Identification and Understanding of Saccharomyces and Oenococcus Interactions in Wine Fermentation

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A thesis submitted for the degree of Doctor of Philosophy

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Thesis submitted for examination: March, 2020

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Abstract

Winemakers are now more frequently choosing to inoculate yeast and bacteria together in a co-inoculation strategy to achieve faster, more efficient fermentations. However, this can be potentially problematic due to yeast-lactic acid bacteria (LAB) incompatibility that can result in stuck fermentations. This PhD thesis examined yeast-LAB compatibility using commercially available strains in co-inoculated fermentations to further understand the complexities of yeast-LAB interactions in wine.

Commercial yeast-LAB pairs (72 in total) were initially screened in a synthetic juice to determine compatible (yeast and LAB able to complete alcoholic and malolactic fermentation) and incompatible (LAB unable to complete malolactic fermentation) pairs. The 72 yeast-LAB pairs were ranked based on fermentation performance, with additional in-depth analysis of the top four and bottom four pairs in a Shiraz juice. Fermentation kinetics and a number of fermentation relevant compounds were measured to elucidate reasons for differences in LAB fermentation performance. This experiment revealed differences in concentrations of H_2S, esters and succinic acid between yeast-alone control fermentations and yeast-LAB co-inoculated fermentations.

In parallel with these studies, a yeast quantitative trait loci (QTL) library was used to determine yeast specific traits that could impact LAB fermentation ability. A QTL was identified which spanned a genomic region containing the gene *SSU1*, known to encode a sulfite exporter (Ssu1p). Follow-up work using hemizygote strains revealed that yeast with *SSU1* haploinsufficiency allowed LAB to perform malolactic fermentation faster than when co-inoculated with wild-type yeast. Considering the

difference in H_2S production and the influence of *SSU1*, a final experiment was performed to assess yeast and LAB sulfur pathway gene regulation in response to co-inoculation.

Quantitative PCR was used to study metabolic links to yeast-LAB compatibility, as well as measurement of glutathione and H_2S. This work involved RNA extraction from mixed yeast-LAB fermentation samples and measurements of H_2S and glutathione over time. When assessing genes involved in sulfur metabolism, differences were observed between yeast only and yeast-LAB fermentations. There were also differences between yeast strains. Additionally, it was observed that there were higher concentrations of glutathione in co-inoculations compared to yeast-only fermentations. Intriguingly, there was a lack of correlation between H_2S production and *CYS3*, *CYS4*, *MET5* and *MET10* gene expression.

Overall the studies carried out in this thesis have highlighted the complexity of yeast-LAB interactions in wine fermentation. This work has provided a starting point for future work investigating yeast-LAB compatibility and the potential role of sulfur in compatibility outcomes.

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.

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I acknowledge the support I have received for my research through the provision of a University of Adelaide Divisional Scholarship and Wine Australia Supplementary PhD Scholarship.

L. J. Bartle

13/03/2020

Date

Acknowledgements

I would like to thank my supervisors Prof Vladimir Jiranek, Dr Joanna Sundstrom, Dr Krista Sumby and Prof Jim Mitchell for guiding, mentoring and encouraging me all throughout my PhD journey. Thank you for making my dreams of overseas work a reality, setting up opportunities for me to collaborate internationally and present my work at conferences. Thank you for pushing me to be a better researcher and to give oral presentations, it gets less frightening each time.

I am so grateful for the contribution each of my supervisors has made to my development as a researcher. I cannot thank you enough.

I would like to acknowledge The University of Adelaide and Wine Australia (AGW Ph1510) for their financial support, by provision of scholarships and operating funds. Additionally I would like to acknowledge the support received from the following travel awards: DR Stranks Travelling Fellowship, Research Abroad Scholarship, Wine Australia Travel Bursary and the Global Learning Travel Grant.

Thank you Dr James Paterson for working with me on the flow cytometry manuscript. You have been a great collaborator.

Thank you Dr Paul Boss and Emily Nicholson from CSIRO for their kindness and guidance and for performing GC-MS which was required for Chapter 4.

Thank you to Dr Philippe Marullo and Dr Emilien Peltier for being amazing researchers to collaborate with at the University of Bordeaux. My time there was so lovely. Thank you for providing yeast strains, statistical guidance, and commentary to enable the work described in Chapter 5. And thank you to Heléne for helping me perform the QTL experiments, those long days we did were totally worth it!

Thank you Dr Duygu Dikicioglu for hosting me at the University of Cambridge and teaching me about metabolic modelling. The time I spent working with you was intense, but fun.

Being a part of the Wine Microbiology Group has been a great experience. So many students and staff have been around at some point during my time as a PhD student. Thank you all, you know who you are. Thank you for your support, friendship and intellectual discussions.

Thank you Prof Kerry Wilkinson for mentoring me, and being a great friend. Your insight and motivation is something to aspire to.

I wouldn’t have gotten this far without the love and support of my family and friends. Thank you Mum and Dad for supporting me through all of my studies. Thank you for Tuesday night dinners, free food always helps a lot! Thank you to my sister, Caroline, for understanding the rollercoaster of emotions a PhD can bring, and thanks to my brother-in-law, Cameron, for the great puns.

A very extra special thank you to my husband, Nathan. Out of everyone, you experienced all my frustrations and accomplishments first-hand. You are the greatest supporter and friend, and your love and support allowed me to get through the hardest times. I am forever grateful to have you as my husband.

My PhD has let me grow as a person, allowed me to work through the toughest situations I have ever faced, and provided me with opportunities I never thought possible. I really did have such a great time!

There were tough times in my PhD...but then there was covid-19.

This is a note to remember that this was a difficult time for so many people, but when everyone works together it helps everyone get through a lot easier.

Be kind.

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Thesis structure and overview

The aim of this study was to untangle the complex interactions between yeast and lactic acid bacteria (LAB) during co-inoculation in wine. Initial work looked at how commercial yeast and LAB behaved during co-inoculation in a chemically defined grape juice, and whether there were any strain specific differences that impacted yeast-LAB compatibility. Malolactic fermentation (MLF) was affected more than alcoholic fermentation (AF) and became the main measure of compatibility. Once compatibility measures were established, the metabolic and genetic influences involved in yeast-LAB compatibility during red wine fermentations were investigated. This thesis is submitted as a combination of published (Chapter 1) or submitted work (Chapter 2) and conventional thesis chapters (3-7).

Chapter 1 is a published literature review that evaluates current knowledge of yeast-bacteria compatibility in wine. Topics summarised include: the roles of various metabolites (i.e. ethanol, glycerol, acetaldehyde, SO_2, fatty acids, bacteriocins, antimicrobial peptides, oxygen, nitrogen and L-malic acid) in compatibility outcomes; physical interactions (i.e. flocculation and biofilm formation); sensory outcomes and gene expression.

As part of defining compatibility, an accurate method to determine LAB viability in wine was investigated. Initial work was directed at replicating previous flow cytometry methods, and troubleshooting when the complications with measuring LAB in this way became evident. A number of experiments were performed to choose suitable fluorescent dyes, including microscopy and flow cytometry. The difficulty of differentiating LAB cells from wine debris and the issue of bacterial chain formation led to the conception of the article that forms **Chapter 2**. This short

article identifies the challenges of enumerating lactic acid bacteria via flow cytometry and a discussion of how current methods could be improved.

Chapter 3 describes the results of screening 72 yeast-bacteria combinations including commercial *Saccharomyces*, non-*Saccharomyces*, *Oenococcus oeni* and *Lactobacillus plantarum* strains in a synthetic juice during co-inoculation fermentations to assess their compatibility (i.e. yeast and LAB ability to complete alcoholic and malolactic fermentations, respectively). Results revealed compatibility was strain specific, with species *Lb. plantarum* unable to survive with any of the tested yeast.

Chapter 4 builds on the findings of Chapter 3, with eight yeast-LAB pairs selected for more in-depth analysis in Shiraz juice fermentations. Multiple assays and analyses were performed to identify compounds that may influence yeast-LAB compatibility. Firstly, the yeast-LAB pairs did not perform the same as in the synthetic medium used for Chapter 3. Some of the yeast-LAB pairs switched compatibility status (i.e. from being incompatible when assessed in synthetic juice, to compatible in Shiraz juice and vice versa). This experiment also revealed that sulfur, in the form of hydrogen sulfide, and succinic acid were influenced by co-inoculation. In addition, there were differences in volatile compounds that related to yeast strain.

To further understand how LAB MLF performance may be affected by yeast genetic background, a quantitative trait loci (QTL) experiment was performed in **Chapter 5**. To determine if a particular yeast genotype, and subsequent phenotype, affected LAB ability to complete MLF during co-inoculation a QTL mapping strategy was applied. The QTL experiment identified a single QTL that included a gene, *SSU1* (YPL092W; sensitive to sulfite: a gene that encodes a membrane sulfite pump for

sulfite efflux), and a translocation (XV-t-XVI) that impacts *SSU1* gene expression and is involved in delayed yeast alcoholic fermentation onset. Follow-up work was performed to elucidate if LAB co-inoculated with yeast containing a single *SSU1* allele, either coupled with the translocation (*SSU1*-t) or wild-type (*SSU1*-wt; sourced from different strains) influenced MLF performance. It was found that *SSU1*-t coincided with longer MLF by SB3 LAB. In addition, yeast with a single *SSU1* allele (translocated or not) allowed LAB to complete MLF faster than LAB co-inoculated with a yeast that had both *SSU1* alleles. In order to further identify how *SSU1* and other sulfur related pathways influence MLF outcomes, two yeast-LAB pairs from Chapter 4 were chosen for a qPCR experiment for gene expression analysis.

In **Chapter 6**, metabolic pathways related to sulfur (including *SSU1*) in both yeast and LAB were chosen for analysis. Previously only yeast gene expression has been analysed for yeast-LAB co-inoculation experiments. This experiment aimed to assess both yeast and LAB gene expression from a single sample.

Chapter 7 discusses future work that could continue on from the findings presented in this thesis, and overall conclusions.

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Chapter 1

Review of literature and introduction
The microbial challenge of winemaking: yeast-bacteria compatibility

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FEMS Yeast Research
2019, Volume 19, Pages foz040

Statement of Authorship

Title of Paper	The microbial challenge of winemaking: yeast-bacteria compatibility
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	FEMS Yeast Research, volume 19, Issue 4, page foz040

Principal Author

Name of Principal Author (Candidate)	Louise Bartle
Contribution to the Paper	Conception of ideas for the paper, collection of references, literature and data interpretation, manuscript preparation and editing
Overall percentage (%)	80%
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: 26/02/2020

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	Krista Sumby
Contribution to the Paper	Conception of ideas and editing and acted as corresponding author.
Signature	Date: 26/02/2020

Name of Co-Author	Joanna Sundstrom		
Contribution to the Paper	Conception of ideas and editing.		
Signature		Date	23/2020

Name of Co-Author	Vladimir Jiranek		
Contribution to the Paper	Conception of ideas and editing.		
Signature		Date	26-2-20

FEMS Yeast Research, 19, 2019, foz040

doi: 10.1093/femsyr/foz040
Advance Access Publication Date: 11 June 2019
Minireview

MINIREVIEW

The microbial challenge of winemaking: yeast-bacteria compatibility

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One sentence summary: Yeast and lactic acid bacteria clearly interact chemically and physically during winemaking, yet the nature of this interaction and its impact on each organism as well as fermentation performance and wine quality are poorly understood.

Editor: Hiroshi Takagi

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ABSTRACT

The diversity and complexity of wine environments present challenges for predicting success of fermentation. In particular, compatibility between yeast and lactic acid bacteria is affected by chemical and physical parameters that are strain and cultivar specific. This review focuses on the impact of compound production by microbes and physical interactions between microbes that ultimately influence how yeast and bacteria may work together during fermentation. This review also highlights the importance of understanding microbial interactions for yeast-bacteria compatibility in the wine context.

Keywords: *Saccharomyces cerevisiae*; lactic acid bacteria; wine; microbial interactions

INTRODUCTION

Winemaking does not involve a microbial monoculture. Wine grapes and the winery carry a range of yeast, bacteria and fungi. Once grapes are ruptured, whether in the vineyard or the winery, the sugar and nutrient-rich environment that is exposed favours an increase in microbial numbers and/or diversity (Ultee *et al.* 2013; David *et al.* 2014; Piao *et al.* 2015; Pinto *et al.* 2015; Godálová *et al.* 2016; Marzano *et al.* 2016; Portillo and Mas 2016; Eder *et al.* 2017; Morgan, du Toit and Setati 2017). In co-existing, these microbes interact with each other in addition to responding to environmental stimuli. Progress of fermentation sees diversity decrease in such populations since only a few species are able to survive the increasingly harsh wine environment (Combina *et al.* 2005; Zott *et al.* 2008; Piao *et al.* 2015; Portillo and Mas 2016).

The major players in the production of wine are yeast and lactic acid bacteria (LAB), the microorganisms responsible for primary (alcoholic) and secondary (malolactic) fermentation, respectively. The microbial activities and presumably the interactions involved in fermentation of juice to wine help produce primary and secondary metabolites (Lee *et al.* 2009) that impact the palatability of the final product. While a variety of organisms may develop in grape must, inoculation with selected strains of *Saccharomyces cerevisiae* yeast and *Oenococcus oeni* LAB are the most commonly used option. This is due to their ability to efficiently convert sugar into ethanol (alcoholic fermentation) and L-malic acid into the less acidic L-lactic acid (malolactic fermentation). However un-inoculated fermentations, whereby the yeast and LAB present on the grapes and winery

Received: 8 May 2019; **Accepted:** 10 June 2019

equipment perform the fermentations, are being increasingly favoured as a way of maintaining or expressing the microbial terroir of the region and for positive modifications to wine flavour. Yeast species commonly found in un-inoculated fermentations, including *Torulaspora delbrueckii*, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) and *Metschnikowia pulcherrima*, can lead to increased acidity, herbaceousness and fruity aromas and a smoother or softer mouthfeel (Gobbi *et al.* 2013; Loira *et al.* 2015; Varela *et al.* 2017). Similarly, *Lactobacillus plantarum* and *Pediococcus damnosus* have great potential to produce desirable esters in wines, although more work is needed to increase their viability during malolactic fermentation (Swiegers *et al.* 2005; Sumby, Grbin and Jiranek 2010, 2014). While increased diversity of microorganisms in wine may maintain terroir characteristics and modify wine flavour, it can also lead to antagonism between members of the microbial community with the potential to negatively impact the fermentation process.

The yeast and LAB making up the community of microorganisms and contributing to wine complexity produce a range of molecules including alcohols, esters, proteinaceous compounds, fatty acids and other organic molecules. Increased microbial diversity may increase the chances of incomplete (stuck) fermentation as different genera can produce inhibitory compounds detrimental to other wine microorganisms. This has raised the importance of development of robust winemaking practices that involve selection of appropriate microbial communities and inoculation procedures to ensure successful fermentations. One way in which the winemaker can influence the microbial community is to inoculate with either a known yeast or LAB strain, depending on their winemaking strategy. However there is a delicate balance between fermentation reliability and allowing the region's natural microbial fingerprint to be expressed in the final wine.

Bacterial inoculation and the timing of inoculation also have the potential to impact on the community structure as LAB cultures are generally inoculated at high cell numbers of at least 1×10^6 colony forming units per mL (Muñoz, Beccaria and Abreo 2014; Tristezza *et al.* 2016; Jiang *et al.* 2018). Typically two bacterial inoculation strategies are used in winemaking: sequential and co-inoculation. Sequential inoculation is the practice of allowing yeast alcoholic fermentation to be completed prior to addition of LAB to complete malolactic fermentation while co-inoculation usually involves the addition of LAB 24–48 hours after yeast inoculation. The growth of co-inoculated LAB starter cultures can be inhibited by the presence of yeast, with the degree of inhibition dependent upon yeast strain and timing of bacterial inoculation (Alexandre *et al.* 2004; Arnink and Henick-Kling 2005; Comitini and Ciani 2007; Mendoza, de Nadra and Farías 2010). Winemakers can obtain some information about compatibility from starter culture manufacturers and industry publications, however, this usually only provides information on yeast and LAB strains produced by each company and does not compare strains of different companies. How compatibility is tested is not always clear either. Additionally, in the literature where commercial yeast and LAB strains are investigated for their fermentation properties, they are not always identified by name, but instead are given arbitrary labels. Thus determining yeast-LAB compatibility can be difficult for winemakers who must carefully select yeast–bacterium pairs for co-inoculation, as any incompatibility can affect both alcoholic fermentation (AF) and malolactic fermentation (MLF).

In co- or sequential-inoculation scenarios there are numerous chemical (i.e. metabolite formation or use) and physical (i.e. cell-to-cell contact) interactions between yeast and LAB that influence their compatibility and fermentation success. This review explores yeast-LAB interactions during winemaking with a focus on the influence of metabolite production and uptake, and physical, cellular contact on the outcome of fermentation in wine. What is currently known about gene expression during co-inoculation of yeast and LAB, and the impact of these chemical and physical processes on wine sensory outcome are also discussed, along with future directions that research in this area may take.

INFLUENCE OF YEAST AND LAB ON METABOLITE PRODUCTION AND UPTAKE

During juice and grape must fermentation, microorganisms constantly change the wine matrix through the production of organic compounds, fatty acids, peptides and antimicrobial compounds. These compounds may be stimulatory, inhibitory or elicit no apparent effect on other microbes present in fermentation, and/or are subsequently involved in yeast-LAB compatibility. Many alcohols, esters and acids have been investigated for their role in final wine quality, however, few have been investigated in relation to their influence on yeast and LAB compatibility.

There are many ways in which compounds can influence yeast–LAB compatibility. Along with metabolite production it is also necessary to consider utilisation of oxygen, assimilable nitrogen and L-malic acid. Here, we discuss the production and utilisation of various compounds and their involvement in microbial interactions (Table 1).

Ethanol and glycerol

Ethanol is one of the most important compounds produced in winemaking with current trends favouring wines lower in alcohol. Although largely driven by consumer demand, the production of wines lower in alcohol has economic benefits for producers too by avoidance of higher duties and taxes (Varela *et al.* 2012). Reduced ethanol in wines also aids survival of LAB since ethanol is a main driver in their demise during alcoholic fermentation and LAB have been reported to be inhibited by as little as 4% (v/v) ethanol in modified FT80 medium (Capucho and San Romão 1994). Some LAB such as *O. oeni* have been successfully evolved in wine-like media with increasing ethanol concentrations to afford tolerance to higher ethanol content (Betteridge *et al.* 2018; Jiang *et al.* 2018). However, LAB ethanol tolerance is confounded by high SO_2 or increasing fatty acid content and in particular a decrease in pH (Capucho and San Romão 1994; Lonvaud-Funel 1995). The low ethanol tolerance of LAB is in contrast to the most ethanol tolerant yeasts, in particular *S. cerevisiae*, which can withstand 15% (v/v) ethanol or more in wine (Gao and Fleet 1988; Pina *et al.* 2004).

Ethanol and glycerol production by yeast occurs through transformation of glyceraldehyde-3-phosphate, an intermediate of glycolysis (Fig. 1). The industrial desire for preferential production of glycerol by *Saccharomyces* has led to the development of *S. cerevisiae* hybrids that alter the glycerol:ethanol ratio in wines (Michnick *et al.* 1997; Goold *et al.* 2017). Lower ethanol and higher glycerol production can allow greater survival of LAB, since LAB utilise glycerol when faced with osmotic stress to maintain cell membrane integrity (van der Heide and Poolman 2000). Additionally, reduced ethanol production provides a more suitable environment for LAB growth and performance since exposure to ethanol increases membrane

Table 1. Summary of molecule and compound production and uptake by wine yeast and/or LAB, and subsequent microbiological effects.

Molecule/compound	Main process	Effects on yeast and/or LAB	Reference(s)
Ethanol	Produced by yeast	• *S. cerevisiae* yeasts can withstand up to 15% (v/v) or more• Concentrations above 4% (v/v) hinder LAB growth and MLF performance • Increases LAB membrane permeability • Decreases LAB ATP production	Gao and Fleet 1988; Capucho and San Romão 1994; Guzzo *et al.* 2000; Da Silveira *et al.* 2003; Pina *et al.* 2004
Glycerol	Produced by yeast	• Can improve LAB survival • LAB utilise glycerol to maintain cell wall integrity	van der Heide and Poolman 2000
Acetaldehyde	Produced by yeast	• Binds free SO_2 • LAB catabolism of SO_2-bound acetaldehyde can inhibit LAB growth	Osborne *et al.* 2000; Wells and Osborne 2012
SO_2	Produced by yeast	• SO_2 at pH < 3.6 becomes more toxic due to its transformation into its molecular form which can readily diffuse into microbial cells • SO_2 works synergistically with ethanol, leading to increased inhibition	Britz and Tracey 1990; Divol, du Toit and Duckitt 2012
Fatty acids	Produced by yeast and *Lb. plantarum*	• Free fatty acids that enter LAB cells cause detrimental ATPase inhibition • *Lb. plantarum* fatty acids disrupt the cellular membrane of sensitive fungi and yeasts	Guilloux-Benatier, Le Fur and Feuillat 1998; Tourdot-Maréchal *et al.* 1999; Sjögren *et al.* 2003; Ryu *et al.* 2014
Bacteriocins	Produced by LAB and engineered yeast	• *Lb. plantarum* can produce bacteriocins that inhibit other LAB • Four *O. oeni* bacteriocin genes have been identified, but no mechanism of action defined • Engineered *S. cerevisiae* can express *Pediococcus* specific bacteriocin PA-1	Schoeman *et al.* 1999; Navarro *et al.* 2000, 2008; Knoll, Divol and du Toit 2008
Antimicrobial peptides	Produced by yeast and LAB	• Uncharacterised AMP's produced by *S. cerevisiae*, 3–10 kDA in size, can inhibit MLF by LAB • *Lactobacillus* from other fermentative environments can produce broad antifungal and antibacterial AMP's	Atanassova *et al.* 2003; Comitini *et al.* 2005; Osborne and Edwards 2006; Mendoza, de Nadra and Farías 2010; Nehme, Mathieu and Taillandier 2010
Oxygen	Utilised by yeast and LAB	• *S. cerevisiae* oxygen use is linked to yeast population growth and nutrient consumption during early AF • Wine LAB in aerobic conditions produce acetic acid rather than lactic acid • Oxygen depletion results in faster LAB growth and preferential lactic acid production	Götz *et al.* 1980; Reguant *et al.* 2005; Boulton *et al.* 2013
Nitrogen and nutrients	Utilised by yeast and LAB	• Yeast deplete nitrogen, in the form of amino acids, which can inhibit LAB growth • Yeast lees provide a site for LAB to scavenge nutrients such as amino acids, sugars and peptides	Fleet, Lafon-Lafourcade and Ribereau-Gayon 1984; Fourcassie *et al.* 1992; Gobert *et al.* 2017
L-malic acid	Utilised primarily by LAB, and some non-*Saccharomyces* yeast	• Can be used as amino acid precursor by LAB • Competition for L-malic acid between LAB and non-*Saccharomyces* yeast may occur, but has not been specifically studied • L-malic acid depletion by LAB decreases chance of spoilage microorganism growth	Rankine 1966; Taillandier, Riba and Strehaiano 1988; Gao and Fleet 1995; Subden *et al.* 1998; Seo, Rhee and Park 2007; Sumby, Grbin and Jiranek 2014; Belda *et al.* 2015

permeability in LAB and decreases adenosine triphosphate (ATP) production to hinder growth and MLF performance (Guzzo *et al.* 2000; Da Silveira *et al.* 2003). This reality provides good reason for utilisation of non-*Saccharomyces* yeasts since they often result in wines lower in ethanol content (Ciani and Maccarelli 1997; Ciani, Beco and Comitini 2006; Jolly, Varela and Pretorius 2014). Though non-*Saccharomyces* yeast are unable to complete AF and tend to leave residual sugar, they are often followed by inoculation with *S. cerevisiae* to complete AF. The use of non-*Saccharomyces* in wines can lead to lower final ethanol concentration since sugars can be converted into metabolic by-products other than ethanol (Giaramida *et al.* 2013; Ciani *et al.* 2016). Reduced ethanol content in wines that have used non-*Sachharomyces* strains followed by *S. cerevisiae* still contained > 10% (v/v) ethanol but also contained higher concentrations of glycerol (Giaramida *et al.* 2013; Belda *et al.* 2015; Englezos *et al.* 2016) compared to *S. cerevisiae* alone. Due to the complexity of metabolite production by non-*Saccharomyces* yeasts and their individual effects on LAB, it is currently unclear how this may affect LAB performance overall.

Ethanol and glycerol production by wine yeast is an important indicator for yeast-LAB compatibility, since the ratios of these compounds can affect survival of LAB during fermentation. Glycerol production by yeast in wine varies by yeast strain and is also dependent on external influences such as agitation, osmotic stress and growth medium (Gardner, Rodrigue and Champagne 1993; Arroyo-López *et al.* 2010; Pérez-Torrado *et al.*

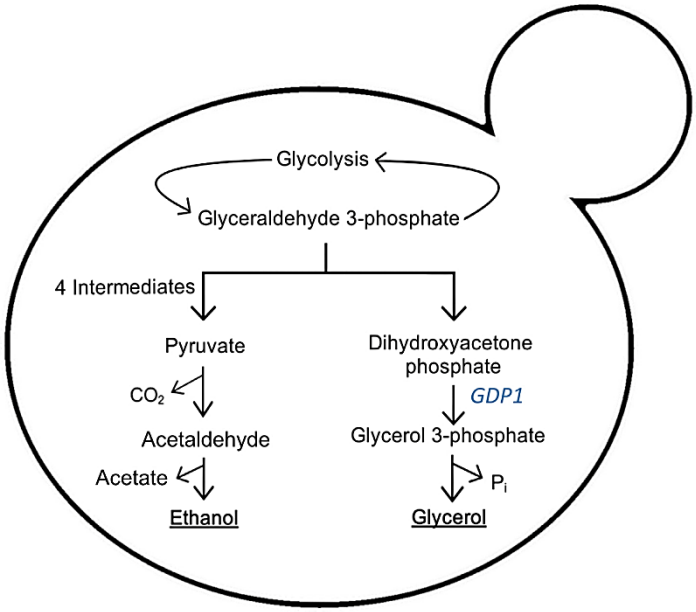

Figure 1. Simplified diagram of the glycerol and ethanol production pathways in *S. cerevisiae*.

2016). Additionally, both *Saccharomyces* and non-*Saccharomyces* strains have different optimal temperatures for glycerol production. When yeast are subjected to osmotic stress, glycerol-3-phosphate dehydrogenase is expressed, which consequently stimulates glycerol synthesis (Fig. 1) (Albertyn *et al.* 1994; Wang *et al.* 2001; Arroyo-López *et al.* 2010). These conditions can be realistically applied to winemaking and therefore utilised to encourage greater glycerol and reduced ethanol concentrations. Although ethanol and glycerol production needs to be considered as a potential reason for MLF failure, there is currently no information on a specific response by yeast in their production of either compound due to the presence of LAB (Tristezza *et al.* 2016; Versari *et al.* 2016; Lasik-Kurdyś, Gumienna and Nowak 2017). This possibility could be investigated by monitoring glycerol-3-phosphate dehydrogenase expression concurrently with glycerol and ethanol concentrations during yeast-LAB co-inoculation compared to a yeast monoculture. Ideally this would be tested in various juice types and with different yeast and bacterial strain combinations to gain a clear idea of whether co-inoculation with LAB influences glycerol and ethanol production by yeasts.

Acetaldehyde, an intermediate compound produced during ethanol synthesis (Fig. 1), also plays a major role in yeast-LAB compatibility.

Acetaldehyde

Acetaldehyde is produced by yeast during alcoholic fermentation at quantities up to 125 $mg.L^{-1}$ (Liu and Pilone 2000) and can stimulate or inhibit the growth of microorganisms. Acetaldehyde binds free SO_2 in wine and can be catabolised by LAB leading to formation of small amounts of ethanol and acetic acid. This is the result of ethanol and acetic acid not being the sole end products of LAB acetaldehyde metabolism, and only accounting for about 60% of metabolised acetaldehyde (Osborne *et al.* 2000). Additionally, LAB catabolism of SO_2-bound acetaldehyde can lead to LAB death, as release of SO_2 from the acetaldehyde is inhibitory to LAB. This process has been labelled as bacteriostatic since the release of SO_2 from acetaldehyde does not necessarily kill LAB, but does inhibit their growth (Wells and Osborne 2012; Fig. 2b). Yeast acetaldehyde production is often positively correlated with SO_2 production and therefore high SO_2 producing strains are not only harmful to LAB solely because of SO_2 toxicity, but the combination of acetaldehyde and SO_2. This correlation between acetaldehyde and SO_2 production is due to the yeast utilising acetaldehyde as a detoxification system, to enable them to survive the increasingly toxic environment (Casalone *et al.* 1992; Aranda *et al.* 2006). Additionally, if acetaldehyde is not produced in the presence of SO_2, then free SO_2 may affect LAB survival at an earlier stage in the fermentation.

Sulfur dioxide

Of all the metabolites that are considered to affect LAB performance in wine, SO_2 is one of the most well known. SO_2 addition by winemakers is common and used to suppress spoilage microorganisms, stop fermentation and minimise or reduce oxidation. Alternatively some microbes can produce SO_2. As such, addition of SO_2 will not be further discussed in this review, since the focus is yeast and bacterial metabolite production.

SO_2 can be present in three forms whose prevalence is pH dependent (Divol, du Toit and Duckitt 2012): molecular SO_2 (most toxic; pH 0–2), bisulfite (antioxidant; pH 2–7) and sulfite (pH 7–10) (Fig. 2a and b). Bisulfite is the most common form in wine due to the pH being between 3 and 4. Sulfur dioxide is an intermediate metabolite in the sulfate assimilation pathway leading to sulfur amino acid synthesis. Under certain conditions, it may be synthesised in excess then excreted into the medium. SO_2 production by yeast is variable and can be anywhere from less than 10 $mg.L^{-1}$ to over 100 $mg.L^{-1}$ (Eschenbruch 1974; Henick-Kling and Park 1994; Osborne and Edwards 2006; Wells and Osborne 2011; Andorrà *et al.* 2018). The production of SO_2 by yeast is

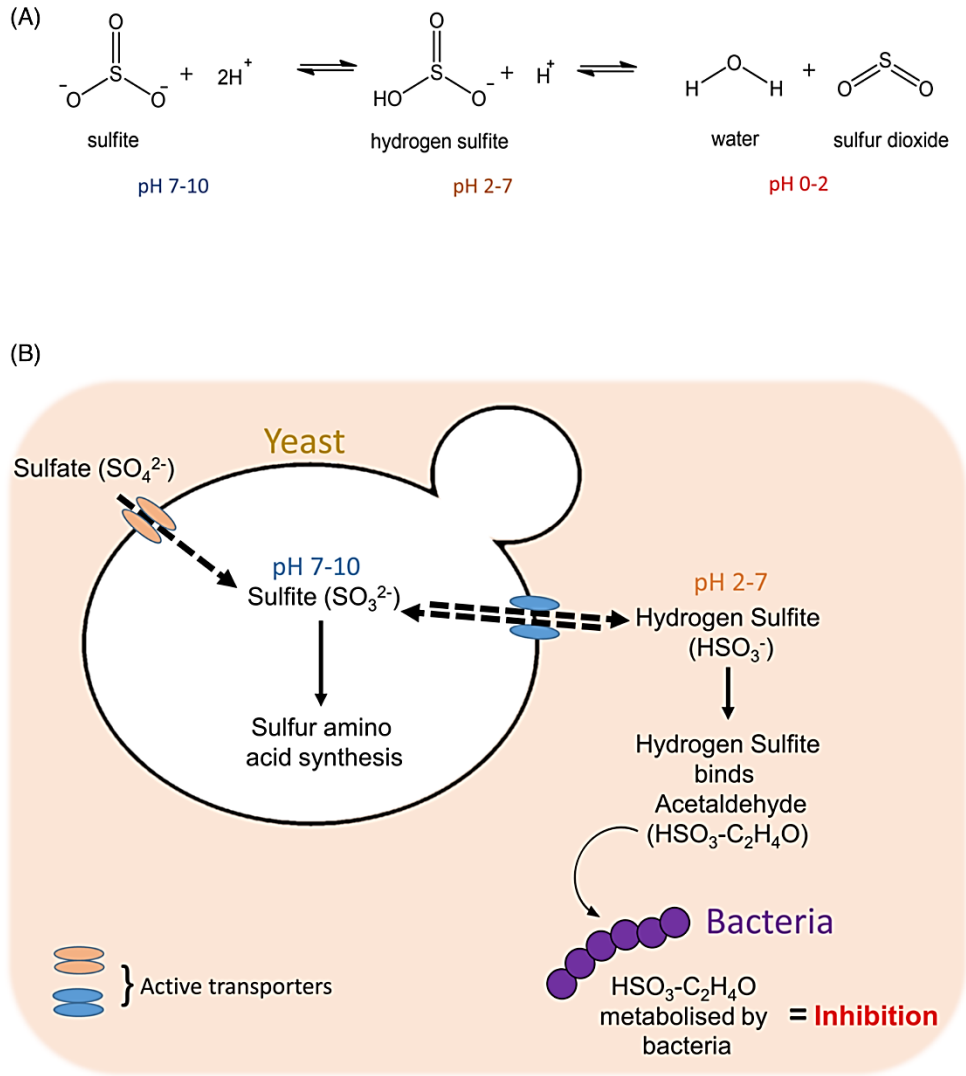

Figure 2. Sulfur compounds found in wine and their metabolism by and effect on wine microbes. **(A)** Equilibrium of sulfur dioxide (molecular SO_2) and sulfite ions (bisulfite and sulfite) in solution. pH impacts the form of SO_2 present, which in wine often lies between pH 3 and 4. **(B)** Simplified sulfate assimilation pathway in wine yeast and effect of SO_2 on LAB. For further information on the sulfate assimilation pathway or sulfur amino acid synthesis see the *Saccharomyces* Genome Database (2007a, 2007b).

influenced by sugar and nitrogen concentration (Osborne and Edwards 2006) and pH (Dott and Trüper 1976), while the amount of free SO_2 is influenced by binding compounds present in the grape must. In fact strains that produce high concentrations of SO_2 tend to also produce high concentrations of acetaldehyde as a self-protective mechanism (Casalone *et al.* 1992). Additionally, low pH (< 3.6) enhances the toxicity of SO_2 by pushing the equilibrium towards molecular SO_2, which is the form that readily diffuses into microbial cells.

Given the importance of yeast SO_2 production on LAB survival, commercial yeasts are often selected for low SO_2 production and suppliers will often quote the propensity for this property to aid strain choice by winemakers. Even with this information winemakers must consider potential additive or synergistic effects due to the presence of other stressors such as ethanol (Britz and Tracey 1990).

Fatty acids

Other molecules to consider when investigating yeast-LAB compatibility are fatty acids. Yeast and LAB in wine fermentations produce a number of fatty acids that contribute to wine aroma and flavour. Medium-chain fatty acids contain carbon chains 10–12 carbon atoms in length and in their free form are toxic to LAB. After entering bacterial cells, free fatty acid molecules dissociate, resulting in a release of hydrogen ions that alter the bacterial transmembrane proton gradient (Guilloux-Benatier, Le Fur and Feuillat 1998). This causes inhibition of ATPase, a crucial

enzyme needed for basic metabolic functions, ultimately leading to death of LAB (Tourdot-Maréchal *et al.* 1999).

Lactobacillus plantarum HD1, isolated from kimchi was found to produce three broad antifungal carboxylic acids: 3-hydroxy decanoic acid, 5-oxododecanoic acid and 3-hydroxy-5-dodecenoic acid (Ryu *et al.* 2014). These compounds may act as detergents, disrupting the cellular membrane of sensitive fungi and yeasts, similar to other 3-hydroxy fatty acids (Sjögren *et al.* 2003). Subsequently there is likelihood that wine LAB may produce fatty acids that affect yeast metabolism.

Nevertheless, production of fatty acids by yeast not only affects LAB growth but can also affect yeast metabolism and survival in wine conditions. Moreover there seems to be no indicative circumstance where yeast and LAB produce medium chain fatty acids as a consequence of co-inoculation.

