knot, *Prunus virginiana*	plant	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 79-116**	UWOPS	Canada, Pinery	North America	black knot, *Prunus virginiana*	plant	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 79-117**	UWOPS	Canada, Pinery	North America	black knot, *Prunus virginiana*	plant	Mix Europe/N. America	Freel et al. 2014 [b]
L. thermotolerans	UWOPS 79-162	UWOPS	Canada, Ontario, Melbourne	North America	black knot, *Quercus rubra*	plant	Mix Europe/N. America	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 79-164**	UWOPS	Canada, Ontario, Melbourne	North America	black knot, *Prunus virginiana*	plant	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 79-195**	UWOPS	Canada, Ontario, Melbourne	North America	black knot, *Prunus virginiana*	plant	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	UWOPS 79-248	UWOPS	Canada, Ontario	North America	frass, Birch	plant	Mix Europe/N. America	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 79-255**	UWOPS	Canada, Ontario	North America	black knot, *Prunus pumila*	plant	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	UWOPS 80-19	UWOPS	Canada, Ontario	North America	black knot, *Prunus virginiana*	plant	Canada trees	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 83-1097.1**	UWOPS	Cayman Islands, Cayman Brac	South/Central America	*Gitona americana*, *Opuntia stricta*	plant	Americas	Freel et al. 2014 [b], Naumova et al. 2007 [c]

L. thermotolerans	**UWOPS 83-1101.1**	UWOPS	Cayman Islands, Cayman Brac	South/Central America	*Gitona americana*, *Opuntia stricta*	insect	Americas	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 85-312.1**	UWOPS	USA, Arizona, Tuscon	North America	*Drosophila carbonaria, Prosopis juliflora*	insect	Americas	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 85-51.1**	UWOPS	USA, Florida, Big Pine Key	North America	*Opuntia cubensis*	plant	Americas	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 90-10.1**	UWOPS	Bahamas, Exumas Cays, Shroud Cay	South/Central America	Columnar cactus	plant	Americas	Freel et al. 2014 [b]
L. thermotolerans	UWOPS 90-1020.1	UWOPS	Bahamas, Exumas Cays, Shroud Cay	South/Central America	coco plum	plant	Americas	Freel et al. 2014 [b]
L. thermotolerans	UWOPS 91-902.1	UWOPS	Hawaii, Saddle Rd Park	Australia/Oceania	flux (white), *Myoporum*	plant	Hawaii/California	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 91-910.1**	UWOPS	Hawaii, Saddle Rd Park	Australia/Oceania	flux (pink), *Myoporum*	plant	Hawaii/California	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 91-912.1**	UWOPS	Hawaii, Saddle Rd Park	Australia/Oceania	flux (white), *Myoporum*	plant	Hawaii/California	Freel et al. 2014 [b]
L. thermotolerans	**UWOPS 94-426.2**	UWOPS	Mexico, Jalisco	South/Central America	distillery, agave must	agriculture/food	Domestic 1	Freel et al. 2014 [b]
L. thermotolerans	YJS4269	na	Ukraine	Europe	*Drosophila sp.*	insect	Americas	na
L. thermotolerans	*Y1017*	IWBT	South Africa	Africa	grapes cv. Chardonnay	grape/wine	Domestic 2	na
L. thermotolerans	*Y1038*	IWBT	South Africa	Africa	grapes cv. Chardonnay	grape/wine	Domestic 2	na
L. thermotolerans	*Y1109*	IWBT	South Africa	Africa	grapes cv. Sauvignon blanc	grape/wine	Domestic 2	na
L. thermotolerans	*Y1202*	IWBT	South Africa	Africa	grapes cv. Sauvignon blanc	grape/wine	Domestic 2	na
L. thermotolerans	*Y1206*	IWBT	South Africa	Africa	grapes cv. Sauvignon blanc	grape/wine	Domestic 2	na
L. thermotolerans	*Y1295*	IWBT	South Africa	Africa	grapes, fermentation (Sauvignon blanc – Chardonnay blend)	grape/wine	Domestic 2	na
L. thermotolerans	*Y905*	IWBT	South Africa	Africa	grapes cv. Chenin blanc	grape/wine	Domestic 2	na
L. thermotolerans	**Yal. 87-1**	na	Russia, Crimea	Russia/Asia	*Quercus sp.* exudate	plant	Europe oak/France grapes	Naumova et al. 2007 [c]
L. thermotolerans	**Yal. 87-2**	na	Finland	Europe	*Quercus sp.* exudate	plant	Other	Naumova et al. 2007 [c]
L. thermotolerans	Yal. 87-5	na	Russia, Crimea	Russia/Asia	*Quercus sp.* exudate	plant	Mix E. Europe	Naumova et al. 2007 [c]
L. thermotolerans	**ZIM 2492**	ZIM	Serbia	Europe	rasberries	agriculture/food	Domestic 1	na
L. thermotolerans	**ZIM 2505**	ZIM	Serbia	Europe	rasberries	agriculture/food	Mix Europe/N. America	na
L. cidri	**CBS 4575T**	CBS-KNAW	na	na	na	na	na	na
L. dasiensis	**CBS 10888T**	CBS-KNAW	na	na	na	na	na	Lee et al. 2009 [e]
L. fantastica	**CBS 6924T**	CBS-KNAW	na	na	na	na	na	na
L. fermentati	**CBS 707T**	CBS-KNAW	na	na	na	na	na	na

L. kluyverii	**CBS 3082T**	CBS-KNAW	na	na	na	na	na	na
L. lanzarotensis	**CBS 12615T**	CBS-KNAW	na	na	na	na	na	Gonzalez et al. 2013 [f]
L. meyersii	**CBS 8951T**	CBS-KNAW	na	na	na	na	na	Fell et al. 2004 [g]
L. mirantina	**CBS 11717T**	CBS-KNAW	na	na	na	na	na	Pereira et al. 2011 [h]
L. nothofagi	**CBS 11611T**	CBS-KNAW	na	na	na	na	na	Mestre et al. 2010 [i]
L. quebecensis	**CBS 14138T**	CBS-KNAW	na	na	na	na	na	Freel et al. 2016 [j]
L. waltii	**CBS 6430T**	CBS-KNAW	na	na	na	na	na	na

Phaff YCC – Phaff Yeast Culture Collection, University of Davis, California, USA; **AWMCC** - AWRI Wine Microorganism Culture Collection, Australia; **CBS-KNAW** - Centraalbureau voor Schimmelcultures – Koninklijke Nederlandse Akademie van Wetenschappen , Netherlands; **CRBOeno** - Centre de Ressources Biologiques OEnologie, France; **DBVPG** - The Industrial Yeasts Collection DBVPG, Italy; **NCYC** - National Collection of Yeast Cultures, UK;, Italy; **BOKU** - Universität für Bodenkultur Wien, Austria; **ISVV** - Institut des Sciences de la Vigne et du Vin, France; **LL** – Landry Lab, Canada; **MUCL** - Mycothèque de l'Université catholique de Louvain, Belgium; **NCAIM** - National Collection of Agricultural and Industrial Microorganisms, Hungary; **ITAP-DEMETER** - Institute of Technology of Agricultural Products, Hellenic Agricultural Organisation, Greece; **NRRL/ARS** - NRRL Agriculture Research Service culture collection, USA; **CRPR** - Centre de Recherche Pernod-Ricard, France; **OSU** – Oregon State University, USA; **UNIFG -** University of Foggia; **UWOPS** - Culture collection of the University of Western Ontario; **IWBT** – Institute for Wine Biotechnology, University of Stellenbosch, South Africa; **ZIM** - Zbirka industrijskih mikroorganizmov, Slovenia. [a] Sipiczki M. Overwintering of vineyard yeasts: Survival of interacting yeast communities in grapes mummified on vines. Frontiers in microbiology. 2016 Feb doi: 10.3389/fmicb.2016.00212; [c] Freel KC, Friedrich A, Hou J, Schacherer J. Population genomic analysis reveals highly conserved mitochondrial genomes in the yeast species *Lachancea thermotolerans*. Genome biology and evolution. 2014 Oct; 6(10):2586-94.; [c] Naumova ES, Serpova EV, Naumov GI. Molecular systematics of *Lachancea* yeasts. Biochemistry (Moscow). 2007 Dec; 72(12):1356-62.; [d] Robinson HA, Pinharanda A, Bensasson D. Summer temperature can predict the distribution of wild yeast populations. Ecology and evolution. 2016 Feb; 6(4):1236-50.; [e] Lee CF, Yao CH, Liu YR, Hsieh CW, Young SS. *Lachancea dasiensis* sp. nov., an ascosporogenous yeast isolated from soil and leaves in Taiwan. International journal of systematic and evolutionary microbiology. 2009 Jul; 59(7):1818-22.; [f] González SS, Alcoba-Flórez J, Laich F. *Lachancea lanzarotensis* sp. nov., an ascomycetous yeast isolated from grapes and wine fermentation in Lanzarote, Canary Islands. International journal of systematic and evolutionary microbiology. 2013 Jan; 63(1):358-63., [g] Fell JW, Statzell-Tallman A, Kurtzman CP. *Lachancea meyersii* sp. nov., an ascosporogenous yeast from mangrove regions in the Bahama Islands. Studies in Mycology 2004 Dec; 50:359-63.; [h] Pereira LF, Costa Jr CR, Brasileiro BT, de Morais Jr MA. *Lachancea mirantina* sp. nov., an ascomycetous yeast isolated from the cachaca fermentation process. International journal of systematic and evolutionary microbiology. 2011 Apr; 61(4):989-92.; [i] Mestre MC, Ulloa JR, Rosa CA, Lachance MA, Fontenla S. *Lachancea nothofagi* sp. nov., a yeast associated with *Nothofagus* species in Patagonia, Argentina. International journal of systematic and evolutionary microbiology. 2010 Sep; 60(9):2247-50.; [j] Freel KC, Charron G, Leducq JB, Landry CR, Schacherer J. *Lachancea quebecensis* sp. nov., a yeast species consistently isolated from tree bark in the Canadian province of Québec. International journal of systematic and evolutionary microbiology. 2015 Oct 1;65(10):3392-9.

S2 Table. Amplification of *L. thermotolerans* microsatellite markers on *Lachancea* species. Numbers are coded as following: 0 - no amplification, 1 - faint band, 2 - medium intensity band, 3 - full intensity band as determined using a microchip electrophoresis system.

Species	Microsatellite loci													
	LT2A	LT3A	LT3B	LT4A	LT5B	LT6B	LT7A	LT8A	LT8B	MA2	MD3	ME11	ME4	MH6
L. cidri	0	3	2	0	3	1	2	0	2	0	1	1	1	0
L. dasiensis	1	2	1	0	3	3	1	0	2	0	0	0	1	0
L. fantastica	0	3	2	0	3	3	2	0	2	1	0	1	0	0
L. fermentati	0	2	2	0	3	2	1	0	0	0	1	0	1	0
L. kluyverii	0	0	1	0	2	3	0	0	2	1	0	1	1	0
L. lanzarotensis	2	2	0	0	1	3	0	1	0	0	0	2	0	0
L. meyersii	0	3	2	0	3	3	1	0	0	0	0	1	1	0
L. mirantina	0	2	0	0	3	3	2	0	3	0	0	0	1	1
L. nothofagi	0	2	0	0	3	3	1	0	2	0	3	1	1	0
L. quebecensis	0	2	1	0	3	3	0	0	1	3	0	1	0	1
L. waltii	0	2	1	0	1	3	1	0	1	0	0	2	1	0
L. thermotolerans	3	3	3	3	3	3	3	3	3	3	3	3	3	3

CHAPTER 5

Oenological phenomes of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation

Statement of Authorship

Title of Paper	Oenological potential of *Lachancea thermotolerans* strains reflects patterns of local divergence and human selection
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Principal Author

Name of Principal Author (Candidate)	Ana Hranilovic
Contribution to the Paper	Conceptualisation of the work; experimental design; experimental work and data collection; data collation, interpretation and analysis; manuscript preparation and editing
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16.2.2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Joanna M. Gambetta
Contribution to the Paper	Volatile composition data processing and analysis, data interpretation; manuscript editing
Signature	Date 20/02/2018

Name of Co-Author	Leigh Schmidtke
Contribution to the Paper	Methodology; volatile composition data processing Manuscript editing
Signature	Date 20/2/2018

Name of Co-Author	Paul Boss		
Contribution to the Paper	Analytical contribution; volatile composition data acquisition Manuscript editing		
Signature		Date	16/2/2018

Name of Co-Author	Paul Grbin		
Contribution to the Paper	Conceptualisation of the work; experimental design Manuscript editing		
Signature		Date	22/02/2018

Name of Co-Author	Isabelle Masneuf-Pomarede		
Contribution to the Paper	Conceptualisation of the work; experimental design Manuscript editing		
Signature		Date	20.2.18

Name of Co-Author	Marina Bely		
Contribution to the Paper	Conceptualisation of the work; experimental design Manuscript editing		
Signature		Date	20.02.2018

Name of Co-Author	Vladimir Jiranek		
Contribution to the Paper	Conceptualisation of the work; experimental design; manuscript editing		
Signature		Date	20-2-18

Name of Co-Author	Warren Albertin		
Contribution to the Paper	Conceptualisation of the work; experimental design, statistical analysis; manuscript editing		
Signature		Date	20.2.18

Oenological phenomes of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation

Ana Hranilovic[1,2], Joanna M. Gambetta[3], Leigh Schmidtke[1,3], Paul K. Boss[4], Paul R. Grbin[2], Isabelle Masneuf-Pomarede[5,6], Marina Bely[5], Warren Albertin[5,7], Vladimir Jiranek[1,2*]

[1] The Australian Research Council Training Centre for Innovative Wine Production, Adelaide, SA, Australia

[2] Department of Wine and Food Science, The University of Adelaide, Adelaide, SA, Australia

[3] National Wine and Grape Industry Centre, School of Agricultural and Wine Science, Charles Sturt University, Wagga Wagga, NSW, Australia

[4] CSIRO Agriculture and Food, Adelaide, SA, Australia

[5] Unité de recherche Œnologie, Institut de la Science de la Vigne et du Vin, University of Bordeaux, Villenave d'Ornon, France

[6] Bordeaux Sciences Agro, Gradignan, France

[7] ENSCBP, Bordeaux INP, Pessac, France

[*] **Correspondence**

Vladimir Jiranek

vladimir.jiranek@adelaide.edu.au

Abstract

The yeast *Lachancea thermotolerans* (previously *Kluyveromyces thermotolerans*) is a species of large, yet underexplored, oenological potential. This study delivers comprehensive oenological phenomes of 94 *L. thermotolerans* strains obtained from diverse ecological niches worldwide, classified in nine genetic groups based on their pre-determined microsatellite genotypes. The strains and the genetic groups were compared for their alcoholic fermentation performance, production of primary and secondary metabolites and pH modulation in Chardonnay grape juice fermentations. The common oenological features of *L. thermotolerans* strains were their glucophilic character, relatively extensive fermentation ability, low production of acetic acid and the formation of lactic acid, which significantly affected the pH of the wines. An untargeted analysis of volatile compounds, used for the first time in a population-scale phenotyping of a non-*Saccharomyces* yeast, revealed that 58 out of 90 volatiles were affected at an *L. thermotolerans* strain level. Besides the remarkable extent of intra-specific diversity, our results confirmed the distinct phenotypic performance of *L. thermotolerans* genetic groups. Together, these observations provide further support for the occurrence of domestication events and allopatric differentiation in *L. thermotolerans* population.

Introduction

The largely untapped biotechnological potential of yeasts other than *Saccharomyces cerevisiae* is triggering rising scientific interest. One remarkable example is *Lachancea thermotolerans*, a ubiquitous species occupying a range of anthropic and wild habitats that cover a large geographic span [1-4]. In particular, this yeast is a common constituent of grape/wine microbiota [5,6], and has thus been explored for its application in oenology. Indeed, multiple studies have evaluated the oenological performance of *L. thermotolerans* isolates [7-10] delivering conclusive results; *L. thermotolerans* does not impart any obvious faults to the wine, rather, it can positively affect its chemical and sensory profile.

In oenological environments, *L. thermotolerans* is a relatively robust fermenter, depending on the strain and physiochemical conditions, capable of achieving up to 13.6% (v/v) ethanol [11]. As typical for non-*Saccharomyces* yeasts, *L. thermotolerans* pure cultures cannot 'complete' wine fermentation (i.e. deplete all sugars), and therefore require sequential or simultaneous addition of another co-starter, generally an *S. cerevisiae* strain [5]. Due to the antagonistic activity of *S. cerevisiae* towards *L. thermotolerans*, mediated by mechanisms of cell-cell contact and secretion of antimicrobial peptides [12], the outcomes of such co-fermentations are inoculation-dependant. The initial absence and/or lower inoculation densities of *S. cerevisiae* allows for the prolonged persistence and, in turn, greater metabolic contribution, of *L. thermotolerans* strains [9,13,14].

The major metabolic contribution of *L. thermotolerans* is lactic acid production concomitant to alcoholic fermentation [5,7-9]. The maximum reported lactic acid concentrations formed during *L. thermotolerans* fermentation exceed 16 g/L [15], thus representing orders of magnitude that are unique among any other non-genetically modified yeasts [16,17]. *S. cerevisiae* wildtype strains, by comparison, in similar conditions normally produce only < 0.4 g/L lactate [16,17]. The resultant acidification is considered to positively affect wine microbial stability and

organoleptic balance, while alleviating the need for external inputs (e.g. tartaric acid) commonly used to acidify grapes from warmer climates/vintages [7,10,18]. Another common characteristic of such grapes is excessive sugar accumulation, leading to undesirably high ethanol concentrations in wines [19]. Several studies reported significantly lower ethanol contents in co-fermentations with *L. thermotolerans* and *S. cerevisiae* that ranged between 0.2% and 0.9% (v/v) less than their respective *S. cerevisiae* monoculture controls [9,10,14,20], thus highlighting the potential of *L. thermotolerans* in production of lower-alcohol wines. Other beneficial/non-detrimental compositional alterations reported in *L. thermotolerans* treatments include increases in glycerol concentration [9,10,13,14], decreases in acetate content [8-10,13,14], partial degradation of malate [21-23] and modulations of both grape- and yeast-derived volatile compounds in wines [10,13,14,22-25].

The extent of intra-specific variability in traits of oenological importance among *L. thermotolerans* strains, however, remains elusive as the previous studies examined only a limited number of strains and/or metabolites, and employed different culture conditions and analytical techniques. Conversely, more insight is available into the genetic diversity of the *L. thermotolerans* population [2,4]. In our recent work we developed a 14-microsatellite genotyping method to study the relationship between 172 isolates from diverse habitats worldwide [4]. The natural isolates were grouped based on their geographic origin, whereas the genetic proximity of isolates from anthropic, in particular oenological environments, suggested domestication events within the species. Plate-based growth assays using different carbon substrates and physicochemical conditions provided further support for the observed clustering [4]. To determine whether, and to what extent, *L. thermotolerans* strains differ in oenologically-relevant traits, and harbour signatures of domestication and/or local divergence, we hereby report a comprehensive phenotypic characterisation of 94 previously genotyped strains in *Vitis vinifera* cv. Chardonnay fermentations.

Results

Fermentation performance of *L. thermotolerans* strains. The tested strains, obtained from diverse ecological niches worldwide, were classified into nine genetic groups (Supplementary Fig. S1; Supplementary Table S1) as determined by microsatellite profiling [4]. Based on the isolation location and/or niche of their constituents, the genetic groups were considered as 'wild' ('Americas', 'Canada trees', 'Hawaii/California', 'Other'), 'domestic' ('Domestic 1', 'Domestic 2') and 'mixed' ('Mix Eastern Europe', 'Mix Europe/North America', 'Europe oak/France grapes'), with a balanced number of strains representing the three classes. The 'wild' groups were comprised predominantly of natural isolates, clustered together based on their geographic origin, while the 'domestic' groups harboured isolates from anthropic, mainly oenological, environments. The remaining groups were 'mixed' with regards the substrate of isolation and/or geographic location of the strains. The strains and the genetic groups were compared for the microbial growth and sugar consumption kinetics, final production of primary and secondary metabolites and pH modulation in 25 mL Chardonnay grape juice fermentations (Supplementary Fig. S2).

All strains were able to proliferate (Fig. 1a) and catabolise sugars (Fig. 1b) despite the extreme conditions inherent to winemaking (e.g. high sugar content, limited assimilable nitrogen, rapid oxygen depletion). Spectrophotometric growth monitoring was not possible for three strains (LL12-031, LL12-056 and UWOPS 79-110) due to the pronounced flocculation. The frequent monitoring of microbial growth (OD_{600}) and sugar consumption allowed for the fermentation kinetics to be subjected to Self-Organizing Map (SOM) analysis. SOM of population growth that best explained the differentiation among genetic groups contained four clusters (Fig. 1a). However, the distribution of the different genetic groups amongst SOM clusters was not significant (Fig. 1c; chi^2 test p-value = 0.19). Conversely, SOM of sugar

consumption kinetics resolved four clusters, which corresponded to low (group 1'), medium-low (group 2'), medium-quick (group 3') and quick (group 4') sugar consumption kinetics (Fig. 1b). The SOM with low fermentation kinetics (group 1') contained 14 strains, i.e. nine 'Americas' and five 'Domestic 1' genotypes. Comparable number of strains displayed medium-low (group 2'; 30) and medium-quick (group 3'; 33) sugar consumption kinetics. These belonged to different genetic groups (Fig. 1d). In contrast, none of the 15 strains displaying quick sugar consumption kinetics (group 4') were 'Americas' and 'Canada trees' strains. Disproportionate distribution of SOM clusters within each genetic group was confirmed by chi^2 test (p-value = 3.10e-5), with an over-representation of low and medium-low fermenters in the 'Americas' and 'Canada trees' groups, and medium-quick and quick fermenters in 'Mix Europe/North America' (Fig. 1d).