Bacteriocins

Though yeast and bacteria can be affected by small molecules, larger molecules such as proteins can also influence compatibility. As a first example, the role of bacteriocins in fermentation is discussed. Bacteriocins are proteinaceous compounds secreted during the exponential phase of bacterial growth (de Arauz *et al.* 2009) and can cause target cells of other species to apoptose (Bruno and Montville 1993). *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus* and *Oenococcus* species have been identified as capable of producing bacteriocins in the context of wine fermentation (Manca De Nadra, Sandino De Lamelas and Strasser De Saad 1998; Navarro *et al.* 2000, 2008; Knoll, Divol and du Toit 2008; Ndlovu *et al.* 2015; Dündar, Salih and Bozoğlu 2016; Lasik-Kurdyś and Sip 2019) but they have instead been studied in greater detail in the dairy industry, where they play an important role in food microbial stabilisation and fermentation (Sobrino-López and Martín-Belloso 2008). Bacteriocins produced by LAB in cheese and fermented milk processing are used for prevention of microbial spoilage, whilst maintaining important sensory properties (Aslim *et al.* 2005; Pisano *et al.* 2015). Analogous studies in wine are lacking.

In red wine fermentation, *Lb. plantarum* is capable of producing bacteriocins that enable inhibition of other LAB strains in the culture (Navarro *et al.* 2000), however, the mechanism of action of these bacteriocins is yet to be determined. It was shown that the excreted bacteriocins were plantaricins, encoded by the *plnA* gene. Plantaricins are thermotolerant and stable in wine conditions, thereby giving advantage to *Lb. plantarum* in un-inoculated fermentations (Navarro *et al.* 2000), although this mechanism could also be utilised for fermentations inoculated with *Lb. plantarum*. While further research into the characterisation of the *Lb. plantarum* bacteriocin has been performed there is still no clear understanding of the overall effects of bacteriocins in red wine fermentations (Navarro *et al.* 2008). In addition to plantaricins, four *O. oeni* bacteriocin genes have been identified, however, no further work has been completed on mechanisms of bacteriocin production in *Oenococcus*, and limited information has been collected about *O. oeni* bacteriocin effects on other LAB (Knoll, Divol and du Toit 2008).

Saccharomyces cerevisiae has previously been engineered to produce pediocin PA-1 (Schoeman *et al.* 1999), a bacteriocin specific for *Pediococcus*. Bacteriocin production by yeast is not a naturally occurring trait, however, the use of bacteriocin-producing yeast could help suppress wine spoilage microorganisms (Borrero *et al.* 2012; Díez *et al.* 2012). It is increasingly important to determine how inclusion of yeast that produce bacteriocins with LAB can benefit wine production. Utilising bacteriocin-producing yeast in fermentations could aid winemakers in stopping MLF during AF, as well as being able to inoculate musts with specific LAB. However, the use of genetically modified organisms is not permitted in most wine producing countries at this time and the impact of bacteriocin production on wine quality has not been studied. While bacteriocin production by yeast is limited to engineered strains, this group of fungi are instead capable of producing peptides that are active against yeast and LAB in wine.

Antimicrobial peptides and compounds

Peptidic fractions produced by *S. cerevisiae* have been shown to inhibit MLF, however, the peptides have yet to be fully characterised (Comitini *et al.* 2005; Osborne and Edwards 2006). Peptidic fractions produced by yeast in wine-like conditions typically fall within the range of 3–10 kDa in size (Mendoza, de Nadra and Farías 2010; Nehme, Mathieu and Taillandier 2010). However, many studies did not characterise the explicit function or activity of the peptides and only report whether or not they exhibit an inhibitory effect on LAB. *Oenococcus oeni* may also produce peptides with antioxidant activity after being exposed to partially fermented grape juice, though the effects of this peptide release on *O. oeni* MLF performance was not measured and the potential for the peptides to affect wine yeast was not discussed (Stivala, Apud and Aredes-Fernández 2018). Further work analysing the peptides by mass spectrometry, crystallography and sequencing may give insight into the structure and activity of the peptidic/protein fractions. Knowing how these antimicrobial peptides (AMP's) cause LAB inhibition or death may allow winemakers to manage AMP's to favour successful MLF.

Lactobacillus species from other fermentative environments produce AMP's with broad antifungal and antibacterial properties. Some of the discovered LAB AMP's share properties similar to known bacteriocin groups, however, the properties cannot be fully attributed to only bacteriocins since they also exhibit broad anti-fungal activity (Atanassova *et al.* 2003). Though antifungal peptides have been discovered in other fermentative environments apart from wine, to date there is no evidence of antifungal activity of LAB AMP's on *S. cerevisiae* or other predominant wine related yeasts. Discovery of LAB AMP's has implications for co-inoculation success, as the introduction of different LAB with potential for AMP-producing properties could impact yeast-LAB compatibility in wine.

Use of new chemistry techniques such as ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC-Q-ToF–MS^2) has enabled discovery and more accurate detection of peptides and compounds in mixtures based on their molecular properties (Liu *et al.* 2016). The use of exometabolomic profiling (analysing compounds in growth medium) has revealed that over 2500 unknown compounds were able to be used to differentiate between yeasts that exhibit a phenotype deemed either compatible or incompatible with MLF by wine LAB. Liu *et al.* (2016) gave insight into how new technology can lead to identification of novel wine compounds and their relation to yeast-LAB compatibility; however, these results were based on data collated from the use of a single juice type and a single LAB strain in sequential inoculation. Though this work reports some interesting findings, there needs to be consideration for the effect of different juice types, vintages, LAB strains and inoculation strategies on metabolite production and subsequent yeast-LAB compatibility. Additionally, the use of new technologies to study AMP's and other compounds leads to reports

of large volumes of unidentified compounds. The reality of this has encouraged the production of new analysis tools, such as the OligoNet web server (Liu *et al.* 2017a). This server has been designed to allow identification of unknown peptides by utilising metabolomics datasets to predict amino acids from mass spectrometry masses. This is useful as peptide annotation is not limited to sequence alignment using peptide databases. OligoNet can also be used to map correlations between peptides to develop potential metabolic pathways and identify biomarkers. As demonstrated by their MLF study, biomarker discovery using OligoNet is especially powerful when matched with phenotypic data, enabling correlation between peptides and desirable traits or outcomes.

[GAR$^+$] prion

In addition to bacteriocins and AMPs, some yeast are able to produce other proteinaceous compounds such as prions. Prions are self-propagating proteins that are passed down from mother to daughter yeast cells, and can change from the natural protein conformation to the prion state (Garcia and Jarosz 2014). *Saccharomyces* yeasts have the heritable [GAR$^+$] (resistant to glucose-associated repression) prion that can mediate glucose utilisation (Walker *et al.* 2016). [GAR$^+$] prion induction allows *Saccharomyces* to override mechanisms involved with preferential glucose utilisation, leading to reduced uptake of glucose and utilisation of more complex carbon sources (Jarosz *et al.* 2014; Walker *et al.* 2016). This process benefits other microorganisms during fermentation as more glucose is available for their utilisation. This mechanism can be induced in response to co-inoculation with LAB, in turn allowing LAB to survive due to increased carbon resources. [GAR$^+$] induction during co-inoculation also inhibits yeast from rapid nitrogen and amino acid depletion, also creating availability of these nutrients for LAB (Ramakrishnan *et al.* 2016). Although this can be seen as a way that LAB purposely mediate other species in their environment to temporarily create better living conditions, it can negatively influence compatibility between yeast and LAB. Induction of the [GAR$^+$] prion by *Saccharomyces* can lead to stuck or sluggish fermentations as yeast utilisation of alternative growth substrates, reduces glucose consumption and ethanol production (Walker *et al.* 2016). The diversity of prions in commercially available *Saccharomyces* strains or spontaneously arising stains from uninoculated fermentations has yet to be determined.

Oxygen uptake

During fermentation yeast and bacteria produce many compounds, but they also utilise a number of key molecules, including oxygen. Initial fermentation conditions are aerobic, allowing growth of several yeast and bacterial species that arise from grapes and winery equipment. Being essential in sterol biosynthesis (Fornairon-Bonnefond *et al.* 2002; Espenshade and Hughes 2007) and thus membrane integrity (Prasad and Rose 1986; Lodolo *et al.* 2008), oxygen utilisation by *S. cerevisiae* in the initial stages of fermentation is important for yeast population growth and their ability to consume sugar and some nutrients. In the latter stages of fermentation, oxygen availability might impact on the catabolism of proline, a process with a strict dependence on oxygen (Ingledew, Magnus and Sosulski 1987). Thus oxygen availability during winemaking (e.g. via pump-overs) may improve biomass yield and/or its activity and, consequently, boost alcoholic fermentation rate when sluggish fermentation is suspected (Blateyron and Sablayrolles 2001). *Oenococcus oeni* and *Lb. plantarum* are facultative anaerobes, having the ability to utilise small quantities of oxygen (Götz *et al.* 1980). LAB have slower growth rates but higher yield under aerobic conditions due to the production of acetic acid rather than lactic acid, resulting in more ATP available for growth (Götz *et al.* 1980; Reguant *et al.* 2005). However, lactic acid is favoured by consumers over acetic acid in wines, so LAB growth under anaerobic conditions is preferred for winemaking. During alcoholic fermentation aerobic conditions are rapidly lost as yeast consume oxygen and begin to produce carbon dioxide that displaces oxygen in the liquid and headspace (Boulton *et al.* 2013). The subsequent anaerobic conditions lead to faster growth of *Oenococcus* and *Lactobacillus* species (Götz *et al.* 1980; Reguant *et al.* 2005) and creation of unfavourable conditions for spoilage aerobes such as acetic acid bacteria (Drysdale and Fleet 1989). The ability of LAB, in particular *O. oeni*, to grow more quickly under anaerobic conditions may in part explain why these LAB are often only detectable after completion of AF. The presence of other LAB species, such as *Lactobacillus* and *Pediococcus*, at the beginning of AF may indicate these LAB have greater oxygen tolerance compared to *O. oeni*, but cannot withstand other selective pressures of AF and MLF. Therefore yeast substantially alter conditions for various LAB to grow by depleting oxygen, and can significantly impact fermentation outcomes.

Nitrogen assimilation and nutrient uptake

Like oxygen, nitrogen and other nutrients play a key role in yeast and LAB health. Nitrogenous compounds including amino acids and ammonium are essential for growth and viability of yeast and LAB in wine fermentations and allow yeast and LAB to grow under stressful conditions. Nitrogen limitation can lead to increased production of hydrogen sulfide by *S. cerevisiae* (Jiranek, Langridge and Henschke 1995a, 1995b) while overall depletion of amino acids by non-*Saccharomyces* yeasts (Gobert *et al.* 2017) can inhibit LAB growth (Fourcassie *et al.* 1992). The requirements LAB have for specific amino acids are complex and strain dependent, but it is certain that they benefit greatly from complex nitrogenous compounds and a large number of individual amino acids (Garvie 1967; Fourcassie *et al.* 1992; Remize *et al.* 2006; Terrade and de Orduña 2009). Determination of the amino acids required for LAB growth is impacted by the methodology, including carryover from nutrient rich media before inoculation into minimal media, as well as the inoculation rate and subsequent growth. This has been addressed by Terrade and de Orduña (2009) leading to identification of more amino acids and other compounds that may be required for LAB growth. Despite this, nutritional requirements identified so far do not cover all available commercial LAB strains, but there are now suitable methods for future determination (Terrade and de Orduña 2009).

Yeast lees in wine provides a source for nutrient scavenging, whereby LAB take up nutrients from dying yeast cells. While it is recognised that yeast leak nutrients such as amino acids, sugars and peptides that LAB can utilise (Fleet, Lafon-Lafourcade and Ribereau-Gayon 1984), the release of nutrients from yeast lees may not necessarily provide a distinct explanation for the commonly observed enhanced growth of LAB toward the end of AF (Patynowski, Jiranek and Markides 2002). Nevertheless this knowledge has led to the commercial availability of yeast-derived nutritional additives that winemakers can use to enhance the growth of LAB for fermentation. In this way, yeast lees may assist the compatibility and success of yeast-LAB co-inoculations.

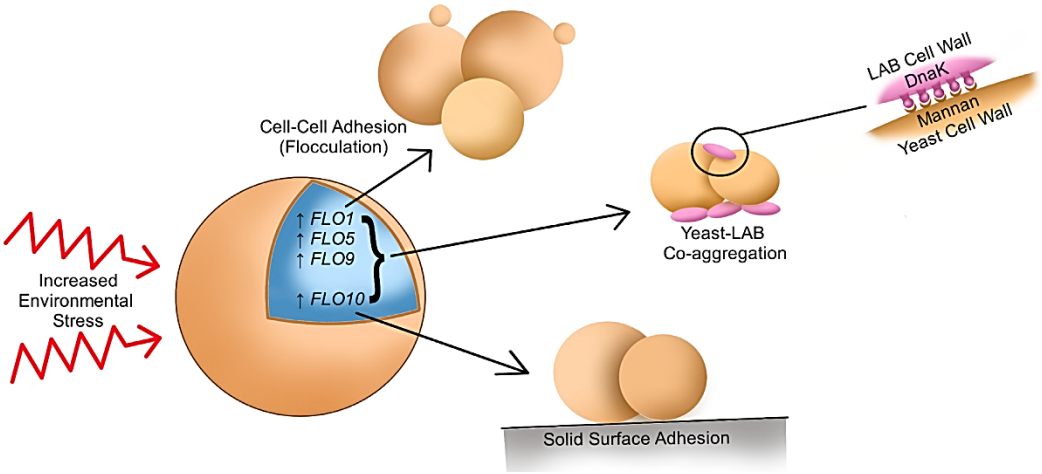

Figure 3. Under increased environmental stress, yeast *FLO* genes are upregulated leading to cell-cell adhesion, yeast-LAB co-aggregation and solid surface adhesion. Yeast-LAB co-aggregation involves LAB cell wall protein DnaK interacting with yeast cell wall mannan.

L-malic acid utilisation

The final compound to be discussed in relation to its utilisation is L-malic acid. This acid is a major contributor to wine acidity, though its concentration in grape must is variable and greatly depends on seasonal temperatures and subsequent grape ripening (Olego *et al.* 2016). Initial L-malic acid concentrations of around 3 $g.L^{-1}$ provide an adequate carbon source for LAB and enable efficient onset of MLF (Fowles 1992; Pretorius 2000). LAB decarboxylate L-malic acid to produce L-lactic acid and CO_2, thereby reducing wine acidity. *Saccharomyces cerevisiae* are typically unable to efficiently degrade or produce L-malic acid due to a lack of a mediated transport system, low substrate affinity for L-malic acid and the mitochondrial location of the malic enzyme (Salmon 1987). Despite this, *S. cerevisiae* may reduce L-malic acid content due to diffusion (Volschenk *et al.* 1997; Nardi *et al.* 2019). However the non-*Saccharomyces* yeast, *Schizosaccharomyces pombe*, can consume a proportion of the initial L-malic acid by converting it into ethanol through malo-ethanolic deacidification (Taillandier, Riba and Strehaiano 1988; Subden *et al.* 1998).

Saguir and Manca de Nadra (2002) identified the importance of L-malic acid for LAB growth in wine since it can be utilised as a precursor for amino acids in low-nutrient conditions, as well as provide biochemical energy needed for amino acid synthesis. There is, however, potential for competition for L-malic acid in fermentations where both non-*Saccharomyces* yeasts and LAB are present. Alternatively, in scenarios where L-malic acid is high (> 4 $g.L^{-1}$) and inhibitory to LAB MLF performance, non-*Saccharomyces* yeasts could be used to reduce L-malic acid thereby allowing onset of MLF by LAB. *Non-Saccharomyces* such as *Sc. pombe, T. delbrueckii, Candida stellata, Candida zemplinina, Hanseniaspora uvarum, L. thermotolerans* and *M. pulcherrima* all have demonstrated an ability to reduce L-malic acid in a synthetic juice medium, juices or wine, with *Sc. pombe* being able to significantly reduce L-malic acid content (Rankine 1966; Gao and Fleet 1995; Seo, Rhee and Park 2007; Belda *et al.* 2015; Minnaar *et al.* 2017; du Plessis *et al.* 2017a; Nardi *et al.* 2019). The extent of L-malic acid utilisation by non-*Saccharomyces* yeasts is strain dependent, as is inhibition of MLF by LAB when non-*Saccharomyces* yeasts are used to complete AF (du Plessis *et al.* 2017a). Though there are varying effects, LAB MLF inhibition is less likely to occur when non-*Saccharomyces* are used for AF, where the opposite effect is commonly observed with *Saccharomyces* strains (Arnink and Henick-Kling 2005; Comitini and Ciani 2007). Although it has been observed that non-*Saccharomyces* strains such as *T. delbrueckii* and *L. thermotolerans* produce less ethanol than *S. cerevisiae* (Ciani, Beco and Comitini 2006; Bely *et al.* 2008), this is not the only factor to be considered for yeast-LAB compatibility. Nevertheless, LAB conversion of L-malic acid into L-lactic acid can microbially stabilise wine, decreasing the chance of spoilage microorganism growth (Sumby, Grbin and Jiranek 2014), leading to optimal conditions for long term wine storage.

PHYSICAL INTERACTIONS AND COMPATIBILITY OUTCOMES

Although metabolite production and utilisation directly affect microorganism performance during fermentation, physical interactions between microorganisms can also determine their fate. Physical interactions between wine microorganisms are an important first step to production of multicellular masses that allow increased stress resistance. The role of flocculation and biofilm formation in yeast-LAB survival during fermentation is discussed below.

Flocculation

Yeast cells flocculate (clump together) during fermentation as a mechanism to withstand increasing environmental stress (Fig. 3; Claro, Rijsbrack and Soares 2007). The mechanism is well known, and involves the encoded products of a number of transcriptionally upregulated *FLO* genes. Expression of *FLO1, FLO5, FLO9* and *FLO10* has been linked to cell-cell adhesion (Verstrepen and Klis 2006; Di Gianvito *et al.* 2017), whereas *FLO11* expression has strong links to adhesion to solid surfaces (Bayly *et al.* 2005; Govender *et al.* 2008). *FLO* gene transcription leads to increased cell surface proteins called flocculins that interact with mannan

on neighbouring yeast cells (Di Gianvito *et al.* 2017). The flocculation mechanism is important for yeast cell populations under conditions of high ethanol and low nitrogen concentrations. For further information, refer to Goossens and Willaert (2010) and Soares (2011).

Yeast surface mannan can also interact with *Lactobacilli* cell wall protein DnaK, a multi-functional protein involved in various stress responses (Fig. 3; Katakura *et al.* 2010). The interaction occurs only under specific conditions and can lead to co-flocculation of yeast and bacteria. Katakura *et al.* (2010) investigated kefir LAB cell surface DnaK interaction with yeast cell wall mannan and suggested such interaction to be influenced by nutrient availability during high stress conditions. Specifically, through their proximity to yeast in a liquid medium, LAB are thought to have increased access to nutrients supplied by yeast compared to a planktonic existence. Similarly, a study of *Lb. plantarum* and *S. cerevisiae* isolated from pot vinegar revealed co-aggregation was the result of interaction of LAB cell surface proteins with yeast cell-bound mannan, although the LAB surface proteins were not identified (Furukawa *et al.* 2011). During these particular instances of co-flocculation it was not identified which *FLO* genes were linked to the overall process. Nevertheless, it was identified that co-aggregation could aid in yeast-LAB mixed biofilm formation (Furukawa *et al.* 2011).

Biofilms

Co-aggregation between yeast and LAB is an important first step for mixed species biofilm formation in various food fermentations. The initial cell-to-cell contact that occurs during co-aggregation involves interactions between yeast cell wall protrusions with the bacterial cell surface. Yeast cell wall protrusions are also involved in cell attachment to solid surfaces and biofilm formation (Guo *et al.* 2000; Reynolds and Fink 2001). Mixed species biofilms have been discussed as an important factor for fermentation success in situations where the starting product is a solid material, such as rice or olives (Furukawa *et al.* 2010; Arroyo-López *et al.* 2012). Biofilms provide protection from external stressors including shear and harsh environmental conditions, as well as allowing survival during low nutrient availability (Garrett, Bhakoo and Zhang 2008; Maragkoudakis *et al.* 2013).

Biofilm formation for yeast in winemaking is a survival mechanism in low nitrogen conditions, as yeast biofilms at the air-liquid interface allow aerobic respiration by cells and use of other available nitrogen and carbon resources (Fidalgo *et al.* 2006; Zara *et al.* 2012). Similarly, for LAB, biofilm formation on tank and oak barrel surfaces has been identified as a stress response to environmental conditions (i.e. low pH, high ethanol, low temperature), that can allow cells to grow more easily (Kubota *et al.* 2008). It is therefore expected that wine yeast and LAB, in particular *Lactobacillus* species, could form mixed species biofilms similar to those observed in rice wine vinegar and beer fermentations. In fact, in the instance of rice wine vinegar, mixed species biofilms formed between *S. cerevisiae* and *Lb. plantarum* were thicker than single species biofilms. It was observed that LAB cells form bridging clamps between large yeast cells, thereby allowing the formation of thicker biofilms (Furukawa *et al.* 2010). These observations suggest that the formation of a mixed species biofilm between yeast and LAB may allow for greater resistance to environmental stress, and therefore better outcomes in LAB and yeast survival during wine fermentations. However, the occurrence and influence of mixed species biofilms in wine needs to be confirmed through further investigation, especially in terms of how they impact fermentation kinetics and wine composition.

YEAST-LAB COMPATIBILITY AND SENSORY OUTCOMES

Volatile compounds produced during fermentation, by both yeast and LAB, are most commonly investigated for their contribution to the sensory outcome of wines. However, there is much less discussion regarding the importance of these compounds for microorganism health, how those compounds affect other microbes (i.e. yeast compounds affecting LAB and vice versa) and the necessity of the production of certain volatiles to maintain a suitable growth environment. Every molecule plays a role in the health of microorganisms and it is recognised that yeast and bacteria found in niche environments have specific metabolic and genetic traits that reflect the stressors they are likely to face in those environments (López-Maury, Marguerat and Bähler 2008; Mitchell *et al.* 2009). There has been considerable work to date in regards to development of microbial strains that are more tolerant to stressors during wine fermentation (Pérez-Torrado, Gimeno-Alcañiz and Matallana 2002; McBryde *et al.* 2006; Cardona *et al.* 2007; Bonciani *et al.* 2016; Betteridge *et al.* 2018; Jiang *et al.* 2018). However, it may be useful to understand the roles of certain metabolic pathways in regard to aspects of yeast and LAB metabolic interactions, to ensure future efficiency in design of experiments for development of robust yeast and LAB strains.

It is known that LAB can modify yeast derived volatile compounds during MLF (both sequential and co-inoculated) and the timing of MLF induction can have a significant effect on the biochemical and sensory properties of the resulting wine. Recent studies have highlighted the variation of a wine's biochemical profile produced as a result of different LAB inoculation procedures (Abrahamse and Bartowsky 2012; Cañas *et al.* 2012, 2015; Knoll *et al.* 2012; Antalick, Perello and de Revel 2013; Tristezza *et al.* 2016). The effect of LAB inoculation has a greater impact on wine sensory properties than yeast treatment alone (du Plessis *et al.* 2017b). Take for example the major sensory compound produced by LAB during MLF, diacetyl, which is formed through the metabolism of citric acid and results in wine with a buttery aroma. Wines produced using sequential inoculation of yeast and LAB, respectively, would be expected to contain LAB produced diacetyl and result in a buttery aroma. Conversely, wines produced using co-inoculation of yeast and LAB together may result in wines with a less buttery aroma, as a result of yeast being able to metabolise diacetyl to acetoin and 2,3-butandiol (Mink *et al.* 2014; Lasik-Kurdyś, Majcher and Nowak 2018).

As highlighted above, distinct changes in biochemical profiles that are dependent on LAB inoculation procedures, ultimately result in alteration of overall wine sensory perception. Examples of this are summarised in Fig. 4, which shows differences in sensory profiles as a result of either sequential or co-inoculation of LAB, or the use of different LAB species. Co-inoculation with yeast and LAB often leads to distinct changes in volatile aroma compounds that enhance the fruity aroma of the finished wine, due to increased ester levels (Sumby, Jiranek and Grbin 2013; Cañas *et al.* 2015; Devi *et al.* 2018). Co-inoculation has also been seen to influence tannin and anthocyanin concentrations in red wine that could lead to lowered astringency and more desirable wine colour, respectively (Abrahamse and Bartowsky 2012; Guzzon *et al.* 2016). For a recent review on the

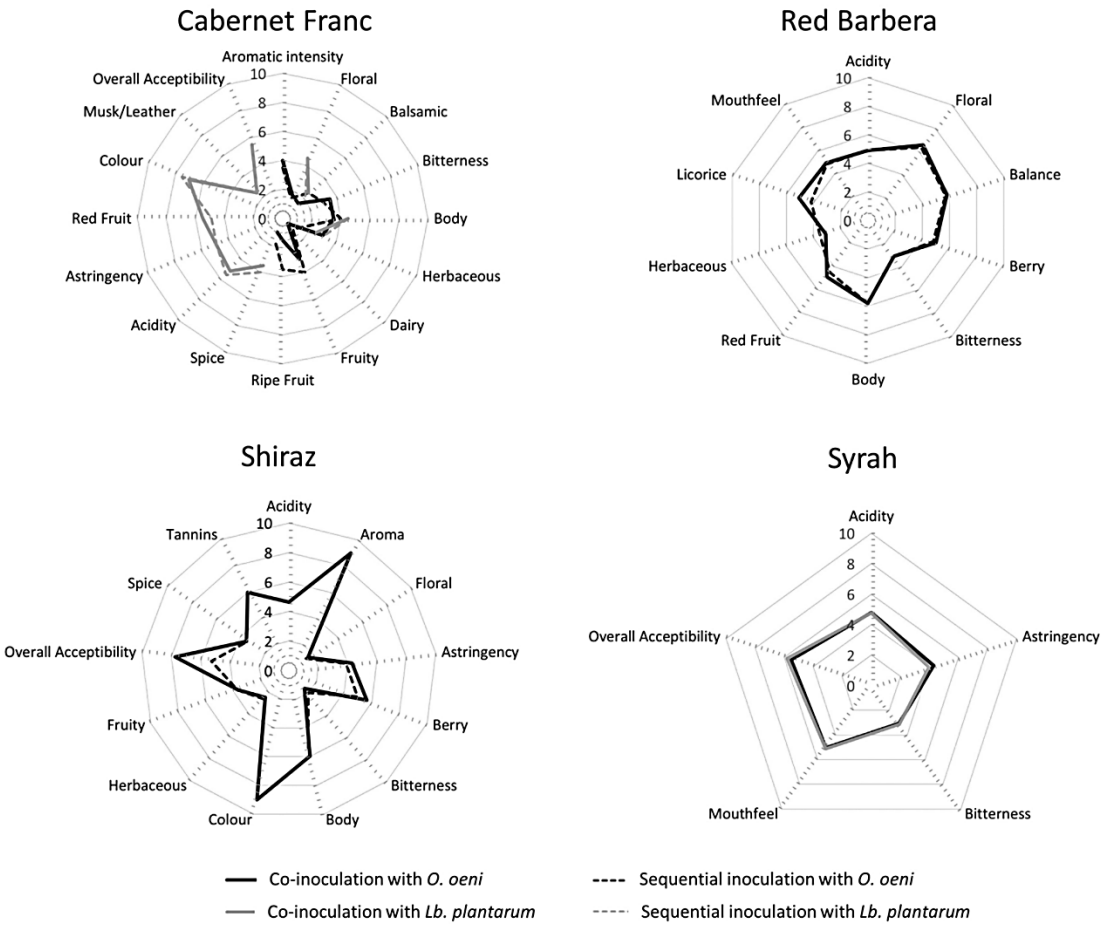

Figure 4. LAB inoculation can influence overall sensory characteristics of red wines as determined by trained tasting panels. Average results of sensory characterisation of wines from multiple reports are summarised here, with data collated from Cañas *et al.* 2015; Versari *et al.* 2016; Minnaar *et al.* 2017; du Plessis *et al.* 2017b; Devi *et al.* 2018 and Nardi *et al.* 2019. Each plot shows the difference in sensory profile as a result of either sequential versus co-inoculation of LAB (Cabernet Franc, Red Barbera or Shiraz) or different LAB species used for MLF (Syrah). Data from Minnaar *et al.* 2017; du Plessis *et al.* 2017b and Nardi *et al.* 2019 were scaled by a factor of 10 to assist in summarising.

modulation of wine sensory profiles by LAB species see Cappello *et al.* (2017).

The optimal MLF strategy for each yeast-LAB combination, to improve wine flavour and quality, appears to be strain dependent and significant variation in resulting wine composition does not always translate to perceivable sensory differences. The effect of interactions between yeast and LAB on the sensory profile of wine therefore requires further research.

GENE EXPRESSION INFLUENCED BY YEAST-LAB CO-INOCULATION

Although metabolite production and physical cellular interactions are major contributors to yeast-LAB compatibility, there are many processes occurring at the genetic level that can influence compatibility outcomes. Transcriptional regulation of genes in yeast and LAB during fermentation can provide insight into how the yeast and LAB respond to environmental stressors. These stressors include nutrient competition, low pH, high ethanol and antagonistic proteins. Only two studies so far have investigated transcriptional changes in both yeast and LAB during fermentations, albeit in Chinese rice wine and during a lactose-based chemostat study, respectively (Mendes *et al.* 2013; Liu *et al.* 2017b). All others have focused on the response of one microorganism or the other (Herve-Jimenez *et al.* 2008; Nouaille *et al.* 2009) including the transcriptional response of *S. cerevisiae* to co-inoculation with *O. oeni* in wine (Rossouw, Du Toit and Bauer 2012). The study by Rossouw, Du Toit and Bauer (2012) uncovered many more questions about interactions between yeast and LAB during co-inoculation. Findings included the potential for yeast and LAB competition for sulfur compounds and the production of unknown antagonistic molecules that resulted in upregulation of yeast genes *SSA4*, *HMS2* and *FYV12* (Rossouw, Du Toit and Bauer 2012). All three genes are related to stress responses, including survival after exposure to killer toxin (Rossouw, Du Toit and Bauer 2012). This raises the question as to what transcriptional responses may be occurring in LAB in response to co-inoculation with yeast, as well as leaving a gap

in knowledge about how *O. oeni* may specifically utilise sulfur compounds.

Similarly, LAB in co-cultivation with yeast in lactose chemostat cultures and Chinese rice wine revealed links between sulfur amino acid related gene regulation and sulfur compound processing. The specific role of sulfur metabolism in relation to yeast-LAB co-cultivation was only explored in depth by Liu *et al.* (2017b), where the authors identified LAB recycling methionine for yeast to use to generate other sulfur compounds. However, in both cases, only LAB of the genus *Lactobacillus* were studied given their roles in dairy and rice wine fermentations. Changes in LAB gene transcription during wine co-inoculation could be similar to those found in dairy and rice wine, although *Lactobacillus* commonly do not survive until the completion of wine fermentations. It would therefore be useful to explore why LAB such as *Lactobacillus* flourish in rice wine and diary cultures to gain insight into how they can be improved for use in wine.

Although these studies reveal the complex relationship between yeast and LAB during fermentation, for wine there is much to explore in regards to LAB gene regulation during co-inoculation. Though the process may prove difficult, analysis pipelines for mixed kingdom transcriptome data have been developed (Westermann, Gorski and Vogel 2012; McClure *et al.* 2013; Westermann *et al.* 2016) and could be applied to yeast-LAB transcriptome experiments.

OUTLOOK

Although we have come a long way in recent years towards our understanding of the role of microbial interactions in winemaking, much remains unknown. This review discusses the many factors that have been reported to influence the compatibility of *Saccharomyces* yeast and LAB during wine fermentation. However it is clear that there are still considerable potential future research directions in this area. This review highlights the influence and significance of microbial production of a range of compounds and proteins on microorganism performance during fermentation and the potential role of physical yeast-LAB interactions on microorganism compatibility. It is expected that further study of the interactions between yeast and LAB in both inoculated and uninoculated ferments will uncover a range of new information on how best to control both AF and MLF. It would appear that it is first necessary to obtain a better understanding of the response of *O. oeni* cells to ethanol and other known and novel inhibitory compounds. Also investigations should continue into the synergistic effect of wine stressors and why some microbes are more affected than others.

Both yeasts and bacteria produce compounds that can alter the wine matrix, influencing the ability of those microorganisms to perform as desired under wine conditions. The production of proteinaceous compounds and organic molecules that can inhibit or promote the growth of yeast and bacteria are of great importance to the wine industry. Simply identifying that these compounds have been produced, however, is not enough. Future research needs to identify the mechanisms by which these compounds are released, and begin to untangle the reasons for production of those compounds. To directly assist winemakers, future work in this area should include: a more comprehensive survey of compatible yeast-LAB than is currently available; identification of novel metabolic functions within yeast that may lead to compatibility or incompatibility with LAB; and discovery of more ways in which LAB can utilise yeast derived metabolites.

FUNDING

This work was supported by Australia's grape growers and winemakers through their investment body, Wine Australia, with matching funds from the Australian Government. LB was supported by joint scholarships from the University of Adelaide and Wine Australia (AGW Ph 1510). JS was supported by Wine Australia project funding (UA1707). JS, KS and VJ are supported by The Australian Research Council Training Centre for Innovative Wine Production (www.ARCwinecentre.org.au; project number IC170100008), which is funded by the Australian Government with additional support from Wine Australia and industry partners. The University of Adelaide is a member of the Wine Innovation Cluster in Adelaide (http://www.thewaite.org/waite-partners/wine-innovation-cluster/).

Conflict of interest. The authors declare no conflict of interest.

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PhD research objectives

As discussed in the review, there are many unexplored areas of yeast-lactic acid bacteria (LAB) interactions in the context of winemaking. It was highlighted that there is a need for a more comprehensive list of compatible yeast and LAB, identification of yeast metabolic functions that may influence compatibility between yeast and LAB, and greater insight into yeast-derived compounds that LAB may utilise.

Therefore, the overarching aim of this thesis was to gain a greater understanding of factors that influence compatibility between yeast and LAB in a winemaking context. Each chapter follows a progression of ideas, as it became clear how complicated the interactions between yeast and LAB were, and the need for improved methods to analyse them became evident.

Initial work focused on addressing the current challenges associated with accurate LAB enumeration using flow cytometry. Following on from this, compatibility of a large number of yeast and LAB pairs was analysed in Chemically Defined Grape Juice Medium. This was followed by more in depth analysis with a reduced number of strains in Shiraz juice. Once it was confirmed that compatibility between yeast and LAB was strain dependent, regardless of ethanol concentration, yeast genetic traits that may impact yeast-LAB compatibility were investigated. Quantitative trait loci (QTL) mapping, followed by a reciprocal hemizygosity assay, was used to determine how yeast-LAB compatibility was affected by yeast with different genetic backgrounds. A yeast gene, *SSU1*, was identified that appears to contribute to an interaction with LAB that influences MLF completion.

Finally, gene regulation during co-inoculation was studied using quantitative PCR, specifically targeting sulfur-metabolism related genes in yeast and LAB. Phenotypic measurements including H_2S, glutathione, alcoholic and malolactic fermentation progress, and yeast and LAB growth were also made. Gene expression differed for yeast-alone compared to yeast co-inoculated with LAB, uncovering the complexity of yeast-LAB interactions during co-inoculation.

The research presented throughout this thesis contributes to the body of knowledge regarding factors that can influence yeast-LAB compatibility during wine fermentation.

Chapter 2

Enumeration of wine microorganisms: challenges and where to from here?

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Cytometry Part A

Submitted: 22nd January 2020

Statement of Authorship

Title of Paper	Enumeration of wine microorganisms: challenges and where to from here?
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Cytometry Part A, submitted on 22nd January 2020

Principal Author

Name of Principal Author (Candidate)	Louise Bartle
Contribution to the Paper	Conception of ideas for the paper, collection of references, literature and data interpretation, manuscript preparation and editing
Overall percentage (%)	85%
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: 2/3/2020

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.