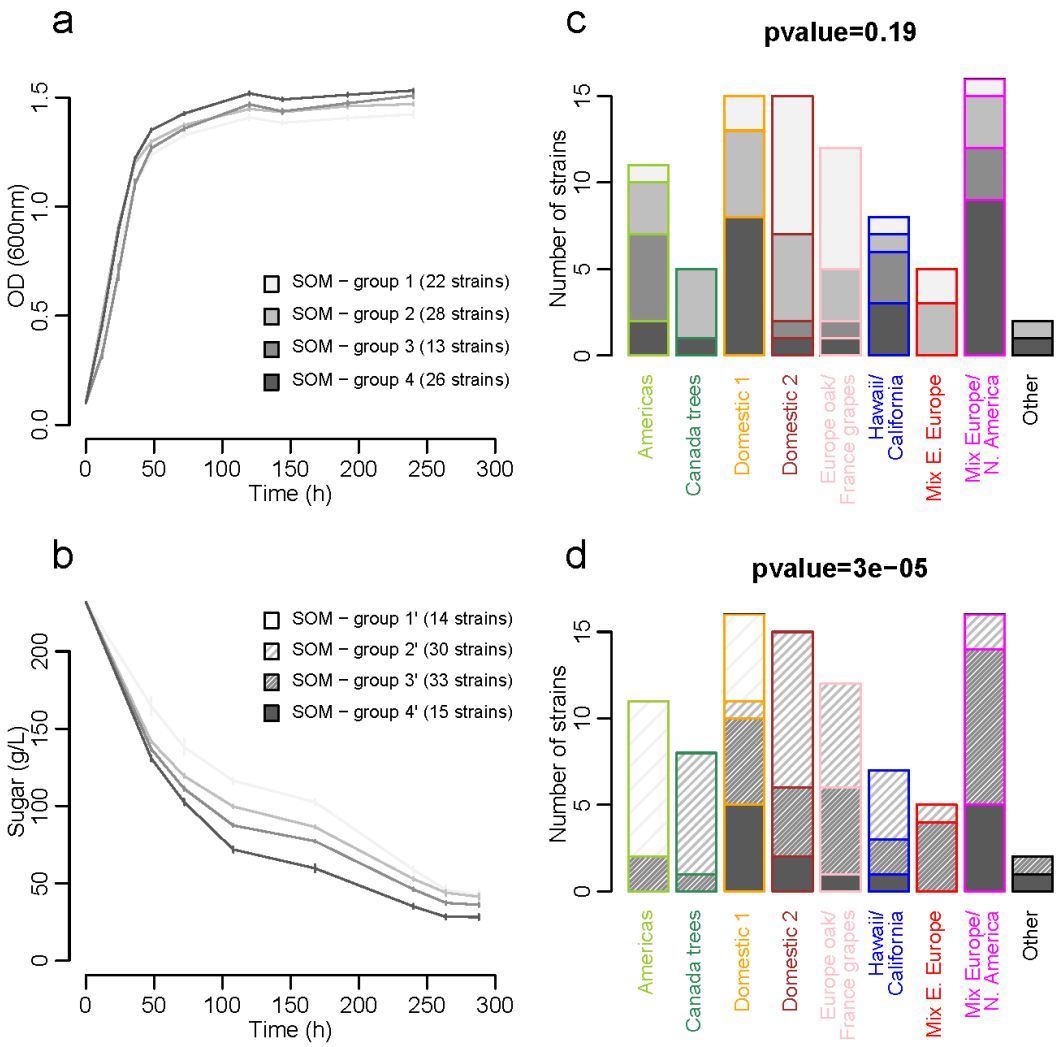

Figure 1. Self-Organizing Maps of growth and sugar consumption kinetics in *L. thermotolerans* fermentations. SOM analysis identified four clusters that best discriminate different genetic groups based on strain growth (a) and sugar consumption kinetics (b). Lines represent mean values and standard errors of SOM clusters. Within each genetic group, the number of strains belonging to different growth- and sugar consumption-related SOM clusters is represented in (c) and (d) barplots, respectively. Distribution of genetic groups within each SOM was determined by chi^2 tests with the corresponding p-values noted in (c) and (d). Colour-coding represents different genetic groups, as per the legends in (c) and (d).

Phenotypic variation in main fermentation metabolites. The extent of final sugar consumption in Chardonnay grape juice fermentations ranged between 161.6 and 223.4 g/L (Table 1). All the strains displayed a glucophilic character, i.e. consumed more glucose than fructose, with variable residual glucose/fructose ratios (Table 1). The achieved ethanol concentrations varied between 7.3 and 10.6% (v/v), however, strains did not significantly differ in their ethanol production capacity (Table 1). Strain 72-132 exhibited extreme glycerol production levels (8.0 g/L), while for most other strains glycerol concentrations and yields were more closely distributed around the mean values (Table 1). Interestingly, the concentrations and yields of glycerol, generally the second most abundant wine fermentation metabolite after ethanol, were lower than those of lactate in 48 strains. The highest lactic acid concentration (12.0 g/L) was produced by 68-140 (Table 1). The same strain consumed the highest concentration of malate, i.e. 0.8 g/L (Table 1). While partial degradation of malate was observed in most treatments, some strains led to an increase in malate of up to 0.3 g/L (LL12_056). In a winemaking context, acetic acid concentrations and yields in all *L. thermotolerans* fermentations were relatively low, and pyruvic acid concentrations ranged between 13 and 78 mg/L (Table 1). A decrease in pH from the initial value of 3.5 was observed in the majority of fermentations (i.e. 68/94). The largest pH drop, that of 0.34 units, observed in strain 68-140 (Table 1). Conversely, a minority of strains elicited de-acidification, with DBVPG 3466 having the highest pH value of 3.81 (Table 1).

Table 1. Analytical properties of Chardonnay wines fermented by different *L. thermotolerans* strains.

Parameter	Minimum [a]		Maximum [a]		Mean [b]
Consumed sugar (g/L)	161.6 ± 1.4	(DBVPG 3464)	223.4 ± 2.9	(NZ156)	199.6 ± 11.4
Glucose (g/L)	3.7 ± 0.5	(DBVPG 3466)	36.2 ± 0.7	(DBVPG 3464)	13.6 ± 6.2
Fructose (g/L)	8.9 ± 1.3	(NZ156)	39.1 ± 1.6	(72-132)	23.2 ± 6.0
G/F [c]	0.16 ± 0.02	DBVPG 3464	0.94 ± 0.01	UWOPS 94-426.2	0.57 ± 0.16
Ethanol (% v/v)	7.3 ± 0.7	(72-132)	10.6 ± 0.7	(CBS 2907)	9.3 ± 0.9
Ethanol yield [d] (g/g)	0.34 ± 0.04	(8/Z-1)	0.40 ± 0.03	(DBVPG 3464)	0.37 ± 0.03
Glycerol (g/L)	3.9 ± 0.1	(DBVPG 3464)	8.0 ± 0.2	(72-132)	5.4 ± 0.6
Glycerol yield [d] (g/g)	0.0205 ± 0.0004	(CBS 137)	0.0478 ± 0.0002	(72-132)	0.0274 ± 0.0039
Lactic acid (g/L)	1.8 ± 0.2	(JCB1)	12.0 ± 0.2	(68-140)	5.8 ± 2.3
Lactate yield [d] (g/g)	0.0086 ± 0.0011	(JCB1)	0.0658 ± 0.0018	(68-140)	0.0291 ± 0.0119
Acetic acid (g/L)	0.06 ± 0.01	(72-137)	0.32 ± 0.01	(UWOPS 85-51.1)	0.20 ± 0.05
Acetate yield [d] (mg/g)	0.30 ± 0.05	(72-137)	1.53 ± 0.03	(UWOPS 85-51.1)	0.98 ± 0.24
FP [e]	0.0067 ± 0.0008	72-137	0.0327 ± 0.0040	LL13-189	0.0213 ± 0.0056
Pyruvic acid (mg/L)	13 ± 1	(MUCL 31342)	78 ± 3	(9/1/Z-4)	44 ± 14
Malic acid (g/L)	3.0 ± 0.1	(68-140)	4.1 ± 0.2	(LL12_056)	3.6 ± 0.3
pH	3.16 ± 0.03	(68-140)	3.81 ± 0.13	(DBVPG 3466)	3.44 ± 0.03

[a] Minimum and maximum values (means of triplicates ± standard errors) associated with the strains in the brackets; [b] Mean values of all observations (± standard errors); [c] G/F - measure of fructophilicity defined as residual glucose and fructose ratio; [d] Yields were calculated from concentrations of respective metabolites and consumed sugar; [e] FP - fermentation purity defined as a ratio of acetic acid and ethanol. Chardonnay grape juice contained 236.4 g/L sugar (1:1 mix of glucose and fructose), 3.8 g/L malic acid and pH 3.5. Differences for parameters in italics were not significant (KW test, p-values > 0.05).

The parameters showing a significant strain effect (Table 1) were further compared at a genetic group level (Fig. 2). ‘Canada trees’ strains were characterised by the lowest extent of sugar consumption as a result of the highest concentrations of glucose and fructose alike (Fig. 2). The ‘Americas’ strains, i.e. another group comprised of natural isolates, were capable of extensive glucose consumption, however, their residual fructose content was higher than in any other group except ‘Canada trees’ (Fig. 2). Consequently, the fructophilicity (G/F) of ‘Americas’ strains was low. The concentrations and yields of glycerol were generally higher for most wild and mixed groups, than for the domestic ones (Fig. 2). ‘Domestic 1’ strains had lower concentrations and yields of lactate and the higher pH values than all other groups except ‘Other’ (Fig. 2). Lactate production was the highest in ‘Domestic 2’, ‘Mix Eastern Europe’ and ‘Mix Europe/North America’ groups. Acetic acid was the lowest in strains belonging to ‘Hawaii/California’ group, and their acetate yields and fermentation purity (FP) were also low (Fig. 2). The levels of malic acid in ‘Canada trees’ and ‘Americas’ wines were higher than in all other groups, and pyruvate concentrations were higher in ‘Domestic 2’ and ‘Mix Eastern Europe’ than in most other groups (Fig. 2).

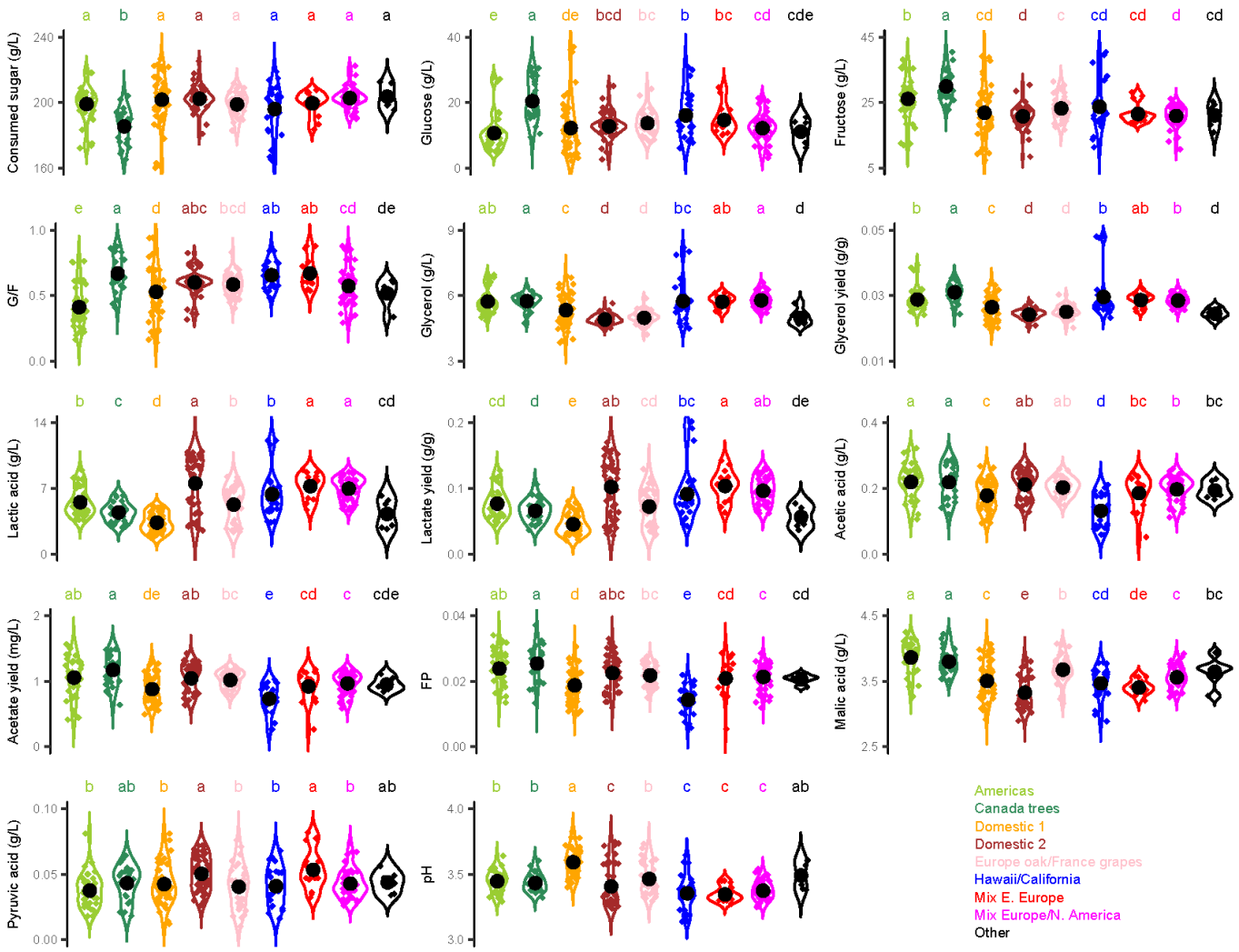

Figure 2. Violin plots for the selected metabolic traits. For each genetic group, numeric values are represented as diamonds, the corresponding probability densities are represented as plain traits, mean and standard error are represented by black circles and segments, respectively (*ggplot2* package, R). Top letters represent significance groups as defined by Kruskal-Wallis test (*agricolae* package, p-value < 0.05 after Benjamini & Hochberg adjustment for multiple comparisons).

Volatile metabolome of *L. thermotolerans* wines. The obtained Chardonnay wines were also analysed for their volatile composition. Out of 90 analysed volatiles, 58 compounds were affected at a strain level (Fig. 3; Supplementary Table S2). The majority of these compounds (35/58) were successfully identified in the NIST database via corresponding mass spectra, Kovats' RI indices and, when available, comparison with pure compounds (Supplementary Table S2). The remaining 23 compounds were unidentified (unknown; 23/58). The identified compounds included higher alcohols (12/58), with the representatives of C_6 (n-hexanol), aryl (2-phenylethanol and 4-methyl-benzenemethanol), branched (isobutanol, isoamyl alcohol, 2-methyl-1-butanol, 3-methyl-1-pentanol, 2-ethyl-hexanol) and non-branched compounds (n-butanol, n-nonanol, n-octanol and n-decanol). A comparable number of ethyl esters was detected (10/58). These included ethyl esters (ethyl propanoate, ethyl octanoate, ethyl decanoate, ethyl 9-decenoate, diethyl succinate), acetates (ethyl acetate, isobutyl acetate, isoamyl acetate and 2-phenylethyl acetate), and a lactate (amyl lactate). Five acids also significantly differed between the strains (5/58; 4-hydroxy-butanoic, hexanoic, octanoic, decanoic and dodecanoic acid). The remaining compounds were classified as aromatic compounds (3/58; 1-ethyl-2,4-dimethyl benzene, 1,2,4-trimethylbenzene and 1,3-bis(1,1-dimethylethyl) benzene), aldehydes (2/58; acetaldehyde and 4-methyl-benzaldehyde), a ketone (1/58; 4-methyl-2-heptanone), a norisoprenoid (1/58; ß-damascenone) and a terpenol (1/58; β-citronellol).

n-Hexanol and n-octanol were the volatiles that displayed the largest strain effect and, after F22, genetic group effect (Fig. 3). These two higher alcohols followed the same trend; their content was lower in 'Domestic 1' wines than those from any of the remaining groups except 'Other', and high in 'Mix Eastern Europe', 'Mix Europe/North America' and 'Domestic 2' groups (Supplementary Fig. S3). The 'Domestic 1' group also produced less n-butanol and several unidentified compounds (e.g. F43 and F50) compared to most other groups.

Conversely, F22 and ß-damascenone were relatively high in the ‘Domestic 1’ group, as were isobutanol, F10 and F56. The ‘Americas’ strains produced high levels of acetaldehyde, ethyl acetate, ethyl propanoate, isoamyl acetate, isoamyl alcohol and ethyl 9-decenoate (Supplementary Fig. S3). Ethyl 9-decenoate was similarly high in ‘Domestic 2’ and ‘Mix Eastern Europe’ wines, which were also characterised by an increase in ethyl decanoate and F86 (Supplementary Fig. S3). The ‘Canada trees’ group was related to a low production of F85, 2-phenylethanol, isobutyl acetate, diethyl succinate, 4-methyl-benzaldehyde and 1,3-bis(1,1-dimethylethyl) benzene, and overproduction of 1-ethyl-2,4-dimethylbenzene (Supplementary Fig. S3). The latter aromatic compound was, in addition to F8, F40 and F46, particularly low in the ‘Hawaii/California’ group (Supplementary Fig. S3). The ‘Mix Europe/North America’ strains produced high levels of 4-methylbenzene methanol and F43 (Supplementary Fig. S3). Hexanoic acid and F83 were also high in this group, as well as in ‘Mix Eastern Europe’, ‘Americas’ and ‘Canada trees’, while 4-hydroxybutanoic acid was low in all these groups but ‘Canada trees’ (Supplementary Fig. S3). The ‘Europe oak/France grapes’ strains yielded less n-decanol and amyl lactate than all groups but ‘Canada trees’ and/or ‘Other’ (Supplementary Fig. S3).

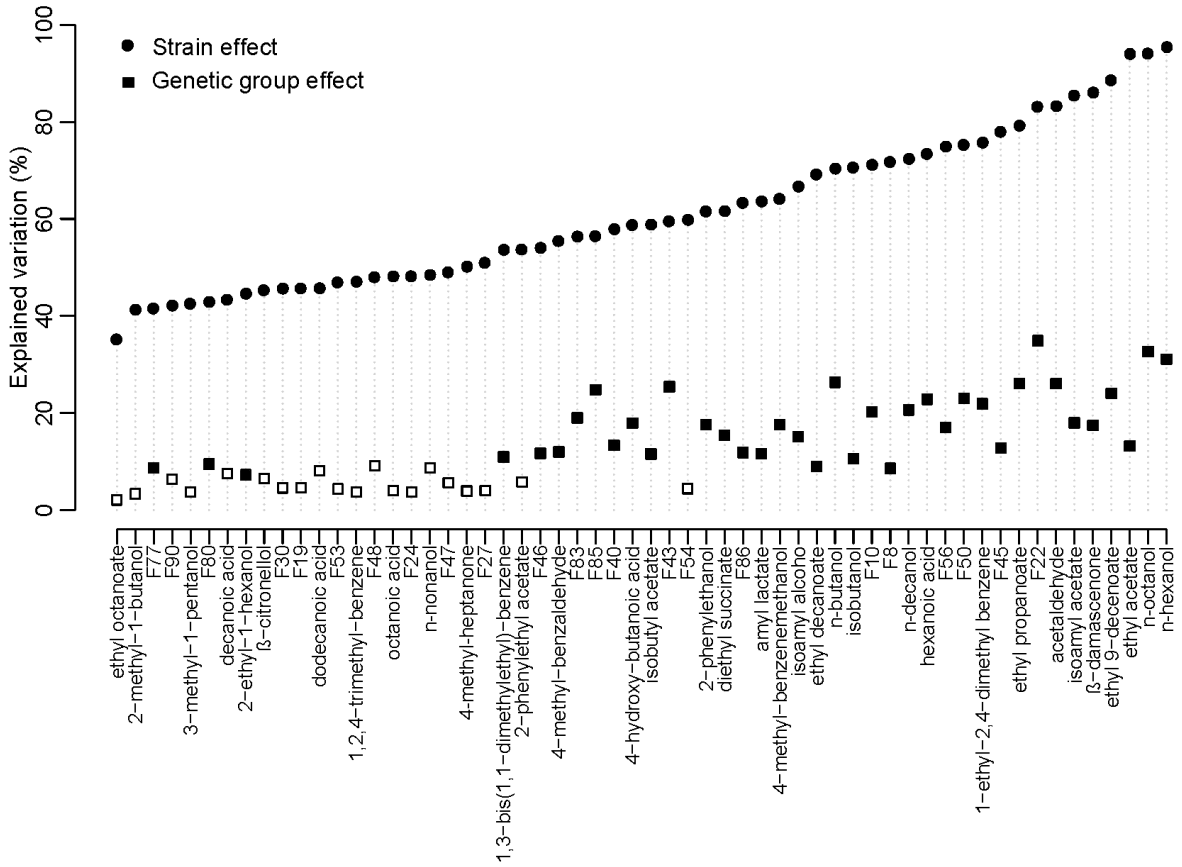

Figure 3. Percentage of variation in volatile compounds explained by either strain or genetic group effect. Only 58 compounds with a significant (p-value < 0.05) strain effect are shown. For the genetic group effect, white squares represent no significant p-values (> 0.05) and coloured squares indicate significant p-values (< 0.05).

Phenotypic differentiation of *L. thermotolerans* genetic groups. To determine whether the obtained metabolic dataset discriminated the *L. thermotolerans* genetic groups, 107 variables were subjected to linear discriminant analysis (LDA). These analysed variables included fermentation kinetics parameters, concentrations of main metabolites, volatile compounds and pH. LDA revealed a clear separation of the 'Canada trees' and 'Domestic 1' groups from all remaining groups (Fig. 4a). Albeit less resolved, a suitable partitioning of strains belonging to

the remaining genetic groups of strains was also obtained, and the co-localisation of ‘Mix Eastern Europe’ and ‘Mix Europe/North America’ groups was congruent with their genetic proximity [4].

The ‘Canada trees’ group were differentiated from other groups based on the low production of the unknown volatile compounds F85, which represented the most important variable for discriminating genetic groups (Figure 4b; Supplementary Fig. S4), as well as the high concentrations of 1-ethyl-2,4-dimethyl-benzene and both grape hexoses (Fig. 2; Supplementary Fig. S3). Low lactate, n-hexanol and n-octanol, and high pH and F22 were amongst the most important variables driving the separation of ‘Domestic 1’ strains (Fig. 2; Supplementary Fig. S3). Glycerol, overall ranked as the second most relevant variable for LDA, was of main importance for ‘Domestic 2’ group, as well ‘Mix Europe/North America’ and ‘Europe oak/France grapes’ (Fig. 4b). A similarly important metabolite for ‘Domestic 2’ and ‘Mix Europe/North America’ was high lactate, followed by low maximum ODs for the former group, and high n-hexanol and n-octanol for both groups (Supplementary Fig. S3). ‘Americas’ strains were discriminated based on the parameters related to their sugar consumption kinetics (i.e. high T50, T30 and max flux; Fig. 1b), and increased production of several volatile compounds (i.e. acetaldehyde, ethyl acetate and isoamyl acetate; Supplementary Fig. S3), and ‘Hawaii/California’ strain primarily due to their low acetic acid production (Fig. 2).

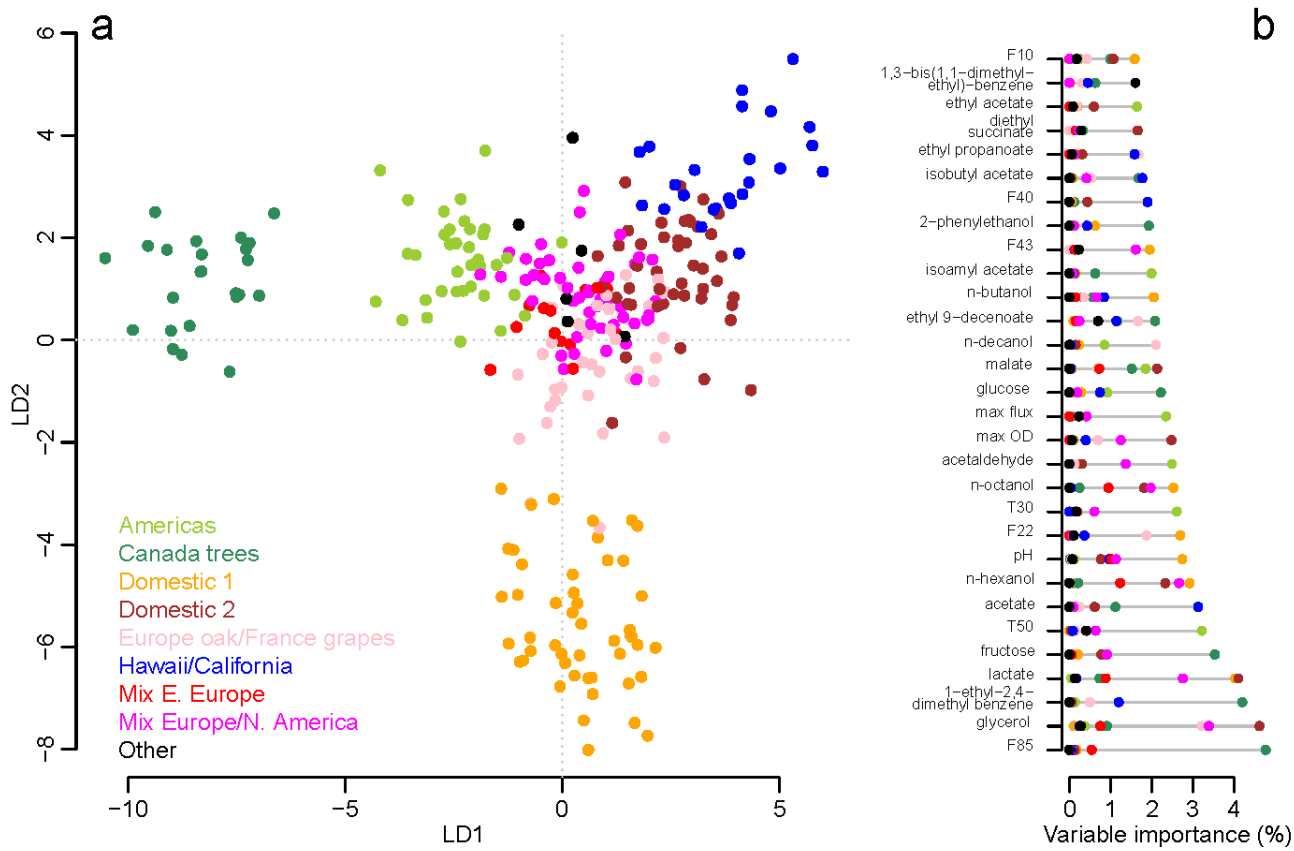

Figure 4. Linear discriminant analysis of nine genetic groups in *L. thermotolerans* based on 107 variables. LDA plot showing the first two axes, i.e. LD1 and LD2 (A). The importance of variables accounting for LDA was computed for each genetic group (B). Only the main 30 variables are shown; all 107 variables are in Supplementary Fig. S4.

Relationships between metabolites. Multiple linear regression analysis was conducted to examine the relationships between the variables of interest (i.e. main fermentation products) and other metabolites as well as pH values. The analysis revealed that the most explanatory variable for pH was lactic acid, accounting for 70% of variation (Fig. 5). Likewise, lactate concentrations explained 73% of variation in pH of the wines. Besides sugar consumption (24% of explained variation), significant contributions to variation in ethanol formation were pH, acetic acid and several volatiles, which all had positive coefficients, except dodecanoic

acid. The extent of sugar consumption was best explained by ethanol production (19% of explained variation), followed by pH, malate and lactate. After ethyl propanoate, pH was also the second most explanatory variable for obtained glycerol concentrations (8% of explained variation; negative coefficient). Several volatile compounds significantly accounted for both sugar consumption and glycerol production. Ethyl acetate and acetaldehyde together explained 38% of acetic acid concentration, and lactate contributed with an additional 8%.

The correlation between pH and lactate was further confirmed by Spearman's correlation test (Fig. 6), which was also used to assess the inter-relationships between consumed sugar concentrations and main fermentation by-products in *L. thermotolerans* fermentations, (i.e. ethanol, lactate, glycerol and acetate) within each genetic group (except 'Other') and the entire dataset (Supplementary Fig. S5-S14). Interestingly, negative correlations were observed between the extent of sugar consumption and lactate production for certain genetic groups, but not globally (Supplementary Fig. S6). Similarly, correlations between lactate and glycerol were detected only within certain groups; positive within four and negative within one ('Hawaii/California'; Supplementary Fig. S12). Both lactate and ethanol, and glycerol and ethanol showed weak negative correlations within the whole dataset, and for several genetic groups individually (Supplementary Fig. S9, S10). No correlations whatsoever were detected for acetate and glycerol production, while acetate and lactate displayed weak positive correlations within the whole dataset and for two genetic groups (Supplementary Fig. S13, S14).

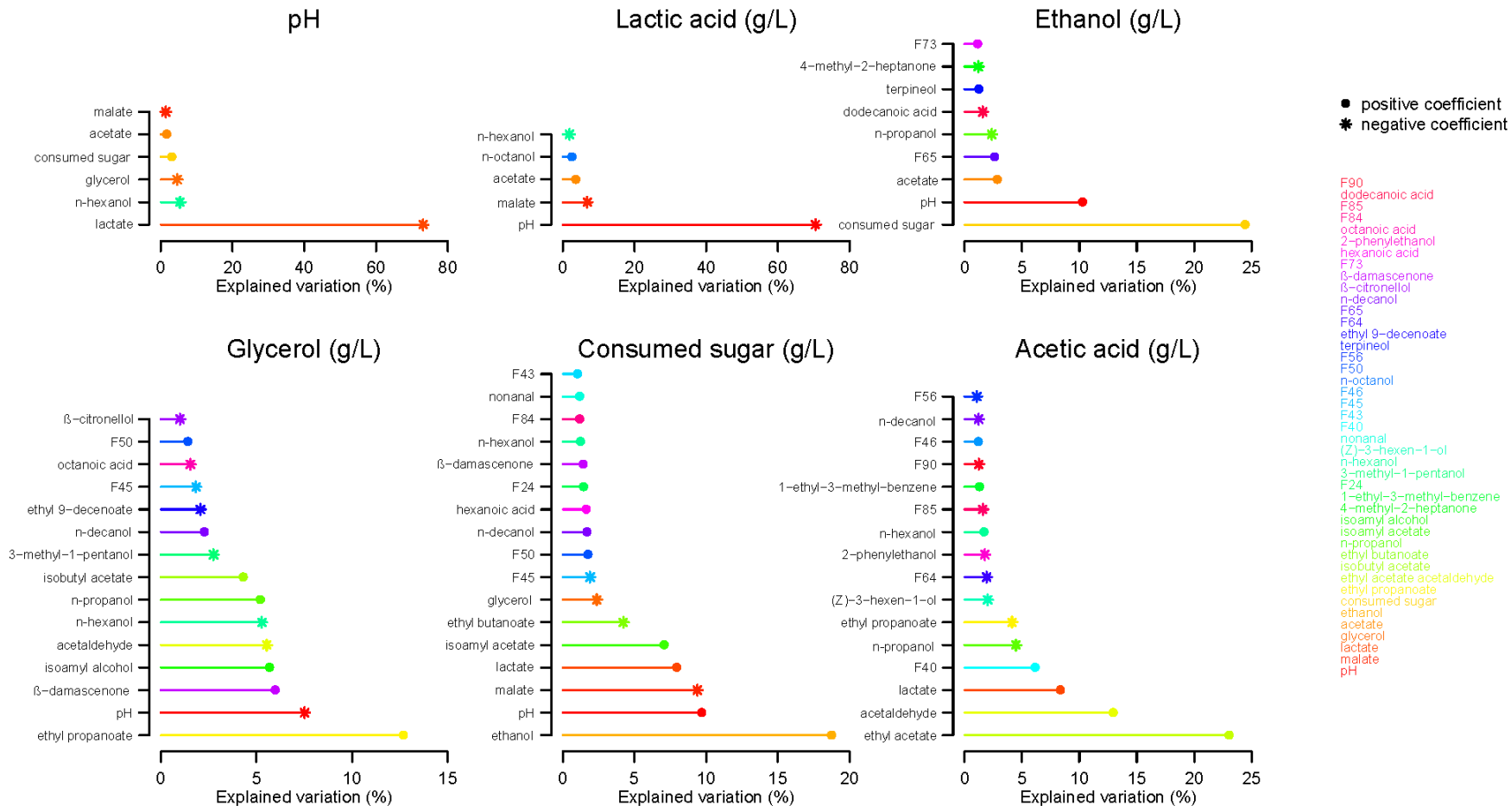

Figure 5. Relationships between metabolites of interest revealed by multiple linear regression analysis. Shown variables significantly (p-value < 0.01) explaining more than 1% of variation.

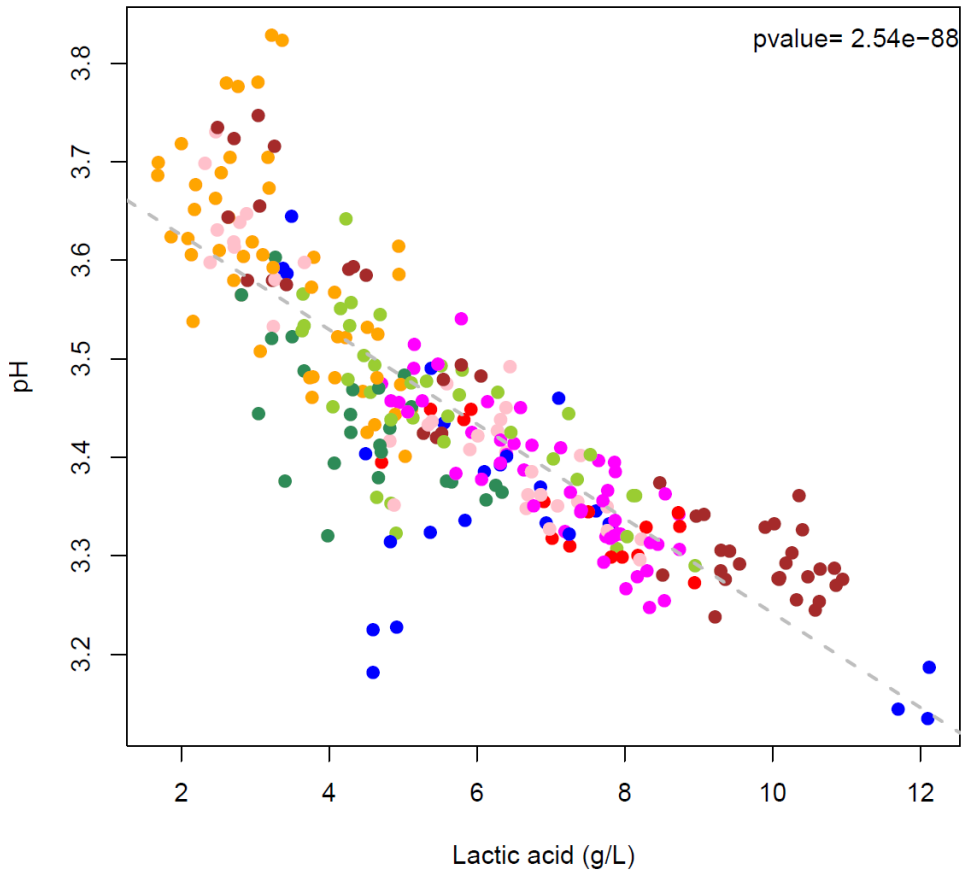

Figure 6. Correlation between lactic acid production and pH values in Chardonnay grape juice (pH 3.5) fermentations. Colour-coding represents *L. thermotolerans* genetic groups (as per Fig. 1, 2, 4).

Discussion

There is a growing interest in the selection and characterisation of non-*Saccharomyces* yeasts to be used in winemaking to build 'complexity' and diversify styles. However, surprisingly few studies systematically explored the concepts of their intra-specific phenotypic variability [26-28]. To our knowledge, the scale and range of this work represents the broadest oenological characterisation of phenotypic variability within a population of a non-*Saccharomyces* yeast. It was designed to assess to what extent strains of *L. thermotolerans* vary

in traits of key importance for fermentation outcome, i.e. microbial growth and sugar consumption kinetics, production of volatile and non-volatile metabolites and the resultant (de)acidification. The comprehensive phenotyping dataset was comprised of 114 measured/derived parameters for triplicate fermentations of 94 recently genotyped *L. thermotolerans* strains, resulting in over 32,100 individual data points. The tested strains were obtained from both natural and anthropic habitats, and distributed across the entire *L. thermotolerans* phylogenetic tree [4] (Supplementary Fig. S1), so as to ascertain if the determined phenotypic variability is fully representative of the species.

The observed phenotypic variability was large and ethanol production was the only primary metabolic trait that did not differ between the strains, possibly due to analytical limitations. Similarly, differences in ethanol content alone, quantified with an equivalent HPLC method, were not detected between 72 *S. cerevisiae* strains in a population-scale phenotyping conducted under winemaking conditions by Camarasa et al. [29], despite significant disparities in strain sugar consumption levels. Regardless, given that *S. cerevisiae* generally yields about 0.47 g of ethanol per 1 g of sugar [30], the ethanol yields of *L. thermotolerans* strains determined here were altogether low (mean value 0.37 g/g; Table 1). Although the comparison of ethanol yields between different conditions and physiological stages is invalid, this attribute warrants further investigation for use of *L. thermotolerans* in the production of wines with lower-ethanol content, as observed elsewhere [9,10,14,20].

In accord with previous reports [8,9,13,15], our results confirmed that the common oenological features of *L. thermotolerans* strains are their glucophilic character, relatively extensive fermentation ability, albeit without 'completion', low production of acetate and formation of lactate. In contrast to previous findings, acidification was found for most, but not all, strains (Table 1). An increase in wine pH has, to our knowledge, thus far not been associated with *L. thermotolerans* fermentations despite the reports of partial degradation of malate [21], as

also witnessed in most of our treatments. Lactate concentrations were, by large, the most explanatory variable for the resultant pH modulation, as shown by multiple linear regression analyses (73% of explained variation; Fig. 5). Although the maximum concentrations of lactate achieved under current conditions (12 g/L) were lower than those from the literature (16.6 g/L) [15], a seven-fold variation was detected for this trait (Table 1).

Lactic acid formation is, in fact, a metabolic hallmark of *L. thermotolerans*, but its physiological role and underlying molecular mechanisms remain poorly understood. From the literature, it is unclear whether in *L. thermotolerans,* as in lactic acid bacteria [16], lactate formation via NAD-dependant lactate dehydrogenase (LDH) serves to re-plenish oxidised NAD^+ depleted through glycolysis (Fig. 7). In yeasts, this is primarily achieved through formation of ethanol via the decarboxylation of pyruvate and the subsequent reduction of acetaldehyde, i.e. alcoholic fermentation [31]. In addition to osmoregulation, glycerol production in *S. cerevisiae* also serves as a redox valve to eliminate excess cytosolic NADH under anaerobic conditions and is coupled with acetic acid production [32,33]. Information of carbon flux and redox balance in *L. thermotolerans* is surprisingly scarce. Our data, nonetheless, highlighted several inter-relationships between metabolites of interest and pH values via multiple linear regression analyses and correlations. Most notable were the significant (and second largest) proportions of variation in ethanol and glycerol production explained by pH values, displaying positive and negative coefficients, respectively (Fig. 5). Moreover, negative (albeit weak) correlations between ethanol (despite the previously discussed analytical constraints) and both lactate and glycerol were observed, while lactate and glycerol correlated differently depending on the *L. thermotolerans* genetic group (no overall correlations, negative within four and positive within one genetic groups; Supplementary Fig. S9, S10, S12). These observations potentially suggest that *L. thermotolerans* strains differ in their metabolic strategies to restore the $NADH/NAD^+$ balance. Furthermore, the contribution of lactate, but not

glycerol, towards variation in acetate (Fig. 5), and the absence of correlations between glycerol and acetate production (Supplementary Fig. S14) indicates that *L. thermotolerans*, as reported for some other non-*Saccharomyces* species such as *T. delbrueckii* [34], differ from *S. cerevisiae* in their metabolic link between glycerol and acetate production. Altogether, these findings invite further investigation of central carbon metabolism in *L. thermotolerans*, in particular the regulatory framework of redox balance, through studies purposely designed to quantify the microbial growth and evolution of metabolites in conjunction with transcriptomics.

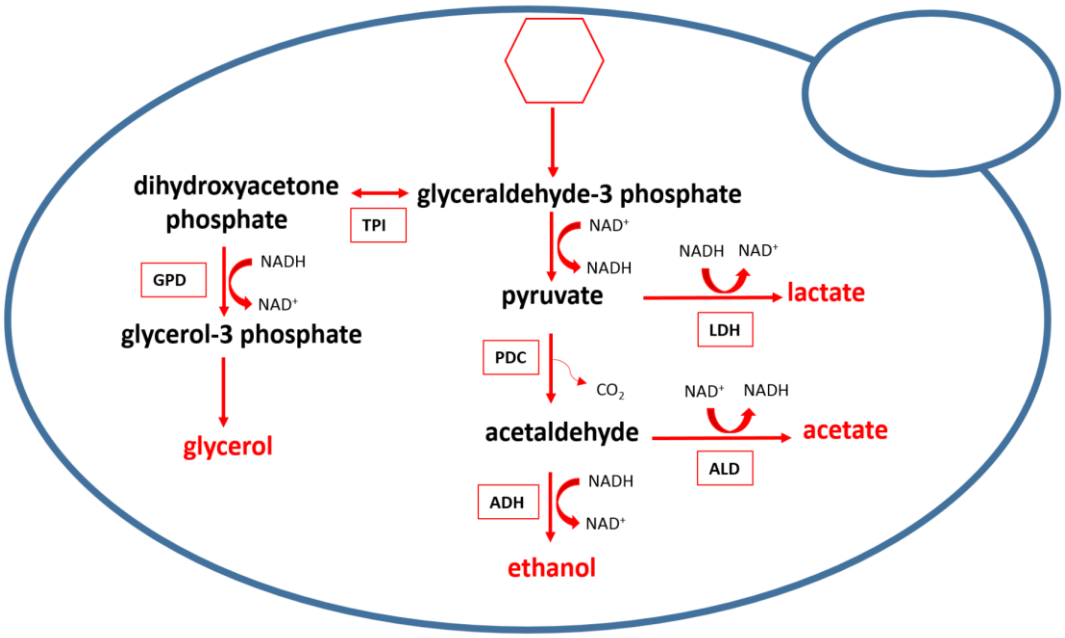

Figure 7. Production of the main fermentation (by)products in *L. thermotolerans*. Hexoses are metabolised via glycolysis to pyruvate through multiple enzymatic steps. Pyruvate is decarboxylated to acetaldehyde (pyruvate decarboxylase; PDC), which is further reduced to ethanol (alcohol dehydrogenase; ADH) or oxidised to acetate (aldehyde dehydrogenase; ALD). A proportion of pyruvate is converted to lactate (lactate dehydrogenase; LDH). Glycerol is produced via dihydroxyacetone phosphate (glycerol-3-phosphate dehydrogenase; GPD;

triosephosphate isomerase; TPI) (adapted from Kegg Pathway Database [52]; http://www.genome.jp/kegg-bin/show_pathway?lth00010).