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Chapter 2

Contextual statement

The first aim of this PhD was to establish a routine flow cytometric method for enumeration of wine lactic acid bacteria, specifically *Oenococcus oeni*. Initial work performed attempted to select appropriate fluorescent stains for *O. oeni*, and subsequently use flow cytometry as a method to accurately and quickly calculate viable *O. oeni* cells. In attempting to evaluate and replicate previously published flow cytometry methods for *O. oeni*, the issue of chain-formation became apparent. Hence, throughout the experiments in later chapters of this thesis, spot-plate counting was used to measure lactic acid bacteria viable cell numbers. Though there are also limitations of spot-plate counting for *O. oeni* enumeration, it was the preferred method over flow cytometry.

The manuscript in this chapter addresses current complications with the use of flow cytometry for enumerating chain-forming bacteria, such as *O. oeni*, in wine samples. A communication to the editor was written to discuss the complications and potential ways forward.

Enumeration of wine microorganisms: challenges and where to from here?

The diversity of yeast and bacteria at the beginning of wine fermentation is well understood (1). These microorganisms originate from the grapes and wine processing equipment. However, it has become a regular practice to inoculate juice with a gold-standard yeast (*Saccharomyces cerevisiae*) for alcoholic fermentation and lactic acid bacteria (LAB, *Oenococcus oeni*) for malolactic fermentation. During the fermentation process, these two highly efficient microorganisms take over and their continued growth is critical to complete the fermentation process. Enumerating yeast and LAB during fermentation is critical for understanding the dynamics of successful and efficient wine production.

Enumerating yeast during fermentation is currently most accurately and quickly done by flow cytometry (2), as they are easy to distinguish from background noise due to their size, intracellular complexity and ease of staining. *O. oeni* has been difficult to distinguish from particulate noise in red wines (2,3) because they are smaller, less complex and do not always stain effectively (4). A recent review discusses staining efficiency and the practicality of different enumeration methods of LAB and yeast to assess viability and vitality (5). The assessment found that dyes used to determine metabolic activity or dyes that rely on membrane fluidity are affected by conditions that LAB are grown in (i.e. high ethanol), and therefore may be unsuitable for wine studies. However, the ability to use other dyes that fluoresce only upon binding of DNA was also discussed. It was also found that application of an established microscopy method, fluorescence *in situ* hybridization (FISH), could be used to identify and enumerate specific microorganisms when coupled with flow cytometry (Flow-FISH; 5). This is a particularly useful technique for wine since there

are few microorganisms that survive the harsh fermentation conditions and the method could also be used to identify spoilage yeast such as *Brettanomyces* (6). Separation of LAB cells from red wine particulates is another reason for combining techniques such as FISH and flow cytometry. The ability to specifically tag microorganisms of interest could allow a better estimate of abundance and viability within a wine sample. There are also a limited number of spoilage microorganisms that are necessary to track, such as the yeast *Brettanomyces bruxellensis* or the acetic acid bacteria *Acetobacter aceti* and *A. pasteurianus*. However, as previously highlighted, Flow-FISH is traditionally expensive and requires technical expertise that is not otherwise required when using dyes and therefore Flow-FISH has not necessarily been applicable to the wine industry (5).

Understanding the staining efficiency of yeast and LAB is an important part of measuring the viability and vitality of these microorganisms. However, the physiology of these yeast and bacterial populations is rarely discussed. Yeast and LAB can form aggregates in the form of tetrads (yeast) or pairs and chains (LAB) (7). Due to their size, yeast tetrads can be distinguished from single yeast cells using flow cytometry (8). However, this has not been the case for LAB. Although there is support regarding the efficiency of staining for LAB, greater understanding of the effect of bacterial chaining is required. The most common LAB found in wine, *O. oeni*, can form chains of >7 cells (7,9). Chaining of bacterial cells affects all types of enumeration methods, including colony counts when spotted on solid agar. The presence of bacterial chains suggests a single colony may arise from multiple cells, therefore estimates of viability are always underestimated. This issue most likely also translates into flow cytometry. Flow cytometry is a useful tool for high throughput study designs, despite the potential for underestimation of abundance.

The underestimation arises from the flow cytometer laser detecting a single particle, and light scattering to obtain a value that is displayed on a plot (Fig. 1).

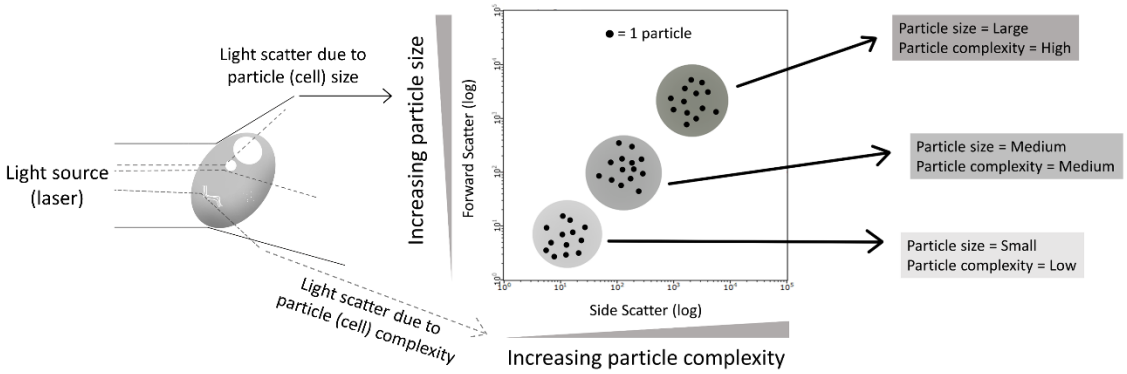

Fig. 1. Overview of particle detection using flow cytometry. As particles pass through the laser light source within a flow cytometer, the light scatters in different ways and passes through different filters to give a specific value. Different values measured by the flow cytometer indicate particle size (forward scatter) and particle complexity (side scatter) and these values are displayed on a plot allowing differentiation of particles (cells) based on size and complexity.

If the bacteria are in chains, then this will be detected as a single particle that would have much larger side scatter and forward scatter values in comparison to a single bacterium. Theoretically, this would explain the “triangle” shaped population quite often seen in flow cytometry cytograms of LAB (Fig. 2).

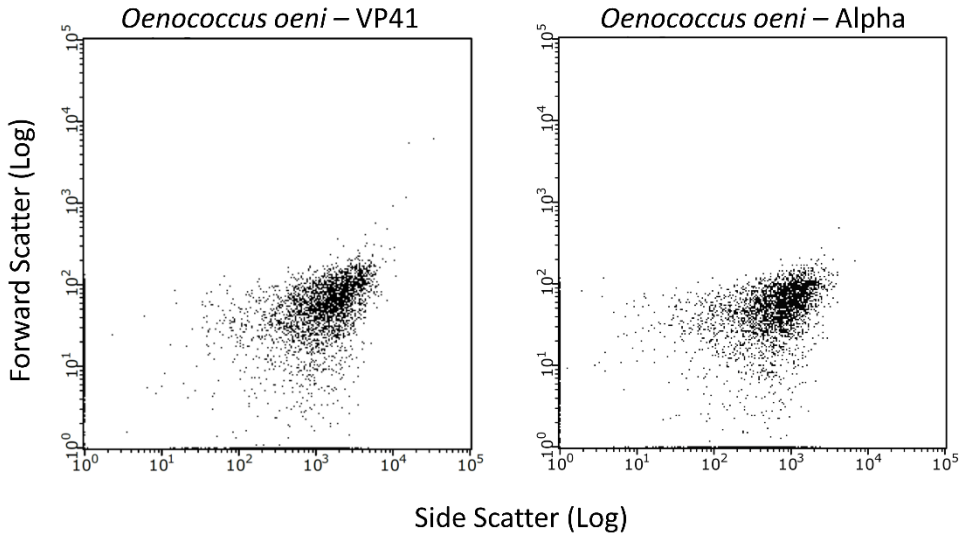

Fig. 2: **Flow cytometry cytogram of pure *Oenococcus oeni* cells**. Two commercial *O. oeni* strains, Lalvin VP41 MBR (Lallemand, Australia) and Enoferm Alpha (Lallemand, Australia), were grown anaerobically in autoclaved Liquid de Man, Rogosa and Sharpe medium (Cat. #AM103, Amyl Media, Victoria, Australia) supplemented with 20% sterile filtered apple juice for four days at 30°C. The samples were diluted 1:10 with TAE and 0.2% SYBR Green-I (Cat. #S7563, Invitrogen, Australia) prior to flow cytometric analysis using a Guava Easycyte 12HT (Millipore) in conjunction with guavaSoft 3.3 flow cytometry software. The threshold was set to 50 on the green-blue channel and samples were collected up to 4,000 events.

However, for now it is not possible to determine how many bacteria are present in a chain based on the side or forward scatter values obtained. Secondly, bacteria that are present on the outer ends of a chain would potentially have larger surface area for dyes to penetrate in comparison to bacteria located within the chain. This may affect the efficiency of dye penetration into the middle cells, also affecting the intensity of the cytometric dye signal.

The development of a method to break chained cells prior to flow cytometry would be one way to combat this issue. However, this presents new challenges to estimating abundance since bacterial de-chaining methods involve sonication which is harsh and may damage or kill cells in the process (10,11).

New technologies are constantly being produced to allow researchers to look deeper into their microbial samples. For example, a flow cytometer that can take an image of every particle that passes through the machine's laser, such as the Amnis (Luminex, Unites States) could aid in tackling problems such as bacterial chaining. In this way, developing methods to enumerate bacteria, whether they are in chains or not, could allow for much more accurate estimates of bacterial abundance. This would create a significant positive impact on the wine industry at the global scale. Additionally, generating a universal method for flow cytometry data presentation could allow research groups to easily interpret each other's results. In this way, discussion may be enhanced between research groups, providing new opportunities for collaboration and support.

As a community, wine scientists may be able to generate a method that is universal. The main benefit being the ability to accurately compare viability and abundance results, and therefore allow better understanding of microbial populations in wines from around the world. It also lays a foundation for assessing wine microbial communities over multiple vintages, generating data that could be used for long term studies of the impact of climate change on winemaking processes.

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Chapter 3

Yeast-bacteria compatibility of industrial strains in a synthetic red juice

1. Introduction

Winemaking is dependent on yeast, either naturally present (un-inoculated fermentation) or intentionally added (inoculated fermentation), to complete alcoholic fermentation (AF). In the instance of red and some white varieties, lactic acid bacteria (LAB) are also needed to complete malolactic fermentation (MLF). AF completion to dryness by yeasts is defined by reduction of total sugars (i.e. glucose and fructose) to < 3 g L^{-1} (Puckette 2015). MLF completion by LAB, where they convert L-malic acid to the less acidic L-lactic acid, is specified as reduction of L-malic acid concentration to < 0.1 g L^{-1} (AWRI 2016). Predicting MLF success is itself dependent on a range of factors including inoculation strategy and compatibility of LAB with yeast. In red winemaking, MLF is primarily employed to ensure microbial stability and reduce acidity, however, LAB can also contribute to wine aroma and mouthfeel by producing esters, polysaccharides and other organic compounds (Gammacurta *et al.* 2018; Sumby, Jiranek & Grbin 2013; Swiegers *et al.* 2005). The concentrations of oenologically important molecules differ depending on yeast and LAB strains present, and differences in production have been reported based on inoculation strategy (Abrahamse & Bartowsky 2012; Antalick, Perello & de Revel 2013; Cañas *et al.* 2012, 2015; Knoll *et al.* 2012; Rossouw, du Toit & Bauer 2012; Tristezza *et al.* 2016). Due to the underlying complexity and biological variability of winemaking there are several factors for winemakers to consider prior to and during fermentation to ensure fermentation success. Additionally, selection

of appropriate winemaking procedures can allow wineries to benefit economically, as well as produce high quality and unique wines.

There are two main inoculation strategies that can be used by winemakers looking to undertake MLF: co-inoculation or sequential inoculation. Co-inoculation involves addition of LAB approximately 24 hours after yeast addition to conduct simultaneous AF and MLF (Antalick, Perello & de Revel 2013; Cañas *et al.* 2012, 2015; Knoll *et al.* 2012). This differs from sequential inoculation, which is the addition of LAB after completion of AF by yeast. Co-inoculation can provide benefits over sequential fermentations, such as reduced overall fermentation time and a greater ability to control fermentation (Abrahamse & Bartowsky 2012; Sumby, Grbin & Jiranek 2014). Addition of LAB at the beginning of AF can allow LAB to grow and conduct MLF efficiently, as the juice contains higher nutrient levels and lower ethanol concentrations. However, the success of co-inoculation greatly depends on the compatibility of yeast and LAB throughout fermentation.

During AF yeast consume glucose, fructose and nitrogenous compounds, produce ethanol and reduce pH, all of which could affect the performance of yeast and LAB during co-inoculation (Bauer, Nel & Dicks 2003; Branco *et al.* 2017; Drysdale & Fleet 1989; Sumby, Grbin & Jiranek 2014). Co-inoculation practices are sometimes avoided by winemakers due to concern about LAB efficiency and MLF speed being impacted through inhibition of LAB by wine yeast (Alexandre *et al.* 2004; Liu *et al.* 2017). Additionally, *O. oeni*, one of the most common wine LAB, is a heterofermenter and may produce unwanted compounds such as acetic acid rather than the desired lactic acid. Whilst some commercial suppliers provide information about yeast strain compatibility with MLF bacteria, there is still uncertainty about the

suitability of specific yeast and LAB pairs used to perform concurrent AF and MLF. This aspect of yeast and LAB strain choice requires more research in order to identify pairs that can conduct co-fermentation efficiently. This is particularly important during compressed vintages when wines need to be produced quickly to provide fermentation capacity and allow harvest and processing of grapes at optimal ripeness. Additional to this, efficient red wine production could aid the growing domestic and international demand for Australian red wines, which accounted for 58% and 39% of export and domestic sales in 2015-2016, respectively (Wine Australia 2017).

The limited information and uncertainty around fermentation outcomes for simultaneous AF and MLF that use specific strains of yeast and LAB has led to the following aims of our study: to generate a list of compatible and incompatible yeast-LAB pairs based on AF and MLF completion; and to elucidate strain compatibility in a synthetic, sterile red juice fermentation. To evaluate strain specific differences in compatibility, this study compared the performance of 72 commercial yeast-LAB pairs during co-inoculation in a chemically defined red grape juice.

2. Materials and methods

2.1. Media

LAB were grown in medium prepared from autoclaved Liquid de Man, Rogosa and Sharpe medium (MRS; catalogue # AM103, Amyl Media, Victoria, Australia; sterilised 121˚C, 0.1 MPa, 20 minutes) supplemented with 20% sterile (0.2 µm filtered) apple juice (MRSAJ). LAB were enumerated on MRSAJ solidified with 2% agar and supplemented with cycloheximide (0.5%) after autoclaving.

Red Chemically Defined Grape Juice Medium (RCDGJM) was prepared as previously described (McBryde *et al.* 2006), with the following changes: addition of equimolar glucose and fructose to a final sugar concentration of 250 g L^{-1}; addition of 1 g L^{-1} L-malic acid to final concentration of 2.5 g L^{-1}; addition of 5% (v/v) grape tannin extract (GSKINEX, Tarac Technologies, Australia) and adjustment to a pH of 3.5.

2.2. Yeast and bacteria strains

Eight commercial yeast and nine commercial bacteria strains were randomly selected for use in this study (Table 1).

Table 1: Commercial yeast and bacteria used in this study

Commercial Name	Name used in this Study	Yeast Species	Supplier
Lalvin EC-1118	EC1118	*Saccharomyces cerevisiae*	Lallemand Inc
ICV D80	D80	*S. cerevisiae*	Lallemand Inc
ICV GRE	GRE	*S. cerevisiae*	Lallemand Inc
NT50	NT50	*S. cerevisiae* x *S. kudriavzevii*	Anchor Yeast
Zymaflore F15	F15	*S. cerevisiae*	Laffort®
Velluto BMV58	Velluto	*S. uvarum*	Lallemand Inc
CONCERTO	Concerto	*Lachancea thermotolerans*	Chr**.** Hansen
Zymaflore Alpha	Alpha Yeast	*Torulaspora delbrueckii*	Laffort®

Commercial Name	Name used in this Study	Bacteria Species	Supplier
Viniflora CH16	CH16	*Oenococcus oeni*	Chr. Hansen
Lactoenos B450 PreAc	450	*O. oeni*	Laffort®
Lactoenos SB3 Direct	SB3	*O. oeni*	Laffort®
Enoferm ALPHA	Alpha LAB	*O. oeni*	Lallemand Inc
O**-**Mega	O-Mega	*O. oeni*	Lallemand Inc
Lalvin VP41 MBR	VP41	*O. oeni*	Lallemand Inc
PN4	PN4	*O. oeni*	Lallemand Inc
Viniflora NoVA	NoVA	*Lactobacillus plantarum*	Chr. Hansen
ML Prime	Prime	*Lb. plantarum*	Lallemand Inc

2.3. Bacteria pre-treatment

LAB were cultured in liquid MRSAJ medium for four days at 30°C under 20% CO_2. Twenty four hours prior to inoculation, LAB were centrifuged at 2,236 x *g* for 5 minutes. The supernatant was removed and cells washed with RCDGJM followed by centrifugation and supernatant removal as above. LAB were re-suspended in fresh RCDGJM and incubated overnight under the same conditions. Prior to inoculation, LAB were adjusted to an OD_{600} of 0.55. OD_{600} was standardised by subtracting the OD_{600} value of un-inoculated growth medium for each sample.

2.4. Concurrent alcoholic and malolactic fermentations

Commercial yeast and bacteria (Table 1) were each assessed in triplicate for compatibility during AF and MLF (n = 216). Yeast were rehydrated and inoculated into RCDGJM following the manufacturers' protocols. Fermentations (100 mL) were conducted at 22°C in glass shake flasks fitted with airlocks.

LAB were inoculated 24 hours post-yeast inoculation by transferring 1 mL of OD_{600} adjusted LAB culture to each flask. Samples (200 µL) were collected from each fermentation at multiple time points for analysis of LAB and yeast viability, sugar and L-malic acid consumption, as described below.

2.5. Yeast enumeration and viability

Yeast were enumerated and viability assessed using flow cytometry. Propidium iodide stain was applied to cells at a final concentration of 0.1 mg mL^{-1} prior to analysis using a Guava Easycyte 12HT (Millipore) in conjunction with guavaSoft 3.3 flow cytometry software. Each sample was analysed for 2 minutes, or up to 1,000 events. Flow cytometry parameters can be found in Table S1 (Appendix A).

2.6. LAB enumeration

Samples were collected throughout fermentation and serially diluted (1:100 in sterile 1 x phosphate buffered saline (PBS) up to 10^{-5}. A 2 µL aliquot of each dilution was transferred to MRSAJ (cycloheximide) agar and incubated anaerobically in either a CO_2 incubation cabinet or a GasPak EZ standard incubation container containing sachets with indicator (BD catalogue # 260671 and # 260001) for 4 days at 30°C. From dilution spot plates, colony forming units (cfu mL^{-1}) were determined.

2.7. Glucose/fructose consumption

Glucose and fructose concentrations were determined enzymatically using commercially available kits (K-FRUGL, Megazyme, Ireland) following methods modified by Walker *et al.* (2014). Glucose and fructose consumption was used as a determinant for alcoholic fermentation progress. Alcoholic fermentation was deemed complete when total glucose plus fructose concentrations were < 3 g L^{-1}.

2.8. L-malic acid concentration

L-malic acid was measured using an enzymatic test kit (catalogue # 4A165, Vintessential laboratories, Australia) with modifications so that a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland) could be used to measure absorbance. Specifically, each well of a 96 well micro-titre plate was dosed with 70 µL buffer (0.1M gly-gly, 0.1M L-glutamate, pH 10), 14 µL nicotinamide adenine dinucleotide (40 mg mL^{-1}), 70 µL distilled water, 0.7 µL glutamate oxaloacetate transaminase (800 U mL^{-1}) and either 5 µL of sample or one of the L-malic acid standards (ranging from 0 – 3.0 g L^{-1}). The plate was incubated at 22 ˚C for 3

minutes and the first absorbance read at 340 nm; 7 µL of the 1:10 diluted L-malate dehydrogenase (12,000 U mL^{-1}) was added and mixed into each well; the plate was incubated at 22 ˚C for 15 minutes before the second absorbance was measured at 340 nm. L-malic acid in each sample was calculated from standard curves prepared with known L-malic acid concentrations. L-malic acid degradation was used as the determinant for MLF progress. MLF was deemed complete when L-malic acid concentrations were < 0.1 g L^{-1}.

2.9. Statistical analysis

R version 3.5.1 was used for all statistical analyses. Significant differences within the data were determined using ANOVA with Tukey's post-hoc test $p < 0.005$. Spearman's rank correlation coefficient and *p* –values were determined using Hmisc package version 4.1-1. Cytoscape (version 3.7.0, Shannon *et al.* 2003) was used to visualise complex statistical relationships among variables. In Cytoscape the variables were represented as nodes and the statistical relationships were represented as lines, referred to as edges, between the nodes.

3. Results and discussion

Eight commercial yeast and nine commercial LAB were assessed for their ability to complete alcoholic and malolactic fermentation during co-inoculation in RCDGJM. In this study, completion of AF and MLF were defined by the following:

1) AF: total sugar concentration reduced to < 3 g L^{-1}
    a. With exception of non-*Saccharomyces* since they are often observed not to complete AF (Ciani, Beco & Comitini 2006; Contreras *et al.* 2014; Jolly, Varela & Pretorius 2014; Soden *et al.* 2000)
2) MLF: L-malic acid concentration reduced to < 0.1 g L^{-1}

In addition to AF and MLF completion, yeast-LAB pair compatibility was defined by the following conditions being met:

1) Completion of MLF
2) Either:
    2.1. *Saccharomyces* completing AF or
    2.2. Non-*Saccharomyces* having no significant difference in final residual sugar concentration between yeast alone and yeast co-inoculated with LAB.

Both *Lb. plantarum* strains were incompatible with all yeast strains used in this study (Table 2) and neither strain was able to sustain growth over the course of the experiment (Figure S1, Appendix A). *Lb. plantarum* strains are becoming popular with winemakers for MLF since they may contribute positively to overall sensory properties of the final wine (Sumby, Grbin & Jiranek 2010, 2014; Swiegers *et al.* 2005). Some *Lactobacillus* species have properties that allow growth and MLF in wine such as tolerance of low pH (< 3.5) and ethanol (up to 13% v/v; G-Alegría *et*

al. 2004), though some *Lactobacillus* are ethanol intolerant (5% v/v; Volschenk, Van Vuuren & Viljoen-Bloom 2006). Although *Lb. plantarum* strains can withstand low pH and high ethanol content, the ability of individual *Lb. plantarum* strains to survive juice and wine conditions, then complete MLF is strain specific (Fras *et al.* 2014; Lerm, Engelbrecht & du Toit 2011). Mixing MRS with wine or using a wine-like medium that contains components similar to MRS have shown to allow *Lb. plantarum* growth and MLF completion (Bravo-Ferrada *et al.* 2013; Fras *et al.* 2014; Iorizzo *et al.* 2016). Therefore, the *Lb. plantarum* strains in this study may have performed better if MRSAJ was mixed with RCDGJM, rather than using RCDGJM alone, though previous works were focused on sequential rather than co-inoculation. Factors influencing *Lb. plantarum* sensitivity to juice and wine have not been fully elucidated, but we theorise it could be due to the complex nutritional requirements of *Lb. plantarum* (Lerm, Engelbrecht & du Toit 2011; Pozo-Bayón *et al.* 2005; Terrade & Mira de Orduña 2009) and sensitivity to a combination of particular juice and wine components other than ethanol, pH and SO_2, that are yet to be identified. Additionally, preliminary results revealed *Lb. plantarum* strain ML Prime was able to complete MLF rapidly when inoculated at a rate of over 1×10^9 cells mL^{-1} (data not shown); however an inoculation rate this high is unreasonable for industry application due to the complexities of scaling up an inoculation rate used for 100 mL fermentations in comparison to hundreds of litres. For these reasons, testing performance of *Lb. plantarum* using a high inoculation rate ($> 1 \times 10^9$ cells mL^{-1}) in a synthetic juice medium such as RCDGJM may not display the true potential of these LAB for co-inoculation in winemaking conditions. Additionally, there is limited information about the overall nutritional requirements of *Lb. plantarum* over the course of wine fermentation so more testing would be required to develop the optimal test medium.

Table 2: Summary of compatible (+) and incompatible (-) yeast-LAB pairs during co-inoculation in 100 mL of RCDGJM. Compatible yeast-LAB pairs, highlighted in green, were classified based on two conditions: 1) completion of MLF; 2) completion of AF (except for non-*Saccharomyces* yeast, where the criterion was no significant difference in final residual sugar concentration between yeast alone and yeast co-inoculated with LAB). The values shown are AF finishing time (days ± SD; rows 1-6) or residual sugar concentration (g L^{-1} ± SD; rows 7 & 8).

	CH16	450	SB3	Alpha LAB	O-Mega	PN4	VP41	NoVA	Prime
EC1118	- 6 ± 0	- 6 ± 0	+ 6 ± 0	- 6 ± 0	- 7 ± 0	+ 6 ± 0.3	+ 6 ± 0	- 6 ± 0	- 5 ± 0
D80	+ 9 ± 0	- 9 ± 0	- 9 ± 0.3	- 10 ± 0.3	- 9 ± 0.3	+ 10 ± 0.3	- 10 ± 0.3	- 9 ± 0	- 10 ± 0
GRE	- 8 ± 0	- 9 ± 0	- 8 ± 0.3	- 7 ± 0	- 8 ± 0	- 8 ± 0	- 8 ± 0	- 8 ± 0	- 8 ± 0
NT50	- 5 ± 0	+ 5 ± 0	- 5 ± 0	+ 4 ± 0.3	+ 5 ± 0	- 5 ± 0	+ 4 ± 0	- 5 ± 0	- 5 ± 0
F15	- 11 ± 0	- 10 ± 0.7	- 11 ± 0	- 11 ± 0	- 10 ± 0	- 10 ± 0	- 11 ± 0	- 10 ± 0.7	- 10 ± 0
Velluto	+ 13 ± 0	- 12 ± 0.7	+ 12 ± 0.7	+ 13 ± 0	- 13 ± 0	+ 13 ± 0.6	+ 13 ± 0	- 14 ± 0.3	- 13 ± 0
Concerto	+ 58.6 ± 1.6	+ 49.6 ± 0.1	+ 35.0 ± 2.3	- 51.6 ± 4.3	+ 52.4 ± 1.7	+ 52.2 ± 4.0	- 49.2 ± 3.6	- 38.7 ± 7.9	- 42.7 ± 2.3
Alpha Yeast	+ 35.4 ± 2.9	+ 42.8 ± 3.0	+ 43.7 ± 1.3	- 44.3 ± 1.0	+ 38.9 ± 2.5	+ 42.4 ± 2.2	- 42.2 ± 0.6	- 40.8 ± 2.3	- 43.4 ± 0.7

S. cerevisiae
S. uvarum
Non-*Saccharomyces*
O. oeni
Lb. plantarum

S. cerevisiae strains GRE and F15 were found to be incompatible with all LAB tested. Of the five *S. cerevisiae* strains tested, GRE and F15 were the third and fifth to complete AF in yeast-only fermentations (Table S3, Appendix A), indicating that AF speed was not directly linked to compatibility outcomes. The negative co-inoculation outcome between GRE and all LAB was unexpected based on its 'co-inoculation friendly' designation by the manufacturer (Lallemand 2019a). It has been reported that GRE and VP41 were compatible in Chambourcin must (Homich *et al.* 2016), thus it is plausible that incompatibility observed in the present work may be the direct result of the medium the yeast and LAB were fermented in, since yeast and bacterial performance are affected by grape cultivar and vintage. This emphasises the complexity of studying yeast and bacterial performance for wines and raises the question of whether an ideal medium to trial and investigate yeast and bacterial efficacy exists. Nevertheless, it is useful to study the complexity of yeast-bacterial compatibility in a simplified, reproducible environment so that major contributors to co-inoculation inhibition or success can be identified.

All yeast-only fermentations reduced L-malic acid from 2.5 g L^{-1} to between 1.6 and 2.1 g L^{-1}. However, yeast L-malic acid consumption could not be correlated to compatibility between yeast and LAB. Yeast L-malic acid consumption has not been linked to MLF inhibition previously, even in fermentations where non-*Saccharomyces* are able to reduce L-malic acid content significantly (du Plessis *et al.* 2017a). In addition, du Plessis and colleagues (2017a) reported that MLF inhibition was yeast strain specific. This is in agreement with these results where yeast L-malic acid consumption had no influence on LAB MLF performance, and MLF inhibition was also strain specific. Hence compatibility outcomes cannot be attributed to individual fermentation measures or metabolites but needs to be

further investigated by detection of a broad range of metabolites or quantifying gene expression of the organisms. This has been done previously for some yeast and LAB (Cañas *et al.* 2015; du Plessis *et al.* 2017b; Nardi *et al.* 2018; Rossouw, du Toit & Bauer 2012; Versari *et al.* 2016), but a fundamental understanding of how metabolites affect bacterial and yeast health, rather than sensory contributions, is lacking.

AF duration for *Saccharomyces* strains GRE, NT50, Velluto, F15 and D80, and residual total sugar for non-*Saccharomyces* strains Concerto and Alpha Yeast, were unaffected by co-inoculation as determined by ANOVA and Tukey post-hoc tests ($p < 0.005$). More specifically residual glucose and fructose concentrations for Concerto and Alpha Yeast were unaffected by co-inoculation (Table S2, Appendix A). Conversely, AF speed for *S. cerevisiae* strain EC1118 was significantly different between the yeast-only control versus co-inoculated situations, except when co-inoculated with O-Mega. EC1118 co-inoculated with the following LAB resulted in slower AF than the yeast-only control: CH16, 450, SB3, Alpha, PN4, VP41 and NoVA, whereas EC1118 co-inoculated with Prime completed AF faster than the yeast alone (Table 2 and S3, Appendix A). AF speed may be influenced by yeast growth, with AF onset determined by yeast switching from respiration in the presence of oxygen, where glucose and fructose are utilised primarily for growth, to fermentation under anaerobiosis where glucose, and less preferably fructose, are converted to ethanol (Alba-Lois & Segal-Kischinevzky 2010; Guillaume *et al.* 2007). Therefore the variation in EC1118 AF speed can be related to the varied maximum yeast growth (Figure 1) and large range of time to reach maximum yeast growth (Table 3) observed for EC1118. EC1118 had the greatest range in maximum yeast concentration which could also be attributed to co-inoculation with different LAB, as

was the time to reach the maximum density. This range was not seen for other yeast. However, the underlying reason as to why EC1118 growth was affected is unclear and requires further investigation. It has been previously reported that yeast AF may be partially impacted by LAB producing inhibitory compounds such as acetic acid (Alexandre *et al.* 2004), or LAB induction of the [*GAR*$^{+}$] prion in yeast (Ramakrishnan *et al.* 2016) that causes yeast to utilise sugars other than glucose, thereby slowing AF. Neither of these mechanisms were measured during this study. Despite LAB having an effect on EC1118 growth and AF speed, there was no direct link between yeast, growth, AF speed and compatibility.

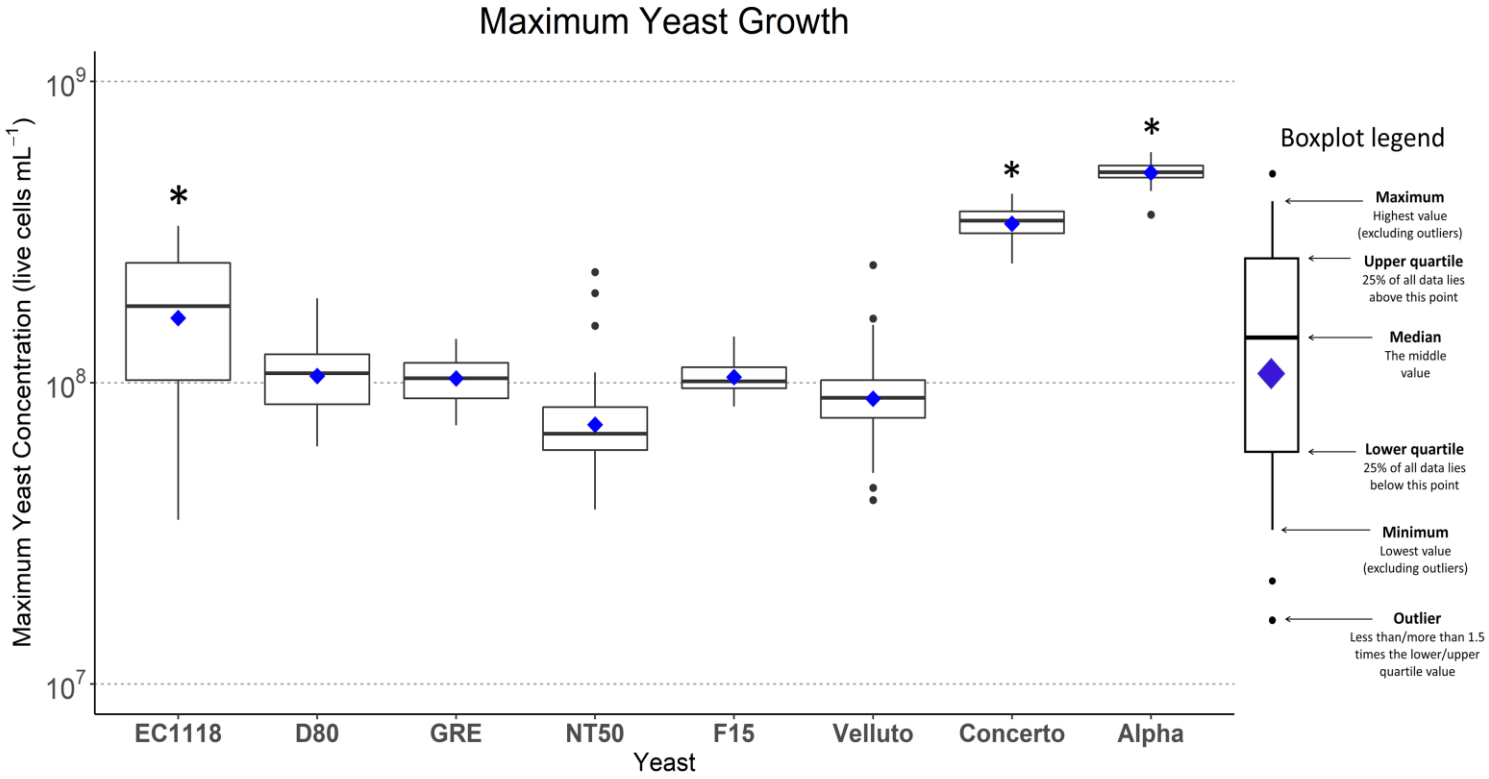

Figure 1: Maximum yeast concentration (live cells mL^{-1}) across all co-inoculations and yeast-only controls. For each yeast strain a boxplot represents the variation of maximum yeast concentrations across co-inoculations and yeast-only controls, while blue diamonds represent the average across all fermentations for each yeast.
*Significantly different average maximum yeast concentration compared to other yeasts (ANOVA, Tukey post-hoc; $p < 0.005$)

Table 3: Range of time (hours) for yeast to reach the maximum yeast concentration (cells mL^{-1}).

Yeast	Time to reach maximum growth (hours) ± SD
EC1118	45 ± 0 - 240 ± 0
D80	80 ± 14 - 96 ± 26
GRE	48 ± 0 - 109 ± 90
NT50	96 ± 0 - 183 ± 14
F15	45 ± 0 - 100 ± 96
Velluto	24 ± 0 - 96 ± 0 *
Concerto	45 ± 0 - 77 ± 28
Alpha Yeast	45 ± 0 - 77 ± 28

*Significant differences were found between yeast strains (ANOVA, Tukey post-hoc $p < 0.005$), but no significant differences were found for any yeast strains between the yeast-only control and yeast co-inoculated with LAB.