In addition to the analysis of primary metabolites, this work, for the first time, implemented a metabolomics approach to study volatile footprints of a non-*Saccharomyces* yeast population. Recently, the volatile metabolome of a commercial *L. thermotolerans* strain was explored in the context of inter-specific comparison of several wine-associated non-*Saccharomyces* yeasts and a *S. cerevisiae* control, using a targeted approach either at an early fermentation stage [35] or an untargeted approach in wines completed with sequentially inoculated *S. cerevisiae* [22,23]. This extensive comparison revealed that, within each matrix (i.e. Syrah and Sauvignon Blanc) and fermentation modality (i.e. pure culture and co-culture), the modulation of wine volatile profiles was species-dependant [22,23,35]. Our data show that a wide array of volatile compounds are also affected at a *L. thermotolerans* strain level (Fig. 3). The high-throughput nature of the applied methodology [36] and an untargeted approach allowed us to study the volatile compounds that might have otherwise been overlooked, yet they significantly differentiated the strains and genetic groups of *L. thermotolerans*. The best examples are the unidentified compounds F22 and F85, which displayed the largest genetic group-effect (Fig. 3) and an importance for genetic group discrimination in LDA (Fig. 4), respectively. Besides the unidentified compounds, the majority of strain-affected volatile compounds represented the main constituents of the so-called secondary, fermentation-derived volatile aroma, i.e. esters and higher alcohols [37,38]. However, the effect on the primary aroma compounds was equally, if not more, pronounced, as some of the most strain-affected compounds were in fact grape-derived (i.e. n-hexanol, n-octanol, ß-damascenone) [39]. The observed variation in volatile composition of wines partially arises from the differential sugar consumption levels (Fig. 5) and, potentially, the matrix effect on the headspace partitioning of

the aroma compounds [40]. However, inter-strain differences in mechanisms involved in the biosynthesis of volatile compounds, including enzymatic activities (e.g. esterase, glucosidase, acetyltransferase), amino acid metabolism and fatty acid synthesis require further investigation.

The genotyping information also enabled us to evaluate the phenotypic variation in the context of genetic structure in *L. thermotolerans*, shaped by domestication and allopatric differentiation [4]. Colonisation of a given ecological niche is known to lead to evolutionary differentiation, harnessing adaptation to specific environmental constraints [41]. If such a niche is anthropic, this process can be seen as domestication, either inadvertent or intentional. Signatures of domestication have been confirmed at a genetic level for several other yeast species, i.e. *S. cerevisiae* [42-44], *S. uvarum* [45] and *T. delbrueckii* [46]. In *S. cerevisiae,* the genetic differentiation of wild and industrial subpopulations (e.g. winemaking, brewing, baking) was found to be largely reflected at the phenotypic level, with industry-specific selection for stress tolerance, sugar consumption and flavour production [44]. Research has, moreover, highlighted the 'degrees' of *S. cerevisiae* domestication; it is the strongest in beer strains, which showed niche specialisation, i.e. decreased ability to grow in nature-like environments as a result of continuous cultivation in mild conditions related to brewing [44]. In contrast, wine strains displayed superior performance across a range of stressors, encountered both in winemaking (e.g. osmotic and ethanol stress) and in nature, likely reflective of the seasonality of winemaking practice [44]. Our previous plate-based phenotyping of *L. thermotolerans* strains using different carbon sources and physicochemical conditions revealed an overall prolific growth of 'domestic' groups (harbouring mainly oenological isolates) that might have contributed to their intra-continental dispersal [4], and in the current study all strains were capable of proliferating in the oenological environment, altogether suggesting an absence of niche specialisation. Nevertheless, the distinct phenotypic performance of *L. thermotolerans* genetic groups, driven by strain fermentation performance and production of (non-)volatile

metabolites, was apparent. Notably, two major genetic groups mainly comprised of natural isolates, 'Americas' and 'Canada trees', showed an overall inferior fermentation performance compared to the 'domestic' and 'mixed' groups, in terms of lower rate/extent of sugar catabolism (Fig. 1, 2). Moreover, the genetic separation of two 'domestic' *L. thermotolerans* groups was also evident at a phenotypic level, as their behaviour for many traits was clearly contrasting. For instance, 'Domestic 1' group formed the lowest concentration of lactate, and thus resulted in the highest pH of wines, while 'Domestic 2' strains showed superior lactate production that induced a pronounced acidification (Fig. 2). This further emphasises the applicability of microsatellite genotyping in selection of fit-for-purpose *L. thermotolerans* starter cultures; a 'Domestic 1' genotype will likely represent a suboptimal choice if the target outcome is (wine) fermentation acidification. Similarly, an overproduction of acetaldehyde and ethyl acetate by 'Americas' strains (Supplementary Fig. S3) potentially excludes their use in wine industry, as the increased concentrations of these compounds are detrimental for wine quality [18]. Together, these observations provide further support for the occurrence of domestication events and geographic differentiation in *L. thermotolerans* population.

In conclusion, this study delivers extensive oenological phenomes of 94 previously genotyped *L. thermotolerans* strains, compared for their overall fermentation performance, production of primary and secondary metabolites and modulations in acidity. As such, it not only paints a comprehensive landscape of intra-specific diversity in *L. thermotolerans,* but also highlights the phenotypic manifestations of the genetic differentiation within this remarkable yeast species.

Materials and Methods

Culture conditions and media. The cryo-cultures (-80 °C in 25% glycerol) of 94 *L. thermotolerans* strains (Supplementary Table S1) with pre-determined microsatellite genotypes [4] were grown on YPD plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar) for 3 days at 24 °C. To establish the inoculation cultures, approximately 10^7 cells were incubated in 900 µL of YPD in each 2 mL well of a 96-well plate agitated on a rotary shaker. After 24 h incubation at 24 °C, cell densities were determined by flow cytometry (Guava easyCyte 12HT, Merck, NJ, USA) to achieve the final inoculation rates of 10^6 cell/mL. The filter-sterilised (0.2 uM) Chardonnay juice was sourced from the Coombe vineyard (Waite Campus, The University of Adelaide, SA). The concentrations of sugars (glucose and fructose; ~180 g/L) and yeast assimilable nitrogen (~160 mg/L) in the juice were increased to 236.4 g/L (equimolar amounts of glucose and fructose) and 300 mg/L using glucose and fructose and diammonium phosphate, respectively. The juice had a pH of 3.5 and contained 3.8 g/L of malic acid.

Fermentation trial set-up and monitoring. A custom-made fermentation platform 'Tee-bot v.2.0', built on EVO Freedom workdeck (Tecan, Männedorf, Switzerland), was used to conduct the fermentation trials. The platform allowed for up to 384 fermentations to be simultaneously conducted with automatic sampling at user-defined intervals. Each fermenter (50 mL) contained a magnetic flea and an airlock with a silicon (sampling) septum on top and was fitted into a custom-made sealed rack forming 96-fermenter blocks. The fermenters were aseptically supplemented with 25 mL of Chardonnay juice and inoculated with pre-established cultures so that each 96-fermenter block contained one biological replicate of *L. thermotolerans* strains, with a row-wise randomisation between the blocks (Supplementary Fig. S2). The approximate liquid to headspace ratio was 3:1. Upon inoculation, the triplicate fermentations were incubated at 24 °C under anaerobic conditions self-induced upon depletion of the initial

oxygen content. The otherwise static fermentations were magnetically stirred during sampling (300 rpm for 2.5 h) so as to ensure yeast cell resuspension. The aliquots (200 µL) were automatically taken at regular intervals (12 or 24 h) into 300 µL 96-well plates to monitor fermentation progress via microbial growth and total sugar consumption. Fermentations were deemed arrested when sugar concentrations did not decline for two consecutive sampling time-points. The final sample was centrifuged (10 min; 3200 × g) in 50 mL tubes and the supernatant decanted into 10 mL tubes and stored at 4 °C until further analysis.

Analytical techniques. Upon sampling, microbial growth was estimated at 600 nm (OD_{600}) upon 30 s resuspension in a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland). The plates were then centrifuged (3 min; 3200 × g) and appropriately diluted for enzymatic determination of total sugar (glucose + fructose) consumption (K-FRUGL kit, Megazyme, Ireland). The pH of wines was measured with a CyberScan 1100 pH meter (Eutech instruments, Thermo Fischer Scientific, MA, USA) and glucose, fructose, ethanol, glycerol, lactic acid, malic acid and acetic acid were analysed by High Performance Liquid Chromatography (HPLC) using a modified method by Frayne [47]. The Agilent 1100 instrument (Agilent Technologies, Santa Clara, CA, USA) was fitted with a HPX-87H column (300 mm × 7.8 mm; BioRad, Hercules, CA, USA) and a 96-well plate sampler (G1367A). Before injection (20 µL), samples (300 µL) were centrifuged (10 min; 1500 × g) using 0.2 µm 96-well plate filter plates (Acroprep™ Advance, Pall Corporation, NY, USA). The eluent was 2.5 mM H_2SO_4, with a 0.5 mL/min flow rate at 60 °C for a 35 min run time. Signals were detected using Agilent G1315B diode array detector (organic acids) and G1362A refractive index detector (hexoses and alcohols). Analytes were quantified using the external calibration curves ($R^2 > 0.99$) in ChemStation software (version B.01.03). The determined concentrations of metabolites were used to derive the following parameters: consumed sugar (g/L); yields (g/g or mg/g) of ethanol, glycerol, acetate and lactate, which were calculated from their respective concentrations (g/L

or mg/L) and sugar consumption extent (g/L); fermentation purity (FP) was expressed as a ratio of acetic acid (g/L) and ethanol (% v/v) and the extent of fructophilicity (G/F) as a ratio of residual glucose and fructose. Concentrations of pyruvic acid in final wines were measured enzymatically (K-PYRUV kit) using a ChemWell 2910 Autoanalyser (Megazyme, Ireland). Solid phase microextraction – gas chromatography - mass spectrometry (SPME-GC-MS) was used to analyse the volatile composition of the wines. Aliquots of the wines (5 mL) were analysed in a 1:2 dilution with deionised H_2O, with 3 g NaCl added to each SPME vial (20 mL) prior to sample addition. The samples were spiked with 10 µL of a methanolic mixture of five internal standards at the specified concentrations: d13-hexanol (920 mg/L; CDN Isotopes Inc., Pointe-Claire, Canada); d11-hexanoic acid (930 mg/L; CDN Isotopes Inc.); d16-octanal (82.1 mg/L; CDN Isotopes Inc.); d9-ethyl nonanoate [48] (9.2 mg/L); d3-linalool (1.73 mg/L; CDN Isotopes Inc.). SPME-GC-MS was carried out using an Agilent 7890A gas chromatograph equipped with a Gerstel (Mülheim an der Ruhr, Germany) MPS2 auto-sampler and using an Agilent 5975C mass spectrometer for peak detection and compound identification. The auto-sampler was operated in SPME mode utilizing a 2 cm, 23-Gauge, divinylbenzene-carboxen-polydimethylsiloxane fiber (50/30 µm DVB-CAR-PDMS; Supelco, Bellefonte, PA) for extraction. Volatile compounds were extracted using agitation (250 rpm) at 40 °C for 30 mins. Chromatography was performed using a ZB-Wax column (Phenomenex, NSW, Australia) of length 30 m, internal diameter 0.25 mm and film thickness 0.25 µm using helium (Ultrahigh Purity; Air Liquide, SA, Australia) as a carrier gas at 1.2 mL/min with constant flow. Volatiles were desorbed from the fibre in the GC-inlet (220 °C) for 1 min and separated using the following temperature program: 35 °C for 1.5 min, increasing at 7° C/min to 245 °C, held isothermally at 245 °C for 3.5 min. The temperature of the transfer line connecting the GC and MS was held at 250 °C. Positive-ion electron impact spectra (70 eV) were recorded in scan mode (range: m/z 35-350, scan rate: 4.45 scans/s).

GC-MS data processing. The GC-MS data was subjected to multivariate curve resolution alternating least squares (MCR-ALS) analysis according to Schmidtke et al. [36] in MATLAB R2017b (Mathworks, Natic, MA, USA). The total ion chromatograms were manually inspected prior to alignment, resulting in 50 time windows. The pre-processing of chromatograms included smoothing and elimination of contamination ions prior to deconvolution. The 90 features (peaks) retained for further analysis were integrated, and their areas were normalised to the geometric mean of the internal standard peak areas. An offset of 1 was applied to each feature peak area prior to logarithmic transformation (base10), mean centring and Pareto scaling were then applied to the block-scaled peak area matrix to obtain the format used for the statistical analysis. The features' mass spectra were exported in a format compatible with the National Institute of Standards and Technology (NIST) Mass Spectral Search Program (demo version). The identification was conducted by matching the mass spectra with the NIST-11 Library, resulting in either confirmed identity (CI), tentative identity (TI) or no identity (NI) of the target compounds. The criteria for TI were the mass spectra match scores ≥ 750 and corresponding Kovats' retention indices (RI), and for CI the same criteria as for TI alongside a comparison with pure compounds. Tentative and confirmed identification was obtained for 15 and 31 compounds, respectively. The identification criteria were not met (NI) for the remaining 44 compounds, denoted as 'unknown'.

Data analysis. Data was analysed with custom-made scripts in R [49]. The microbial growth data (OD_{600} readings) were fitted into a logistic model as per Albertin et al. [50], allowing for the extraction of four population dynamics parameters: lag phase duration (lag OD, h), the maximum growth rate (r OD, maximum number of division/h), the maximum growth (max OD), and the growth time without the lag phase (growth time, h). The sugar consumption data was fitted using a Local Polynomial Regression (*loess* function) to estimate the time required for consumption of 5% (lag AF, h), 30% without the lag AF (T30, h) and 50% without the lag

AF (T50, h) of initial sugars, and maximum sugar consumption rate (max flux; maximum g/L sugar consumed per h). Growth and sugar consumption parameters were used to identify outliers (*outlier* function; package psych). Outliers for growth encompassed triplicates of 51-160 and YJS4206, two replicates of YJS4246, and one replicate of each MS91Z4 and YJS4295. For sugar consumption, outliers were triplicates of 51-160 and Y72_132, two replicates of YJS4206, and one replicate of YJS4219. The unsupervised learning analysis self-organising map (SOM) was used for dimensionality reduction of both sugar consumption and growth kinetics (*som* function, som package [51]). SOM was performed on mean kinetics per strain upon excluding the outliers, as they may bias the mapping. Several combinations of x-dimension (1-4) and y-dimension (1-4) of the maps were performed. For each combination, a chi^2 test was performed to determine whether the corresponding SOM allowed for the discrimination of strains' genetic groups. For growth kinetics, the lowest p-value (0.19) was obtained for four clusters (x=2, y=2). For sugar consumption kinetics, the lowest p-value (3.10e-5) corresponded to a 4-cluster map (x=1, y=4).

The variation in each measured and derived parameter (114 parameters) was tested following two factors: strain factor and genetic group factor as determined in Hranilovic et al. [4]. For each parameter, the factor effect was tested by either ANOVA (to estimate the percentage of variation explained by each factor) or Kruskal-Wallis (KW) to determine the significance groups (R package agricolae). For both factors and both analyses, the p-values were corrected for multiple tests (*p.adjust* function, Benjamini & Hochberg correction). LDA was performed using *lda* function (R package MASS). Since collinear variables may blur the analysis, combined variables (e.g. G/F, yields) were excluded and the data matrix for LDA thus contained 282 rows (experiments) and 107 variables. A classification algorithm was used (random forest implemented on *cforest* function, R package party) to identify which variables accounted the most for genetic group discrimination, and the importance of each variable was

computed using the *varimp* function (R package party). Multiple linear regression analysis was performed to examine the relationships between parameters of interest and the remaining variables. An initial model was performed (*lm* function) containing all explaining variables, followed by a stepwise algorithm (*step* function, mode in both direction), which was used to choose a model based on the Akaike Information Criterion (AIC). Correlations between metabolites of interest were assessed using Spearman's test.

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Author Contributions

AH, PRG, IMP, MB, WA, VJ conceptualised and designed the study; AH, PKB acquired the data; AH, JG, LS, WA analysed the data; WA performed the statistical analysis; AH wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated and analysed during the current study are available from the corresponding and leading authors on a reasonable request.

Supplementary Information

Oenological phenomes of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation

Ana Hranilovic[1,2], Joanna M. Gambetta[3], Leigh Schmidtke[1,3], Paul K. Boss[4], Paul R. Grbin[2], Isabelle Masneuf-Pomarede[5,6], Marina Bely[5], Warren Albertin[5,7], Vladimir Jiranek[1,2*]

[1] The Australian Research Council Training Centre for Innovative Wine Production, Adelaide, SA, Australia

[2] Department of Wine and Food Science, The University of Adelaide, Adelaide, SA, Australia

[3] National Wine and Grape Industry Centre, School of Agricultural and Wine Science, Charles Sturt University, Wagga Wagga, NSW, Australia

[4] CSIRO Agriculture and Food, Adelaide, SA, Australia

[5] Unité de recherche Œnologie, Institut de la Science de la Vigne et du Vin, University of Bordeaux, Villenave d'Ornon, France

[6] Bordeaux Sciences Agro, Gradignan, France

[7] ENSCBP, Bordeaux INP, Pessac, France

[*] **Correspondence**

Vladimir Jiranek

vladimir.jiranek@adelaide.edu.au

Supplementary Table 1. List of the studied *L. thermotolernas* strains. Strains of different geographic and isolation niches were divided into nine genetic groups according to Hranilovic et al. (2017).

Strain	Source	Isolation location	Isolation niche	Genetic group	References
11/2-112	University of Debrecen	Slovakia, Mala Trna	grapes, mummified	Mix Europe/N. America	2, 5
11/Z-1	University of Debrecen	Slovakia, Mala Trna	grapes, mummified	Mix Europe/N. America	2, 5
14/1/Z-2	University of Debrecen	Hungary, Erdöbénye	grapes, mummified	Mix E. Europe	2, 5
2/2/Z-8	University of Debrecen	Hungary, Sárazsadány	grapes, mummified	Mix Europe/N. America	2, 5
5/1/Z-7	University of Debrecen	Hungary, Vinicky	grapes, mummified	Mix Europe/N. America	2, 5
50-15	Phaff YCC	USA, California	*Drosophila pseudoobscura*	Americas	2
51-160	Phaff YCC	USA, California	*Drosophila azteca*	Americas	2
51-171	Phaff YCC	USA, California	*Drosophila pseudoobscura*	Hawaii/California	2
60-260	Phaff YCC	USA, California	*Aulacigaster sp.*on *Ulmus carpinifolia* exudate	Americas	2
61-245	Phaff YCC	USA, California	*Aulacigaster sp.*	Americas	2
61-518	Phaff YCC	USA, California	*Drosophila melanogaster*	Americas	2
68-140	Phaff YCC	USA, California	bees	Hawaii/California	2
72-132	Phaff YCC	Hawaii, Ahumoa	*Myoporum sandwicens*exudate	Hawaii/California	2
72-137	Phaff YCC	Hawaii, Ahumoa	*Myoporum sandwicens*exudate	Hawaii/California	2
8/2/Z-3	University of Debrecen	Slovakia, Černochov	grapes, mummified	Mix Europe/N. America	2, 5
8/Z-1	University of Debrecen	Slovakia, Černochov	grapes, mummified	Mix E. Europe	2, 5
9/1/Z-4	University of Debrecen	Slovakia, Mala Trna	grapes, mummified	Mix E. Europe	2, 5
AWRI 2009	AWMCC	Australia, South Australia	grapes	Domestic 1	2
CBS 10516	CBS-KNAW	Ukraine	*Quercus sp.* exudate	Europe oak/France grapes	2

CBS 10517	CBS-KNAW	Ukraine	*Quercus sp.* exudate	Other	2
CBS 10518	CBS-KNAW	Ukraine	*Quercus sp.* exudate	Mix E. Europe	2
CBS 137	CBS-KNAW	Netherlands	date	Domestic 1	1, 2
CBS 2860	CBS-KNAW	Italy, Sardinia	grape must	Domestic 1	1, 2
CBS 2907	CBS-KNAW	South Africa	soil	Domestic 2	1, 2
CBS 5464	CBS-KNAW	Australia	cotton seed	Hawaii/California	2
CBS 6292	CBS-KNAW	Australia	na	Europe oak/France grapes	2
CBS 6340T	CBS-KNAW	Russia	mirabelle plum conserve	Domestic 1	1, 2, 3
CBS 6467	CBS-KNAW	Japan	tree exudate	Other	2
CBS 7772	CBS-KNAW	Brazil	*Uca sp.*	Americas	1, 2
CL 41	University of Leon	Spain	grapes	Domestic 2	2
CL 43	University of Leon	Spain	grapes	Hawaii/California	2
CONCERTO™	CHR Hansen	'Mediterranean country'	na	Domestic 1	2
CRBO L0672	CRBOeno	France, Bordeaux	grapes, fermentation	Mix Europe/N. America	2
DBVPG 10092	DBVPG	Algeria	soil, apple orchard	Domestic 2	2
DBVPG 2551	DBVPG	Italy, Piemonte	wine cv. Barbera	Domestic 2	1, 2
DBVPG 2700	DBVPG	Spain, La Mancha	grapes cv. Airen	Mix Europe/N. America	1, 2
DBVPG 3464	DBVPG	Spain, La Mancha	grapes	Domestic 1	1, 2
DBVPG 3466	DBVPG	Spain, La Mancha	grapes	Domestic 1	1, 2
DBVPG 3469	DBVPG	Spain, La Mancha	grapes	Mix Europe/N. America	1, 2
DBVPG 4035	DBVPG	ex Yugoslavia	grapes, must	Domestic 1	1, 2
DBVPG 6322	DBVPG	Italy	grapes	Domestic 2	2
DBVPG 6326	DBVPG	Italy	grapes, raisins	Domestic 2	2
Fin. 89-2	na	Finland	*Quercus sp.*exudate	Europe oak/France grapes	1, 2, 3
FRI10C.1	NCYC	UK, Fritham	*Quercus sp.*	Europe oak/France grapes	2, 4
HU 2511	BOKU	Austria	grapes	Domestic 2	2

ISVV Ltyq25	ISVV	France, Sauternes	grapes, high sugar must	Europe oak/France grapes	2
ISVV Ltyq3	ISVV	France, Sauternes	grapes, high sugar must	Europe oak/France grapes	2
ISVV Ltyq36	ISVV	France, Sauternes	grapes, high sugar must	Europe oak/France grapes	2
JCB1	ISVV	France, Sauternes	grapes, high sugar must	Domestic 1	2
KEH.34.B.3	na	USA, Missouri	grapes, fermentation	Canada trees	1, 2
LEVULIA® ALCOMENO	AEB	France, Burgundy	grapes, fermentation	Europe oak/France grapes	2
LL12-031	LL	Canada	*Quercus sp.*tree bark	Canada trees	2
LL12-040	LL	Canada	*Acer sp.* bark	Mix Europe/N. America	2
LL12-041	LL	Canada	*Quercus sp.*bark	Mix Europe/N. America	2
LL12-056	LL	Canada	planted *Quercus sp.*bark	Canada trees	2
LL13-038	LL	USA, Massachusetts	*Quercus sp.*bark	Mix Europe/N. America	2
LL13-189	LL	Canada, New-Brunswick	*Quercus sp.*bark	Canada trees	2
LL13-194	LL	Canada, New-Brunswick	*Quercus sp.*bark	Canada trees	2
MB10D.1	NCYC	France, Montbarri	*Quercus sp.*	Europe oak/France grapes	2, 4
MUCL 31341	MUCL	Italy	wine	Domestic 1	1, 2
MUCL 31342	MUCL	Italy	wine	Domestic 1	2
MUCL 47720	MUCL	Italy	wine	Domestic 1	2
NCAIM Y.00775	NCAIM	Hungary, Babat	*Carpinus betulu* exudate	Mix Europe/N. America	2
NCAIM Y.00798	NCAIM	Hungary, Csikóváralja	brown rotten *Quercus sp.*	Mix Europe/N. America	2
NCAIM Y.00873	NCAIM	Hungary, Budapest	rotten material of a cavity of *Betula pendula*	Mix E. Europe	2
NCAIM Y.01703	NCAIM	Hungary, Nagyeged	grapes	Mix Europe/N. America	2
NRLL Y-2193	NRRL/ARS	USA, California	*Drosophila pseudoobscura*	Americas	2
NRLL Y-27329	NRRL/ARS	USA, West Virginia	grapes	Domestic 2	2

NZ156	CRPR	New Zealand	grapes cv. Chardonnay	Domestic 1	2
OCK6C.1	NCYC	UK, Ocknell	*Quercus sp.*	Europe oak/France grapes	2, 4
OSU A	OSU	USA, Oregon	grapes	Mix Europe/N. America	2
PLU5B.1	NCYC	UK, East Sussex	*Quercus sp.*	Europe oak/France grapes	2, 4
PYR14B.1	NCYC	Greece, Pyradikia	*Quercus sp.*	Domestic 1	2, 4
T 13/17 F	University of the Republic	Uruguay	grapes cv. Tannat	Domestic 2	2
TAX9D.1	NCYC	Greece, Taxiarchis	*Quercus sp.*	Mix Europe/N. America	2, 4
UNIFG 16	UNIFG	Italy	wine	Domestic 2	2
UNIFG 17	UNIFG	Italy	wine	Domestic 2	2
UNIFG 18	UNIFG	Italy	wine	Domestic 2	2
UNIFG 22	UNIFG	Italy	wine	Domestic 2	2
UNIFG 26	UNIFG	Italy	wine	Domestic 1	2
UNIFG 28	UNIFG	Italy	wine	Domestic 2	2
UNIFG 32	UNIFG	Italy	wine	Domestic 2	2
UWOPS 79-110	UWOPS	Canada, Ontario	black knot, *Prunus virginiana*	Canada trees	1, 2
UWOPS 79-164	UWOPS	Canada, Ontario	black knot, *Prunus virginiana*	Canada trees	1, 2
UWOPS 79-195	UWOPS	Canada, Ontario	black knot, *Prunus virginiana*	Canada trees	1, 2
UWOPS 83-1097.1	UWOPS	Cayman Islands	black knot, *Prunus virginiana*	Americas	1, 2, 3
UWOPS 83-1101.1	UWOPS	Cayman Islands	*Gitona americana*, *Opuntia stricta*	Americas	1, 2
UWOPS 85-312.1	UWOPS	USA, Arizona	*Drosophila carbonaria*, *Prosopis juliflora*	Americas	1, 2
UWOPS 85-51.1	UWOPS	USA, Florida	*Opuntia cubensis*	Americas	1, 2
UWOPS 90-10.1	UWOPS	Bahamas, Exumas Cays	Columnar cactus	Americas	1, 2
UWOPS 91-910.1	UWOPS	Hawaii, Ahumoa	flux (pink), *Myoporum*	Hawaii/California	1, 2
UWOPS 91-912.1	UWOPS	Hawaii, Ahumoa	flux (white), *Myoporum*	Hawaii/California	1, 2
UWOPS 94-426.2	UWOPS	Mexico, Jalisco	distillery, agave must	Domestic 1	1, 2

Yal. 87-1	na	Russia, Crimea	*Quercus sp.* exudate	Europe oak/France grapes	2, 3

Phaff YCC – Phaff Yeast Culture Collection, University of California, Davis, USA; **AWMCC** – AWRI Wine Microorganism Culture Collection, Australia; **CBS-KNAW** – Centraalbureau voor Schimmelcultures – Koninklijke Nederlandse Akademie van Wetenschappen, Netherlands; **CRBOeno** - Centre de Ressources Biologiques OEnologie, France; **DBVPG** The Industrial Yeasts Collection DBVPG, Italy; **NCYC** – National Collection of Yeast Cultures, UK;, Italy; **BOKU** – Universität für Bodenkultur Wien, Austria; **ISVV** – Institut des Sciences de la Vigne et du Vin, France; **LL** – Landry Lab, Canada; **MUCL** – Mycothèque de l'Université catholique de Louvain, Belgium; **NCAIM** – National Collection of Agricultural and Industrial Microorganisms, Hungary; **NRRL/ARS** – NRRL Agriculture Research Service Culture collection, USA; **CRPR** – Centre de Recherche Pernod-Ricard, France; **OSU** – Oregon State University, USA; **UNIFG –** University of Foggia; **UWOPS** – Culture Collection of the University of Western Ontario; na – not available; [1] Freel, K. C., Friedrich, A., Hou, J. & Schacherer, J. Population genomic analysis reveals highly conserved mitochondrial genomes in the yeast species *Lachancea thermotolerans. Genome Biol. Evol.* **6**, 2586-2594, doi:10.1093/gbe/evu203 (2014); [2] Hranilovic, A., Bely, M., Masneuf-Pomarede, I., Jiranek, V. & Albertin, W. The evolution of *Lachancea thermotolerans* is driven by geographical determination, anthropisation and flux between different ecosystems. *Plos One* **12**, e0184652, doi:10.1371/journal.pone.0184652 (2017); [3] Naumova, E. S., Serpova, E. V. & Naumov, G. I. Molecular systematics of *Lachancea* yeasts. *Biochemistry (Moscow)* **72**, 1356-1362 (2007); [4] Robinson, H. A., Pinharanda, A. & Bensasson, D. Summer temperature can predict the distribution of wild yeast populations. *Ecol. Evol.* **6**, 1236-1250 (2016); [5] Sipiczki, M. Overwintering of vineyard yeasts: Survival of interacting yeast communities in grapes mummified on vines. *Front. Microbiol.* **7**, 212, doi:10.3389/fmicb.2016.00212 (2016).

Supplementary Table 2. Analysed volatile compounds in *L. thermotolerans* wines displaying significant (p-value < 0.05) and non-significant strain effect divided into chemical classes. The tentative identification (TI) was accomplished via corresponding Kovats' retention indices (RI) obtained with an equivalent stationary phase (in italics) and/or mass spectra match scores ≥ 750, and confirmed (confirmed identification; CI) via comparison with pure compounds. The unknown compounds (no identification, NI) are numbered based on the chromatographic elution profile.

No.	Compound	Formula	CAS	Kovats' RI	Identification
Significant strain effect					
	Alcohols				
1	butanol	$C_4H_{10}O$	71-36-3	1143	CI
2	isobutanol	$C_4H_{10}O$	78-83-1	1089	CI
3	isoamyl alcohol	$C_5H_{12}O$	123-51-3	1222	CI
4	2-methyl-1-butanol	$C_5H_{12}O$	137-32-6	1219	CI
5	hexanol	$C_6H_{14}O$	111-27-3	1349	CI
6	3-methyl-1-pentanol	$C_6H_{14}O$	589-35-5	*1334*[h]	TI
7	2-ethyl-1-hexanol	$C_8H_{18}O$	104-76-7	*1483*[i]	TI
8	octanol	$C_8H_{18}O$	111-87-5	1560	CI
9	2-phenylethanol	$C_8H_{10}O$	60-12-8	1901	CI
10	4-methyl-benzenemethanol	$C_8H_{10}O$	589-18-4	*1967*[a]	TI
11	nonanol	$C_9H_{20}O$	143-08-8	1647	CI
12	decanol	$C_{10}H_{22}O$	112-30-1	1755	CI
	Esters				
13	ethyl acetate	$C_4H_8O_2$	141-78-6	882	CI
14	ethyl propanoate	$C_5H_{10}O_2$	105-37-3	954	CI
15	isobutyl acetate	$C_6H_{12}O_2$	110-19-0	1011	CI
16	isoamyl acetate	$C_7H_{14}O_2$	123-92-2	1111	CI
17	diethyl succinate	$C_8H_{14}O_4$	123-25-1	1670	CI
18	amyl lactate	$C_8H_{16}O_3$	6382-06-5		TI
19	2-phenylethyl acetate	$C_{10}H_{12}O_2$	103-45-7	1802	CI
20	ethyl octanoate	$C_{10}H_{20}O_2$	106-32-1	1414	CI
21	ethyl decanoate	$C_{12}H_{24}O_2$	110-38-3	1629	CI
22	ethyl 9-decenoate	$C_{12}H_{22}O_2$	67233-91-4	*1688*[b]	TI

	Acids				
23	4-hydroxy-butanoic acid	$C_4H_8O_3$	591-81-1		TI
24	hexanoic acid	$C_6H_{12}O_2$	142-62-1	1860	CI
25	octanoic acid	$C_8H_{16}O_2$	124-07-2	2076	CI
26	decanoic acid	$C_{10}H_{20}O_2$	334-48-5	2295	CI
27	dodecanoic acid	$C_{12}H_{24}O_2$	143-07-7	2488	CI
	Aromatic hydrocarbons				
28	1,2,4-trimethyl-benzene	C_9H_{12}	95-63-6	*1277*[c]	TI
29	1-ethyl-2,4-dimethyl benzene	$C_{10}H_{14}$	874-41-9	*1348*[d]	TI
30	1,3-bis(1,1-dimethylethyl)-benzene	$C_{14}H_{22}$	1014-60-4		TI
	Aldehydes				
31	acetaldehyde	C_2H_4O	75-07-0	*744*[e]	TI
32	4-methyl-benzaldehyde	C_8H_8O	104-87-0	*1656*[f]	TI
	Norisoprenoid				
33	β-damascenone	$C_{13}H_{18}O$	23726-93-4	1830	CI
	Terpenols				
34	β-citronellol	$C_{10}H_{20}O$	106-22-9	1779	CI
	Ketone				
35	4-methyl-2-heptanone	$C_8H_{16}O$	6137-06-0	*1206*[g]	TI
	Unknowns				
36-58	F8, F10, F19, F22, F24, F27, F30, F40, F43, F45, F46, F47, F48, F50, F53, F54, F56, F77, F80, F83, F85, F86, F90				NI
Non-significant strain effect					
	Alcohols				

59	propanol	C_3H_8O	71-23-8	1030	CI
60	3-ethoxy-1 propanol	$C_5H_{12}O_2$	111-35-3	1377	CI
61	(Z)-3-hexen-1-ol	$C_6H_{12}O$	928-96-1	1396	CI
	Esters				
62	ethyl butanoate	$C_6H_{12}O_2$	105-54-4	1028	CI
63	ethyl hexanoate	$C_8H_{16}O_2$	123-66-0	1592	CI
	Aromatic hydrocarbons				
64	1-ethyl-3-methyl-benzene	C_9H_{12}	620-14-4	*1224*[c]	TI
65	2-ethyl-1,4-dimethyl-benzene	$C_{10}H_{14}$	1758-88-9	*1343*[d]	TI
	Aldehydes				
66	nonanal	$C_9H_{18}O$	124-19-6	1375	CI
67	2,5-benzaldehyde	$C_9H_{10}O$	5779-94-2		TI
	Ketone				
68	2-nonanone	$C_9H_{18}O$	821-55-6	1375	CI
	Terpenols				
69	terpineol	$C_{10}H_{18}O$	8006-39-1	1690	CI
	Unknowns				
70-90	F5, F13, F17, F20, F26, F31, F32, F41, F42, F52, F55, F64, F65, F66, F72, F73, F74, F79, F81, F84, F87				NI

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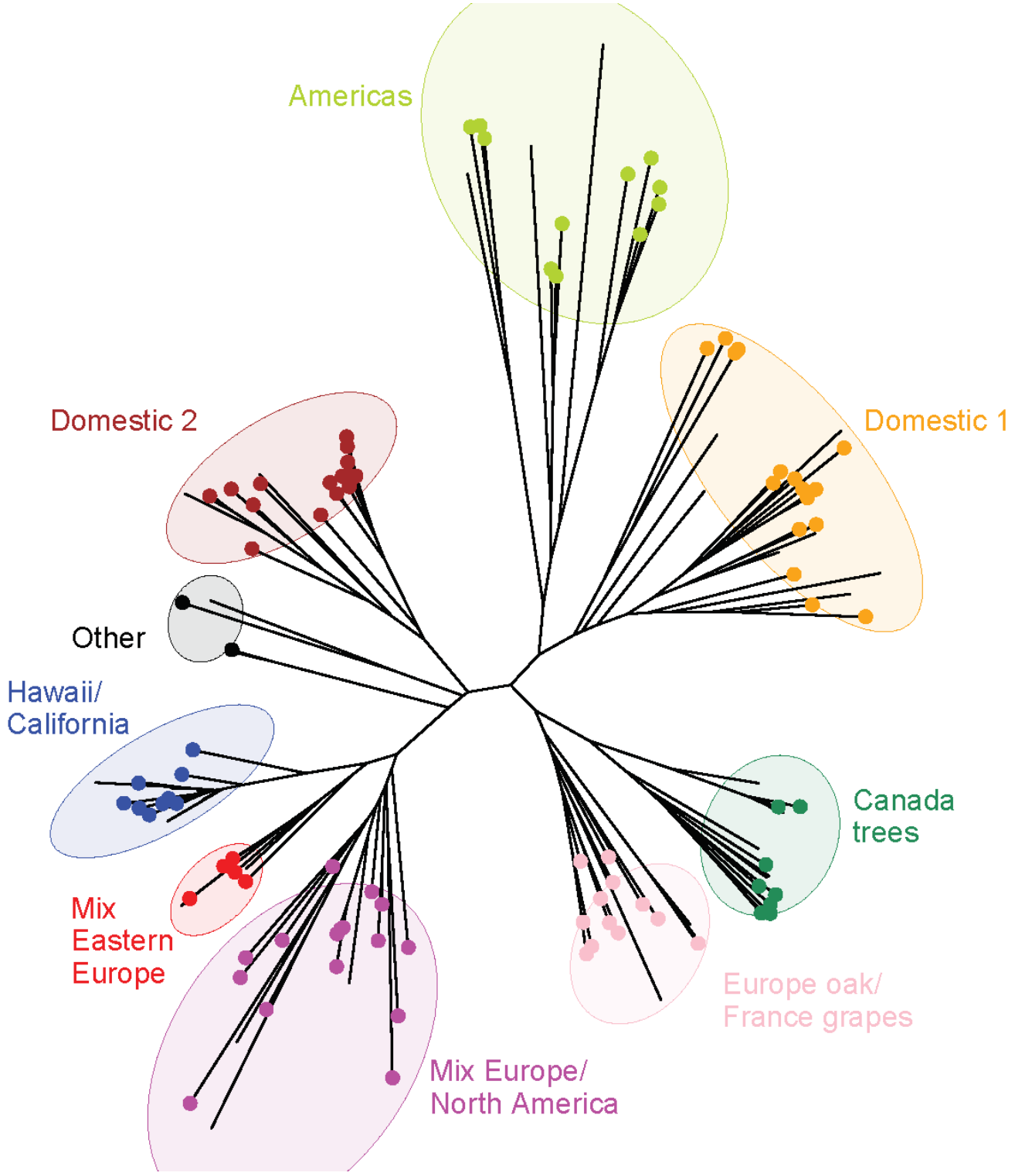
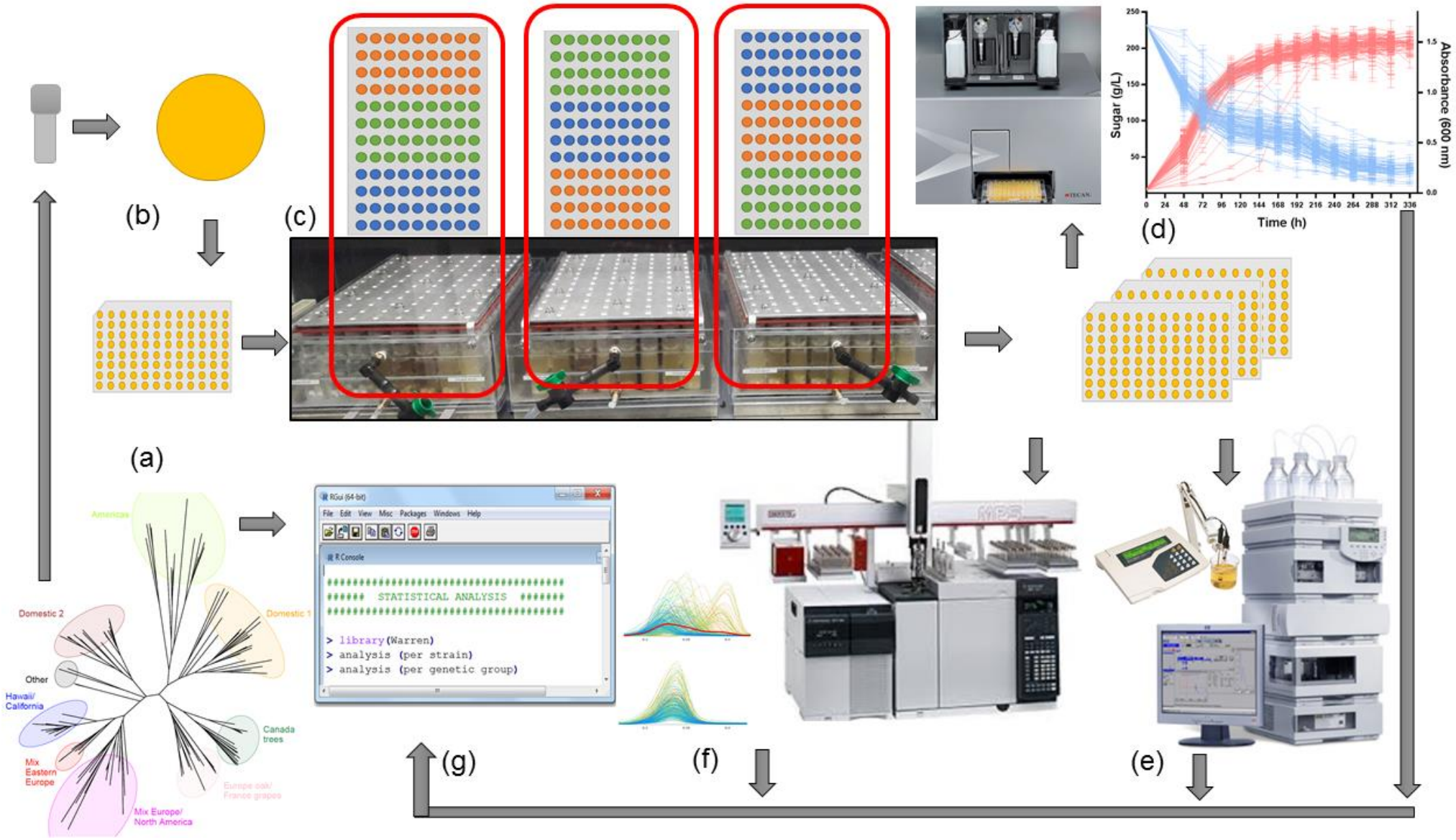
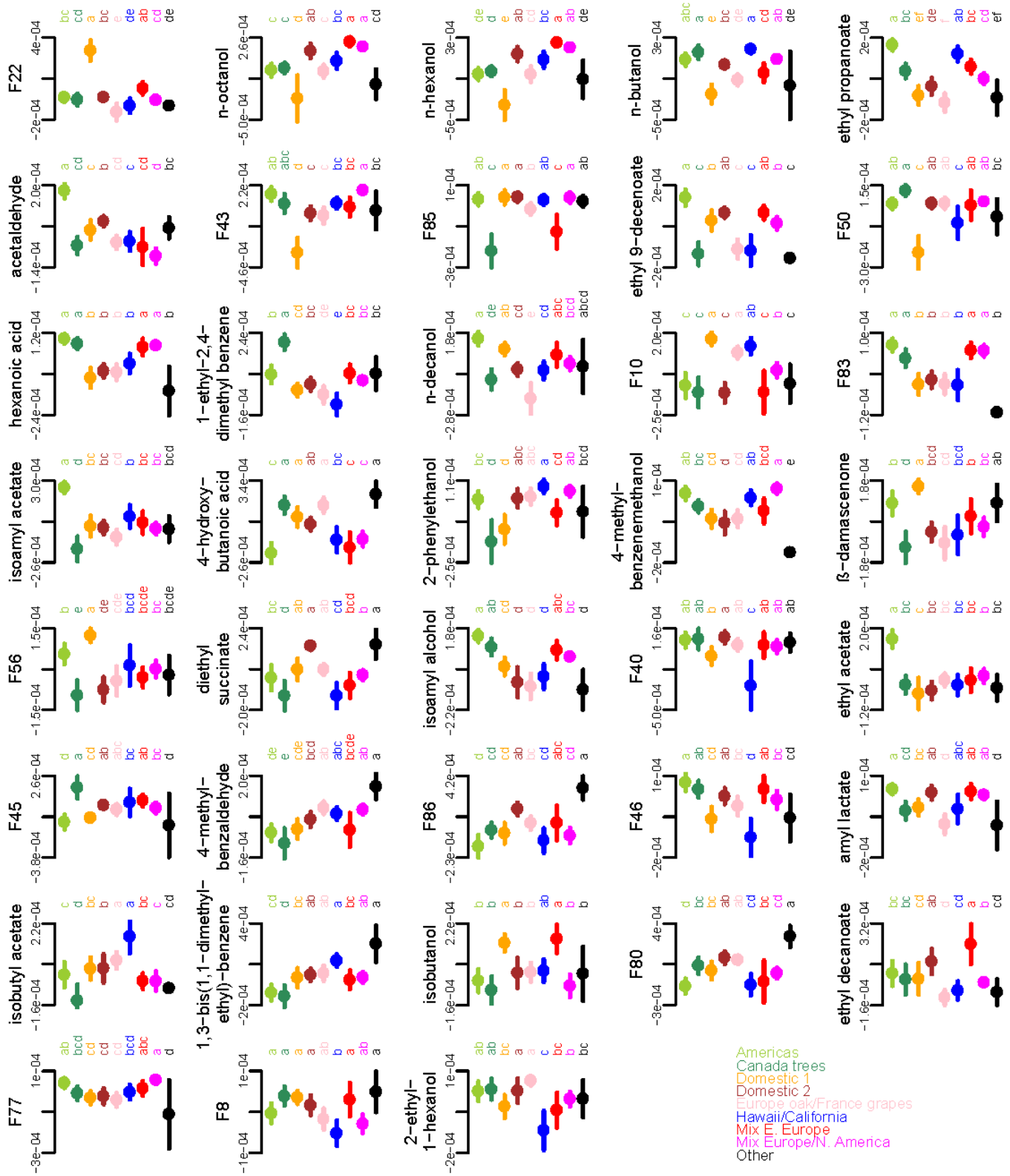