There were differences in AF durations and total residual sugar concentrations between yeast (Table 2). It was expected that *S. cerevisiae* strains would complete AF the fastest, followed by *S. uvarum*. Slower fermentation by *S. uvarum* has been reported repeatedly, with observation that *S. uvarum* is more likely to complete AF at lower temperatures (i.e. 13 °C), but not at a faster rate than *S. cerevisiae* (Magyar & Tóth 2011; Masneuf-Pomarède *et al.* 2010; Varela *et al.* 2016). The mechanism behind slower AF kinetics of *S. uvarum* is unknown.

Non-*Saccharomyces* were not expected to reduce total sugar concentration to less than 3 g L^{-1} due to their sensitivity to ethanol at concentrations as low as 6% (Pina *et al.* 2004; Pina, António & Hogg 2004) and reported inability to complete AF alone. This was confirmed by viability data showing an increase in the percentage

of dead Concerto and Alpha Yeast cells at the time residual sugar concentration plateaued (Figure S2, Appendix A). There were higher levels of fructose at the end of fermentations by Concerto and Alpha Yeast (Table S2, Appendix A) that could have contributed towards AF inhibition. Fructose and ethanol act synergistically, which causes yeast stress for both *Saccharomyces* and non-*Saccharomyces* in conditions where ethanol is 9% or greater (v/v; de la Torre-González *et al.* 2016). Therefore, the added stress of residual fructose in the medium could be a major factor contributing to the inability of many non-*Saccharomyces* to complete AF.

Maximum yeast growth (Figure 1) was highest for the non-*Saccharomyces* strains Concerto and Alpha Yeast. These yeasts were able to reach a significantly higher density of live cells mL^{-1} in comparison to *Saccharomyces* strains (ANOVA, Tukey post-hoc p <0.005). This may be partly due to the size of *T. delbrueckii* and *L. thermotolerans* cells which are 2-7 µm in length compared to *S. cerevisiae* that can grow up to 10 µm, leading to the conclusion that smaller cells could occupy the same space at a higher density. Though logical, there is no evidence to show that this is the case, and no other proposals have been published as to why these non-*Saccharomyces* may grow to such high densities during fermentation.

Yeast cell density could not be correlated to compatibility outcome, even though it would be reasonable to assume that a higher density of yeast could compete and deplete nutrients faster. While yeast density was significantly higher for Concerto and Alpha Yeast, they were compatible with only five of the nine LAB used in this study. Concerto (*L. thermotolerans*) and Alpha Yeast (*T. delbrueckii*) are able to produce a number of compounds that could both inhibit or promote MLF by LAB (Balmaseda *et al.* 2018; Morata *et al.* 2018). For example the use of

non-*Saccharomyces* for co-inoculation may improve MLF outcomes, because the production of inhibitory compounds such as ethanol and medium-chain fatty acids is generally lower (Belda *et al.* 2015; Contreras *et al.* 2015), while beneficial compounds like pyruvic acid and glycerol are higher compared to *S. cerevisiae* (Balmaseda *et al.* 2018; Belda *et al.* 2015; Benito *et al.* 2016). However, *L. thermotolerans* can produce high concentrations of lactic acid that may inhibit LAB and MLF (Benito 2018; Morata *et al.* 2018). Though these metabolites weren't measured in the present work, it was observed that compatibility between non-*Saccharomyces* and LAB strains are strain specific and therefore non-*Saccharomyces*-LAB compatibility warrants further investigation.

MLF completion was affected by yeast-LAB pairs (Figure 2) and in multiple cases was not able to be fully attributed to LAB inoculation rate even though there were significant differences in LAB inoculation rates between some experimental subsets (Table S4, Appendix A). L-malic acid degradation by LAB was sometimes variable between biological replicates, which led to large standard deviations of the mean. There was no apparent explanation for such differences. Inclusion of more biological replicates could be useful for future work involving yeast-LAB co-inoculation to allow for such biological differences; however this impacts the cost and practicality of experiments.

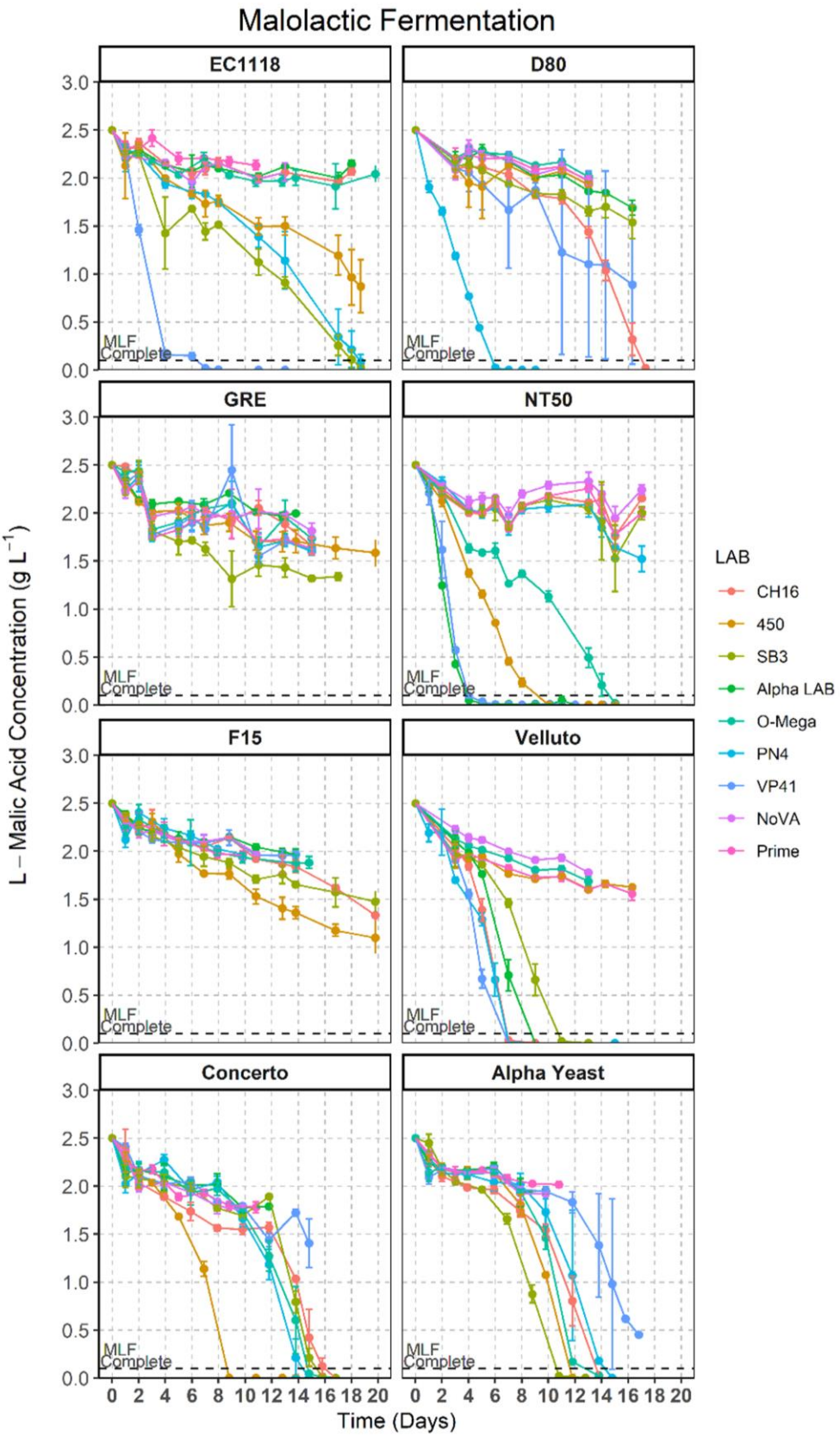

Figure 2: Malolactic fermentation profiles for each yeast-LAB pair tested in this study. Plots are separated by yeast strain used for co-inoculation, and colours indicate the LAB strain conducting MLF. The dashed line specifies 0.1 g L^{-1} L-malic acid, which was considered the end point for MLF. Values are the mean of triplicates and error bars are the standard deviation.

The amount of time taken for LAB to reach high cell numbers (i.e. 1 x 10^6 cells mL^{-1}) was also not a reliable indicator for MLF completion. LAB density of 1 x 10^6 cells mL^{-1} is considered the necessary critical mass for initiation and completion of MLF (Lonvaud-Funel 1999). When inoculating fermentations with LAB, there is an observable drop in cfu mL^{-1} before LAB recover and begin MLF. Although it is observed often (Knoll *et al.* 2012; Ong 2010; Tristezza *et al.* 2016; Zapparoli, Tosi & Krieger 2006), there is currently no reported method to stop this initial decrease in viable cell number, though maintaining the initial critical mass would be highly desirable. The ability of LAB to reach the critical density more quickly should indicate that MLF would be likely to finish. However, in this study, even though some of the LAB were able to reach critical density in as little as 45 hours (Table 4), it did not guarantee MLF completion (Table 2). Considering this, it is important not only for LAB to recover post-inoculation and reach critical density, but to maintain that critical density to ensure MLF completion (Durieux, Nicolay & Simon 2000; Guerrini *et al.* 2002). This further highlights the need for a greater understanding of nutritional requirements and wine stress resistance of *Lb. plantarum* strains as they were mostly unable to reach critical density post-inoculation or maintain growth over the course of fermentation.

Table 4: Time (hours ± SD) for LAB to reach 1 x 10^6 cfu mL^{-1}. Initial inoculation rates were excluded as there was an initial drop in cfu mL^{-1} followed by a recovery period. Yeast-LAB pairs deemed to be compatible are highlighted in green. The same superscripted letters indicate no significant difference between times to reach critical density ($p < 0.005$). *NR represents 1 x 10^6 cfu mL^{-1} was not reached.

		CH16	450	SB3	Alpha LAB	O-Mega	PN4	VP41	NoVA	Prime
	EC1118	NR	48 ± 0 ^{E}	48 ± 0 ^{E}	NR	NR	304 ± 55.4 ^{A,B,C}	48 ± 0 ^{E}	NR	NR
	D80	48 ± 0 ^{E}	435 ± 0 ^{A}	168 ^{B,C,D,E}	NR	NR	56 ± 13.9 ^{E}	108 ± 84.9 ^{C,D,E}	NR	48 ± 0 ^{E}
	GRE	NR	NR	48 ± 0 ^{E}	NR	NR	48 ± 0 ^{E}	48 ± 0 ^{E}	NR	48 ± 0 ^{E}
	NT50	48 ± 0 ^{E}	48 ± 0 ^{E}	NR	48 ± 0 ^{E}	255 ± 97.0 ^{B,C,D}	407 ^{A,B}	48 ± 0 ^{E}	NR	NR
	F15	NR	45 ± 0 ^{E}	NR	NR	NR	NR	NR	NR	NR
	Velluto	48 ± 0 ^{E}	NR	168 ± 0 ^{B,C,D,E}	96 ± 0 ^{D,E}	NR	48 ± 0 ^{E}	80 ± 27.7 ^{D,E}	NR	48 ^{E}
	Concerto	251.7 ± 179.0 ^{B,C,D}	45 ± 0 ^{E}	259 ± 0 ^{A,B,C,D}	NR	291 ± 55.4 ^{A,B,C}	259 ± 0 ^{A,B,C,D}	355 ± 0 ^{A,B}	NR	NR
	Alpha Yeast	187.7 ± 123.6 ^{B,C,D,E}	212 ± 0 ^{B,C,D,E}	45 ± 0 ^{E}	NR	259 ± 0 ^{A,B,C,D}	275.3 ± 72.9 ^{A,B,C}	355 ± 0 ^{A,B}	NR	NR

S. cerevisiae
S. uvarum
Non-*Saccharomyces*
O. oeni
Lb. plantarum

Though LAB density is an important factor for determining MLF completion, it does not necessarily correlate with MLF speed. It was observed that the time to reach critical density (Table 4) could not be used to predict MLF completion time (Figure 2). Therefore the results demonstrate that LAB growth data cannot be used to accurately predict MLF outcomes. Moreover, MLF fermentation completion times varied significantly across yeast-LAB pairs (Figure 2), an observation that has also been reported for other yeast-LAB combinations (Arnink & Henick-Kling 2005; Knoll *et al.* 2012; Tristezza *et al.* 2016). In some instances, LAB began reducing L-malic acid concentration substantially towards the end of experimentation (Figure 2); however, the length of time it took for these LAB to start MLF is unacceptable. MLF that does not start quickly increases the risk of contamination in a winemaking setting, since SO_2 is withheld at this stage to encourage MLF onset. This lack of SO_2 may allow contamination by unwanted microbial species such as *Brettanomyces bruxellensis* and *Acetobacter aceti* that can produce off-flavours or toxins (Bartowsky & Henschke 2008; Romano *et al.* 2009). Thus, LAB that are able to start MLF in a timely manner are considered better candidates for co-inoculation.

To identify links between alcoholic and malolactic fermentation, a Spearman's rank correlation test was performed to evaluate the strength and direction of association between AF and MLF (Figure 3). Correlation tests are a useful means of evaluating trends in large datasets. These correlations are displayed in the form of a network to enable easier interpretation. Nodes (circles) are used to identify the different measures for AF and MLF, and edges (lines connecting nodes) are used to visualise the correlations between AF and MLF measures. Spearman's rank correlation coefficient revealed significant ($p < 0.005$) positive and negative correlations between AF and MLF related measures. AF and MLF outcomes were

ranked based on completion outcome as follows: 1 = fast completion (< 163 hours); 2 = medium (< 336 hours), 3 = slow (< 600 hours) and stuck (> 600 hours or did not complete). AF outcome negatively correlated with 10% malic acid conversion, indicating that fermentations where AF was fast and initial malic acid degradation was slow. There were positive correlations between 20%, 50% and 80% AF and MLF, which is the opposite of initial L-malic acid degradation and overall AF outcome. This could be explained by the overall MLF kinetics, since MLF proceeds quickly once LAB critical density is reached and maintained. The discrepancy in the 10% malic acid degradation could also be explained by the ability of yeast in this study to degrade L-malic acid and may not be indicative of LAB influence at all. It is also important to note that these correlations are somewhat weak with values falling between -0.36 and +0.33. Correlation is an effect size and so the strength of the correlation can be described as ranging from very weak to very strong. Very strong correlations have values closer to -1 and +1, but either way these are correlations and only imply a possible connection between AF and MLF measures and do not imply causation.

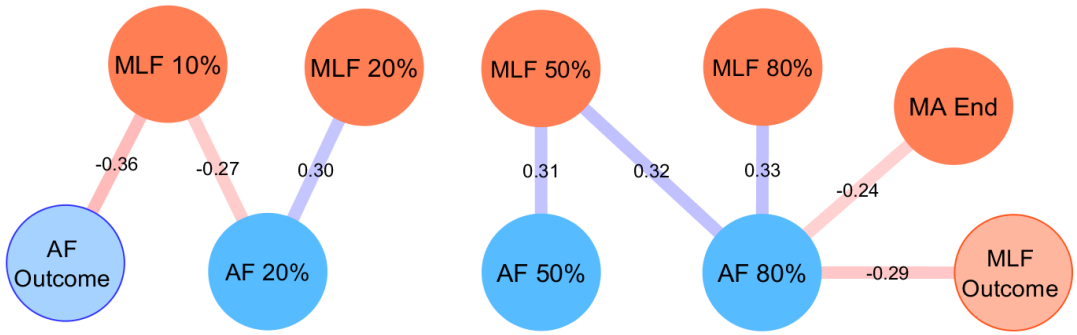

Figure 3: Network displaying significant (p < 0.005) Spearman's rank correlation coefficients between malolactic fermentation (orange nodes) and alcoholic fermentation (blue nodes) related measures. Red lines between nodes are negative correlations and blue lines are positive correlations. The numbers on each line are the Spearman's rank correlation score, which lie on a scale of -1 to +1. AF and MLF outcome comprise of ranked values: 1 = fast completion (< 163 hours); 2 = medium (< 336 hours), 3 = slow (< 600 hours) and stuck (> 600 hours or didn't finish). Percentage completion is time in hours taken for samples to reach 10%, 20%, 50% or 80% of the total L-malic acid or total sugar concentration. MLF 10% and MLF or AF 20%, 50%, and 80% are time to reach 10%, 20%, 50% and 80% MLF or AF completion (hours), respectively. MA End is L-malic acid concentration at the end of the experiment.

4. Conclusions

Ultimately, this data represents how complicated interactions in co-inoculation are. It is inherently difficult to predict which yeast and LAB may work well together since compatibility outcomes may be affected by numerous intrinsic and extrinsic factors such as grape cultivar, pH, SO_2 production, oxygenation, microbial competition, nutrient requirements. This experimental subset opens a forum for acknowledging the challenge of compatibility prediction in winemaking, since predictability becomes more challenging as other factors are introduced (i.e. using juice or must, volume scale-up, non-sterile conditions). It is also understandable that winemakers continue to use inoculation strategies and microbes that have been successful during previous vintages, however it would also be useful to conduct a survey of strategies and strains used. This study has generated a list of 24 compatible and 48 incompatible commercial yeast-LAB pairs in a synthetic juice fermentation. Though there weren't any strong correlations to allow a significant understanding of why these yeast and LAB were compatible or not, we were able to uncover strain specific differences. To achieve a more detailed understanding of compatibility for a subset of these yeast-LAB pairs, an in-depth analysis of fermentation in a more complex environment (i.e. grape juice) should be undertaken. Additionally, more work is needed to understand the impact of yeast-LAB co-inoculation at the molecular scale.

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Chapter 4

Yeast-bacteria compatibility of industrial strains in Shiraz juice

1. Introduction

Commercial red winemaking relies on the ability of yeast and lactic acid bacteria (LAB) to successfully complete alcoholic (AF) and malolactic (MLF) fermentation, respectively. Overall fermentation speed is becoming more important as the occurrence of compressed vintages increase (Jarvis *et al.* 2019; Webb *et al.* 2012). Compressed vintages cause economic pressure within wineries, but this pressure can be mitigated by fast and efficient fermentation. Co-inoculation of yeast and LAB can decrease overall fermentation time since yeast and LAB are able to simultaneously perform AF and MLF; however, yeast-LAB compatibility is crucial to ensure timely fermentation completion.

Yeast-LAB compatibility is influenced by yeast and LAB strain, matrix composition, and the production of compounds that may antagonise or stimulate AF and MLF (Da Silveira *et al.* 2003; Nehme, Mathieu & Taillandier 2010; Sumby, Grbin & Jiranek 2014; van der Heide & Poolman 2000; Wells & Osborne 2012; Chapter 3). Chapter 3 revealed that measuring AF and MLF progress alone cannot be used to indicate compatibility between yeast and LAB. Additionally, AF speed and yeast cell density are not reliable indicators for yeast-LAB compatibility (Chapter 3). In order to further understand the basis of compatibility, it is necessary to conduct experiments in a complex matrix such as grape juice, which can provide precursors for compounds that would not be present in a synthetic juice. In addition, understanding the roles of particular metabolites utilised and produced over the course of fermentation could provide invaluable information for better control over yeast and LAB fermentation performance.

Overall the complexity of yeast-LAB relationships is not well understood, but strong antagonists for yeast and LAB during fermentation have been identified. For LAB, ethanol, SO_2 and fatty acids can inhibit bacterial growth and MLF performance (Betteridge *et al.* 2018; Capucho & San Romão 1994; Lonvaud-Funel 1995). Ethanol causes an increase in membrane permeability and decrease in ATP production (Da Silveira *et al.* 2003; Guzzo *et al.* 2000) that can lead to death of LAB cells during fermentation. Whereas SO_2 in its molecular form can easily cross the cellular membrane and have a toxic effect in LAB (Bartle *et al.* 2019a; Divol, du Toit & Duckitt 2012), as well as having a synergistic effect with pH and ethanol, inhibiting LAB growth and MLF (Britz & Tracey 1990; Lonvaud-Funel 1995). In addition, fatty acids produced by yeast can enter LAB cells, inhibit ATPase, and cause cell death (Guilloux-Benatier, Le Fur & Feuillat 1998; Tourdot-Maréchal *et al.* 1999). Alternatively, LAB production of acetic acid can inhibit yeast growth and slow AF (Drysdale & Fleet 1988).

Although there are a number of defined compounds and growth conditions that affect yeast and LAB, there needs to be greater consideration of the role of volatile compounds in yeast and bacterial growth. Wine research is focused on sensory outcomes of volatile compounds produced using different yeast-LAB pairs, however, there is much more to learn about the role of such compounds in yeast-LAB compatibility.

Considering the well-defined role of some compounds and conditions on yeast-LAB compatibility the aims of this study were to:

1) Evaluate the performance of commercial yeast and LAB in co-inoculated sterile Shiraz juice fermentations.
2) Identify volatile compounds that may contribute to LAB antagonism.

3) Evaluate the combinatorial role of volatile and non-volatile compounds, pH and microbial growth in yeast-LAB compatibility.

To evaluate the potential role of volatile compounds and the combination of other factors and how they influence yeast-LAB compatibility, eight commercial yeast-LAB pairs were tested in a sterile Shiraz juice. Gas chromatography-mass spectrometry (GC-MS) was used to measure volatile compounds at the end of fermentations. H_2S was measured over the course of fermentation. High performance liquid chromatography (HPLC), among other enzymatic assays, were used to determine the concentrations of a number of compounds either throughout fermentation or at fermentation completion.

2. Materials and methods

2.1. Shiraz juice preparation

Shiraz grapes (2017 vintage, Coombe vineyard Waite Campus, Urrbrae, South Australia) were harvested, de-stemmed, crushed and macerated at 0°C for 7 days to enable polyphenolic extraction. Shiraz must was pressed and the juice was stored at -20°C until required. No SO_2 or antibacterial agents were added to the juice during pressing. Prior to experimentation Shiraz juice was filtered using an in-line groundwater filter (0.45 µm; catalogue # FHT45, Air-Met Scientific, Victoria, Australia) to remove grape matter and solids. The juice was adjusted to 250 g L^{-1} total sugar, 2.5 g L^{-1} L-malic acid and pH 3.5 followed by filter sterilisation (0.2 µm). Initial measurements of total sugar were estimated by refractometry, and sugar reduced by addition of water. L-malic acid was measured using L-malic acid assay (described in section 2.3) and increased by addition of pure L-malic acid. pH was decreased by addition of tartaric acid.

2.2. Yeast and bacteria strains and fermentation conditions

Eight commercial yeast-LAB co-inoculation pairs were chosen from a preliminary list of 72 yeast-LAB pairs. Briefly, 72 yeast-LAB were co-inoculated into sterile synthetic juice and assessed for compatibility. The four fastest completers of MLF and the four pairs that had the highest residual L-malic acid were determined as the most and least compatible, respectively. Full details are provided in Chapter 3.

The eight selected yeast-LAB pairs were analysed for compatibility in sterile Shiraz juice co-inoculations. Yeast and bacteria used in this study are listed in Table 1, and the yeast-bacteria combinations tested are listed in Table 2.

Prior to inoculation, yeast were rehydrated following manufacturers specifications. LAB were rehydrated in sterile water before being cultured in liquid MRSAJ medium for 4 days at 30°C. Twenty-four hours before inoculation into fermentations, LAB were centrifuged at 2,236 x *g* for 5 minutes. The supernatant was discarded and the cell pellet washed with sterile Shiraz juice before being incubated overnight in fresh sterile Shiraz juice at 30°C. Prior to inoculation, LAB were adjusted to OD_{600} 0.55 after subtracting an OD_{600} Shiraz juice blank value from each sample. Successively, 1.5 mL of adjusted LAB culture was transferred to each corresponding fermentation using a sterile 21-gauge needle inserted through a sampling port located on the side of the fermentation vessel.

Table 1: Yeast and bacteria tested in this work.

Yeast	Species	Supplier	Name used in this study
Lalvin EC-1118	*Saccharomyces cerevisiae*	Lallemand Inc	EC1118
ICV D80	*S. cerevisiae*	Lallemand Inc	D80
NT50	*S. cerevisiae* x *S. kudriavzevii*	Anchor Yeast	NT50
Velluto BMV58	*S. uvarum*	Lallemand Inc	Velluto
Bacteria			
Viniflora CH16	*Oenococcus oeni*	Chr. Hansen	CH16
Enoferm ALPHA	*O. oeni*	Lallemand Inc	Alpha
O-Mega	*O. oeni*	Lallemand Inc	O-Mega
Lalvin VP41 MBR	*O. oeni*	Lallemand Inc	VP41
PN4	*O. oeni*	Lallemand Inc	PN4

Table 2: Yeast-bacteria combinations tested for co-inoculation compatibility in sterile Shiraz juice fermentations.

Yeast	Bacteria
EC1118	CH16 Alpha
D80	PN4 O-Mega
NT50	CH16 Alpha VP41
Velluto	VP41

Fermentations were conducted at a volume of 150 mL in glass shake flasks fitted with an airlock and kept at a constant temperature of 22°C. Fermentations were performed in triplicate for each yeast-LAB pair and yeast-only controls. Samples (200 µL) were collected from each fermentation at multiple time-points for analysis of yeast and LAB growth, sugar consumption, L-malic acid utilisation, total nitrogen consumption and hydrogen sulfide production. After completion of AF by yeast-only controls and overall fermentation by yeast-LAB pairs, 50 mL of culture was collected and centrifuged (5 minutes at 2,236 x *g*). End-point samples were used for the following analyses: pH, free and total SO_2, ethanol concentration, density, organic acid content, succinic acid concentration, amino acid content and volatile compound concentrations.

2.3. Enzymatic assays for glucose/fructose, L-malic acid, free amino nitrogen and succinic acid

An enzymatic kit (K-FRUGL, Megazyme, Ireland) was used to determine glucose and fructose concentrations over the course of alcoholic fermentation. Kit methods were modified according to Walker *et al.* (2014), and AF considered complete when combined glucose and fructose concentrations reached < 3 g L^{-1}.

L-malic acid concentration was determined using a test kit (catalogue # 4A165, Vintessential laboratories, Australia) modified for use with a 96-well plate spectrophotometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland). Specific methods are described in Chapter 3 and MLF was considered complete when L-malic acid concentration reached < 0.1 g L^{-1}.

Free amino nitrogen (excluding proline and ammonia) were determined using o-phthaldialdehyde and N-acetyl-L-cysteine (NOPA; Dukes & Butzke 1998).

Succinic acid concentration at fermentation completion was determined using an enzymatic kit (K-SUCC, Megazyme, Ireland) in conjunction with a ChemWell 2910 Automated EIA and Chemistry Analyzer (Megazyme, Ireland).

2.4. Yeast and bacterial viability

Yeast viability and number (cells mL^{-1}) were determined using flow cytometry. Samples were diluted with 1 x PBS to < 500 cells μL^{-1}, followed by staining with propidium iodide to a final concentration of 0.1 mg mL^{-1}. Stained cells were analysed using a Guava Easycyte 12HT (Millipore) in conjunction with guavaSoft 3.3 flow cytometry software. Each sample was analysed up to 1,000 events or for 2 minutes, whichever occurred first.

LAB culturable cell number (cfu mL^{-1}) was determined using serial dilution spots on MRSAJ agar (2%) supplemented with cycloheximide (0.5%). Samples were serially diluted 1:10 in sterile 1 x PBS up to 10^{-5}. 2 µL aliquots of each dilution were transferred to MRSAJ agar (+ cycloheximide) and incubated anaerobically in a GasPak EZ standard incubation container containing sachet with indicator (catalogue # 260671 and # 260001, BD, Australia) for 4 days at 30°C.

2.5. Hydrogen sulfide, pH, SO_2, ethanol and density

H_2S production was measured using silver nitrate H_2S detector tubes with a detection range of 25-1000 ppm (catalogue # 120SF, Kitagawa America LLC, USA,).

pH was measured using a CyberScan pH 1100 (Eutech Instruments, Thermo Fisher Scientific) prior to yeast inoculation and at fermentation completion.

Free and total SO_2 concentrations were measured by aspiration and titration following the method developed by Rankine & Pocock (1970).

Final ethanol concentration and liquid density were determined by an Alcolyser Wine ME/DMA 4500M (Anton Paar, Australia).

2.6. HPLC for amino acid and organic acid concentrations

HPLC was used to detect the presence of three organic compounds and a range of amino acids, listed in Table 3. For organic acids the system utilised an Aminex HPX-87H column (300 mm × 7.8 mm; BioRad) and was performed at 60°C with 2.5 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1}. Peaks were detected with an Agilent DAD G1315B Diode Array detector for organic acids. Samples were quantified by comparison with prepared standards using Chem Station software version B.01.03 (collection) and B.03.01 (analysis; Agilent).

Amino acid analysis was performed as described by Culbert *et al.* (2017), using an AccQ-Fluor kit (Waters Corporation) and Agilent 1200 series HPLC with fluorescence detector.

Table 3: Compounds detected by HPLC and GC-MS. Amino acids were analysed during and at fermentation completion, whereas organic compounds and compounds analysed by GC-MS were measured solely at fermentation completion.

Equipment	Compound detected	
HPLC – Organic compounds	Acetaldehyde Acetic acid Glycerol	
HPLC – Amino Acids	β-Alanine γ-Aminobutyric acid (G-Aba) Alanine Arginine Asparagine Aspartic acid Glutamine Glutamic acid Glycine Histidine Hydroxyproline	Isoleucine Leucine Lysine Methionine Phenylalanine Proline Serine Threonine Tyrosine Valine
GC-MS	1-butanol 1-hexanol 2-methylbutanoic acid 3-(methylthio)-1-propanol Acetic acid Benzyl alcohol Decanoic acid Ethyl acetate Ethyl butanoate	Ethyl decanoate Ethyl octanoate Ethyl propanoate Hexanoic acid Hexyl acetate Isobutanol Octanoic acid Phenyl ethyl alcohol

2.7. GC-MS

GC-MS and subsequent peak analysis were performed by Emily Nicholson and Dr Paul Boss from CSIRO.

Shiraz juice and fermentation samples were diluted to either 1:10 or 1:100 and 10 mL were transferred to GC-MS vials containing 3 g sodium chloride. 50 µL and 10 µL of mixed internal standards (Table S1, S2, Appendix B) were added to each juice and fermentation sample, respectively. Prepared samples were subjected to solid-phase microextraction and GC-MS (SPME-GC-MS) analysis. SPME-GC-MS was carried out using an Agilent 7890A gas chromatograph (Palo Alto, CA, USA) equipped with a Gerstel MP2 autosampler (Mülheim an der Ruhr, Germany) and a 5973N mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) for peak detection and compound identification. The autosampler was operated in SPME mode utilising a divinylbenzene-carboxen-polydimethylsiloxane fiber (2 cm, 23-gauge, 50/30 µm DVB-CAR-PDMS; Supelco, Bellefonte, PA, USA) for extraction. After 5 minutes incubation volatile compounds were extracted using agitation (300 rpm) at 50°C for 30 min. Chromatography was performed using a ZB-Wax column (30 m, 0.25 mm i.d., 0.25 µm film thickness) using helium as a carrier gas at 1.5 mL min^{-1} (constant flow). Volatiles were desorbed from the fiber 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^{-1} 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 per sec). The compounds measured are listed in Table 3. Peaks were analysed using Agilent MassHunter Quantitative Analysis B.07.01 and concentration of the compounds listed in Table 3

were determined by comparison with internal standards (Table S1, S2, Appendix B).

2.8. Statistical analysis

R version 3.5.1 was used for all statistical analyses and graphs. Significant differences were determined using ANOVA with Tukey's post-hoc test $p < 0.005$. Spearman's rank correlation coefficient and *p*-values were determined using stats package version 3.6.1.

3. Results and discussion

Six of the eight yeast-LAB pairs were able to complete both AF and MLF (Table 4), with two LAB able to complete MLF in eight and six days (D80/O-Mega, NT50/VP41). It was observed that four of the eight pairs switched compatibility status when tested in Shiraz juice compared to synthetic juice (Chapter 3; Table 4). In comparison to CDGJM, Shiraz juice likely contains a number of compounds that could influence MLF and AF. This is supported by Liu *et al.* (2016) where 2,500 unknown compounds and 800 putative markers for MLF completion were found using an untargeted metabolomic approach.

AF completion speed did not correlate with LAB MLF completion. It was expected that faster AF would negatively affect MLF progression and increase total fermentation time, as fast AF would likely result in quicker ethanol production and nutrient depletion. However, it was observed that NT50/VP41 had the second fastest overall fermentation completion time despite NT50 performing AF the fastest. In addition, D80 and Velluto both had slower AF compared to NT50, but co-inoculation with PN4 and VP41 resulted in sluggish MLF. From these results it

appears that AF speed alone does not account for incompatibility with LAB. This was also the case for fermentations performed in Negroamaro (Tristezza *et al.* 2016), Riesling (Knoll *et al.* 2012) and Tempranillo (Cañas *et al.* 2012) where yeast AF completion was unaffected by co-inoculation and MLF completion time varied for different LAB.

Table 4: Alcoholic and malolactic fermentation (AF and MLF) completion time (days).

Yeast	LAB	AF (days ± SD)	MLF (days ± SD)	Time to complete* both AF and MLF (days)	Compatibility in Shiraz juice	Compatibility in synthetic juice (Chapter 3)
D80	—	7.7 (± 0.6) ^{A,B}	—	7.7		
	PN4	8.0 (± 0) ^{A}	13.0 (± 0) ^{A}	13.0	Compatible	Compatible
	O-Mega	8.0 (± 0) ^{A}	6.0 (± 0) ^{B}	8.0	Compatible	Incompatible
EC1118	—	6.7 (± 0.6) ^{C,D}	—	6.7		
	Alpha	7.0 (± 0) ^{B,C}	14.0 (± 0) ^{A,C}	14.0	Compatible	Incompatible
	CH16	7.0 (± 0) ^{B,C}	13.7 (± 0.6) ^{A,C}	13.7	Compatible	Incompatible
NT50	—	6.0 (± 0) ^{D}	—	6.0		
	Alpha	6.0 (± 0) ^{D}	DNC	DNC	Incompatible	Compatible
	CH16	6.0 (± 0) ^{D}	DNC	DNC	Incompatible	Incompatible
	VP41	6.0 (± 0) ^{D}	8.0 (± 0) ^{D}	8.0	Compatible	Compatible
Velluto	—	8.0 (± 0) ^{A}	—	8.0		
	VP41	8.0 (± 0) ^{A}	14.7 (± 0.6) ^{C}	14.7	Compatible	Compatible

* AF was complete when total sugar reached < 3 g L^{-1} and MLF was complete when L-malic acid was < 0.1 g L^{-1}. Values are the mean of triplicates and SD is the standard deviation from the mean. DNC = did not complete. Significant differences between completion times within each column are indicated by different letters (ANOVA, Tukey Post-hoc $p < 0.005$).

3.1. LAB and yeast growth

LAB growth during fermentation can provide information about their ability to perform MLF, since MLF onset is reliant on LAB reaching a critical density of 1×10^6 cells mL^{-1} (Lonvaud-Funel 1999). O-Mega, PN4 and VP41 were all able to reach critical density after 48 hours. Conversely, Alpha and CH16 co-inoculated with EC1118 only reached critical density after 192 hours and when co-inoculated with NT50 critical density was not reached at all (Figure 1). Alpha and CH16 were able to complete MLF in 14 days when co-inoculated with EC1118, which is comparable to PN4 co-inoculated with D80 (13 days) and VP41 with Velluto (14 days), despite PN4 and VP41 reaching critical density after only 48 hours. These results reveal that time to reach critical density cannot be used as an indicator for MLF efficiency, as was also discussed in Chapter 3. A drop in LAB viable cell number occurred 12 hours post-inoculation (Figure 1). This drop in viable cell number has been consistently reported (Chapter 3; Ong 2010; Knoll *et al.* 2012; Tristezza *et al.* 2016; Zapparoli, Tosi & Krieger 2006), with no explanation. All LAB populations decreased to < 1% of their original viable population number, but the amount of decrease did not correlate with compatibility outcome (Table S3, Appendix B). However, in agreeance with results from Chapter 3, LAB ability to recover from the initial drop in density, then reach and maintain critical density, remain the most important factors for MLF completion in this study.