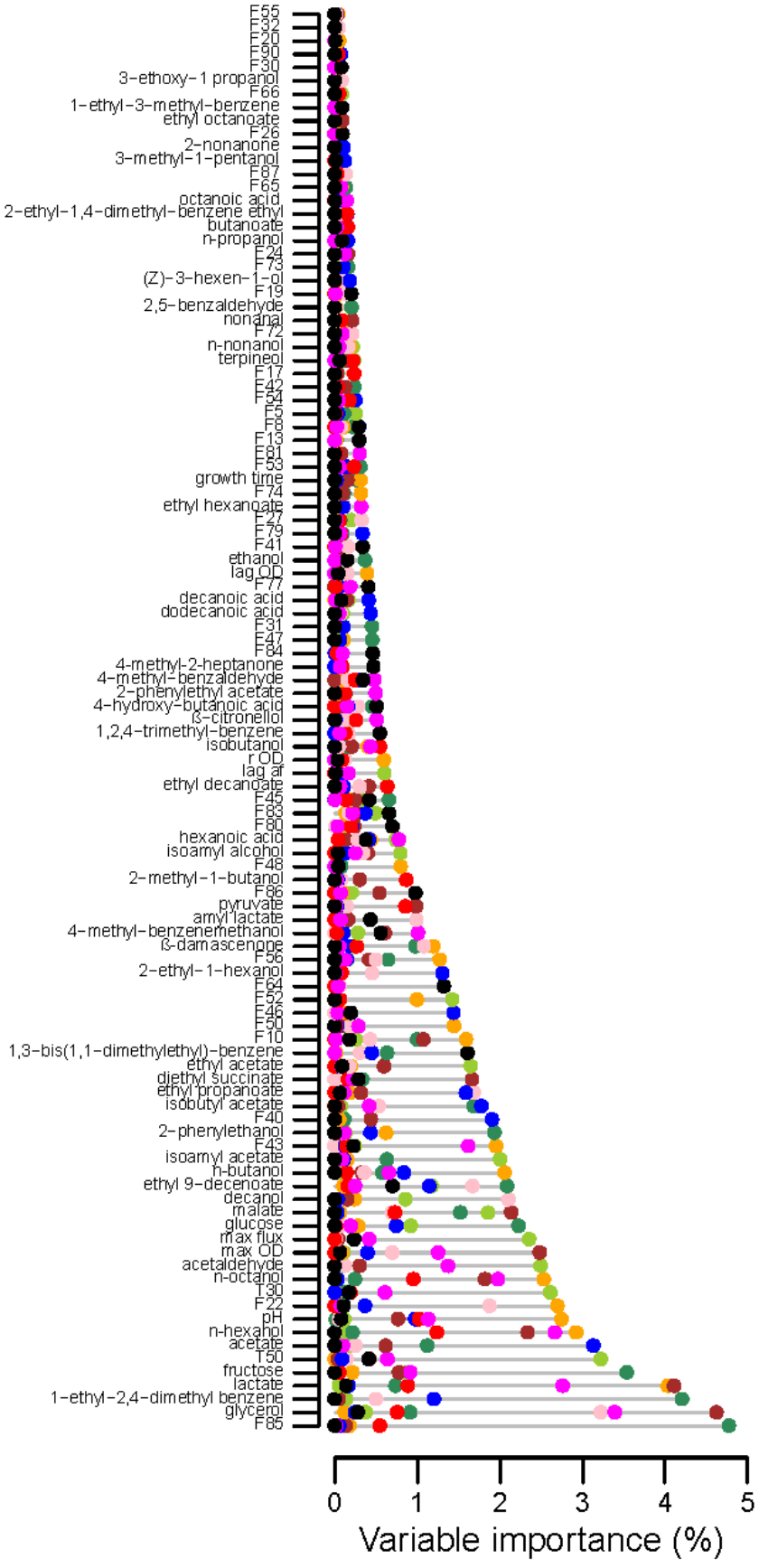
Supplementary Figure 1. Genetic relationships between 94 phenotyped *Lachancea thermotolerans* determined using 14 microsatellite markers.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
Americas
Domestic 2
Domestic 1
Other
Hawaii/
California
Canada
trees
Mix
Eastern
Europe
Europe oak/
France grapes
Mix Europe/
North America
RGui (64-bit)
File Edit View Misc Packages Windows Help
R Console
STATISTICAL ANALYSIS
> library(Warren)
> analysis (per strain)
> analysis (per genetic group)
Sugar (g/L)
Absorbance (600 nm)
Time (h)
0 24 48 72 96 120 144 168 192 216 240 264 288 312 336
250
200
150
100
50
1.5
1.0
0.5
0.0

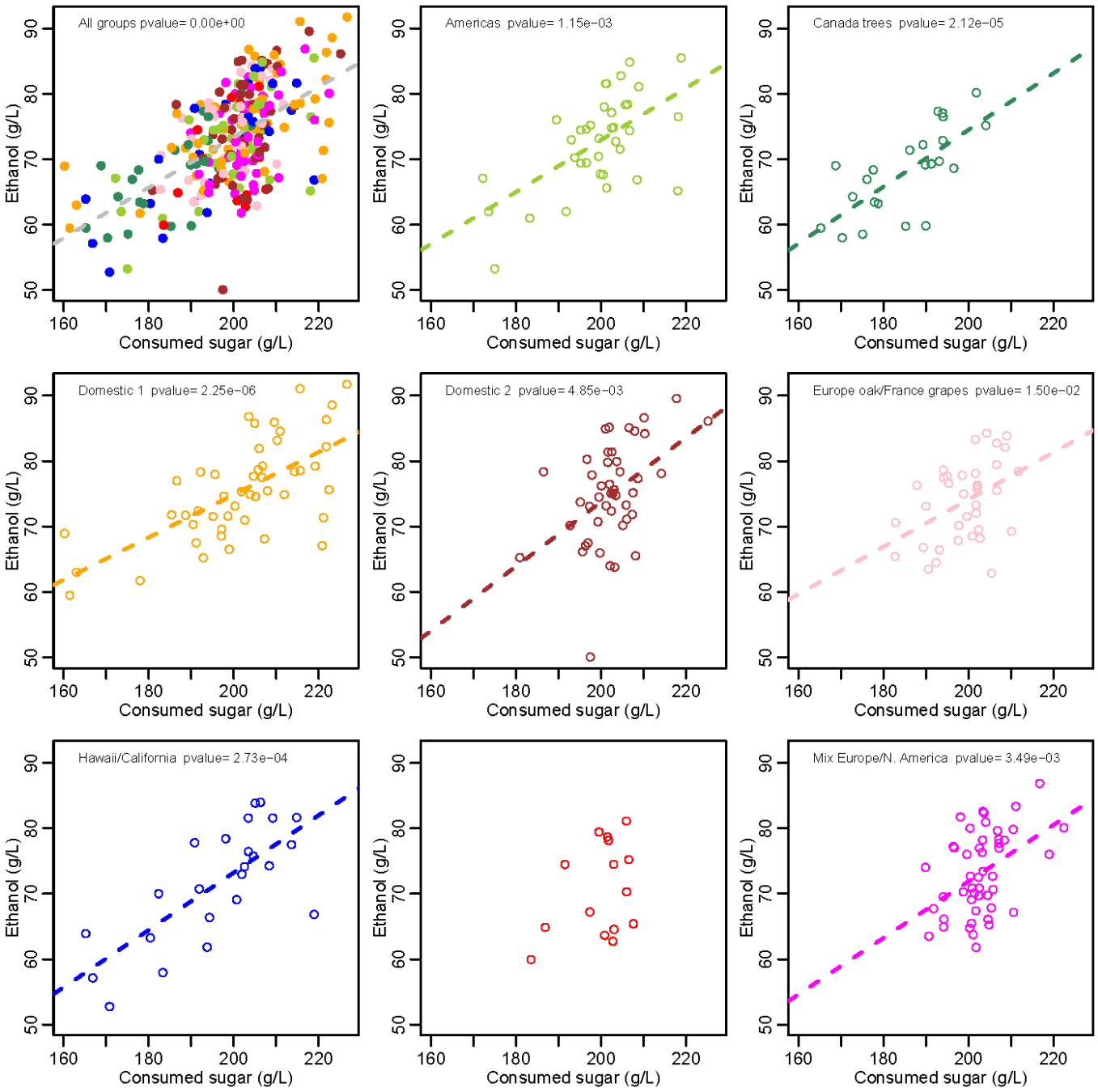
Supplementary Figure 2. Schematic representation of the *L. thermotolerans* phenotyping workflow: The cryo-cultures of 94 *L. thermotolerans* strains belonging to nine genetic groups (a) according to Hranilovic et al. (2017) were grown on YPD agar plates and in YPD broth to establish the inoculation cultures for Chardonnay grape juice fermentations (b). The triplicate fermentations were set up in 'Tee-bot v.2.0' using three 96-fermentor blocks (c). Each block contained one biological replicate, with row-wise randomisation between the blocks (indicated with orange, green and blue colour). Such randomisation was maintained for all downstream analysis. Fermentations were monitored regularly via OD_{600} and sugar consumption (d). The final wines were analysed for their pH values, and concentrations of organic acids, hexoses and alcohols via HPLC (e), and volatile composition via SPME-GC-MS (f). All the measured and derived parameters were subjected to appropriate univariate and multivariate statistical analysis (g).



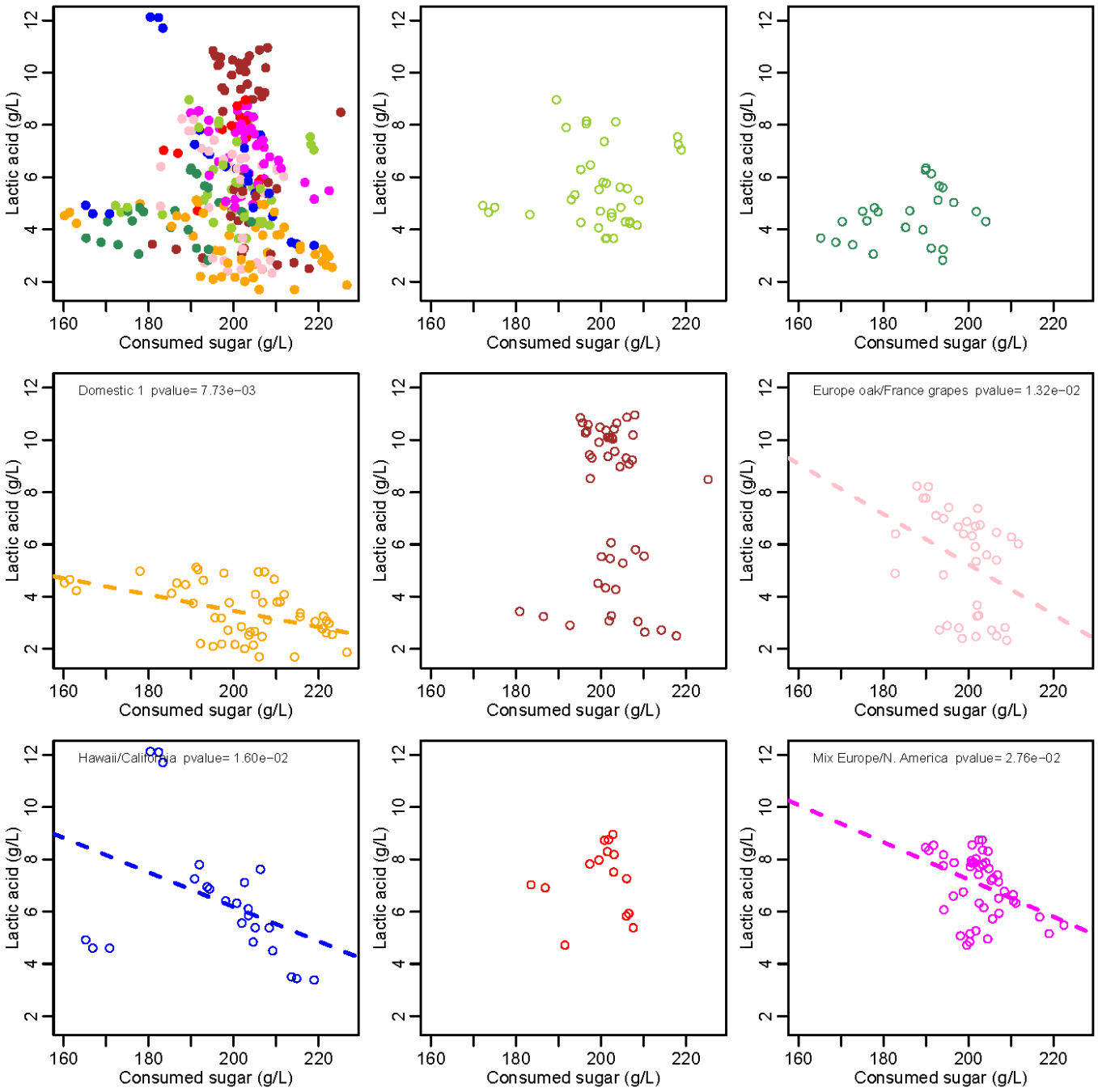
Supplementary Figure 3. The analysed volatile compounds displaying a significant genetic group effect. Dots and bars represent means and standard errors, respectively. Top letters represent significance groups as defined by Kruskal-Wallis test (*agricolae* package, p-value < 0.05 after Benjamini & Hochberg adjustment for multiple comparisons).



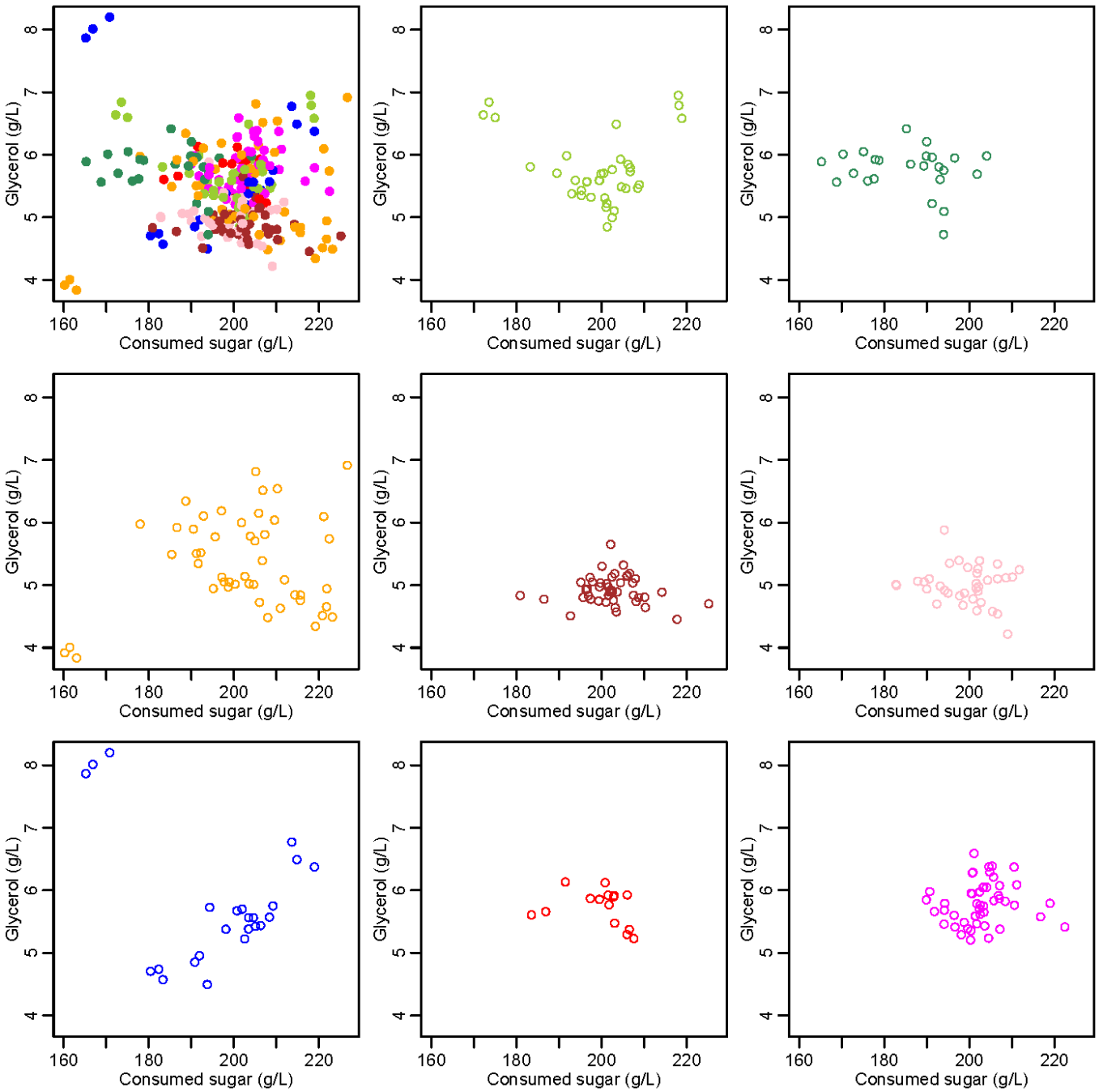
Supplementary Figure 4. The importance of all 107 variable subjected to LDA.



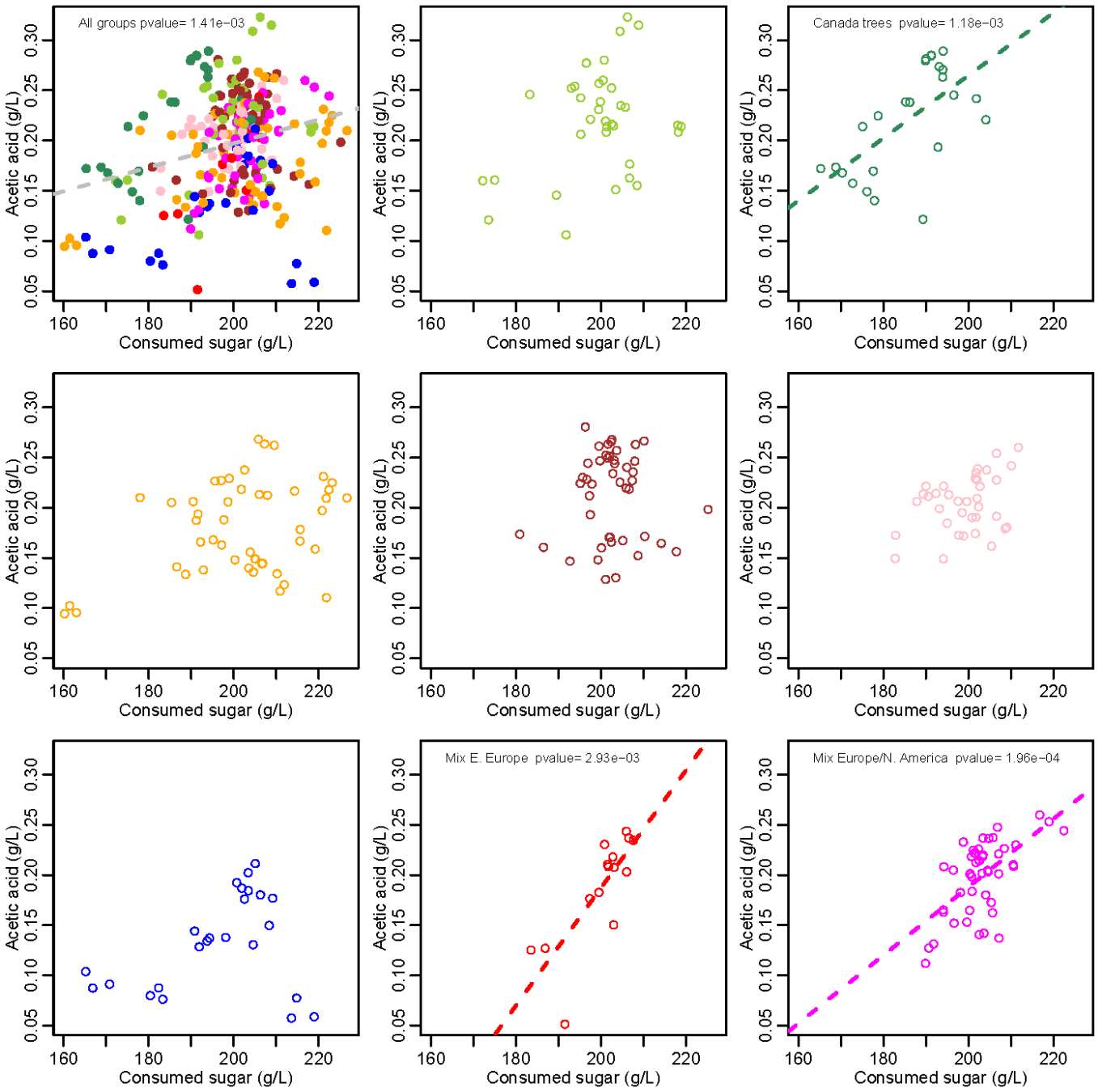
Supplementary Figure 5. Spearman's correlation test between the selected metabolites: consumed sugar and ethanol.



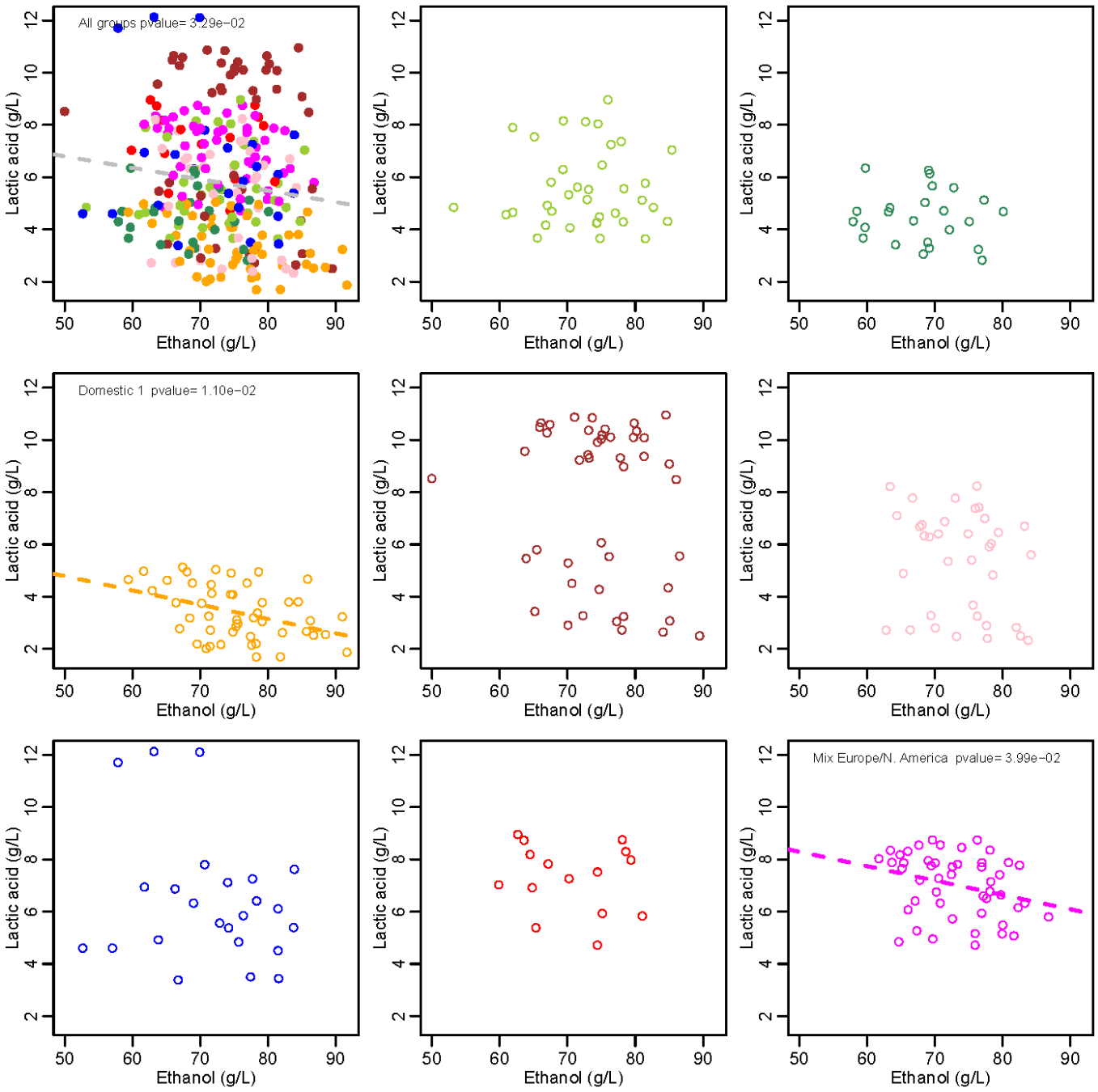
Supplementary Figure 6. Spearman's correlation test between the selected metabolites: consumed sugar and lactic acid.



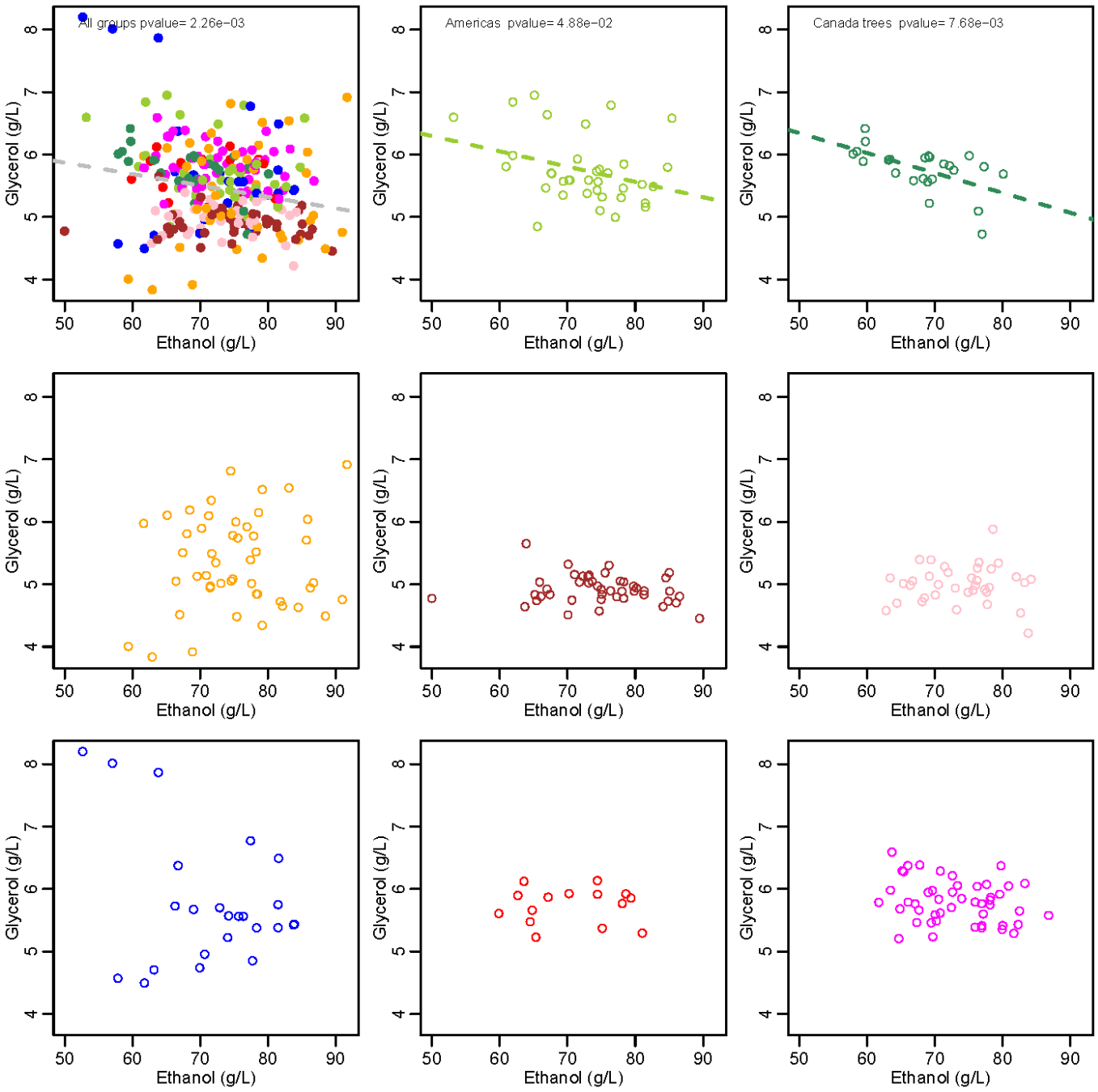
Supplementary Figure 7. Spearman's correlation test between the selected metabolites: consumed sugar and glycerol.



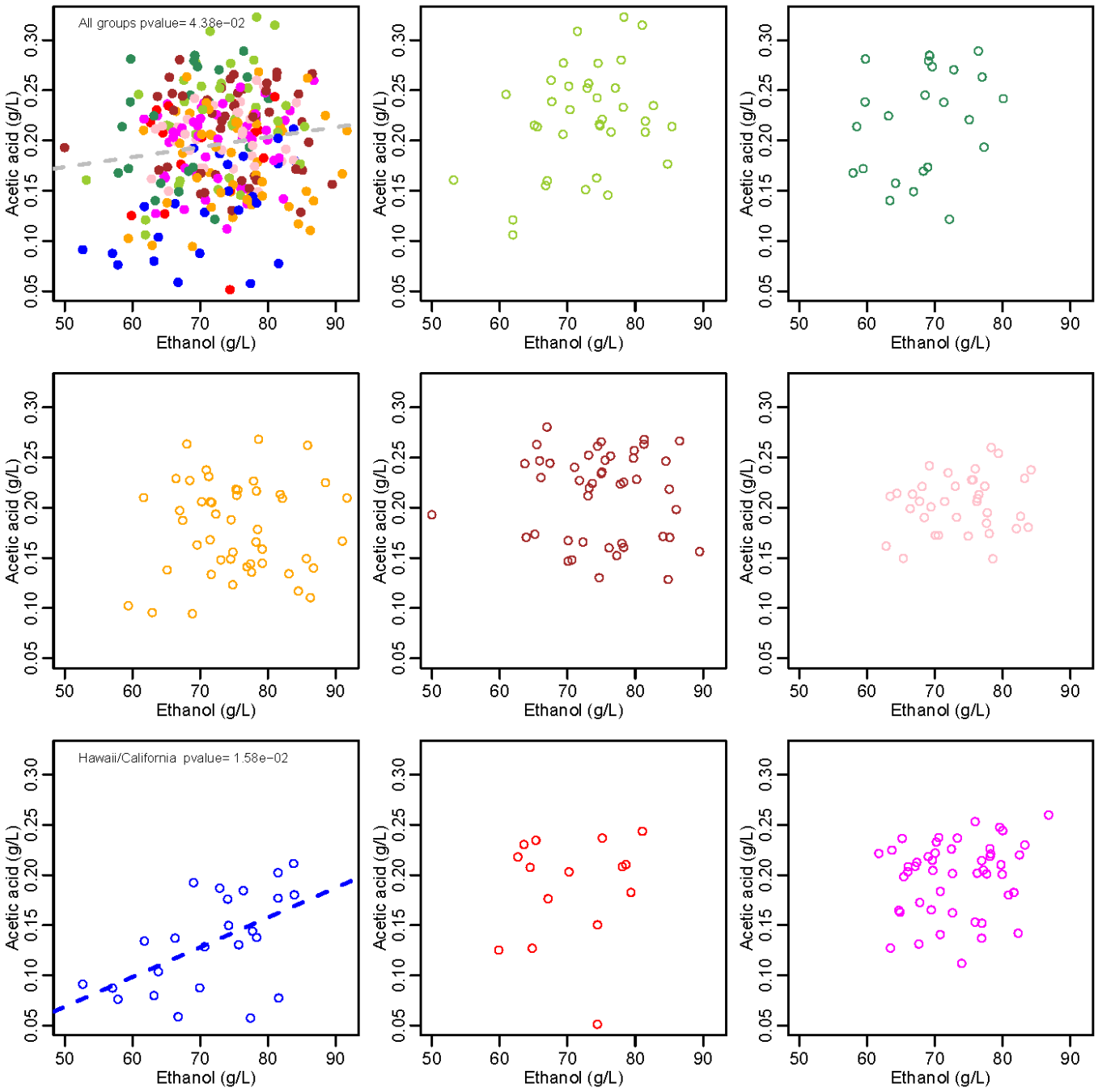
Supplementary Figure 8. Spearman's correlation test between the selected metabolites: consumed sugar and acetic acid.



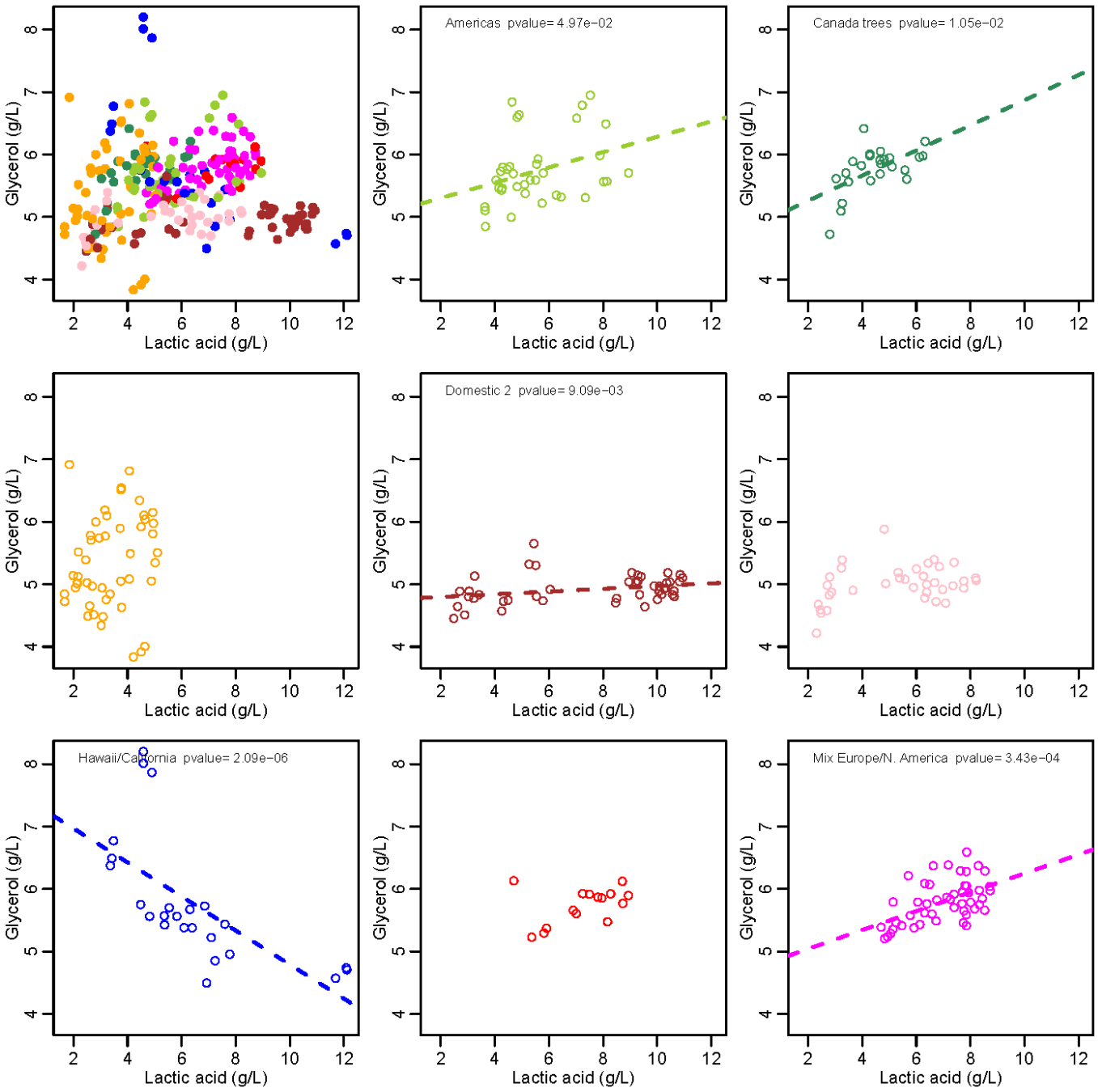
Supplementary Figure 9. Spearman's correlation test between the selected metabolites: ethanol and lactic acid.



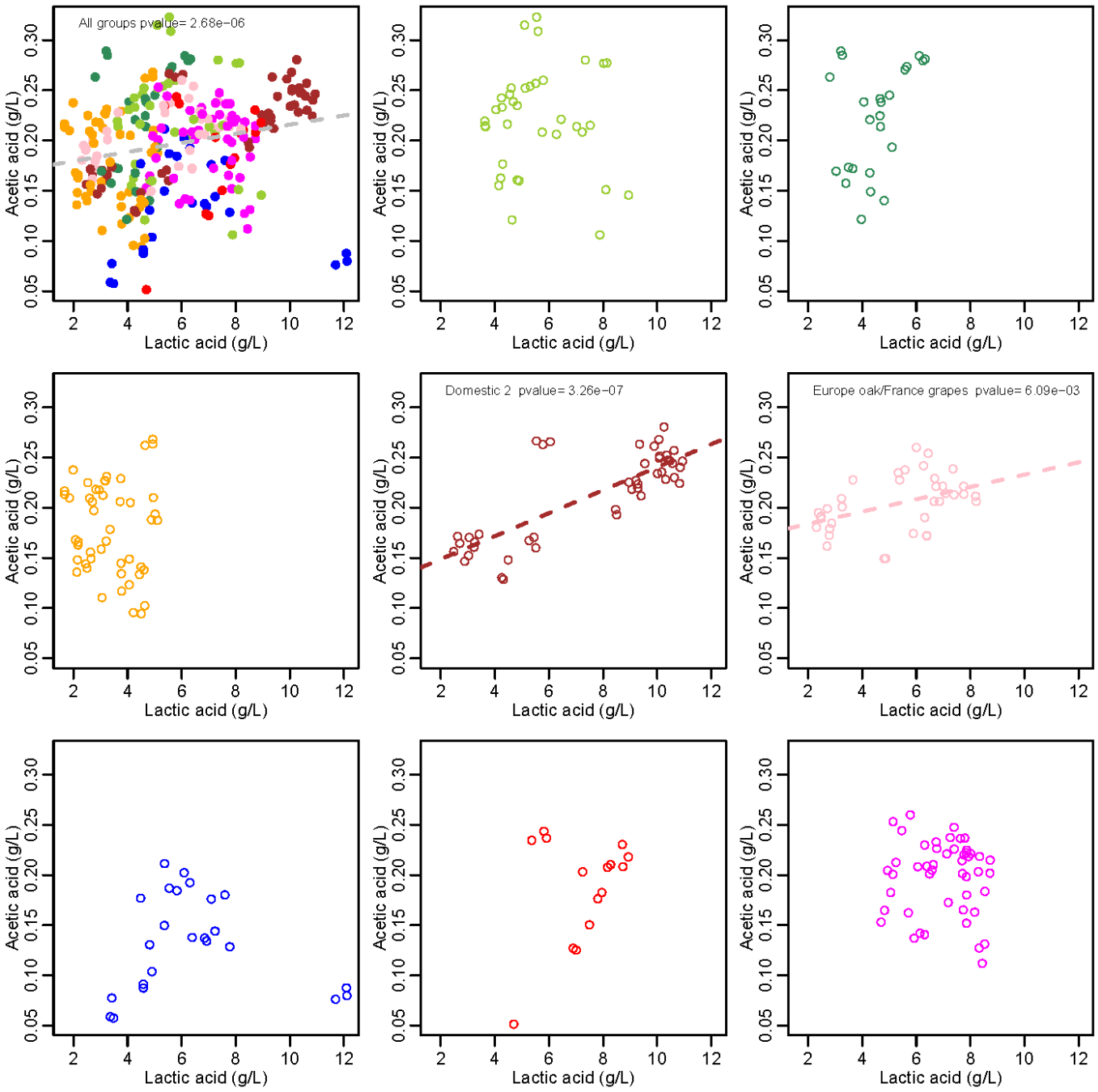
Supplementary Figure 10. Spearman's correlation test between the selected metabolites: ethanol and glycerol.