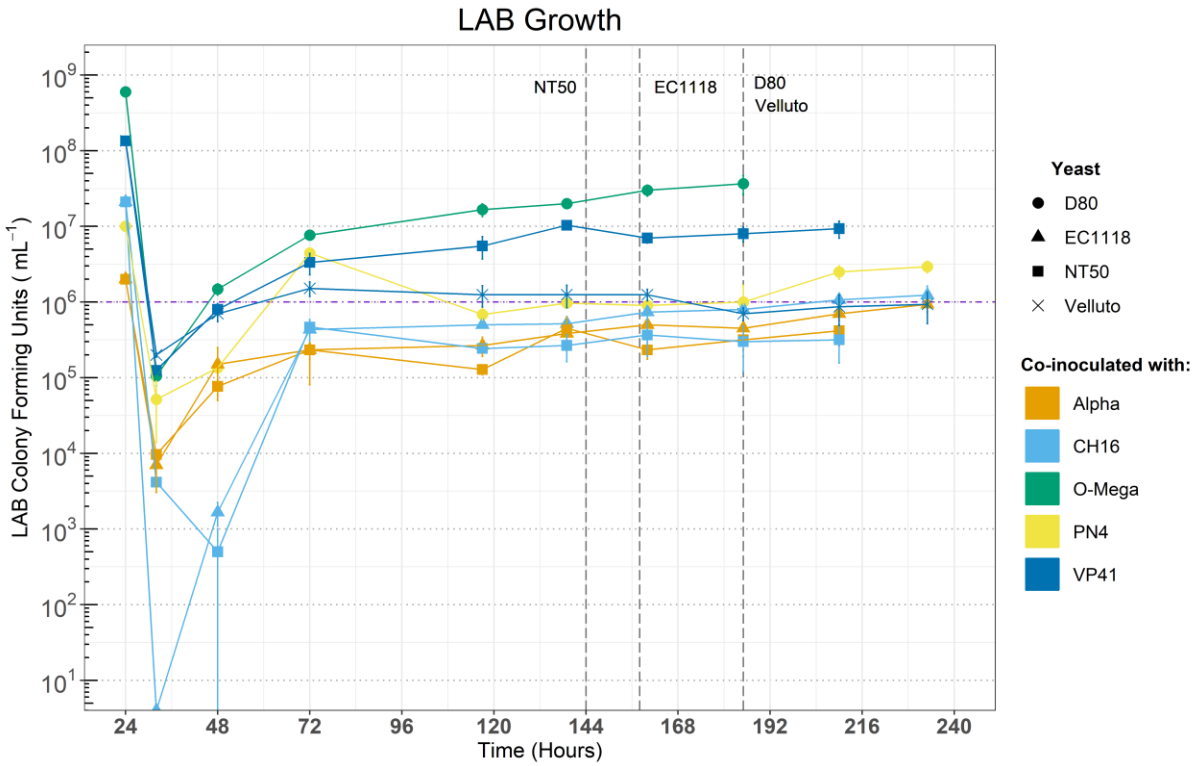

Figure 1: LAB growth (cfu mL^{-1}) over the course of fermentation. Symbols indicate yeast strain and colour indicates LAB strain. Vertical dashed lines indicate average AF completion time of the specified yeast. The horizontal dashed line at 1 x 10^6 cfu mL^{-1} indicates the critical density for MLF onset. Values are the mean of triplicates and error bars are the standard deviation.

VP41 co-inoculated with Velluto (*S. uvarum*) resulted in lower VP41 cell numbers compared to co-inoculation with NT50 (Figure 1). Growth inhibition of VP41 co-inoculated with Velluto may have been a result of Velluto's higher cell density compared to all other yeast (Figure 2), since this would potentially result in faster depletion of nutrients. The ability of *S. uvarum* to grow to a higher density than *S. cerevisiae* has been observed before (López-Malo, Querol & Guillamon 2013).

In addition to high cell numbers and nutrient depletion, *O. oeni* MLF inhibition in wines fermented by *S. uvarum* has been partly attributed to delayed nutrient release (Zapparoli *et al.* 2003), which is supported by the low percentage of dead yeast observed for Velluto in this work (Figure 3A).

Although there were no differences in nitrogen release between yeast until 192 hours (Figure 3B), other vitamins and nutrients are released by dead cells that could be scavenged by LAB (Bartle *et al.* 2019a; Fleet, Lafon-Lafourcade & Ribereau-Gayon 1984). Contrary to VP41 fermentations, Alpha and CH16 had higher cell numbers and completed MLF when co-inoculated with EC1118, which had a higher average maximum cell concentration than NT50 (Figure 2). This observation is not explained by utilisation of nitrogen over the course of fermentation since there were no differences in nitrogen use between yeast (Figure 3B). NT50 also had a higher percentage of dead cells than EC1118, therefore the differences cannot be attributed to differences in potential nutrient release.

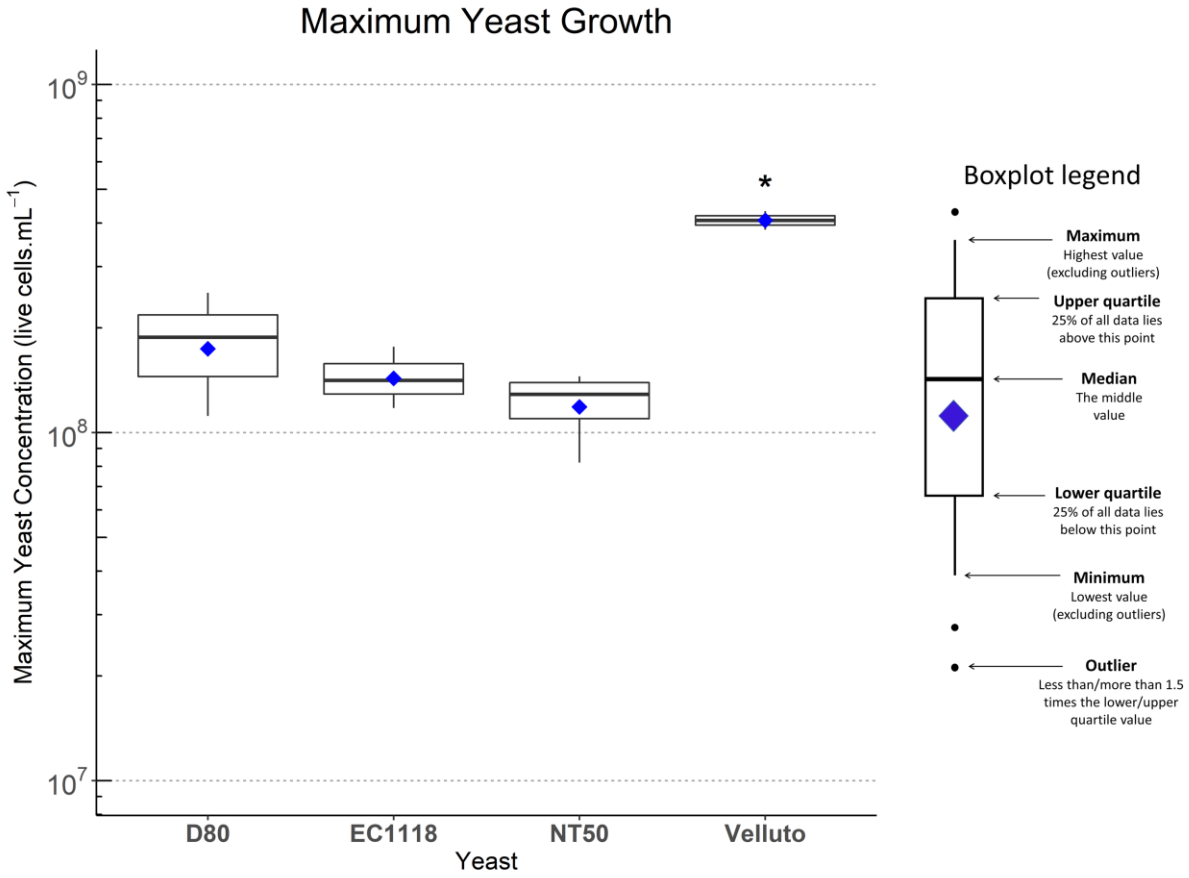

Figure 2: Maximum yeast concentration (viable cells mL^{-1}) for all fermentations. Each boxplot displays the variation of maximum yeast density across all co-inoculated and yeast-only controls. Blue diamonds are the average concentration for each yeast. *Significant difference between yeast average concentration (ANOVA, Tukey post-hoc; $p < 0.005$).

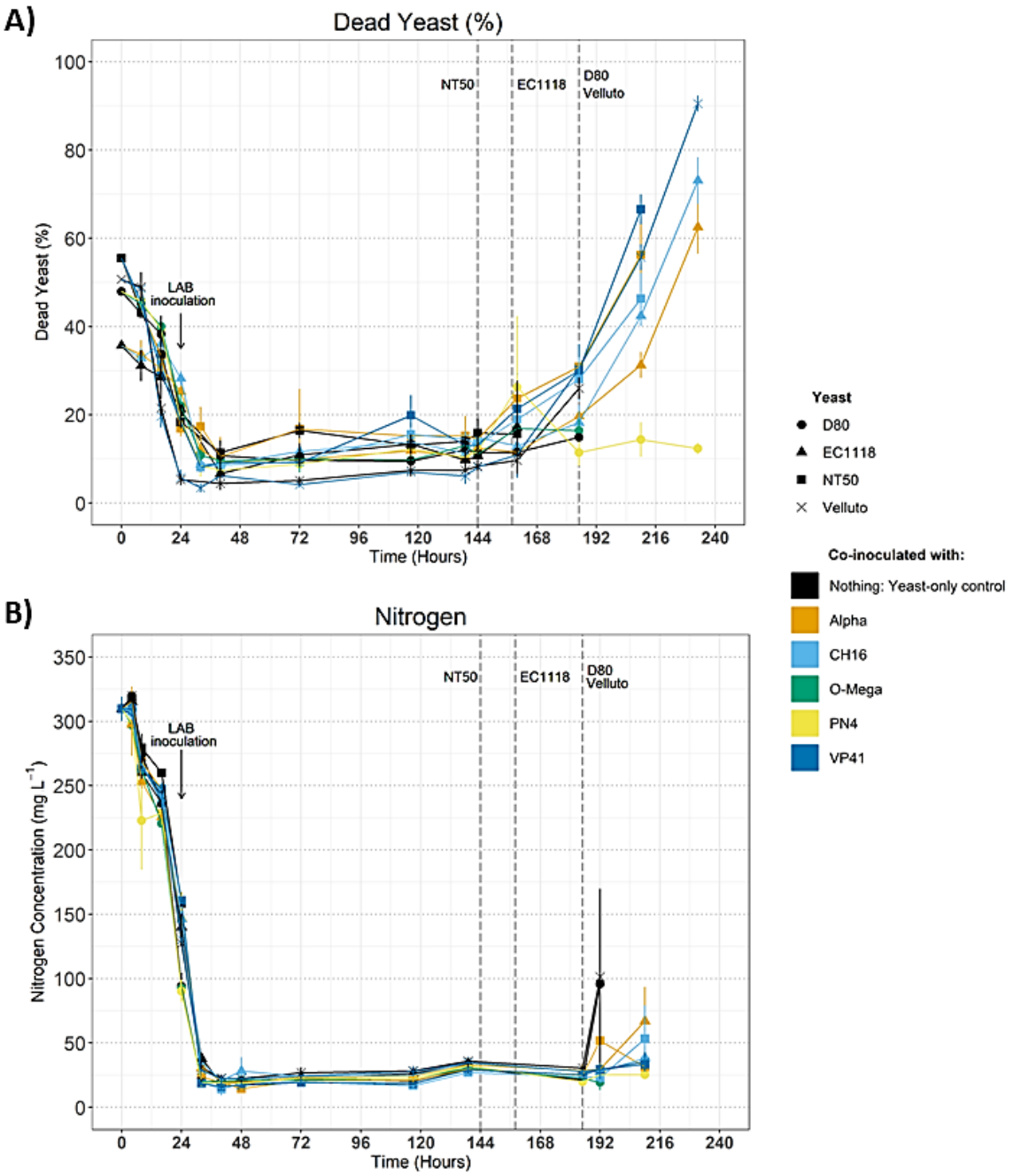

Figure 3: A) Yeast dead cell % measured over the course of fermentation. **B)** Free amino nitrogen (mg L^{-1}; excluding proline and ammonia) concentration measured throughout fermentation using NOPA. Vertical dashed lines indicate average AF finishing time for the specified yeast. Arrow indicates LAB inoculation. Values are the mean of triplicates and error bars are the standard deviation.

The percentage of dead yeast cells varied for each strain with co-inoculation fermentations having a higher percentage of dead yeast at the end of AF compared to yeast alone (Figure 3A). NT50 and Velluto yeast had higher percentages of dead cells in co-inoculations after 144 hours of fermentation compared to EC1118 and D80 co-inoculations. This may have been because NT50 and Velluto yeast were unable to withstand the ethanol content in combination with increased pH and lactic acid content compared to the two *S. cerevisiae* strains, D80 and EC1118.

Amino acids were measured by HPLC after both AF and MLF or end of the experiment if MLF did not complete. As expected, there was a high concentration of proline in the juice (Huang & Ough 1989), and a minimal amount was consumed throughout fermentation (Table S4, Appendix B; Long *et al.* 2018). Most amino acids, excluding proline, were depleted at the end of AF with no significant differences between yeast alone and co-inoculation (Table S4, Appendix B). There was a greater abundance of amino acids after completion of MLF by EC1118/Alpha, EC1118/CH16 and Velluto/VP41 in comparison to D80/O-Mega and D80/PN4 (Table S4, Appendix B). Each of the fermentations with a higher amino acid content at the end of MLF completed MLF after 14 days, allowing time for dead cells to liberate amino acids into the wine, compared to the fermentations where MLF finished after six and eight days. The percentage of dead yeast supports this result, since D80 had fewer percentage of dead yeast at the end of MLF compared to EC1118 and Velluto (Figure 3A). A higher percentage of dead yeast cells may have the potential for release of amino acids into the wine that could aid LAB growth and MLF. Release of amino acids from dead LAB could also occur, but dead LAB were not measured in this work. Considering all amino acids (except proline) were depleted at the end of AF for all yeast (6-8 days) and nitrogen release

occurred after 192 hours of fermentation (Figure 3B), yeast-LAB compatibility and MLF duration could not be correlated with amino acid content or nitrogen release.

3.2. pH, SO_2, acetaldehyde, ethanol and glycerol

All co-inoculations had a higher pH than yeast-only controls at the end of the experiment (Table 5). This is expected since the conversion of L-malic acid to L-lactic acid results in an increase in pH. Even in co-inoculations where MLF was incomplete, partial conversion of malic acid would still result in a pH change. Other alkaline compounds, such as amines, also contribute to the pH increase (Moreno-Arribas *et al.* 2003). The smaller increase in pH for EC1118, NT50 and Velluto may be in part due to conversion of 0.6 – 1 g L^{-1} malic acid by these yeasts (Table S5, Appendix B). D80 had a decrease in pH compared to the starting juice, most likely due to the presence of other acids in combination with malic acid.

SO_2 production by yeasts differed by yeast and LAB strain, however, no clear trend was evident for SO_2 production that aided in explaining yeast-bacteria compatibility (Table 5). Co-inoculations of LAB with EC1118 and Velluto resulted in less SO_2 than the yeast only control. This may be partially explained by the fermentation length, since SO_2 may be liberated from the fermentation over a longer period of time in the co-inoculations. This is contrary to D80/O-Mega that had slightly higher SO_2 and longer total fermentation compared to D80/PN4 (Table 4, 5). It is unclear whether the yeast are producing less SO_2 when co-inoculated with LAB, or SO_2 is binding other compounds or being internalised by LAB. This is especially so in the case of Velluto where there was 5.6 g L^{-1} less SO_2 at the end of the co-inoculation fermentation with VP41 compared to the yeast-alone, a significant amount that cannot be explained solely by liberation. It is also unclear to what extent LAB could

uptake SO_2 in its bound form. The opposite effect of SO_2 was observed for co-inoculations of LAB with D80 and NT50, where SO_2 was higher in co-inoculations compared to yeast alone. It cannot be elucidated if SO_2 was higher due to yeast production, liberation from dead LAB or other modes of SO_2 production. Additionally, the trend of SO_2 production did not correlate with acetaldehyde concentration, a known strong binder of SO_2 in wine (Bartle *et al.* 2019a ; Osborne & Edwards 2006). SO_2 can also bind carbonyl compounds such as glucuronic and pyruvic acids (Barbe *et al.* 2000; Wells & Osborne 2012); however, we were unable to specify what compounds were bound to SO_2. In future it would be useful to measure SO_2 evolution over the course of fermentation, though we currently do not have a method to enable this for small scale fermentation trials.

Acetaldehyde concentration differed depending on yeast strain, and was significantly higher in co-inoculated fermentations, except for NT50 (Table 5). There were no significant differences in acetaldehyde concentration for different LAB co-inoculated with the same yeast. Under test conditions in basic growth medium, *O. oeni* can metabolise acetaldehyde, generating ethanol and acetic acid (Osborne *et al.* 2000). During fermentation *O. oeni* acetaldehyde metabolism could result in wines with a final lower acetaldehyde concentration (Burns & Osborne 2015; Jackowetz & Mira de Orduña 2012; Pan *et al.* 2011). Therefore the results obtained in this work are not in agreeance with previous findings, where co-inoculation resulted in wines with lower acetaldehyde content. In addition, the increased acetaldehyde in co-inoculations found in the present work coincided with higher acetic acid concentration, which is discussed further in section 3.4. Though acetaldehyde may be broken down to acetic acid and ethanol, it remains unclear why both acetic acid and acetaldehyde levels were higher after co-inoculation compared to yeast-alone fermentations.

Table 5: pH, total SO_2 and ethanol concentration measured in the starting juice and at the end of the experiment. Values are averages of triplicate values ± standard deviation. Bold indicates yeast-only fermentations. Different letters indicate significant differences within each column (ANOVA, Tukey post-hoc, $p < 0.005$).

Yeast	Bacteria	pH	Total SO_2 (mg L^{-1})	Acetaldehyde (g L^{-1})	Ethanol (% v/v)	Glycerol (g L^{-1})
Starting Juice		3.5	0	0.04	0	0
D80	**None**	**3.4 (± 0.1) ^{A}**	**0.3 (± 0.5) ^{A}**	**0.18 (± 0.01) ^{A}**	**15.2 (± 0.1) ^{A}**	**9.5 ± (0.03) ^{A}**
	O-Mega	3.6 (± 0.1) ^{B,E}	1.1 (± 0.5) ^{A}	0.26 (± 0.0) ^{B}	15.1 (± 0.0) ^{A}	9.5 ± (0.02) ^{A}
	PN4	3.8 (± 0.1) ^{C,F}	1.3 (± 0.5) ^{A}	0.29 (± 0.02) ^{B}	15.7 (± 0.0) ^{B,C,D}	9.5 ± (0.13) ^{A}
EC1118	**None**	**3.6 (± 0.1) ^{A,B}**	**3.2 (± 1.4) ^{A,B}**	**0.55 (± 0.0) ^{C}**	**15.4 (± 0.0) ^{A,B,C,D}**	**10.6 ± (0.10) ^{B}**
	Alpha	3.9 (± 0.0) ^{C}	1.3 (± 0.5) ^{A}	0.62 (± 0.0) ^{D}	15.8 (± 0.0) ^{B,C}	10.6 ± (0.02) ^{B}
	CH16	3.9 (± 0.1) ^{C,D}	1.3 (± 0.5) ^{A}	0.63 (± 0.01) ^{D}	15.8 (± 0.0) ^{B}	10.6 ± (0.10) ^{B}
NT50	**None**	**3.5 (± 0.1) ^{B}**	**7.5 (± 1.7) ^{C}**	**0.71 (± 0.01) ^{E,F}**	**15.2 (**± 0.1) ^{A}	**12.2 ± (0.02) ^{C}**
	Alpha	3.7 (± 0.1) ^{D,E,F}	8.8 (± 2.1) ^{C}	0.70 (± 0.0) ^{F}	15.3 (± 0.1) ^{A,D}	12.1 ± (0.10) ^{C}
	CH16	3.7 (± 0.0) ^{B}	9.3 (± 0.5) ^{C}	0.70 (± 0.0) ^{E,F}	15.3 (± 0.2) ^{A,C,D}	12.2 ± (0.09) ^{C}
	VP41	4.0 (± 0.1) ^{C}	9.1 (± 0.5) ^{C}	0.74 (± 0.01) ^{E,G}	15.4 (± 0.2) ^{A,B,C,D}	12.2 ± (0.09) ^{C}
Velluto	**None**	**3.7 (± 0.0) ^{B,F}**	**6.1 (± 0.9) ^{B,C}**	**0.77 (± 0.01) ^{G}**	**15.0 (± 0.1) ^{A}**	**12.7 ± (0.06) ^{D}**
	VP41	3.9 (± 0.1) ^{C,D}	0.5 (± 0.5) ^{A}	0.83 (± 0.01) ^{H}	15.3 (± 0.3) ^{A,D}	12.6 ± (0.14) ^{D}

Ethanol concentration ranged from 15.0-15.8 % (v/v) across all fermentations, with D80/PN4 having the only significant difference in comparison with yeast alone (Table 5). Higher concentration of ethanol is known to contribute to problematic and sluggish MLF and in the instance of D80/PN4 compared to D80/O-Mega, could explain the slower MLF result. This could also be true for EC1118/Alpha and EC1118/CH16 where ethanol reached 15.8% (v/v), the highest of all fermentations, as well as slower MLF (Table 4). Velluto had a lower ethanol concentration than the *S. cerevisiae* strains, but, as discussed earlier, may inhibit MLF progress due to delayed nutrient release.

Ethanol combined with SO_2 causes a combinatorial stress on LAB. Ethanol causes increased cell membrane permeability allowing easier entry of molecular SO_2 into LAB cells that inhibits growth and impairs cellular function (Da Silveira *et al.* 2003; Guzzo *et al.* 2000; Wells & Osborne 2012). The combination of ethanol and SO_2 partially explains the difference in Alpha and CH16 ability to complete MLF with EC1118, and not with NT50. These results further highlight VP41's overall high tolerance to multiple stressors, including ethanol and SO_2. In fact, VP41 performed faster MLF when SO_2 and ethanol were higher, further indicating that Velluto may have delayed nutrient release (Zapparoli *et al.* 2003) that subsequently affects VP41 MLF progress.

Glycerol concentration at the end of fermentation was significantly different between yeast strains, but was unaffected by co-inoculation (Table 5). As expected, glycerol production was higher for NT50 and Velluto. This agrees with other studies where *S. cerevisiae* x *S. kudriavzevii* hybrid and *S. uvarum* produced higher concentrations of glycerol in comparison to pure *S. cerevisiae* (Arroyo-López *et al.* 2010; Bertolini *et al.* 1996; González *et al.* 2007). Higher glycerol production by *S. cerevisiae* hybrid and *S. uvarum* strains is due to a difference in regulation of the

glycerol:ethanol metabolic pathway equilibrium that allows a proportionally higher production of glycerol under conditions of low pH (< 3.6), and high sugar concentration (> 200 g L^{-1}; Arroyo-López *et al.* 2010). Glycerol can be protective to LAB as they use it to maintain cell wall integrity under osmotic stress (van der Heide & Poolman 2000). However, these results indicate that glycerol production is not influenced by co-inoculation, and therefore cannot be used for predicting co-inoculation success.

3.3. Hydrogen sulfide

Hydrogen sulfide (H_2S) production differed between yeast only fermentations, and yeast co-inoculated with bacteria (Figure 4). Different levels of H_2S production by yeast is a well-known phenomenon (Huang *et al.* 2017; Spiropoulos & Bisson 2000; Spiropoulos *et al.* 2000), and often commercial yeast manufacturers report H_2S production potential (i.e. low, medium or high) of each yeast strain (Lallemand 2019b). H_2S has a very low odour detection threshold of 1.6 µg L^{-1} equivalent to 0.0016 ppm and can impart an off-putting rotten egg aroma (Swiegers & Pretorius 2007). H_2S is involved in sulfur amino acid synthesis and the sulfate assimilation pathway within yeast (*Saccharomyces* Genome Database 2007a, 2007b). During AF where there is sufficient nitrogen, H_2S can be incorporated into sulfur-containing amino acids methionine and cysteine, but under low nitrogen conditions yeast may break down sulfur-containing amino acids to utilise nitrogen, liberating H_2S (Jiranek, Langridge & Henschke 1995a, 1995b). A considerable amount is known about H_2S production by wine yeast (Huang *et al.* 2017), but very little is known about the role of H_2S in *O. oeni*. The influence of H_2S on LAB during co-inoculation is something that warrants further exploration. H_2S has been identified as a signalling molecule for yeast that leads to population biological rhythm synchrony in response to

chemical stressors (Sohn, Murray & Kuriyama 2000). Therefore the increased production of H_2S during co-inoculations with some LAB may be the result of LAB derived compounds causing stress to yeast, leading to yeast H_2S signalling. The involvement of sulfur compounds in yeast-LAB interactions has been proposed based on yeast gene upregulation of sulfur related metabolic processes during co-inoculation with LAB (Rossouw, du Toit & Bauer 2012). Despite the intriguing findings, no further work has been reported on the role of sulfur compounds in yeast-LAB interactions.

Previously, H_2S levels have been recorded after yeast-bacteria co-inoculation fermentation completion in red wine (Antalick, Perello & de Revel 2013), but to date no study has measured H_2S production over the course of AF and MLF. In addition to this, our work also includes measurement of H_2S production over time for different yeast-LAB combinations. Unlike yeast co-inoculated with CH16, O-Mega and VP41, co-inoculations of yeast with Alpha LAB did not have an observable increase of H_2S in comparison with the yeast only controls (Figure 4). These results identify that yeast H_2S production can be influenced by LAB strain.

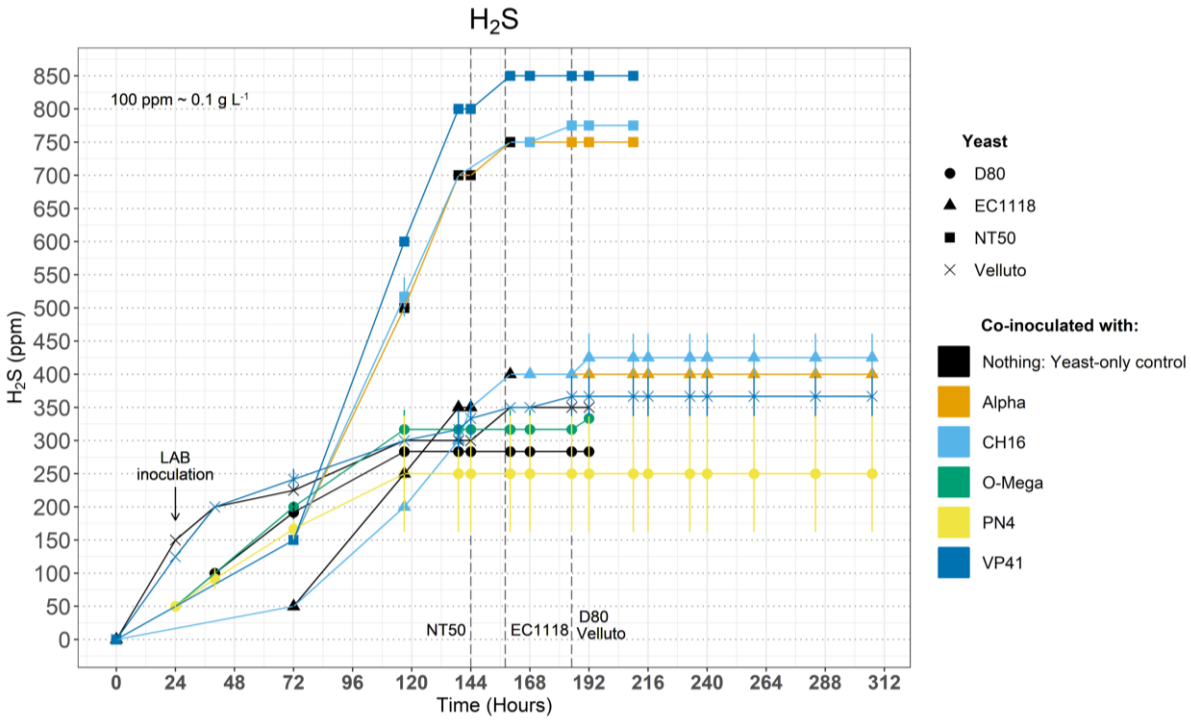

Figure 4: Hydrogen sulfide production over the duration of AF and MLF. H_2S was measured daily by recording H_2S values indicated on silver nitrate H_2S detector tubes (detection range 25-1000 ppm). The first measurement after inoculation was taken at first detection of H_2S production. Vertical dotted lines represent average AF finishing times for indicated yeast. Values are the mean of triplicates and error bars are the standard deviation.

The ability of different LAB to influence specific yeast processes has been observed before with yeast [*GAR*$^+$] prion induction (Ramakrishnan *et al.* 2016). Similar to the H_2S results here, [*GAR*$^+$] prion is not induced by all *Oenococcus* strains, and therefore it is not surprising that differences in H_2S production could also be influenced by co-inoculation with different LAB.

In addition to H_2S production, a number of other volatile compounds may play a vital role in yeast-LAB compatibility.

3.4. GC-MS

The concentration of volatile compounds was influenced by yeast strain. All NT50 fermentations had significantly higher concentrations of ethyl propanoate, 1-butanol, hexyl acetate (Figure 5), and significantly lower concentrations of acetic acid (Figure 6) compared to all other fermentations.

All fermentations had similar concentrations of decanoic acid, octanoic acid (Figure 6), 1-hexanol, 2-methylbutanoic acid, 3-(methylthio)-1-propanol, benzyl alcohol, ethyl acetate and ethyl octanoate (Table S7, Appendix B).

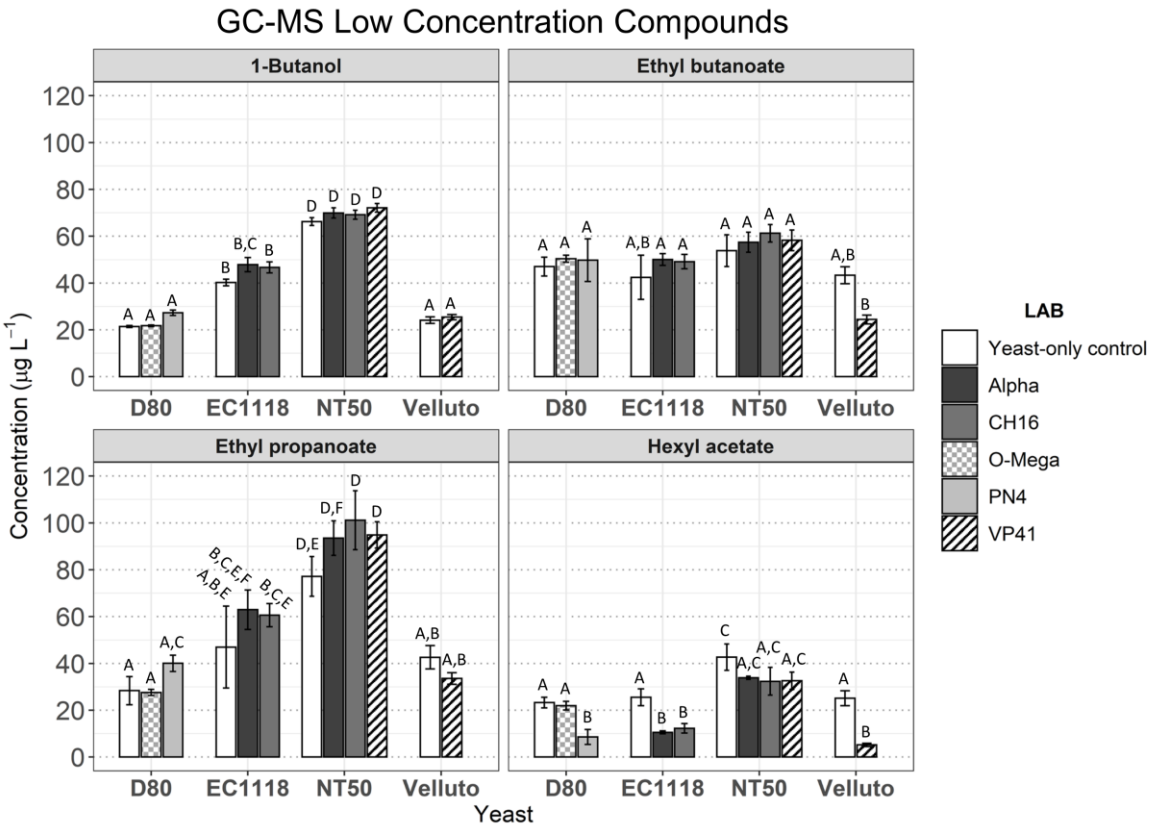

Figure 5: Low concentration volatile compounds measured at fermentation completion using GC-MS. Values are the mean of triplicates and error bars are the standard deviation. Different letters indicate significant differences (ANOVA, Tukey post-hoc $p < 0.005$)

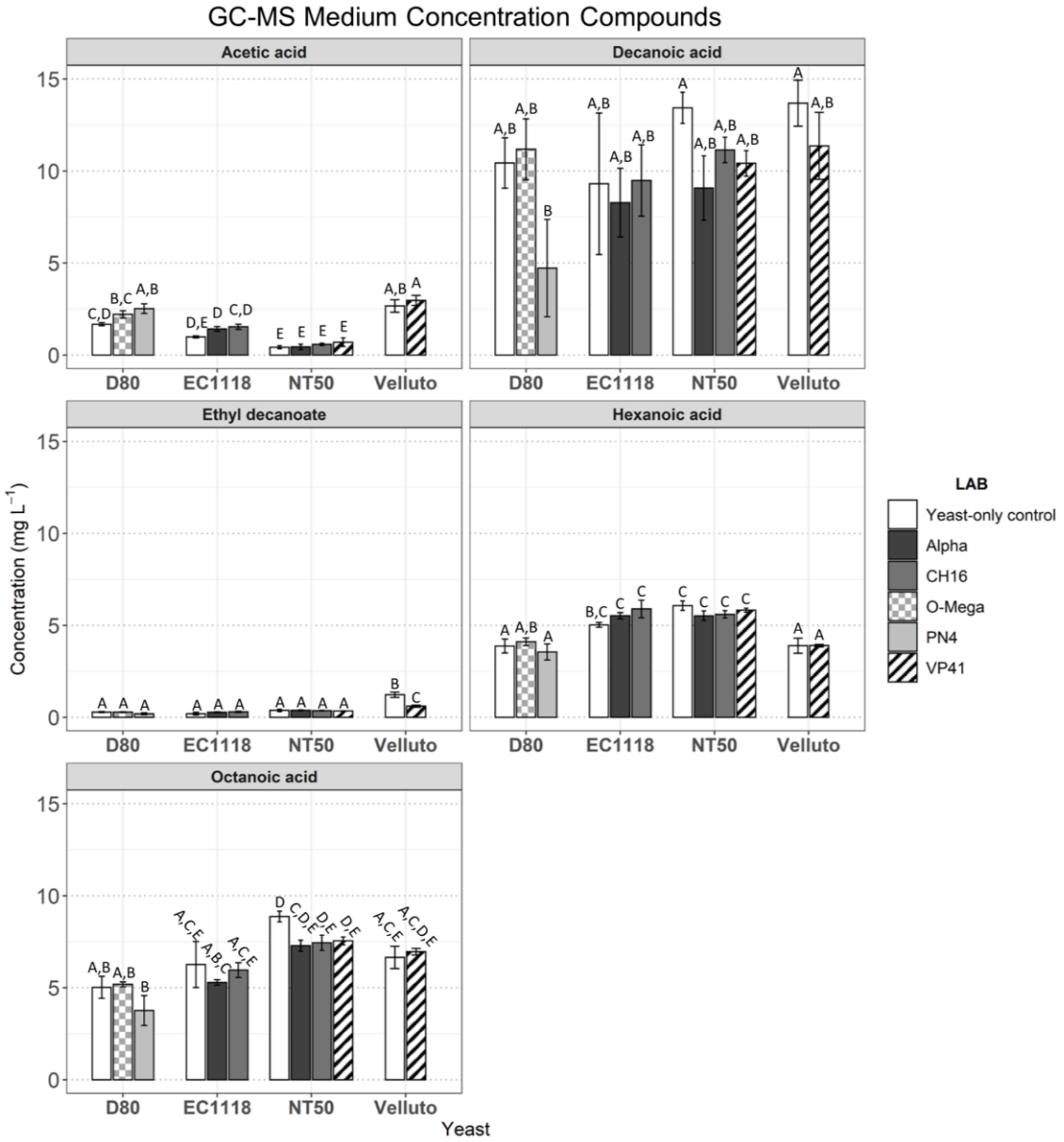

Figure 6: Medium concentration volatile compounds measured at fermentation completion using GC-MS. Values are the mean of triplicates and error bars are the standard deviation. Different letters indicate significant differences (ANOVA, Tukey post-hoc $p < 0.005$)

In the case of NT50, it may be that the combination of alcohols, acids and esters is the major reason for incompatibility with CH16 and Alpha. Volatile compounds are usually reported for their sensory impact on wines and not their role in fermentation completion. However, it should be considered that the production of esters by yeast and bacteria occurs for a reason. In the case of esters, it involves the formation of a volatile, hydrophobic compound from an alcohol and a carboxylic acid, with the release of water. For *S. cerevisiae* it has been hypothesised that esterification is used as a detoxification system (Saerens *et al.* 2010), though no current work has successfully tested this hypothesis. This hypothesis arises from the fact that carboxylic acids and alcohols affect membrane fluidity and internal pH, inhibiting growth and cellular functions (Henderson & Block 2014; Pampulha & Loureiro-Dias 2000). Esters are able to move across the cell membrane more easily, thereby relieving the effects of carboxylic acids and alcohols. Since LAB also produce esters the detoxification hypothesis could also be applied to them. For LAB in particular, it is not unreasonable to theorise that production of different levels of alcohols and carboxylic acids during fermentation could have a combinatorial impact on LAB growth and performance, as LAB may not be able to keep up with the rate of alcohol and acid production. LAB also show different tolerance to various carboxylic acids, with a combination of decanoic, hexanoic and octanoic acids exhibiting a more fatal outcome for LAB in comparison to each individual acid (Lonvaud-Funel, Joyeux & Desens 1988). As for alcohols, the negative impact of ethanol on microbial health is quite clear (Betteridge *et al.* 2018; Olguín *et al.* 2015), but the impact of other alcohols on microbes is yet to be determined.

The ability of alcohols to pass through the cellular membrane is dependent on molecule size and polarity. Ethanol is a small, highly polar molecule that passes through the membrane passively and rapidly (Yang & Hinner 2015) and therefore

affects microbial cells easily. The highest concentration of another higher alcohol, isobutanol, was produced by D80 (Figure 7). *S. cerevisiae* isobutanol production varies by strain, ranging from < 20 to > 200 mg L^{-1} (Mateos, Pérez-Nevado & Fernández 2006; Romano *et al.* 2003a, 2003b). There was no relationship between yeast-LAB compatibility and isobutanol concentration. Phenylethyl alcohol concentration was significantly higher for Velluto than all other yeast (Figure 7). This is a long-known trait of cryotolerant yeasts such as *S. uvarum* (Bertolini *et al.* 1996; Masneuf-Pomarède *et al.* 2002). Despite the differences in concentration of alcohols for each yeast (Figure 5, 7), the effects of alcohols, other than ethanol, on LAB growth and metabolism are unknown and the individual concentrations of each alcohol did not correlate to co-inoculation outcomes.

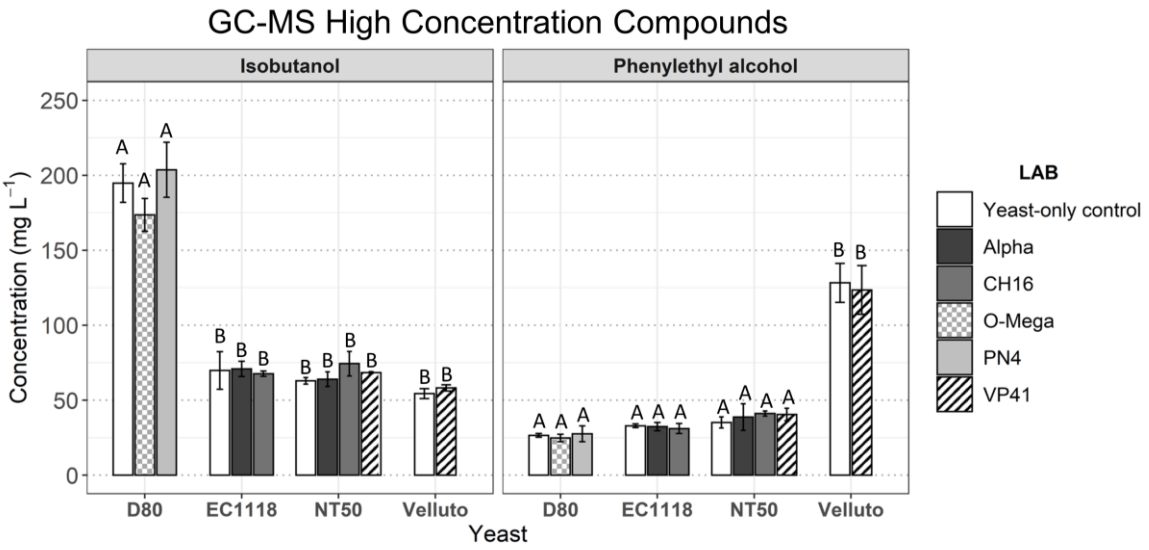

Figure 7: High concentration volatile compounds measured at fermentation completion using GC-MS. Values are the mean of triplicates and error bars are the standard deviation. Different letters indicate significant differences (ANOVA, Tukey post-hoc $p < 0.005$)

Fermentations with Velluto had the highest levels of ethyl decanoate (Figure 6). Esters such as ethyl decanoate are produced by a reversible reaction where alcohols react with carboxylic acids, forming an ester and water. Considering this process, ethyl decanoate can be produced from a reaction between ethanol and decanoic acid. Thus, Velluto could have produced higher concentrations of decanoic acid throughout fermentation (Figure 6). Decanoic acid works synergistically with ethanol and low pH to inhibit intracellular ATPase (Carrete *et al.* 2002), and could partly explain the slower MLF of VP41 when co-inoculated with Velluto. The decanoic acid concentration at fermentation completion was not higher for Velluto than for other yeast strains, although it is possible that more decanoic acid was produced, and subsequently esterified during Velluto fermentations. In future, measurements of carboxylic acids, alcohols and esters could be performed over the course of fermentation to try to look for higher production of ester precursors.

The levels of acetic acid varied for each yeast-bacteria pair and yeast alone (Figure 6). During AF acetic acid can diffuse into yeast cells where the higher internal pH leads to disassociation of the acid, causing cytoplasmic acidification (Thomas, Hynes & Ingledew 2002). This acidification can inhibit AF and other cellular processes within the yeast. Velluto and D80 fermentations had extended AF duration (Table 4) that could be partly attributed to acetic acid concentration, since Velluto and D80 fermentations also had the highest end concentrations of acetic acid (Figure 6).

3.5. Succinic acid

Succinic acid concentration at the end of fermentation was different between yeast strains, but not significantly different after co-inoculation (Figure 8). Velluto had the highest concentration of succinic acid at fermentation completion, though it was not statistically different from other yeast. Nevertheless, the ability of *S. uvarum* to produce higher succinic acid than *S. cerevisiae* is common (Bertolini *et al.* 1996; Giudici *et al.* 1995) since high succinic acid production is a phenotype of cryotolerant strains.

Initially succinic acid concentration was measured using HPLC, however it was determined that succinic acid was co-eluting with other compounds due to the inexplicably high concentrations (Bartle *et al.* 2019b). These initial results revealed a trend that aligned with the compatibility results. After measuring succinic acid concentration using an enzymatic assay, the values were approximately ten times lower than previously measured concentrations. We were unable to identify the co-eluting compound or compounds observed during HPLC and in future, using HPLC to measure succinic acid in red wine fermentations should be treated with caution. The succinic acid trend was still apparent after using the enzymatic kit, but to a lesser extent compared with the HPLC results. The LAB that completed MLF had similar or lower succinic acid concentration than yeast-only controls at the end of fermentation, and LAB that were unable to complete MLF had higher succinic acid concentrations.

Though the differences were statistically insignificant, the observation of different succinic acid concentrations based on MLF completion requires more discussion. There are a few plausible explanations for this trend. Firstly, LAB that were able to complete MLF may have taken up succinic acid from the environment. Since NT50/Alpha and NT50/CH16 LAB growth was still occurring, it's possible that

succinic acid was being utilised after the onset of MLF because succinic acid is an intermediate of the citric acid cycle that is needed for growth. However, succinic acid is also a competitive inhibitor of the malolactic enzyme (Lonvaud-Funel & De Saad 1982). It is possible that the inability of Alpha and CH16 to reach critical density and complete MLF when co-inoculated with NT50 was due to a combination of increased ethanol-induced membrane fluidity and inhibited growth, while production of succinic acid inhibited the malolactic enzyme. The ethanol-succinic acid combination may also explain why Alpha and CH16 successfully completed MLF with EC1118, since EC1118 did not complete AF as quickly, or produce as much succinic acid as NT50. Additionally, VP41 was able to reach critical density by 72 hours, and completed MLF slower in combination with Velluto compared to NT50, which correlates to the higher concentration of succinic acid produced by Velluto. It is important to consider that each LAB may have a different tolerance to particular compounds, as is the case for ethanol and SO_2 (Betteridge *et al.* 2018; G-Alegría *et al.* 2004; Jiang *et al.* 2018).

In order to fully understand how succinic acid and its potential synergism with ethanol can affect co-inoculation success, measurement of succinic acid and ethanol concentration over the course of fermentation should be performed. Since ethanol also has a synergistic effect in combination with pH and SO_2, measuring and mapping metabolic processes and metabolite production could lead to invaluable insights into the complex metabolite network that underpins compatibility outcomes.

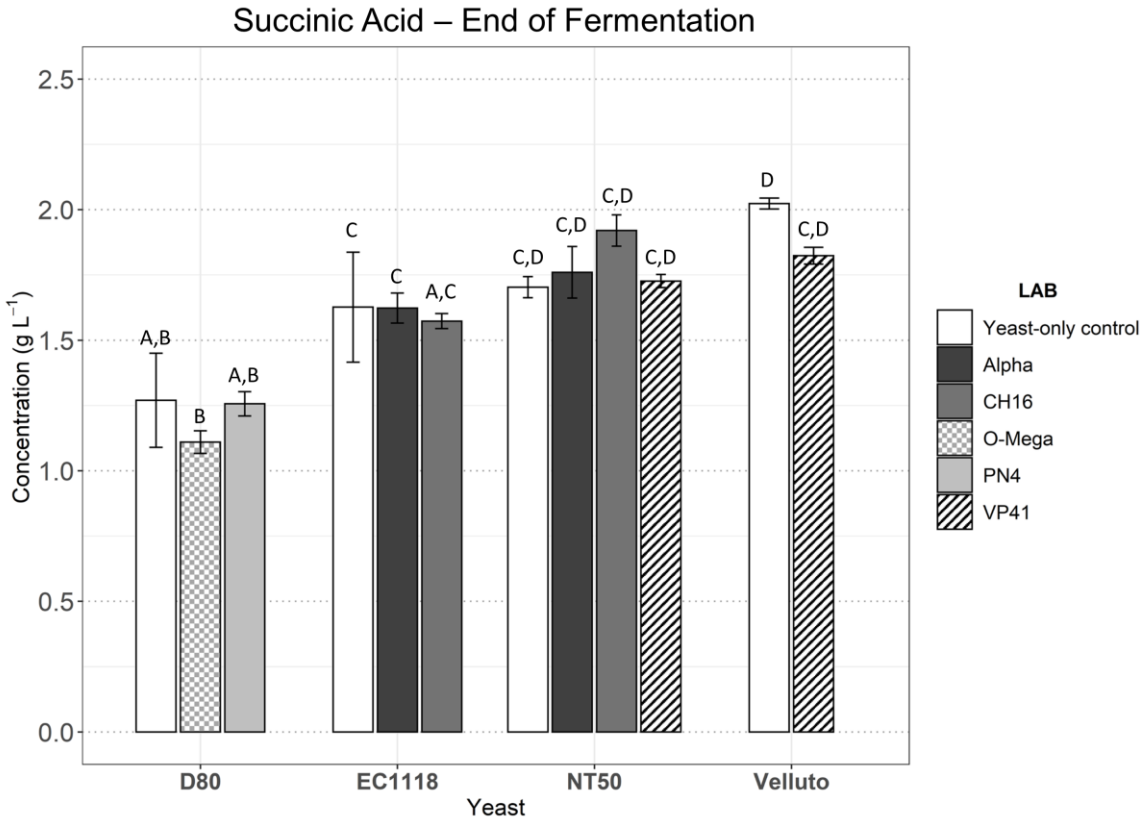

Figure 8: Succinic acid concentration measured at fermentation completion by enzymatic assay. Values are the mean of triplicates and error bars are the standard deviation. Significant differences are indicated by different letters (ANOVA, Tukey post-hoc $p < 0.005$)

3.6. Matrix effect

The use of juice provides a more complex matrix compared to the use of a synthetic medium, as discussed earlier. The results presented here demonstrate the usefulness of using a matrix that more closely mimics the conditions of large scale winemaking. However, to ensure sterility, the skins were removed which is not what occurs at an industrial scale. This process may have altered the outcome, as the skins provide other compounds, mainly polyphenols, which can alter yeast and LAB fermentation performance (García-Ruiz *et al.* 2011; Sidari, Caridi & Howell 2014). Phenolic compounds are released from the skins throughout fermentation, providing the deep red colour that is expected of red wines. For yeast, the presence of phenolic compounds may induce biofilm formation (Sidari, Caridi & Howell 2014), while for LAB, phenolic compounds may cause membrane disruption and subsequent growth inhibition (García-Ruiz *et al.* 2011). The cold maceration step in preparation of the juice in this work enabled some polyphenolic extraction from the grapes, but not to the extent that is observed during industrial winemaking when skins remain during much of fermentation. Removing the skins also reduced the likelihood of yeast biofilm formation, as the skins provide additional surfaces for cell attachment.

Inclusion of grape skins and potential yeast biofilm formation would increase experimental complexity when seeking to delineate factors influencing yeast-LAB compatibility. However, further investigation into biofilm formation and its specific impact on yeast-LAB compatibility may prove useful for both researchers and industry. In future, therefore, the use of small scale winemaking techniques that include grape skins could offer more comparable results to industrial winemaking. Further, performing fermentations in a non-sterile environment would enable

exploration of interactions between inoculated and indigenous microorganisms, along with the potential influence of biofilm formation on yeast-LAB compatibility.

4. Conclusions

This work demonstrates the complexity of yeast-LAB compatibility in Shiraz juice fermentation. Under the tested conditions, there are no clear and defined metabolites that can be used as solid indicators for compatibility. This work did reveal the potential for compounds such as hydrogen sulfide, esters and succinic acid to contribute to MLF success. Much more work is needed to elucidate the role of hydrogen sulfide in co-inoculation, and such work should include evaluation of hydrogen sulfide signalling between LAB and yeast. The formation of esters and their role in bacterial detoxification also warrants further investigation. There is much more to learn about general detoxification systems within *O. oeni*, and how this information could be applied to choosing yeast and LAB pairs for successful co-inoculation. It is clear from this work (Chapters 3 and 4) that yeast-bacteria compatibility is not only dependent on one or a few compounds, but the complex relationship between multiple compounds and conditions. There are numerous other compounds, both known and unknown, that are produced throughout fermentation that could also impact the ability of MLF to be completed by LAB. Additionally it may be the timing of production of compounds that underpins yeast-LAB compatibility, not just whether they are produced or not. Understanding the complexity of yeast-LAB relationships shows great promise for gaining more control of fermentation, as well as tailoring sensory attributes of wine. Future work should include in depth analysis of gene regulation within both yeast and *O. oeni* in response to co-inoculation to uncover the roles of esters and sulfides in stress response, as well as other metabolic processes.

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Chapter 5

Genetic determinism of yeast-bacteria interactions: another pleiotropic effect of the *SSU1* gene revealed by QTL mapping

1. Introduction

Fermented beverages are the result of biotransformation of complex matrices by microbial communities that can include moulds, yeast, bacteria and bacteriophage (Bokulich & Bamforth 2013; Mounier *et al.* 2008; Renouf, Claisse & Lonvaud-Funel 2007). Within these communities, the rate of growth and metabolic activity of each microbial species depends on the biochemical composition of the medium, physicochemical conditions of the process (e.g. converting sugars to ethanol during juice fermentation), and physiological state of the microbes. Additionally, microbial species in fermented beverages may interact with each other in a direct or indirect manner (e.g. cell-to-cell contact or metabolite production).

Fermenting grape juice is a fast changing environment that is especially interesting for studying how the two most common wine microbes, the yeast *Saccharomyces cerevisiae* and lactic acid bacterium *Oenococcus oeni*, coexist and interact. Microbial interactions can affect the final composition of volatile compounds (Renault *et al.* 2015, 2016) and wine sensorial complexity (Tempère *et al.* 2018). The importance of microbial interactions in wine is evident from the wide number of studies focusing on co-inoculated or sequential inoculation of *S. cerevisiae* and *O. oeni*, with the aim of decreasing overall fermentation time while maintaining or increasing wine quality (Abrahamse & Bartowsky 2012; Cañas *et al.* 2012, 2015; Chasseriaud *et al.* 2018; Knoll *et al.* 2012; Renault *et al.* 2015, 2016).

The mechanisms of yeast-lactic acid bacteria (LAB) interactions during juice fermentation have been reviewed recently (Bartle *et al.* 2019a). Broadly, microbial

interactions may include cell-cell contact (Nissen, Nielsen & Arneborg 2003, 2004; Renault, Albertin & Bely 2013) or production of small metabolites (Renault *et al.* 2009; Sadoudi *et al.* 2012) and macromolecules (Comitini *et al.* 2005; Jarosz *et al.* 2014) that can inhibit and/or activate the growth and activity of interacting microbes. Understanding the molecular mechanisms of yeast-LAB interactions is a challenging task, but the benefits of such work include optimisation of yeast-LAB co-inoculation strategies for implementation in wineries.

S. cerevisiae and *O. oeni* interactions can affect their ability to complete alcoholic (AF) and malolactic fermentation (MLF), respectively (Bartle *et al.* 2019a). Yeast may produce metabolic compounds that can inhibit LAB growth, including ethanol (Capucho & San Romão 1994; Gao & Fleet 1995; Guzzo *et al.* 2000), SO_2 (Osborne & Edwards 2006), short and medium-chain fatty acids (Alexandre *et al.* 2004; Capucho & San Romão 1994), and antimicrobial peptides (Atanassova *et al.* 2003; Mendoza, de Nadra & Farías 2010; Nehme, Mathieu & Taillandier 2010). Yeast and LAB also have the potential to interact physically in the form of mixed species biofilms (Bartle *et al.* 2019a) or through co-aggregation (Furukawa *et al.* 2011), though to date there have not been reports of this occurring between *S. cerevisiae* and *O. oeni*.

In addition to chemical and physical interactions, *S. cerevisiae* gene expression has been reported to be affected by co-inoculation with *O. oeni* (Rossouw, du Toit & Bauer 2012). *S. cerevisiae* differential gene expression in response to co-inoculation with *O. oeni* included up-regulation of genes related to yeast stress response and possible competition for sulfur compounds compared to *S. cerevisiae* alone (Rossouw, du Toit & Bauer 2012). Several studies have also reported strain specificity of yeast and LAB compatibility during co-inoculation (Abrahamse & Bartowsky 2012; Antalick, Perello & de Revel 2013; Arnink & Henick-Kling 2005;

Comitini & Ciani 2007; Rossouw, du Toit & Bauer 2012; Tristezza *et al.* 2016). Considering this, the intraspecific genetic variability of interacting species requires further investigation and analysis. To our knowledge, the identification of genetic variations that explain a “strain compatibility” effect are yet to be reported.

For *S. cerevisiae,* the genetic determinism of any complex trait can be investigated by mapping quantitative trait loci (QTLs) in a segregated progeny (Liti & Louis 2012). In the context of wine, this strategy has been used for elucidating the genetic basis of many traits of industrial interest (Peltier *et al.* 2019) including acetic acid production (Salinas *et al.* 2012), rate of nitrogen uptake (Brice *et al.* 2014; Jara *et al.* 2014), resistance to stuck fermentation (Marullo *et al.* 2019), resistance to low pH (Martí-Raga *et al.* 2017) and the production of aroma compounds (Eder *et al.* 2018; Huang, Roncoroni & Gardner 2014; Roncoroni *et al.* 2011; Steyer *et al.* 2012). To date, QTL mapping has been performed for single pure cultures focusing on traits related to yeast fitness or effect on wine quality. However, this strategy may be applied to any trait resulting in a measurable phenotypic variability. In the present work, we applied a QTL mapping strategy to delineate how *S. cerevisiae* genetic variability may affect the success of malolactic fermentation in co-inoculated fermentations with a commercial strain of *O. oeni.*

2. Materials and methods

2.1. Media

Shiraz Juice: Shiraz grapes (2017 vintage, Coombe vineyard, Waite Campus, Urrbrae, South Australia) were harvested, de-stemmed, crushed and left to macerate at 0°C for 7 days to enable polyphenolic extraction. Shiraz must was pressed and the juice stored at -20°C until required. No SO_2 or antibacterial agents

were added to the juice during pressing. Prior to experimentation Shiraz juice was filtered (0.45 µm, FHT45, Air-Met Scientific, Victoria, Australia) to remove grape matter and solids . The juice was adjusted to 250 g L^{-1} total sugar by addition of water, 2.5 g L^{-1} L-malic acid by addition of pure L-malic acid and pH 3.5 by addition of tartaric acid, followed by addition of 100 mg L^{-1} diammonium phosphate. Finally, the juice was filter sterilised (0.2 µm).

Liquid de Man, Rogosa and Sharpe medium (MRS; catalogue # AM103, Amyl Media, Victoria, Australia), supplemented with 20% apple juice (MRSAJ) was used for growing bacteria prior to inoculation. MRS was sterilised (121˚C, 0.1 MPa, 20 minutes) and sterile filtered apple juice (0.2 µm) added post sterilisation before use. MRSAJ with agar (2%) and addition of cycloheximide (0.5%) following sterilisation of the medium, was used for enumeration of bacteria.

All yeast strains were initially streaked for single colonies on YPD agar (2% glucose, 2% peptone, 1% yeast extract, 2% agar) and grown at 28°C, before growth of single isolates in YPD (2% glucose, 2% peptone, 1% yeast extract) at 28°C overnight. If required, Geneticin (G418, 100 µg mL^{-1}; catalogue # G8168, Sigma-Aldrich, Australia) was added to YPD cultures to select for strains carrying the KanMX deletion cassette.

2.2. Strains and Fermentations

2.2.1. Yeast strains

Strains used in this work are listed in Table 1. QTL analysis was performed using the SBxGN yeast background. SBxGN is the F1-hybrid of SB and GN strains, two diploid fully homozygous strains derived from the wine starters Actiflore BO213 and Zymaflore VL1, respectively (Peltier *et al.* 2018b). The population used for QTL mapping was constituted of 67 haploid progeny clones derived from the hybrid BN, an isogenic variant of SBxGN (Marullo *et al.* 2007). These haploid meiotic progenies have been previously genotyped by whole genome sequencing (Martí-Raga *et al.* 2017).

The effect of the gene *SSU1* was assayed using the reciprocal hemizygosity assay by deleting each parental copy of *SSU1* individually in the SBxGN F1 hybrid (Steinmetz *et al.* 2002). The reciprocal hemizygous hybrids SΔG092 and GΔS092 were previously obtained as described by Zimmer and colleagues (2014). The reciprocal hemizygous hybrids MΔF092 and FΔM092 were obtained following the same protocol by transforming the hybrid M2xF15. Two hemizygotes with each parental allele were phenotyped.

Yeast live cell concentrations were determined by flow cytometry. Yeast were diluted 100 times in sterile phosphate buffered saline (PBS) solution then stained with propidium iodide at a final concentration of 0.1 mg mL^{-1}. Samples were analysed using a Millipore Guava Easycyte 12HT flow cytometer (Millipore). Yeast concentrations were adjusted to inoculate sterile Shiraz juice at a final rate of 5×10^6 live cells mL^{-1}.

Table 1: Yeast strains used in this study

Strain	Comment	Genotype	Origin
SB	Monosporic clone of Actiflore BO213 (Laffort, France)	*HO/HO, diploid*	Peltier *et al.* (2018b)
GN	Monosporic clone of Zymaflore VL1 (Laffort, France)	*HO/HO, diploid*	Peltier *et al.* (2018b)
SBxGN	F1 hybrid SBxGN	*HO/HO, diploid*	Peltier *et al.* (2018b)
BN	F1 hybrid hoSBxGN	*HO/ ho::kanMx4, diploid*	Marullo *et al.* (2007)
pop BN	67 progeny clones of BN (hoSBxGN). Labelled with prefix "CM" followed by an ID number	*ho::kanMx4, haploids*	Marullo *et al.* (2007)
M2xF15	F1 hybrid M2xF15	*HO/HO, diploid*	Huang, Roncoroni & Gardner (2014)
SΔG092	Hemizygote hybrid isogenic to SBxGN	*ho/ho, YPL092^{SB}::kanMX4/ YPL092^{GN}, diploid*	Zimmer *et al.* (2014)
GΔS092	Hemizygote hybrid isogenic to SBxGN	*ho/ho, YPL092^{GN}::kanMX4/ YPL092^{SB}, diploid*	Zimmer *et al.* (2014)
MΔF092	Hemizygote hybrid isogenic to M2xF15	*HO/HO, YPL092^{M2}::kanMX4/ YPL092^{F15}, diploid*	This study
FΔM092	Hemizygote hybrid isogenic to M2xF15	*HO/HO, YPL092^{F15}::kanMX4/ YPL092^{M2}, diploid*	This study

2.2.2. Bacteria

Freeze-dried SB3 (Laffort, France) was grown anaerobically in MRSAJ for four days at 30°C 20% CO_2. Twenty-four hours prior to inoculation, bacteria were centrifuged at 2,236 x *g,* the supernatant was discarded and the cell pellet washed in sterile Shiraz juice before overnight incubation in fresh sterile Shiraz juice at 30°C. Bacteria were adjusted to an OD_{600} of 0.55 immediately prior to inoculation. For QTL library fermentations, 200 µL of bacterial culture was added to each fermentation vessel manually through a silicone septa with a 21-guage needle. For the hemizygote fermentations, 200 µL of bacterial culture was transferred from a 96-well deep well plate to each fermentation vessel using the tee-bot automatic inoculation system (developed after performing the QTL experiment).

2.2.3. Fermentations

Fermentations were conducted using an automated fermentation platform built on an EVO Freedom workdeck (Tecan, Männedorf, Switzerland; Figure 1). The system enabled 384 concurrent fermentations at a volume of up to 25 mL. Full details of the system were described by Hranilovic *et al.* (2018) and can also be found on the University of Adelaide Biotechnology and Fermentation Facility website (https://sciences.adelaide.edu.au/agriculture-food-wine/research/biotechnology-and-fermentation-facility).

Fermentation vessels were filled with 20 mL of sterile Shiraz juice and inoculated with yeast (5 x 10^6 live cells mL^{-1}) followed by LAB inoculation 24 hours later. Sampling occurred daily, and fermentations were homogenised by stirring prior to sampling. For the QTL mapping experiment, both parental strains (SB and GN), the hybrid BN and the 67 haploid progeny were fermented as pure cultures (in

duplicate) or co-inoculated with SB3 (in triplicate). To test the effect of *SSU1*, hemizygote hybrids and F1- hybrids (SBxGN and M2xF15) were assessed in triplicate for both pure and co-inoculated fermentations with SB3.

Figure 1: 384 fermentation Tee-bot system. Set-up included 4 x 96 tube blocks each temperature controlled by water baths and individual tube mixing by magnetic stir bars (top). Fermentations were sampled aseptically using an automated system (bottom).

2.3. Fermentation monitoring

2.3.1. Glucose and fructose consumption

Glucose and fructose concentrations were determined enzymatically using commercially available kits (K-FRUGL, Megazyme, Ireland) following methods modified by Walker *et al.* (2014). Glucose and fructose consumption was used as a determinant for alcoholic fermentation progress. Alcoholic fermentation was deemed complete when total glucose plus fructose concentration was < 3 g L^{-1}.

The amount of glucose/fructose consumed over time was modelled by local polynomial regression fitting with the R-loess function setting the span parameter to 0.8. Five parameters were extracted from the model, which are described in Table 2.

2.3.2. L-malic acid concentration

L-malic acid was measured using an enzymatic test kit (4A165, Vintessential laboratories, Australia) with modifications so that a plate-reader/spectrophotometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland) could be used to measure absorbance. Specifically, each well of a 96 well micro-titre plate was dosed with 70 µL buffer (0.1M gly-gly, 0.1M L-glutamate, pH 10), 14 µL nicotinamide adenine dinucleotide (40 mg mL^{-1}), 70 µL distilled water, 0.7 µL glutamate oxaloacetate transaminase (800 U mL^{-1}) and 5 µL sample or one of the L-malic acid standards (ranging from 0 - 3.0 g L^{-1}). The plate was incubated at 22˚C for 3 minutes and the first absorbance was read at 340 nm; 7 µL of the 1:10 diluted L-malate dehydrogenase (12,000 U mL^{-1}) was added and mixed into each well; the plate was incubated at 22˚C for 15 minutes before the second absorbance was measured at 340 nm. L-malic acid in each sample was calculated from standard curves prepared

with known L-malic acid concentrations. L-malic acid degradation was used as the determinant for MLF progress. MLF was deemed complete when L-malic acid concentration was < 0.1 g L^{-1} and designated *tend-MLF* (Table 2).

L-malic acid end point parameters were determined for yeast alone and yeast-SB3 co-inoculation fermentations. These parameters were: percentage of L-malic acid consumed or produced by yeast alone in relation to the starting L-malic acid concentration of 2.5 g L^{-1}, and percentage of L-malic acid consumed by yeast and LAB in co-inoculated fermentations. These were assigned *Pct_malic_AF* and *Pct_malic_co*, respectively.

To estimate the overall L-malic acid reduction by LAB when co-inoculated with yeast, the average concentration of L-malic acid for co-inoculated fermentations at the end of experimentation was subtracted from the average L-malic acid concentration for corresponding yeast-alone fermentations. This parameter was designated *Malic_acid_LAB_consumed*.

A summary of all parameters assessed in this study can be found in Table 2.

Table 2: AF and MLF measures used to perform QTL mapping, BN progeny evaluation or statistical analysis for comparison of hemizygote strains with their corresponding SBxGN or M2xF15 hybrids. Abbreviations, if assigned, are shown below:

AF measures	Abbreviation
Time to complete AF (hours)	tend-AF
Time to reach equivalent of 35% CO_2 (175.53 g L^{-1} total sugar)	t35-AF
Time to reach equivalent of 50% CO_2 (143.62 g L^{-1} total sugar)	t50-AF
Time to reach equivalent of 80% CO_2 (79.79 g L^{-1} total sugar)	t80-AF
Slope between t50 and t80	s50-80-AF
Percentage L-malic acid consumption or production by yeast alone	Pct_malic_AF

MLF measures	Abbreviation
Residual L-malic acid concentration for yeast alone fermentations (g L^{-1})	Not assigned
Percentage L-malic acid consumption for yeast-LAB co-inoculation fermentations	Pct_malic_co
Estimated overall L-malic acid consumed by LAB (g L^{-1})	Malic_acid_LAB_consumed
Time to complete MLF (hours)	tend-MLF

2.4. Statistical Analysis

All statistical analyses were performed using R versions 3.4.4 or higher, with expert assistance from Dr Philippe Marullo and Dr Emilien Peltier at the University of Bordeaux. Kendall correlation coefficient test was performed using R/stats package v3.6.2. The QTL mapping analysis was performed with the R/qtl package (Broman *et al.* 2003) by using the Haley-Knott regression model that provides a fast approximation of standard interval mapping (Haley & Knott 1992). A threshold corresponding to a 5% and 10% false discovery rate (FDR) was computed by performing 1000 permutations in order to assess the significance of the LOD score for QTL peaks (Churchill & Doerge 1994). The overall procedure was described by Peltier *et al.* (2018b) for multiple environments.

Linear modelling was performed to evaluate the effect of allele, yeast background and translocation on MLF and AF parameters using the following formula:

$$y_{ij} = m + B_i + T_j + B_i T_j + \in_{ij}$$

Where y_{ij} is the value for the background i (i = 1,2) with translocation j (j = 1,2), m is the overall mean, B_i is the yeast background effect, T_j is the translocation effect, $B_i T_j$ is the interaction effect between yeast background and translocation, $\in_{ij}$ is the residual error. Tukey post-hoc test (α = 0.05) was used to elucidate differences between ANOVA test groups.

3. Results

3.1. Biometric assessment of MLF completion in the SBxGN progeny population

In order to identify QTLs influencing the completion of MLF, L-malic acid consumption by *O. oeni* was measured in co-inoculated Shiraz grape juice fermentations. *S. cerevisiae* alone was able to consume a fraction of L-malic acid (Figure 2A). The concentration of residual L-malic acid at the end of AF in yeast-alone fermentations ranged from 1.41 g L^{-1} to 2.75 g L^{-1}, which corresponded to between 44% consumption and 10% production of L-malic acid in respect to the starting concentration of 2.5 g L^{-1} (Figure 2B; Table S1, Appendix C). The ability of yeast to either consume or produce this amount of L-malic acid is in agreement with previous findings (Delcourt *et al.* 1995; Peltier *et al.* 2018a; Yéramian, Chaya & Suárez Lepe 2007).

The continuous distribution of L-malic acid consumption or production observed among the yeast progeny suggests that this trait is controlled by many genes. A study detailing those genes is currently under preparation (Peltier *et al.* personal communication, February 2020).

In the present study, the focus was the impact of yeast genotype on LAB MLF efficiency. Therefore, we measured L-malic acid consumption over time for fermentations co-inoculated with *S. cerevisiae* strains and LAB SB3 (Figure 2C). As expected, L-malic acid consumption was much higher for many of the yeast-LAB co-inoculated fermentations. However, SB3 was only able to complete MLF in 39 of the 71 co-inoculated fermentations (Figure 2). Since LAB were only able to complete MLF when co-inoculated with some of the SBxGN progenies, this provided evidence of strong yeast-LAB interactions.

Though there were differences in residual L-malic acid across fermentations with different yeast strains, the ability of yeast to consume L-malic acid (as seen for yeast-alone fermentations) did not seem to impact MLF completion time by SB3 in co-inoculations. Kendall rank correlation coefficient revealed only a weak positive correlation (0.21, $p = 0.009$) between the amount of L-malic acid consumed by yeast and SB3 MLF completion time.