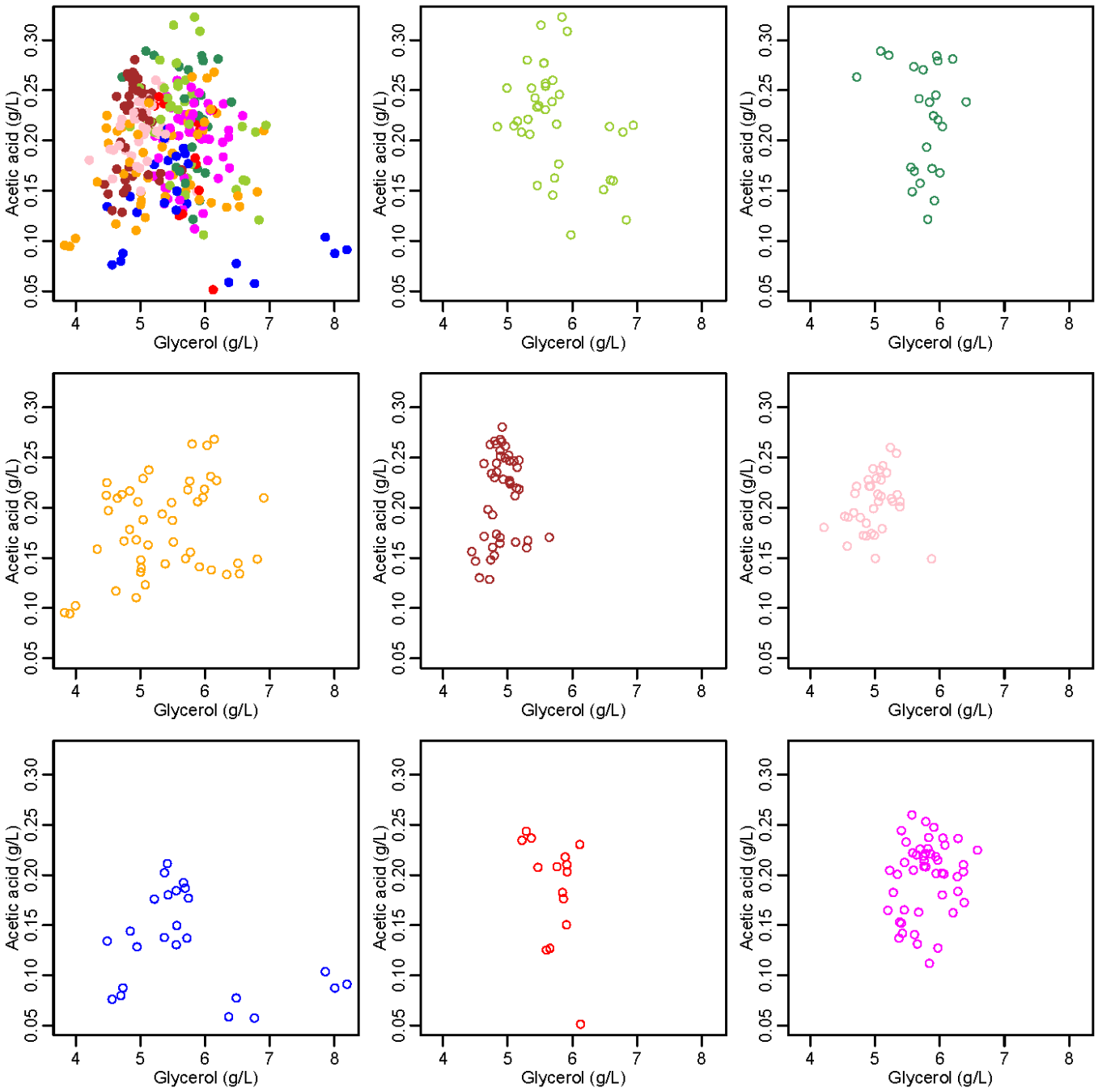
Supplementary Figure 11. Spearman's correlation test between the selected metabolites: ethanol and acetic acid.



Supplementary Figure 12. Spearman's correlation test between the selected metabolites: lactic acid and glycerol.



Supplementary Figure 13. Spearman's correlation test between the selected metabolites: lactic acid and acetic acid.



Supplementary Figure 14. Spearman's correlation test between the selected metabolites: glycerol and acetic acid.

CHAPTER 6

Concluding Remarks and Future Directions

6.1. Conclusions

As seen from the increasing number of reports over the last two decades (Figure 1), non-*Saccharomyces* yeasts have become a 'hot' topic in oenology. The aim of this work was to further understand the potential of different non-*Saccharomyces* yeasts in managing wine ethanol and flavour balance. Four individual studies were designed for this purpose, allowing a number of conclusions to be drawn.

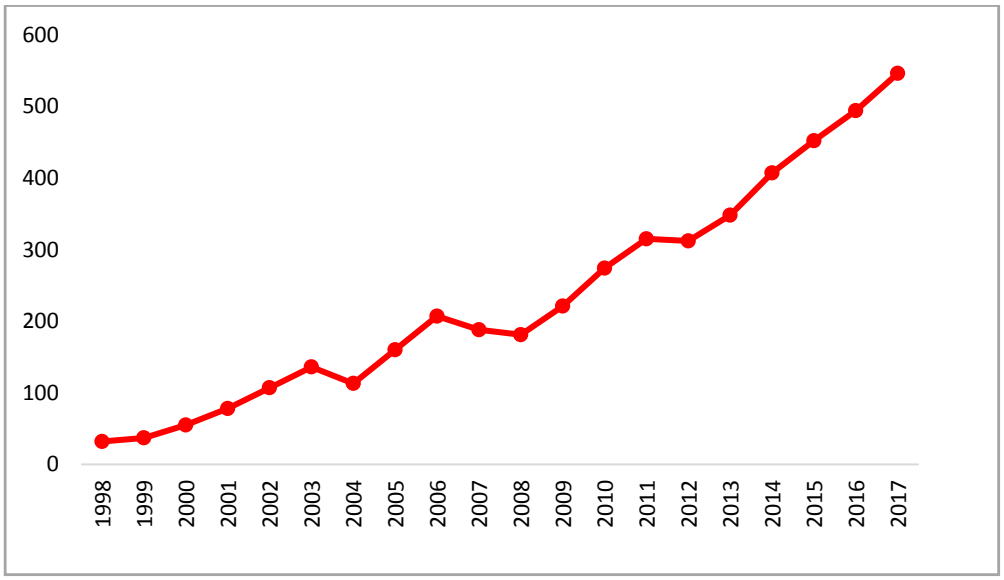


Figure 1. Google Scholar Search results for 'non-*Saccharomyces* wine' query within the last two decades (https://scholar.google.com.au/).

6.1.1. Chemical and sensory profiling of Shiraz wines co-fermented with commercial non-Saccharomyces inocula

The objective for this winemaking trial was to compare the existing non-*Saccharomyces* starter cultures in sequential fermentations of Shiraz at two maturity levels. This approach was based on previous reports (and manufacturers' claims) of the positive

metabolic contribution of non-*Saccharomyces* starters to wine chemical and sensory properties. In particular, this experiment sought to determine whether non-*Saccharomyces* starters could compensate for the sub-optimal flavour ripeness of earlier-harvested fruit (24 °Brix), but also how they would perform at technical ripeness (29 °Brix). Eight yeast treatments trialled in pilot scale fermentations included three *Torulaspora delbrueckii* strains, one strain each of *Lachancea thermotolerans* and *Metschnikowia pulcherrima*, and an initially un-inoculated treatment, which were all inoculated with *Saccharomyces cerevisiae* after 60 h, as well as a commercial blend of two non-*Saccharomyces* species and a *S. cerevisiae*, and a *S. cerevisiae* monoculture. The fermentations were regularly monitored for their sugar consumption kinetics, and the resultant wines were subjected to comprehensive chemical profiling in terms of basic chemistry, volatile profile and phenolic composition, and descriptive sensory analysis. The results showed a pronounced matrix-derived modulation of wine profiles, which was reflective of the maturity level of the grapes. However, within each harvest date, inoculum-derived differences were also apparent as the yeast treatments had a significant impact on a range of compositional and, in turn, sensory parameters of the wines. The latter were of particular interest. At earlier harvest stage, the wines produced with a *S. cerevisiae* monoculture were perceived as 'vegetal, 'acidic' and 'bitter', while certain non-*Saccharomyces*, in particular *T. delbrueckii*, treatments were related to more favourable sensory attributes (i.e. 'floral', 'red fruit', 'aroma intensity', 'spice'). However, these treatments were related to incomplete fermentations in higher ripeness conditions. The protracted or incomplete fermentation kinetics were in fact observed with the co-inocula recommended for the use in high sugar and/or low acidity grapes, including *T. delbrueckii* and *L. thermotolerans* due to their ability to decrease volatile acidity and increase total acidity, respectively. Inarguably, sugar levels in grapes at the second harvest (i.e. 29 °Brix) were rather extreme because of the unusually warm and compressed nature of the 2015 vintage in South Australia (Schelezki et al., 2018), a

phenomenon that is becoming increasingly common. Thus, as hypothesised, certain non-*Saccharomyces* yeast showed promise in enhancing the quality of wines produced from earlier-harvested grapes and as such represent a complementary approach in managing wine ethanol concentrations. The importance of a suitable yeast choice for high sugar/alcohol fermentation was also highlighted, much as the need to develop improved methods to control ethanol levels in wines. One approach that has been already explored, but has yet to deliver the strains for the industry, is the selection of low-ethanol producing non-*Saccharomyces* yeasts. The following study (Chapter 3) was therefore designed to deliver such strain(s).

6.1.2. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing

In accord with several previous selection programs targeting lower-ethanol non-*Saccharomyces* starters (Contreras et al., 2014, Quiros et al., 2014), a pre-screening of an in-house microbial culture collection highlighted this phenotype among *M. pulcherrima* isolates. Six *M. pulcherrima* strains were therefore trialled in fermentations of a synthetic grape juice with *S. cerevisiae* sequentially inoculated after seven days. The best-performing candidate, strain MP2, was chosen for further characterisation. This was carried out in both synthetic and white grape juice (~255 g/L total sugar), with a progressively delayed *S. cerevisiae* sequential inoculation timing (occurring between three days and the time required for MP2 to consume 50% of initial sugar). The analysis of main metabolites was undertaken prior to sequential inoculations and upon fermentation completion, enabling us to study the evolution of metabolites in MP2 alone, and their subsequent modulation in co-cultures. Depending on the inoculation delay, MP2 white wines contained between 0.6% and 1.2% (v/v) less ethanol than

the *S. cerevisiae* monoculture, with even larger decreases achieved in a synthetic grape juice. The lower-ethanol production of the selected MP2 strain was therefore confirmed in multiple conditions. Surprisingly, sugar consumption in MP2 led to the accumulation of an unknown UV-detectable metabolite, which was depleted upon *S. cerevisiae* sequential inoculation. This metabolite was identified as fumaric acid via HPLC-MS analysis, suggesting that TCA cycle by-products are among the potential carbon sinks in MP2 wines. The MP2 treatments also had higher concentrations of glycerol and lower concentrations of acetic acid compared to the *S. cerevisiae* controls. Together, these observation highlighted how little we know, not only about the regulation of central carbon metabolism in non-*Saccharomyces* yeasts, but also about the interaction occurring between different yeasts under winemaking conditions. Moreover, the analysis of volatile compounds revealed distinct profiles of volatile in the lower-alcohol wines, with compositional alterations arising from the *S. cerevisiae* inoculation delay. No apparent aroma off-flavours were detected in the MP2 wines, which were generally characterised by increased concentrations of acetate esters, higher alcohols and monoterpenoids. Accordingly, the selected and characterised MP2 strain showed promise when used in conjunction with *S. cerevisiae* as a means of modulating ethanol level and balancing flavour profile upon fermenting grapes with a high sugar content. The selected strain was delivered to the industry partner for further oenological characterisation (UA170889 Material Transfer Agreement between BioLaffort and the University of Adelaide).

6.1.3. The evolution of *Lachancea thermotolerans* is driven by geographic determination, anthropisation and flux between different ecosystems

The final two studies were carried to better understand the concepts of intra-specific diversity among non-*Saccharomyces* yeasts. Such diversity was apparent in the previous two

studies; three tested *T. delbrueckii* co-inocula showed distinct performance in Shiraz vinifcations in the first study, and six *M. pulcherrima* characterised in the second study also differed in their fermentation kinetics and metabolite production. However, another species was chosen for the next set of experiments – *L. thermotolerans*. This yeast harbours several metabolic traits that are of value in oenology, especially in the context of climate change. It abundantly produces lactic acid (thereby acidifying the fermenting grape juice/must) and can also lead to decreased ethanol content and modulated flavour in wines (Jolly et al., 2014). The availability of a high quality genome sequence of *L. thermotolerans* type strain allowed us to mine the genome for microsatellites to be used for genotyping of a collection of strains catalogued as *L. thermotolerans* acquired from multiple generous labs and culture collections worldwide. The newly-developed 14-microsatellite genotyping method was used to examine the relationships between 172 *L. thermotolerans* strains sourced from both human-related and natural habitats. This exercise revealed that the natural isolates were mainly grouped together based on their geographic origin ('wild' groups: 'Americas', 'Canada trees', 'Hawaii/California'), and separately from those from the anthropic environments ('domestic' groups: 'Domestic 1', 'Domestic 2'). This observation was strongly suggestive of domestication events within the species. Several genetic groups comprised of isolates with mixed origins in terms of geography and/or isolation habitat were also differentiated ('mixed' groups: 'Mix Eastern Europe', 'Mix Europe/North America', 'Europe oak/France grapes'). Such clustering, validated by several statistical methods (e.g. population structure analysis, PCA, AMOVA, F-statistics), indicated that the evolution of *L. thermotolerans* subpopulations is driven by geographic origin ('wild' groups), anthropisation ('domestic' groups) and flux between different ecosystems, presumably mediated by macroorganisms such as insects, birds and humans. The genotyping study was complemented with a set of agar plate-based growth assays using different carbon sources and physicochemical conditions, so as to test whether the

genetic differentiation was mirrored at a phenotypic level. The phenotyping experiment revealed a high degree of intra-specific diversity, and an overall prolific growth of the 'domestic' groups across different conditions. Evidence for a narrower ecological adaptation was also suggested; e.g. a superior growth of 'Canada trees' strains at 8 °C. Thus, the genetic differentiation was supported to a degree at a phenotypic level. However, we were intrigued to know whether/how this diversity translates into the oenological context. Therefore, the final experiment of this project was designed to test this.

6.1.4. Oenological phenomes of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation

A subset of 94 previously genotyped *L. thermotolerans* strains was characterised in Chardonnay grape juice (236 g/L sugars, pH 3.5) fermentations. Strains were compared for their growth and sugar consumption kinetics, production of volatile and non-volatile metabolites and the resultant modulation of pH. The availability of genotyping data for the tested strains was valuable not only to ascertain that the phenotypic variability is fully representative of the species, but also to study the traits of interest in the context of the described genetic structure in *L. thermotolerans.* All tested strains were capable of proliferating and catabolising sugars despite the harsh conditions inherent of winemaking. However, the rate and/or extent of sugar consumption was lower for strains belonging to two major 'wild' genetic groups, i.e. 'Americas' and 'Canada trees'. Significant differences were detected in all measured and derived parameters related to production of primary metabolites except ethanol

concentrations and yields. Despite the detected variability, the common oenological features of *L. thermotolerans* strains were their glucophilic character, relatively extensive fermentation capacity (albeit without completion), low production of acetic acid and formation of lactic acid. A seven-fold variation was observed in concentration of lactate, significantly affecting the pH of the wines, which ranged between 3.16 - 3.81. Volatile profiles of the wines also differed greatly; the strain and group-affected compounds included the common yeast-derived volatiles (e.g. esters and higher alcohols), but also a range of grape-derived (e.g. hexanol, ß-damascenone) and unidentified compounds. Linear discriminant analysis was performed to assess whether the obtained metabolic dataset discriminated the genetic groups in *L. thermotolerans,* revealing suitable partitioning of the groups. The separation of groups was driven by distinct growth and fermentation kinetics parameters, and production of (non)-volatile metabolites by the strains. For example, the 'Americas' strains showed slower fermentation kinetics and overproduction of several potential wine off-flavours (e.g. ethyl acetate, acetaldehyde), while the 'Domestic 1' and 'Domestic 2' groups elicited increases and decreases in pH, respectively. Thus, the applicability of microsatellites in marker-assisted selection of *L. thermotolerans* starters suitable for different wine styles was also highlighted. Together, these results provided a population-wide insight into the extent of phenotypic variability in oenologically-relevant traits in *L. thermotolerans,* whilst adding support for the occurrence of domestication events and allopatric differentiation within this remarkable yeast species.

6.2. Future directions

Based on the studies above, further research should cover the following oenological and fundamental aspects.

6.2.1. Oenological aspects

The findings on the potential of certain non-*Saccharomyces* yeast treatments to enhance the quality of earlier-harvested grapes requires further validation across a range of conditions. In particular, it remains to be evaluated if such behaviour will also occur in more pronouncedly under-ripe grapes, with potential ethanol concentrations around 10 % (v/v). As several non-*Saccharomyces* species (e.g. *T. delbrueckii*) are generally capable of achieving similar ethanol yields without the addition of *S. cerevisiae* starters, another research avenue could explore the use of non-*Saccharomyces* pure cultures in the production of lower-alcohol wines. Likewise, further characterisation of the *M. pulcherrima* MP2 strain, selected for its ability to decrease wine ethanol content in co-cultures with *S. cerevisiae*, is required to assess its performance in non-sterile large-scale fermentations. Sugar consumption in MP2 fermentations led to an accumulation of fumaric acid. Given the potentially negative impact of fumarate on lactic acid bacteria (Cofran and Meyer, 1970), it remains to be evaluated whether this strain is suitable for production of wines destined for malolactic fermentation. In general, the effect of non-*Saccharomyces* yeasts on lactic acid bacteria represents another relevant yet poorly studied aspect of oenology. Presumably, the lower initial fermentation rates displayed in sequential fermentations of non-*Saccharomyces* and *S. cerevisiae* could benefit the proliferation of simultaneously-inoculated lactic acid bacteria, but this hypothesis remains to be validated. The lower-alcohol wines produced with co-cultured MP2 and *S. cerevisiae* differed greatly from the *S. cerevisiae* control not only in ethanol level, but also in their volatile composition. It remains to be investigated how this chemical modulation impacts the sensory properties of the

lower-alcohol MP2 wines. Sensory effects of the *L. thermotolerans* strains also remain to be evaluated, especially in the context of abundant lactic acid formation exceeding concentrations of 10 g/L. Prior to this, the compatible *S. cerevisiae* strains and inoculation regimes for production of 'dry' *L. thermotolerans* mixed-fermented wines needs to be established and tested at an industrial scale.

6.2.2. Fundamental aspects

The lack of fundamental understanding of primary and secondary metabolism in different non-*Saccharomyces* species is striking. The knowledge and techniques developed for characterising *S. cerevisiae* are yet to be fully translated into the non-*Saccharomyces* research arena. In this context, further work is required to elucidate a number of phenomena observed in our studies and in the literature, including lactic acid formation by *L. thermotolerans* and lower-ethanol production by *M. pulcherrima*. Labelled isotope flux studies are needed to fully evaluate the carbon sinks in yeasts that partially divert carbon from ethanol to other end-products. The development of transformation systems, that are available for some but not all wine-associated non-*Saccharomyces* (Varela and Borneman, 2017), will not only provide greater understanding of metabolic peculiarities of different species, but also aid the development of improved starters. In the era of 'omics', transcriptomics is to be pursued to evaluate the response of different species and strains to (a)biotic stressors, including the effect of yeast co-cultures. Implementation of these methods has already begun, delivering fascinating results on the mutual metabolic stimulation of non-*Saccharomyces* and *S. cerevisiae* species (Tronchoni et al., 2017, Curiel et al., 2017), but it is still at its infancy. Yeast interactions, indeed, require thorough research. It still remains elusive why some fermentations succeed, while others under same conditions fail. For example, in our sequential fermentation

trial with six different *M. pulcherrima* strains, five treatments went to completion. Conversely, fermentation with one *M. pulcherrima* strain arrested, as did the *T. delbrueckii* treatments in higher ripeness Shiraz fermentations. A metabolomics approach should complement such studies. Transcriptomics and metabolomics are also to be pursued to understand the development of aroma compounds in non-*Saccharomyces* pure cultures, which can be significantly affected at a strain level as seen from the oenological characterisation of *L. thermotolerans*. Finally, full genome sequences are available for hundreds of *S. cerevisiae* strains, but a limited number of non-*Saccharomyces* representatives have thus far been fully sequenced, and for some species reference genomes are still lacking. Whole-genome sequencing of the genotyped *L. thermotolerans* population would offer a powerful dataset to study the evolution and ecology of this species. The oenological phenomes of 94 *L. thermotolerans* strains, covering 114 phenotypic traits for each strain tested in triplicate, represent an immensely valuable asset for such future studies.

In the light of Pasteur's quote 'There are no such things as applied sciences, only applications of science', the oenological and fundamental approach are in fact inseparable, and further research in the non-*Saccharomyces* field will thus benefit the research community and wine industry alike.

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