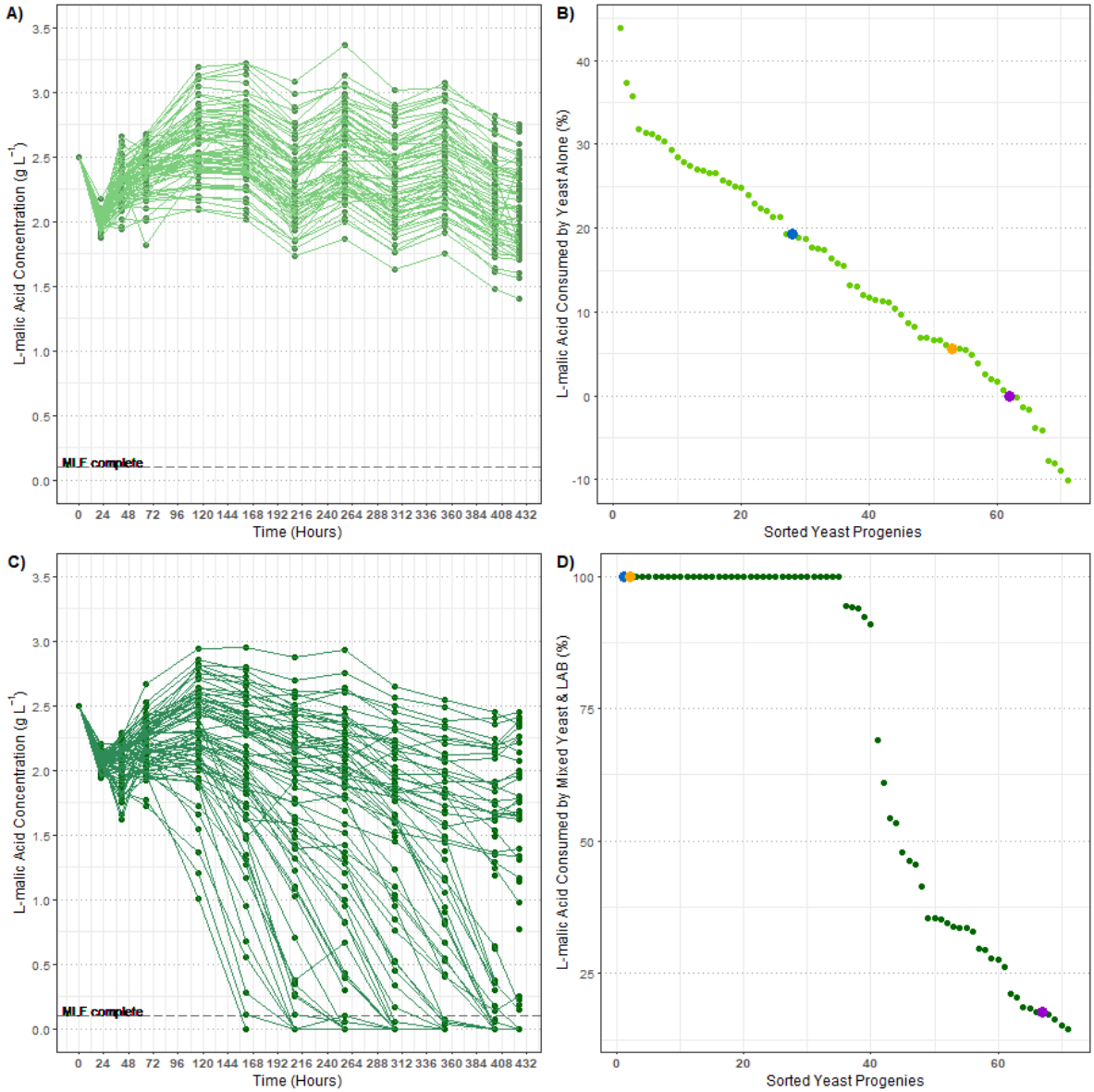

Figure 2: A) L-malic acid concentration measured over the course of the experiment for yeast-alone fermentations for the population of 67 SBxGN yeast progeny. Values are the mean of duplicates. **B)** All yeast-alone strains ranked by percentage of L-malic acid consumption (positive %) or production (negative %), measured at the end of the experiment in relation to the starting L-malic acid concentration of 2.5 g L^{-1}. Percentages were calculated from the mean of duplicates. Colours indicate yeast parental strains: BN (orange), SB (blue) and GN (purple). All other yeast progeny are shown in green. **C)** MLF progress measured for yeast co-inoculated with SB3 LAB. Values are the mean of triplicates. The horizontal line at 0.1 g L^{-1} indicates when MLF was deemed complete. **D)** All yeast strains in co-inoculations with SB3 LAB were ranked by the percentage of L-malic acid consumed, measured at the end of the experiment. Percentages were calculated from the mean of triplicates. Colours are the same as panel B.

3.2. QTL mapping

To determine the concentration of L-malic acid consumed only by LAB at the end of the experiment, average final yeast-LAB L-malic acid was subtracted from average final yeast-alone L-malic acid concentration. This new parameter, *malic_acid_LAB_consumed,* provides a more accurate measure for SB3 MLF efficiency after co-inoculation with different yeast strains. Additionally, this parameter has a nearly continuous distribution (Figure 3A) among the SBxGN progeny. Genetic regions linked to the variation of this trait were tracked by applying a linkage analysis. Despite the small number of progeny tested, three peaks were detected (Figure 3B). One peak, located on *S. cerevisiae* chromosome XVI, achieved a LOD score of 7.58 which is highly significant with respect to the threshold value of 4.58 that was estimated by 1000 permutations with an FDR of 5%. Two other peaks, located on *S. cerevisiae* chromosome XV and chromosome XIII, had lower LOD scores of 4.02 and 3.95, respectively. These LOD scores are close to the threshold value of 4.00 which corresponds to an FDR of 10%.

For *S. cerevisiae* chromosome XVI, the best marker for the QTL peak was located at genomic position XVI_374156 and was therefore named XVI_374. Due to the density of markers surrounding XVI_374 (6 markers within 817 bp) there was high confidence in the specificity of *SSU1* being the target of the QTL peak. SB3 co-inoculated with yeast meiotic clones with the SB allele at this position consumed more L-malic acid than when co-inoculated with clones with the GN allele (Figure 3C). Interestingly, this phenotypic discrepancy is not due to the ability of yeast to consume L-malic acid. In yeast-alone fermentations the inheritance of XVI_374 from either SB or GN did not alter the percentage of L-malic acid consumed by the yeast (Figure 3D). In contrast, most of the strains containing the yeast SB allele for this QTL allowed SB3 to complete MLF (Figure 3C, 3D). Altogether, this data

provides clear evidence that genetic regions of the *S. cerevisiae* genome have a direct impact on the metabolic activity of LAB during co-inoculation.

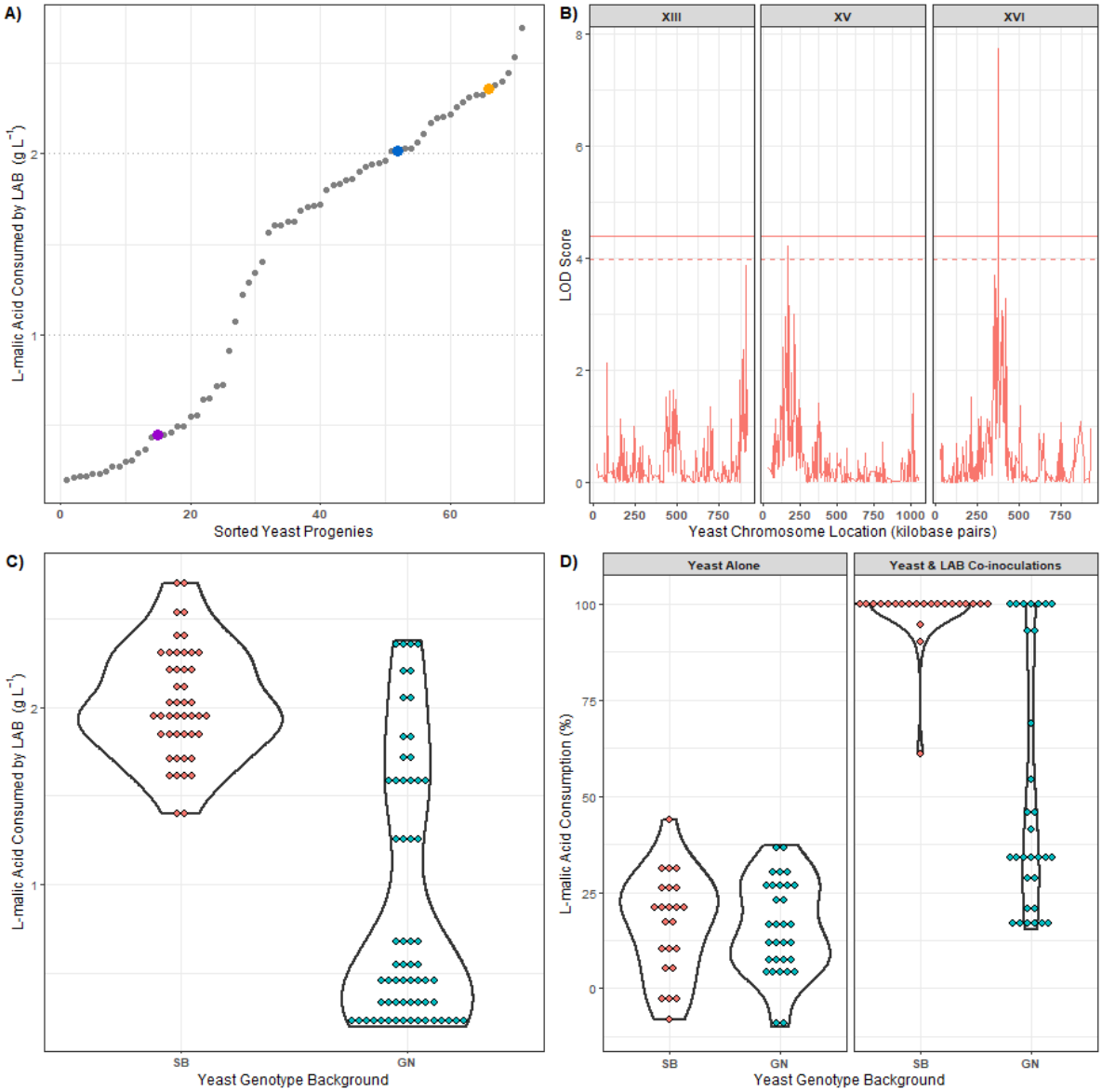

Figure 3: A) Yeast ranked by the concentration of L-malic acid that was able to be consumed by SB3 during co-inoculation with each yeast strain. Values are the mean of triplicates. Colours indicate yeast parental strains: BN (orange), SB (blue) and GN (purple). All other yeast progeny are shown in grey. **B)** Genomic location of QTL peaks for the parameter *malic_acid_LAB_consumed*. Threshold values are estimated from 1000 permutations and 5% FDR, indicated by the solid horizontal line. The dotted horizontal line indicates a LOD score threshold of 4. Significant (peak above threshold) and potential (peaks near a LOD score of 4) QTLs were found on chromosomes XIII (left), XV (middle) and XVI (right). **C)** Distribution of yeast progenies with respect to the concentration of L-malic acid consumed by SB3 in co-inoculations with each yeast strain. Progenies are grouped by yeast background (SB, left; GN, right). **D)** Distribution of yeast progenies based on percentage of L-malic acid consumed (measured at the end of experimentation) for yeast alone (left panel) or when co-inoculated with SB3 (right panel). Progenies are grouped by yeast background.

The peak at position XIII_909421 did not reach the threshold, but did fall within the 10% FDR and therefore warrants discussion. This marker spans a region containing *S. cerevisiae* gene YMR317W, which encodes a protein of unknown function.

The genomic positions of markers XV_162503 and XVI_374156 encompass the well-documented translocation break point between chromosomes XV and XVI (Zimmer *et al.* 2014; Figure 4) that segregate in the SBxGN progeny. This translocation impacts the *SSU1* gene that encodes Ssu1p, a transmembrane sulfite efflux pump (Peltier *et al.* 2018b; Zimmer *et al.* 2014).

To determine the influence of *SSU1* and the translocation on MLF outcome, reciprocal hemizygosity assay RHA was used, which involved generating two hemizygote yeast with one functional parental *SSU1* allele (i.e. SBxGN with either an SB or GN *SSU1* allele; Figure 4).

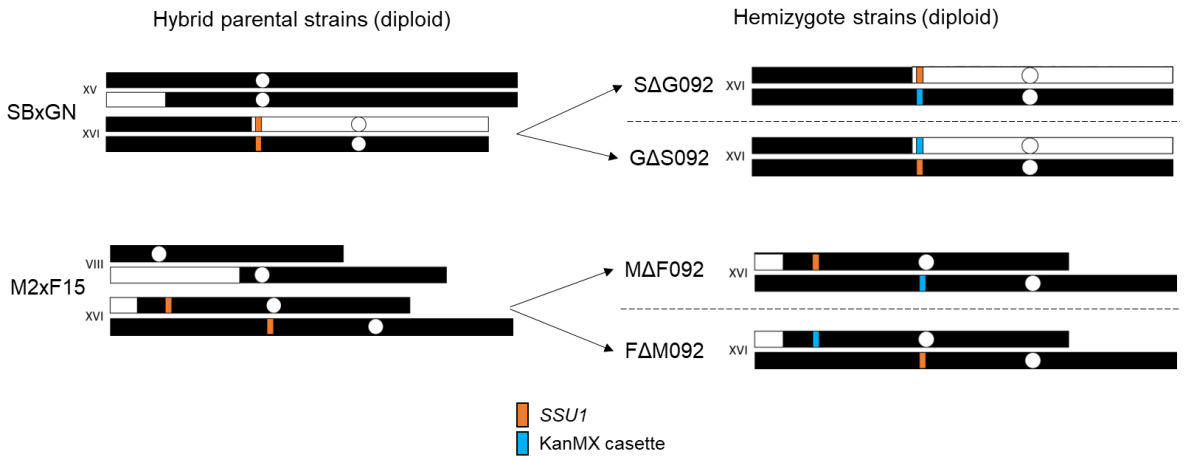

Figure 4: Representation of the translocations located in SBxGN and M2xF15 that result in increased *SSU1* gene expression due to reduced proximity between *SSU1* and promotor regions. **A)** SBxGN has an XV-t-XVI translocation that leads to a single copy of wild-type XV and XVI chromosomes (all black) and reciprocal XV and XVI translocated chromosomes (black and white). Hemizygote strains SΔG092 and GΔS092 with a single *SSU1* allele (orange) were generated to perform a reciprocal hemizygosity assay. **B)** M2xF15 has a VIII-t-XVI translocation that leads to a single copy of wild-type VIII and XVI chromosomes (all black) and reciprocal VIII and XVI translocated chromosomes (black and white). In addition to SΔG092 and GΔS092 hemizygote strains MΔF092 and FΔM092 were also created. Hemizygote strains were created by replacing a single copy of *SSU1* with a KanMX cassette (blue).

3.3. Functional study of a QTL closely related to the *SSU1* gene

To determine the impact of the yeast *SSU1* allele (translocated or wild-type) on SB3 MLF, hemizygote yeast containing either the GN *SSU1*-translocation (*SSU1*-t) or SB *SSU1*-wild-type (*SSU1*-wt) allele in the SBxGN background (SΔG092 and GΔS092 respectively) were co-inoculated with SB3. Unexpectedly, there was no significant difference in MLF completion time when SB3 was co-inoculated with the hemizygote strains (Figure 5). A difference in MLF completion was expected for the hemizygote strains because in the QTL study, SBxGN progeny with GN inheritance, and therefore the translocation (XV-t-XVI), did not allow SB3 to complete MLF as often as progeny with SB inheritance (Figure 3D). Additionally, previous work revealed SΔG092 yeast had a shorter lag phase of growth and increased viability in comparison to GΔS092 (Zimmer *et al.* 2014), leading to the hypothesis that LAB co-inoculated with SΔG092 would negatively impact SB3 MLF performance. Hence it was expected that SB3 co-inoculated with SΔG092 would result in slower MLF than co-inoculation with GΔS092.

To further explore how presence of *SSU1*-t may affect SB3 ability to complete MLF, hemizygote yeast strains were also constructed in the M2xF15 background (MΔF092 and FΔM092). Similar to GN, F15 also has a translocation, VIII-t-XVI (Roncoroni 2014), albeit a different translocation to GN. The VIII-t-XVI translocation has been previously reported to generate the *SSU1-R* allele (Goto-Yamamoto *et al.* 1998; Pérez-Ortín *et al.* 2002), which leads to increased *SSU1* expression (Park & Bakalinsky 2000; Pérez-Ortín *et al.* 2002), akin to XV-t-XVI. Additionally, yeast with VIII-t-XVI have similar growth kinetics and response to SO_2 as yeast with XV-t-XVI (Zimmer *et al.* 2014), making them comparable for this study. Here, however, unlike SΔG092 and GΔS092, there was a difference in MLF completion time for MΔF092 and FΔM092, with the expected result of SB3 completing MLF faster when

co-inoculated with the *SSU1*-wt strain FΔM092 (Figure 5). This result may be partially explained by overall genetic differences between SBxGN and M2xF15 (Peltier *et al.* 2018b), as well as differences between the translocations. Nevertheless, the difference in MLF for MΔF092 and FΔM092 co-inoculation fermentations indirectly supports the QTL findings.

Although the pattern for MLF completion with hemizygote strains differed, it was clear that *SSU1* haploinsufficiency had a large impact. SB3 co-inoculated with hemizygote strains resulted in 72 to 96 hours faster MLF completion in comparison to their respective hybrid strains (Figure 5).

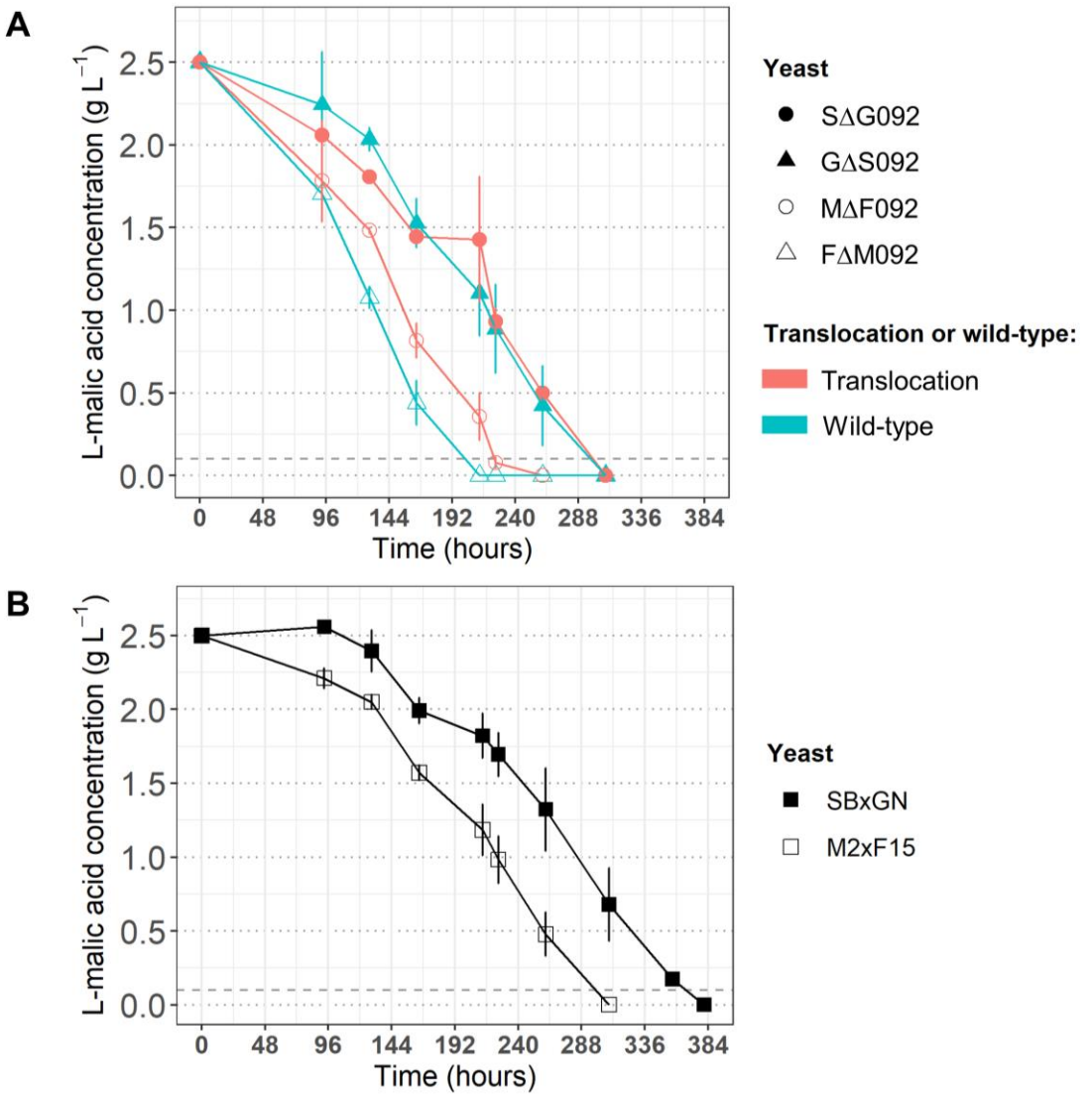

Figure 5: A) Malolactic fermentation by SB3 during co-inoculation with hemizygote yeast strains SΔG092 (filled circle), GΔS092 (filled triangle), MΔF092 (empty circle) and FΔM092 (empty triangle). Colour indicates the presence of a translocation (red) or wild-type (blue) *SSU1*. **B)** Malolactic fermentation by SB3 during co-inoculation with SBxGN (filled squares) and M2xF15 (empty squares). Values are the mean of triplicates and error bars are the standard deviation.

Statistically, AF completion time was significantly slower for SΔG092 compared to SBxGN and GΔS092, though observationally they were very similar (Figure 6). SΔG092 had significantly slower AF completion and t35-AF when co-inoculated with SB3 (Figure 6; ANOVA, Tukey post-hoc, $p < 0.05$, Table S2, Appendix C). In contrast, FΔM092 completed AF later than M2xF15 and MΔF092, and SB3 co-inoculation had no effect on AF (Figure 6; ANOVA, Tukey post-hoc, $p < 0.05$; Table S2, Appendix C). When comparing all the yeast, AF completion time and s50-80-AF (slope between t50-AF and t80-AF; Table 2) were significantly affected by translocation and background (ANOVA, Tukey post-hoc, $p < 0.05$; Table S3, Appendix C). Specifically, presence of the translocation exhibited different AF effects, with SΔG092 having slower AF, while MΔF092 had faster AF in comparison to the *SSU1*-wt GΔS092 and FΔM092 strains, respectively. For the current study, it was unable to be discerned why such a difference in AF kinetics occurred.

Overall it was observed that the yeast had greater impact on SB3 MLF ability compared to the impacts of co-inoculation on AF. In consideration of this, the difference between SB3 MLF completion when co-inoculated with MΔF092 and FΔM092 may be a direct result of difference in AF rate. Though it has been reported previously that the VIII-t-XVI and XV-t-XVI can both result in reduced lag phase and increased viability (Zimmer *et al.* 2014), this result was not observed in our work (Figure S1, Appendix C). This led to the conclusion that faster AF by MΔF092 impacted the ability of SB3 to complete MLF in comparison to co-inoculation with FΔM092. It was also unable to be discerned if the outcome was related to SO_2 efflux by MΔF092, as the measurement method (Rankine & Pocock 1970) revealed no differences between the yeast strains, though the SO_2 concentration may have been below the detectable limit.

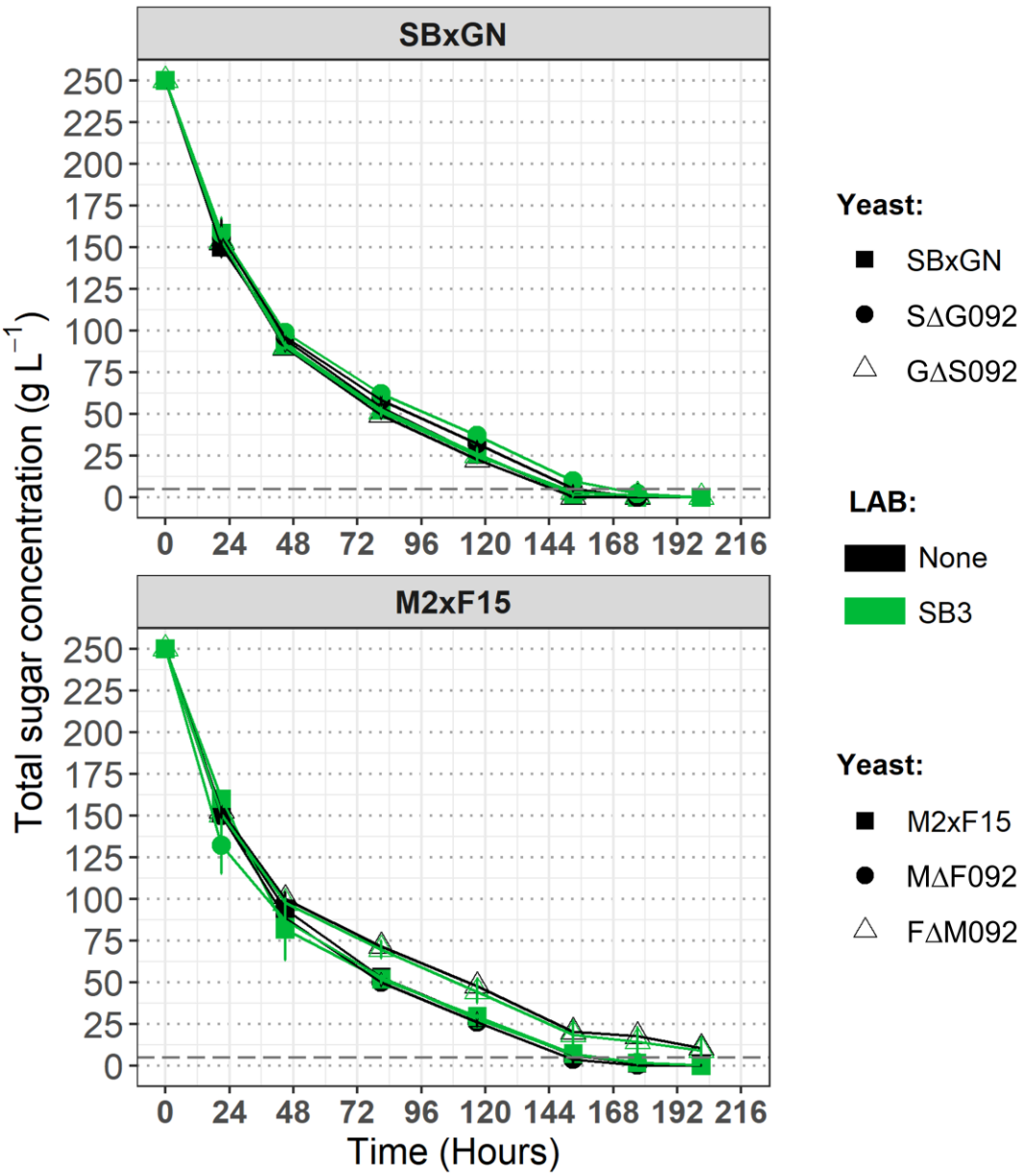

Figure 6: Alcoholic fermentation performed by SBxGN, SΔG092, GΔS092 (top), M2xF15, MΔF092 or FΔM092 (bottom). Shape indicates yeast strain, colour indicates yeast alone (black) or yeast co-inoculated with SB3 (green). The horizontal dashed line at 3 g L^{-1} indicates AF completion. Values are the mean of triplicates and error bars are the standard deviation.

4. Discussion

For the first time a QTL was identified for yeast-LAB interactions during co-inoculated fermentation. QTL mapping has been used numerous times for *S. cerevisiae* to determine the genotypic traits that influence yeast AF (Marullo *et al.* 2019), acetic acid production (Salinas *et al.* 2012), nitrogen uptake (Brice *et al.* 2014; Jara *et al.* 2014) and aroma compound production (Eder *et al.* 2018; Huang, Roncoroni & Gardner 2014; Roncoroni *et al.* 2011; Steyer *et al.* 2012) which are important for fermentation progress and wine quality. However, to the best of our knowledge, QTL mapping has not been used to study yeast-bacteria interactions in wine.

QTLs have also been used for a range of microbial and plant species to elucidate genetic differences that relate to a particular phenotype (Chen *et al.* 2010; Huang, Roncoroni & Gardner 2014; Marullo *et al.* 2007; Weng *et al.* 2008). For the methods used in the current work, the most closely related studies involve QTL mapping for plant responses to pathogens (Chen *et al.* 2010; Decroocq *et al.* 2005; Eun *et al.* 2016). Similar to the methods in our study, the plant QTL progenies were exposed to a pathogen, then the genotype relating to the phenotype of pathogen resistance was mapped. This has been performed numerous times, successfully unveiling plant genotypic links to pathogen resistance (Chen *et al.* 2010; Decroocq *et al.* 2005; Eun *et al.* 2016). Similarly, in the current work, *S. cerevisiae* SBxGN progeny (pop BN) were co-inoculated (i.e. exposed) to LAB during fermentation. The phenotype of LAB completing MLF was used to map yeast genotypes that corresponded to LAB MLF completion or inhibition. Though slightly more complex than plant-pathogen QTL studies, the overall concept was similar. In future, QTL studies with a larger number of yeast progeny could be used to further understand

how yeast genotypic differences specifically enable or hinder LAB MLF during co-inoculation.

Previously, studies investigating yeast-LAB interactions during juice fermentation relied on AF and MLF kinetics and production of volatile and non-volatile compounds (Arnink & Henick-Kling 2005; Comitini & Ciani 2007; Mendoza *et al.* 2010; Nehme, Mathieu & Taillandier 2008, 2010). Many combinations of yeast and LAB, either sequentially or co-inoculated in juice or wine, have revealed that yeast-LAB compatibility is strain specific (Comitini *et al.* 2005; Comitini & Ciani 2007; Muñoz, Beccaria & Abreo 2014). Considering the differences reported for different yeast and LAB strains, and production of different metabolites by yeast strains, there is no question of the influence that yeast genetic makeup has on co-inoculation outcomes. In addition to the studies on strain combination and metabolite production, further work has revealed gene expression differences within *S. cerevisiae* in response to co-inoculation with *O. oeni* (Rossouw, du Toit & Bauer 2012). Though insightful, none of these works have identified specific genetic differences between yeast strains that may influence compatibility with *O. oeni*. Hence the present work has laid a foundation for understanding how *S. cerevisiae* genetic makeup can impact MLF outcomes during co-inoculation with *O. oeni.*

The QTL peak at position XIII_909421 spans a region containing *S. cerevisiae* gene YMR317W, which encodes a protein of unknown function. Though this peak did not reach the threshold, it has potential for impacting MLF by SB3. Although intriguing, it is currently unknown how the presence of this gene may impact MLF and yeast-LAB interactions, but in future larger QTL studies may uncover its overall impact.

The QTL identified on chromosome XVI spans the genomic region containing the *SSU1* (YPL092W) gene and a well-known translocation, XV-t-VXI (Peltier *et al.* 2018b; Treu *et al.* 2014; Zimmer *et al.* 2014). *SSU1* encodes Ssu1p, an intermembrane transporter that is responsible for *S. cerevisiae* sulfite efflux (Park & Bakalinsky 2000). The efficiency of Ssu1p is important for *S. cerevisiae* performance, since sulfite export is used as a defence mechanism in response to excessive sulfite that can be detrimental to yeast cells (Park & Bakalinsky 2000). In terms of co-inoculation, yeast efficient in sulfite export could negatively impact *O. oeni*, since sulfite can inhibit *O. oeni* internal ATPase (Carrete *et al.* 2002), thereby inhibiting growth and MLF.

The translocation XV-t-VXI results in a decrease in distance between the *ADH1* promoter region and *SSU1* (Zimmer *et al.* 2014). This decrease in distance has been reported to lead to increased expression of *SSU1* and reduced lag phase duration (Peltier *et al.* 2018b; Zimmer *et al.* 2014). Similarly to XV-t-XVI, XIII-t-XVI has also been well-defined in wine yeast (Pérez-Ortín *et al.* 2002). In the current work, XIII-t-XVI was located in MΔF092. XIII-t-XVI results in the *SSU1-R* allele, where the transcriptional activator Fzf1p promotor region is in closer proximity to *SSU1* (Pérez-Ortín *et al.* 2002). *SSU1-R* also results in increased *SSU1* expression, making it an ideal candidate for comparison.

To elucidate if the QTL found truly related to MLF outcome in co-inoculation, a reciprocal hemizygosity assay was performed. This method involves the use of hemizygote hybrid strains containing a single parental allele of the gene of interest. In this work, hemizygote hybrid strains SΔG092, GΔS092, MΔF092 and FΔM092 were constructed from SBxGN and M2xF15 diploids, respectively. SΔG092 and MΔF092 contained the translocated allele (*SSU1*-t), while GΔS092 and FΔM092 had the wild-type allele (*SSU1*-wt). It was hypothesised that SB3 co-inoculation with

S∆G092 and M∆F092 would result in slower MLF (due to increased *SSU1* expression) than co-inoculations with G∆S092 and F∆M092. However, this only occurred for M∆F092; SB3 co-inoculated with S∆G092 and G∆S092 had similar MLF. This discrepancy for the SBxGN hemizygotes could be a result of the use of haploid strains in the QTL mapping experiment and diploid strains in the reciprocal hemizygosity assay. Diploid strains likely have different metabolic capabilities and differences in transcriptional regulation, however it is not known what the actual effects may be.

The SBxGN hemizygote strains did not support the hypothesis that SB3 co-inoculated with yeast harbouring *SSU1*-t would result in slower MLF compared to co-inoculations with yeast containing *SSU1*-wt. However, the result obtained using the M2xF15 hemizygotes provide indirect support of this hypothesis. The difference in result between the M2xF15 and SBxGN hemizygotes may be a result of the different translocations. *SSU1* expression for VIII-t-XVI has been shown to be 3-fold less than *SSU1* expression with the XV-t-XVI translocation under the same growth conditions (Zimmer *et al.* 2014). In comparing the M2xF15 hemizygote strains with SBxGN strains, this is in agreement with Zimmer *et al.* (2014), since MLF was slower with SBxGN hemizygote strains. The difference in overall genetic makeup of M2xF15 strains and SBxGN strains may also explain why SB3 performed differently for the hemizygotes. Generally, SB3 was able to complete MLF faster with M2xF15, M∆F092 and F∆M092 compared to SBxGN, S∆G092, and G∆S092. Nevertheless, there is evidence that the presence of a XV-t-XVI or XIII-t-XVI translocation could inhibit MLF by LAB. Though it is important to consider that wild-type strains, though potentially more compatible for MLF, may have the trade-off of a slower lag phase.

From this work, the greatest impact was *SSU1* haploinsufficiency. Hemizygote strains SΔG092 and GΔS092 displayed no adverse differences in AF. But for FΔM092 AF was sluggish. In terms of MLF, the impact was significant. SB3 co-inoculation with SΔG092, GΔS092, MΔF092 and FΔM092 resulted in a 72 to 96 hour decrease in MLF completion time compared to SBxGN and M2xF15, respectively. The generation of *SSU1* haploinsufficient strains could be useful for industry, especially in winemaking where SO_2 addition is avoided. In this scenario, yeast with *SSU1* haploinsufficiency may not be adversely affected by decreased ability to export sulfite, since sulfite exposure would arise from yeast (or other microbes) sulfite production in the fermentation. With this, yeast strains with *SSU1* haploinsufficiency may enable greater compatibility with LAB, resulting in overall faster fermentation. However, much more work is needed to confirm how *SSU1* haploinsufficiency may impact sensorial properties of wine, and if this effect can be repeated in different juice types and yeast strains.

5. Conclusions

For the first time, yeast genetic background was assessed for its role in yeast-LAB compatibility during fermentation. The impact of *SSU1* haploinsufficiency on LAB ability to complete MLF was clear, but there is much more work needed to understand the role of XV-t-VXI and XIII-t-XVI on MLF outcomes. The influence of *SSU1* in this work adds to the understanding of the pleiotropic role of *SSU1,* since it was reported to impact yeast AF, growth and SO_2 production, and now also has the potential to impact co-inoculation outcomes with LAB. This work starts to unravel the complexity of *S. cerevisiae* genetic differences that can lead to a phenotype that impacts *O. oeni* during co-inoculation. Understanding the delicate interplay between genotype and phenotype can create opportunities for wine yeast manufacturers to

develop yeast that work effectively with LAB, without negatively impacting yeast AF performance.

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Chapter 6

Saccharomyces sulfur pathway regulation in response to co-inoculation with *Oenococcus oeni*

1. Introduction

As has been demonstrated in Chapters 3, 4 and 5, yeast and lactic acid bacteria (LAB) compatibility during wine fermentation is inherently complex. One relationship that emerged from this work was the potential role of sulfur related metabolites in LAB performance. In Chapter 4, H_2S measurements taken throughout co-inoculation fermentations revealed that yeast H_2S production differed based on the LAB strain it was co-inoculated with. In some cases this resulted in higher H_2S concentrations compared to yeast only controls. In Chapter 5 quantitative trait loci (QTL) analysis revealed a single QTL that was linked to the total amount of L-malic acid LAB strain SB3 was able to consume when co-inoculated with yeast. The QTL encompassed a translocation and the *SSU1* gene. Follow-up analysis using hemizygote yeast with a single *SSU1* allele revealed that *SSU1* haploinsufficiency enabled faster MLF by LAB strain SB3. Ssu1p is responsible for sulfite efflux in *S. cerevisiae* (Zimmer *et al.* 2014), thus inability to efficiently export sulfite could enable surrounding LAB to continue MLF without sulfite inhibition.

The metabolism of sulfur compounds by both yeast and LAB during fermentation and their effect on co-inoculation success was a tantalising prospect. Therefore, potential interactions were initially investigated *in-silico* by reviewing the available literature and then tested by qPCR analysis. Sulfur in the form of molecular SO_2 inhibits LAB growth and MLF performance by binding acetaldehyde that is then internalised by LAB, eliciting a bacteriostatic effect after the sulfur ions are released from acetaldehyde (Bartle *et al.* 2019a; Osborne, Dubé Morneau & Mira de Orduña

2006; Wells & Osborne 2012). Sulfur in other forms, such as H_2S, is undesirable for wine when produced late in alcoholic fermentation (AF) due to its offensive aroma (Franco-Luesma *et al.* 2016). Wine yeast are capable of releasing excess H_2S under low nitrogen conditions (Huang *et al.* 2017; Jiranek, Langridge & Henschke 1995b), as a direct result of insufficient levels of O-acetyl homoserine (OAS) available to bind H_2S for amino acid synthesis (Jiranek, Langridge & Henschke 1995b). The process of H_2S production via the sulfate assimilation pathway within *S. cerevisiae* during fermentation has been well documented (Figure 1) and was reviewed recently (Huang *et al.* 2017).

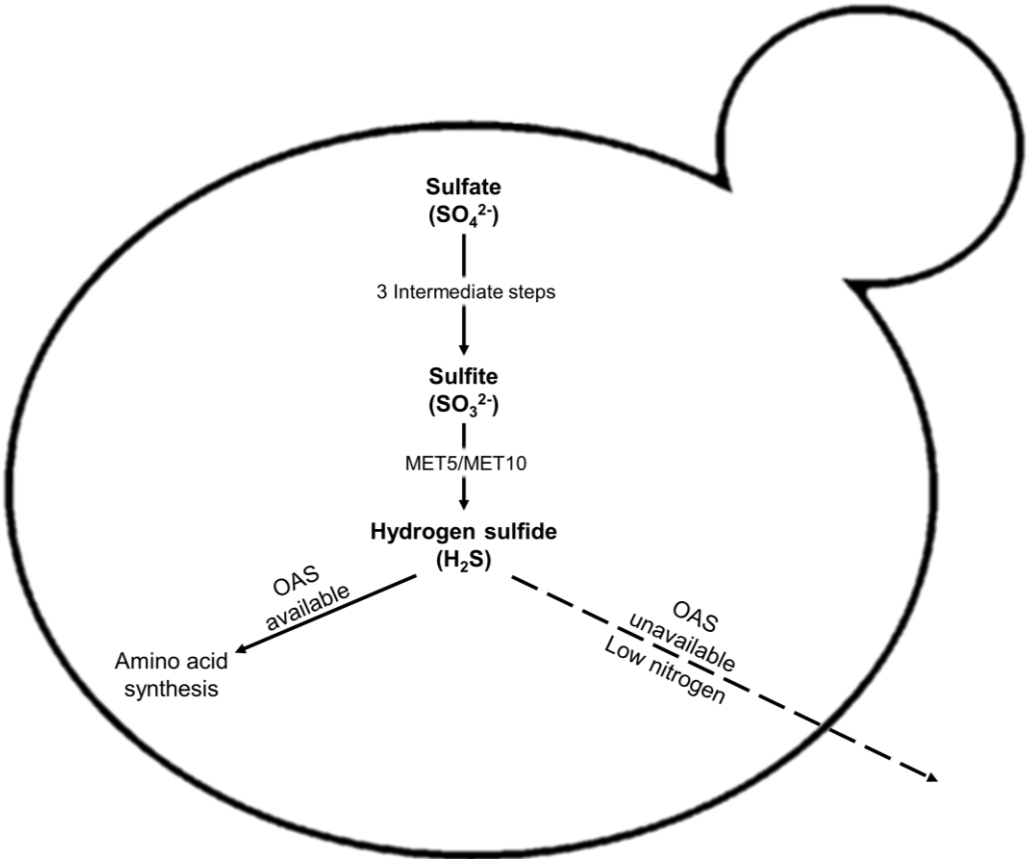

Figure 1: Simplified sulfate assimilation pathway in *S. cerevisiae*. When O-acetyl homoserine (OAS) is available it binds with H_2S for amino acid synthesis. Under low nitrogen conditions, such as the end of alcoholic fermentation, O-acetyl homoserine is unavailable to bind with H_2S and excess H_2S is liberated from the cell. More detailed information can be found on the *Saccharomyces* Genome Database (2007a).

In contrast to yeast, the role of H_2S in *O. oeni* health is unclear. Metabolically, H_2S can be utilised to form cysteine within *O. oeni*, similar to *S. cerevisiae*. An *in-silico O. oeni* metabolic model demonstrated that under ethanol stress, *O. oeni* consumed cysteine faster than other amino acids, therefore highlighting the importance of cysteine in *O. oeni* stress resistance (Contreras *et al.* 2018). It is plausible that *O. oeni* may obtain cysteine from cysteine-containing precursors in conditions where external cysteine is low, such is the case for juice and wine (Huang *et al.* 2017). One of these precursors, glutathione (GSH; glutamyl-L-cysteinylglycine), is commonly found in wine, however there is currently no known link between glutathione and cysteine pathways within *O. oeni* (Kanehisa Laboratories 2019). Therefore, this work aimed to gain information about glutathione concentration over the course of co-inoculation and related gene regulation within yeast and LAB.

As well as being a cysteine precursor, glutathione is known to be protective for yeast and *O. oeni* when they are exposed to the harsh conditions of wine (low pH, increasing ethanol, oxidative stress; Inoue *et al.* 1999). The metabolic processes required for production and metabolism of glutathione are well documented for *S. cerevisiae* (Figure 2A; Avery & Avery 2001; Elskens, Jaspers & Penninckx 1991; Mehdi & Penninckx 1997; Inoue *et al.* 1999; Penninckx 2002). In *S. cerevisiae*, *CYS3* and *CYS4* encode cystathionine γ-lyase and cystathionine β-synthase which catalyse the reactions: homocysteine → cystathionine and cystathionine → cysteine, respectively (*Saccharomyces* Genome Database 2008). Cysteine may be used for glutathione synthesis, or may also be produced by glutathione breakdown (*Saccharomyces* Genome Database 2007c). *GSH1* and *GSH2* encode γ-glutamylcysteine synthetase and glutathione synthetase, respectively, catalysing the formation of γ-glutamyl-L-cysteine, followed by GSH synthesis (*Saccharomyces*

Genome Database 2007c). *ECM38, DUG2 and DUG3*, that encode glutathione gamma-glutamate hydrolase and a complex comprised of Dug2p and Dug3p respectively, breakdown GSH to L-cysteinylglycine (Ganguli, Kumar & Bachhawat 2007; *Saccharomyces* Genome Database 2007c). Subsequently L-cysteinylglyicine can be broken down to cysteine via Dug1p enzyme, encoded by *DUG1* (Ganguli, Kumar & Bachhawat 2007).

Recently, analogous pathways in *O. oeni* have also been discovered, though *O. oeni* is only able to metabolise glutathione and currently has not shown the ability to synthesise it (Figure 2B; Margalef-Català *et al.* 2017). *cydC* and *cydD* encode a dimeric transporter, CydDC, which is capable of transporting GSH and cysteine into the cell (Pophaly *et al.* 2012). *gshR* and *gpo* encode glutathione reductase and glutathione peroxidase, respectively, that breakdown GSSG to GSH, or generate GSSG from GSH (Figure 2B; Smirnova & Oktyabrsky 2005; Margalef-Català *et al.* 2017).

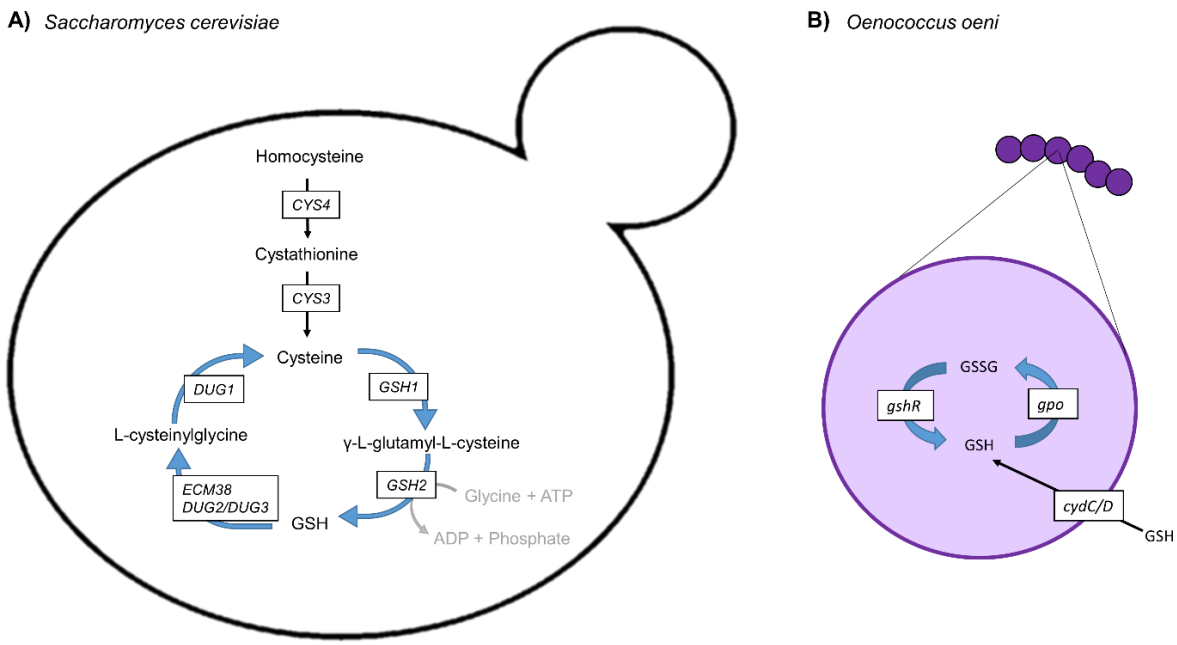

Figure 2: Glutathione (GSH) metabolism in *S. cerevisiae* (A) and *O. oeni* (B). Diagrams are based on information from the *Saccharomyces* Genome Database (2007c, 2008), Ganguli, Kumar & Bachhawat (2007) and Margalef-Català *et al.* (2017).

In addition to the role of sulfur in yeast-LAB compatibility, there is little information about the influence of co-inoculation on *O. oeni* gene expression. Gene regulation related to the stress response of *O. oeni* to wine conditions has been studied in great depth, however, these studies focus on sequential inoculation or specific stressors (i.e. ethanol, pH, SO_2) in other growth media (Betteridge *et al.* 2018). As for yeast, there have been a limited number of studies that have explored global transcriptional changes within *S. cerevisiae* when co-inoculated with *O. oeni* (Rossouw, du Toit & Bauer 2012). It was reported that regulation of sulfur-related pathways was increased in response to co-inoculation (Rossouw, du Toit & Bauer 2012), but at the time there was no other supporting information about the role of sulfur in yeast-LAB interactions.

Assessment of literature regarding *S. cerevisiae* and *O. oeni* AF and MLF and co-inoculation has highlighted the importance of sulfur metabolism for both *S. cerevisiae* and *O. oeni*. Previous chapters have also pointed to a possible role of sulfur metabolism in yeast-LAB compatibility. Accordingly, this chapter describes steps taken to gain a greater understanding of the effect this might have during co-inoculation of yeast and LAB in juice.

Two commercial *S. cerevisiae* and two commercial *O. oeni* strains were both sequentially inoculated and co-inoculated in sterile Shiraz juice. Production of key metabolites, including H_2S, was measured throughout co-inoculation and gene expression was measured at set time points (48 hours and 96 hours post LAB inoculation). Genes chosen for analysis were either identified as being involved in a sulfur related pathway within yeast or LAB, or were previously reported reference and stress response genes. The results from this work have deepened our current understanding of yeast sulfur metabolism during co-inoculation with *O. oeni*, as well as opened avenues for future work aimed at delineating yeast-LAB compatibility.

2. Materials and methods

2.1. Shiraz juice preparation, yeast and bacteria strains and fermentation conditions

Sterile Shiraz juice was prepared as described in Chapter 4. Considering the results from Chapter 4, two yeast-LAB pairs were chosen for in depth analysis. Yeast strains NT50 and EC1118 were chosen for their levels of H_2S production: NT50 produced the highest amounts of H_2S of all yeast and EC1118 the second highest. LAB strains VP41 and Alpha were also selected for their apparent contribution to H_2S: yeast cultures co-inoculated with VP41 had higher H_2S than the corresponding yeast-only controls, while strains co-inoculated with Alpha had no difference compared to the corresponding yeast-only controls.

Yeast were rehydrated and inoculated into sterile Shiraz juice according to the manufacturer's protocols. Prior to inoculation, LAB were grown in MRSAJ for 4 days at 30°C, 20% CO_2, centrifuged for five minutes at 2,236 x *g*, washed with sterile Shiraz juice and grown overnight in sterile Shiraz juice. LAB OD_{600} was adjusted to 0.55 and inoculated into fermentations at a final dilution of 1:100.

Volume and fermentation conditions were the same as described in Chapter 4: 150 mL sterile Shiraz juice in shake flasks fitted with a glass airlock and temperature kept at 22°C. For sequential fermentations, LAB were inoculated 2 days post-AF completion. For co-inoculation fermentations, LAB were inoculated 24 hours post-yeast inoculation. In both instances LAB were inoculated into the flasks through a sampling port with a sterile 21-guage needle. Sequential, co-inoculation and yeast-only fermentations were performed with 6 biological replicates.

Samples (200 µL) were collected at time-points over the course of fermentation for analysis of yeast and LAB growth, AF and MLF progress, free amino nitrogen

consumption, hydrogen sulfide and total glutathione production. Larger volume samples (1 mL) were collected at 48 and 96 hours post-LAB inoculation for transcriptional analysis.

2.2. Enzymatic assays for glucose, fructose, L-malic acid, nitrogenous compounds and total glutathione

Glucose, fructose, L-malic acid and free amino nitrogen (excluding proline and ammonium) enzymatic assays were performed following methods described in Chapter 4.

Total glutathione was also determined by enzymatic assay (catalogue # CS0260, Sigma-Aldrich, Australia). The optimal sample concentration was determined by testing a range of sample dilutions (1:5, 2:5, 3:5, 4:5 diluted with 5% 5-sulfosalicylic acid (SSA) and undiluted). A sample was deemed suitable if the measured increased in absorbance was steady over 5 minutes, comparable to the standard curve. Total glutathione was measured for four co-inoculation and three sequential time-points: 1, 3, 5 and 7 days and 11, 13 and 15 days, respectively. Samples were measured in duplicate for each time-point. To perform the assay, 150 µL of working mixture containing 95 mM potassium phosphate buffer (0.95 mM EDTA, pH 7.0), 0.16 U mL^{-1} glutathione reductase and 0.04 mg mL^{-1} 5,5'-dithiobis (2-nitrobenzoic acid) was incubated with 6 µL of sample and 4 µL of 5% SSA, or 10 µL of standard only (0, 3.125, 6.25, 12.5, 25 or 50 µM glutathione) at room temperature for 5 minutes. 50 µL of NADPH (0.16 mg mL^{-1}) were added to each well and mixed by pipette before measuring absorbance (412 nm) at 1 minute intervals for five minutes. At each absorbance reading, the 0 µM GSH standard value was

subtracted from all other measured values. The standard curve was generated by using the following calculation for standard samples:

The standard curve was used to calculate the ΔA_{412} minute^{-1} for 1 nM of GSH. The concentration of GSH in each unknown sample was calculated using the following equation:

$$x = \frac{y \times \text{dilution of sample}}{z \times \text{volume of sample}}$$

Where x is the concentration of GSH in the sample (nM), y is ΔA_{412} minute^{-1} for the sample and z is ΔA_{412} minute^{-1} for 1 nM GSH.

2.3. Hydrogen sulfide

Hydrogen sulfide was measured using silver nitrate H_2S detector tubes with a detection range of 25-1000 ppm (catalogue # 120SF, Kitagawa America LLC, USA).

2.4. Total RNA extraction

An initial trial was performed to determine the volume of sample required to have amplification of both yeast and LAB cDNA from a mixed yeast and LAB fermentation sample. The trial samples were taken from three replicate yeast-LAB co-inoculation fermentations in Shiraz juice, 24 hours post-LAB inoculation. Sample volumes of 10, 5, 2, and 1 mL were taken from each fermentation, and the RNA extracted as detailed below. The most appropriate sample volume was 1 mL. This volume was used for all subsequent RNA extractions.

RNA was extracted from samples at two time points (6 biological replicates for 2 yeast-LAB pairs and 2 yeast-only controls; 48 and 96 hours post-LAB inoculation).

Samples were collected aseptically and centrifuged for two minutes at 20,238 x *g*. The supernatant was removed and the pellet was resuspended in 1 mL of 80% -20°C ethanol. Samples were stored at -80°C prior to Trizol treatment.

Samples were centrifuged for 30 seconds at 3,824 x *g*, the supernatant was removed and the cell pellet was resuspended in 1 mL of Trizol reagent (catalogue # 15596018, Invitrogen, Australia). The samples were frozen in liquid nitrogen for 30 seconds, then stored at -80°C before continuing.

The samples were defrosted, followed by the addition of glass beads (catalogue # G8772, Sigma-Aldrich, Australia) up to 50% of the volume. Cells were lysed by vortexing in 30 second increments 3 times, separated by 30 second intervals on ice to prevent samples from overheating. Samples were incubated at 65°C for 3 minutes, then 200 µL of chloroform was added, followed by vortexing for 15 seconds and incubation at room temperature for 5 minutes. Samples were centrifuged at 20,817 x *g*, 4°C for 10 minutes, and the colourless liquid phase transferred to a new tube containing 500 µL isopropanol. The tubes were inverted 6 times and incubated at room temperature for 10 minutes, then centrifuged 20,817 x *g*, 4°C for a further 10 minutes. The supernatant was removed and the pellet washed twice with 1 mL 80% -20°C ethanol, by addition of ethanol, centrifugation (20,817 x *g*) at 4°C for 10 minutes, then removal of ethanol. After the second ethanol wash step, the ethanol was removed and the pellet allowed to air-dry in a laminar flow for up to 10 minutes. The RNA pellet was dissolved in 75 µL diethyl pyrocarbonate treated water (catalogue # AM9916, Invitrogen, Australia) and stored at -80°C.

2.5. RT-qPCR

RNA samples were treated with Turbo DNase (catalogue # AM1907, Invitrogen, Australia), to remove genomic DNA, following the manufacturers protocol for routine treatment. After Turbo DNase treatment, RNA quality and concentration were measured using a NanoDrop 1000 and NanoDrop One spectrophotometer (Thermo Scientific). For samples undergoing downstream yeast gene analysis (yeast-alone and co-inoculation samples), 250 ng input RNA was used for cDNA synthesis. Samples that were to be analysed for LAB genes (co-inoculation samples only) had 400 ng input RNA for cDNA synthesis.

cDNA was synthesised using the iScript cDNA Synthesis Kit (catalogue # 1708891, Bio-Rad). Genomic DNA contamination was checked using real-time qPCR.

2.5.1. Reference and candidate gene selection

Reference genes for both yeast and bacteria were selected based on previous work (Beltramo *et al.* 2006; Desroche, Beltramo & Guzzo 2005; Sumby, Grbin & Jiranek 2012; Vaudano *et al.* 2011). Candidate genes were selected after extensive analysis of sulfur metabolic pathways present in yeast and LAB. In consideration of the glutathione results, metabolic pathways that involved glutathione breakdown or metabolism were assessed. This lead to selection of *GSH1, GSH2, ECM38, DUG1, DUG2, DUG3* and *OPT1* yeast genes, which encode proteins that catalyse reactions in the glutathione pathway for yeast or glutathione transport (Figure 3A). For *O. oeni,* four genes were selected: *gshR, gpo, cydC* and *cydD.* These genes are involved in the cyclic synthesis of glutathione (GSH) and glutathione disulfide (GSSG), or possible glutathione transport (Figure 3B).

In addition to glutathione, the role of cysteine in yeast and LAB was intriguing. For yeast, the involvement of cysteine in H_2S production by yeast and the integration of cysteine into pyruvate for bacteria led to the selection of the following candidate genes: *CYS3* and *CYS4* (for yeast) and *pepN*, *cysE*, *cysK*, *cbl* and *cgl* (for bacteria; Figure 3).

To further explore sulfur-related gene expression, *SKP2*, *JLP1*, *MET5* and *MET10* were selected for yeast, and *tauE* was selected for bacteria. In particular, *MET5 and MET10* were chosen for their role in yeast H_2S production.

SSU1 was also chosen based on findings in Chapter 5, which revealed a potential role of *SSU1* expression for compatibility between *S. cerevisiae* and *O. oeni*.

In addition to glutathione, sulfur and cysteine related genes, stress-related genes were also selected based on previous findings. For yeast, these included *FYV12* and *MMP1*, while for bacteria *ctsR*, *groES*, *dnaK*, *grpE*, *trxA* and *hsp18* were selected.

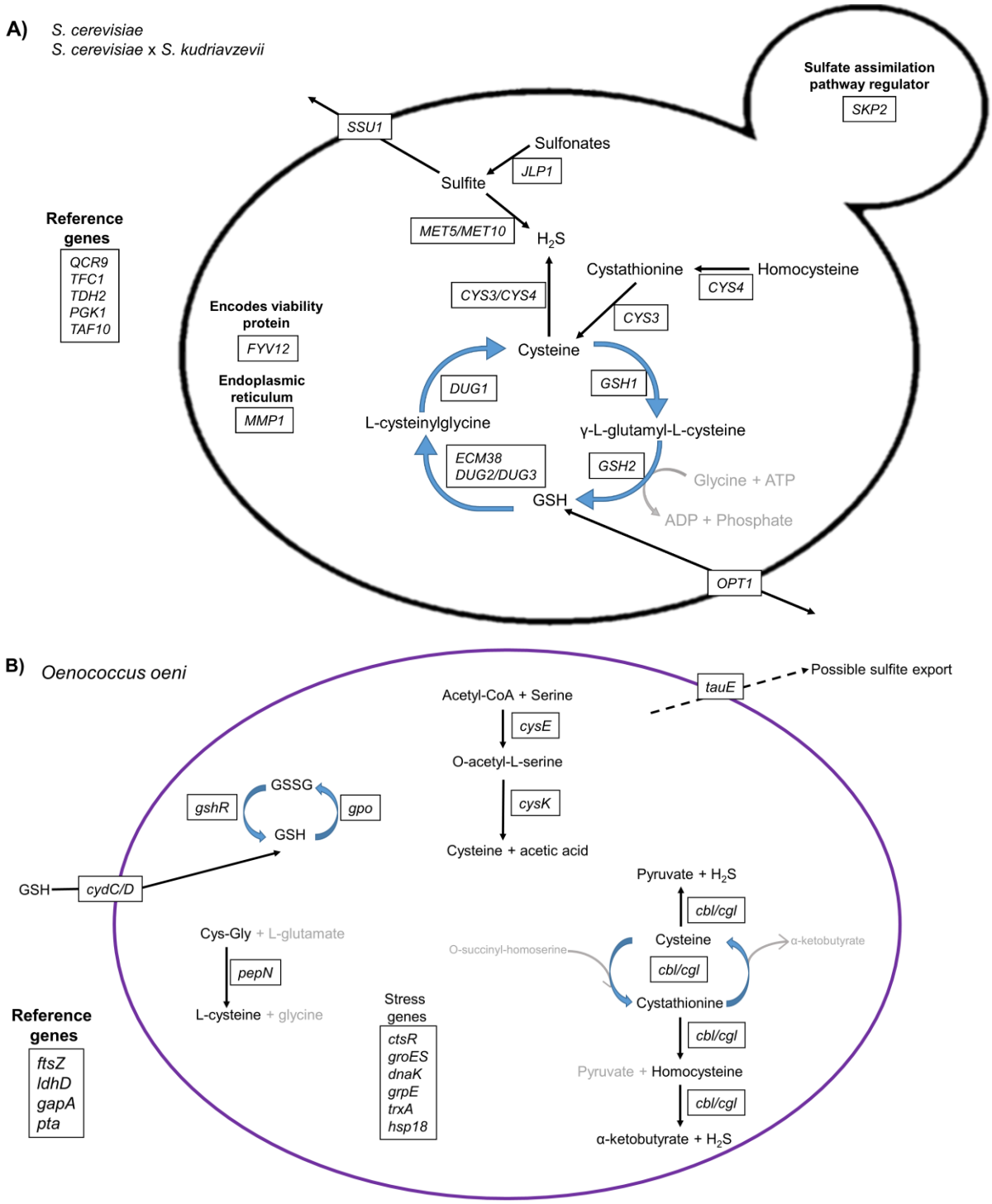

Figure 3: Metabolic pathways and related genes chosen for analysis for *S. cerevisiae* and *S. cerevisiae* x *S. kudriavzevii* strains **(A)** and *O. oeni* **(B)**. Genes are named within boxes. Reference genes are listed outside of the cell. Gene descriptions are listed in Table 1.

2.5.2. Primer design and qPCR

Primers for real-time qPCR (Table 1) were designed following the Qiagen guidelines for primer design (https://www.qiagen.com/us/service-and-support/learning-hub/molecular-biology-methods/pcr/#PCR%20primer%20design). Reference genes for yeast and LAB were selected based on previous work (Table 1; Beltramo *et al.* 2006; Desroche, Beltramo & Guzzo 2005; Sumby, Grbin & Jiranek 2012; Vaudano *et al.* 2011). Primer design software (IDT OligoAnalyzer) was used to select primer sequences. The length of PCR products ranged between 86 and 126 bp for yeast targets and 91 and 196 bp for LAB targets. Gene specificity of the designed primers was determined using NCBI BLAST. All primers were purchased from Sigma-Aldrich (Australia).

Real-time qPCR was performed on a Bio-Rad CFX96 real-time PCR system with PowerUp SYBR Green mastermix (catalogue # A25778, Applied Biosystems, Australia) in 96-well plates (catalogue # HSP9601, Bio-Rad, Australia). Reaction volume was 10 µL and consisted of 5 µL PowerUp SYBR mastermix, 0.5 µL each of forward and reverse primers (final concentration 0.5 µM), 3 µL nuclease free water (catalogue # 10977015, Invitrogen, Australia), and 1 µL cDNA (1:2 diluted for yeast or undiluted for LAB). cDNA was amplified by real-time qPCR using specified primers (Table 1). A no-template control was included for each primer pair in every PCR run. Thermal cycling conditions were as follows: Uracil-DNA Glycosylase (UDG) activation at 50°C for 2 minutes, DNA polymerase activation at 95°C for 2 minutes, 50 cycles denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. A melt curve was performed after each run to confirm the specificity of each primer pair. The melt curve conditions were: incrementing 1.6°C/second from 60°C to 95°C and 95°C to 60°C, followed by dissociation by incrementing 0.5°C/second to 95°C.

qPCR normalisation was carried out using qbase_plus software (Biogazelle).

Cycle threshold (Ct) values were only included for analysis if at least 3 out of 6 replicates amplified. After normalisation, all values were increased by 0.01 to allow for calculation of log2 fold change.

For yeast reference genes, five were tested and the best two were selected for further analyses. The two reference genes were selected based on amplification consistency and reliability.

2.5.3. Genomic DNA extraction and primer efficiency

EC1118 and NT50 were rehydrated according to the manufacturer's instructions, then streaked for single colonies on YPD agar (2%) and allowed to grow overnight at 28°C. To extract gDNA, a single colony was resuspended in 100 µL 20 mM NaOH, then heated at 94°C for 10 minutes (Hranilovic *et al.* 2017) in a Thermocycler (Bio-Rad, Australia).

VP41 and Alpha were rehydrated in sterile water (25°C) for 10 minutes. 100µL of rehydrated LAB was heated at 95°C for 10 minutes in a Thermocycler (Bio-Rad, Australia).

Primer efficiency was evaluated by performing qPCR on six 1:2 serial dilutions of yeast or LAB gDNA with each individual yeast or LAB-specific primer pair, respectively. Regression curves of the Ct values vs the log (gDNA dilution) were used to determine a slope value. Efficiency was calculated using the following formula (Ginzinger 2002):

Efficiency (%) = $((10^{-1/\text{slope}}) - 1) \times 100$

Table 1: Reference genes, candidate genes and primer pairs used in this work. Primers were designed using the IDT OligoAnalyzer tool, or following previous works. Rows highlighted with blue are the tested reference genes for yeast or LAB.

Primer target	Description	Forward primer	Reverse primer	Primer reference
Yeast Genes				
QCR9	ubiquinol-cytochrome C oxidoreductase[1]	ATCTTTGCAGGT GCCTTTGT	GCAGCTATTCG AGCCTTGAC	Vaudano *et al.* (2011)
TFC1	Transcription factor class C[1]	CCCAGAAGTTCA GTGGAATAC	TGGTGGCTTTG GTACATTC	This study
TDH2	Triose-phosphate dehydrogenase[1]	CGTCGAAGTTGT TGCTTTG	GAAACTTCACC AGCGTATCT	This study
PGK1	3-phosphoglycerate kinase[1]	TCCCATTGGACG GTAAGA	AGAAGCCAAGA CAACGTATC	This study
TAF10	TATA binding protein-associated factor[1]	TAGTGGATGATG GGAGTGAA	ATTACTGCATC GGGAATGATAG	This study
CYS3	Cystathionine gamma-lyase[1]	TCCCATGCGGTC TCTATC	CGTTAGTGAAG GAGGTTTCC	This study
CYS4	Cystathionine beta-synthase[1]	GAGATTCCTGGT GCTGTTATAC	GTCTTCTAGCT GTCTTTGGATT	This study
DUG1	Encodes Dug1p[1]	GAAGGTGGTTCC ATTCCTATC	TCATCGCCTCT ACCCATT	This study
DUG2	Encodes Dug2p[1]	TGGTGGTAATCC TGTTGTATTC	AACGTCATAAT GCCCATACC	This study
DUG3	Encodes Dug3p[1]	GTACCCACCAAC AGCATATT	GCGAGGGTTGA ACTTCTTAG	This study
ECM38	Encodes ECM38p[1]	GACCGCATAGAA CTGGAAAG	AACTACGCTCTT GGGAAATAC	This study
FYV12	Function required for yeast viability/survival after K1 killer toxin exposure (protein function unknown) [1]	ACAGGAAACCCG GATGA	TGGGCGTACAA GGTAAGA	This study
GSH1	Gamma glutamylcysteine synthetase[1]	CCTTTCAGGCAC CCAATATC	TCGGCTAGCCA ACCTTTA	This study
GSH2	Glutathione synthetase[1]	GGACACAGAGCA GGAAATAG	GAGCCAGATAA TTGAGTGAGTA A	This study
OPT1	Oligopeptide transporter[1]	CGTCCAAATCTAT GCCACTATC	GGTTGATCGGT GGTACATAAG	This study

Primer target	Description	Forward primer	Reverse primer	Primer reference
MET5	Sulfite reductase beta subunit[1]	ACCACTTGAAGG TATCGTTATT	CTTCGACACCC ATATCATCTAC	This study
MET10	Sulfite reductase alpha subunit[1]	AGGTTATGAGATT GGTGAAGTC	CGCCGATGTGT GTGATAATA	This study
SSU1	Plasma membrane sulfite pump[1]	GCTGCCCGTAAA TCTTCATTA	CTAGAGCCGAG TTTGATTCTTC	This study
JLP1	alpha-ketoglutarate dioxygenase[1]	CCTGATGGTGGT GGAGATA	ATTCTCTGCCT GTTCCTTTG	This study
MMP1	High-affinity S-methylmethionine permease[1]	GCCGAGACTGAG TTTGCTCT	ACCAGTAGAGT TAGGCCCCC	This study
SKP2	F-box protein/involved in sulfur metabolism enzyme regulation[1]	TACCGCTTACTTT GGGAGAG	CCTCATCTGTCT CATCAACAC	Designed by Krista Sumby
LAB genes				
ftsZ	Filamenting temperature-sensitive mutant Z-GTPase[2]	TGCCGGATCGAC ACCTGA	CGGACGAGTAA CAACGCCAAC	Sumby, Grbin & Jiranek (2012)
ldhD	d-Lactate dehydrogenase[2]	CAAAGTTTCCGG TATGGTAATG	TCATCCAAACG AGCATCAG	Desroche, Beltramo & Guzzo (2005)
gapA	d-Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) [2]	TCCACGCTTACA CATCGACTCA	CGCTGAGCATG ACCATTCAAC	Sumby, Grbin & Jiranek (2012)
pta	Phosphotransacetylase[2]	CATGGCTGAGAT TGCCGTTC	TCTCCTGCGCC AGCTTAGT	Sumby, Grbin & Jiranek (2012)
ctsR	Master regulator of stress response[2]	CTCAGTCAGGAC GAAATCACC	AAGGGTAAAAC GGGTGTTGA	This study
grpE	GrpE, heat shock chaperone class II[2]	CGCAGGCAGAAA AGAACAATC	ATCGGAAACAG CTGAAGACG	Forward primer: Desroche, Beltramo & Guzzo (2005) Reverse primer: this study

Primer target	Description	Forward primer	Reverse primer	Primer reference
trxA	Thioredoxin[2]	ATCCGGCGTGACTGTGACT	AAGTCCATTTGCCGTTTCCT	This study
hsp18	Stress protein Lo18[2]	CGGTATCAGGAGTTTTGAGTTC	CGTAGTAACTGCGGGAGTAATTC	Beltramo *et al.* (2006)
cydD	Thiol reductant ABC exporter subunit CydD[2]	GATATCGTAAATCGACAATGCG	AGAGACATCTTCCCTTCCATC	This study
cydC	Thiol reductant ABC exporter subunit CydC[2]	GGTTTGGATACTCCGATGAG	CGTTCGGTTAAAGGATCGAG	Forward: This study Reverse: Margalef-Català *et al.* (2017)
gpo	Glutathione peroxidase[2]	CAGGAGCGATTGGAAAATCT	TTTTCTGGATCGGTCTTTGG	Margalef-Català *et al.* (2017)
pepN	Aminopeptidase[2]	GCAGGCTTTCCCTTGTATT	CGTACTTCCGGCATGTTT	This study
cysE	Serine acetyltransferase[2]	TCGATCATGGATTGGGTGTAGT	TATCGGCAATGTGAGGATGAC	This study
tauE	Possible sulfite transporter[2]	GGGATATAGGGCGACAGTAAT	ATTCTAGGCTCATTGGGCTACT	This study
cbl	Cystathionine β-lyase[2]	CCGCCATCAGTTCAGTTT	TTCAACAAGCGGTAGGTTC	This study
cgl	Cystathionine γ-lyase[2]	ACAAGGTCGCTGGAAATG	CCAGCTTTCCCTTCTTCTAAA	This study
groES	GroES, heat shock chaperone class I[2]	TGTGGCAATTTCGGAGAC	AACTTGAGAACCGGCATATT	This study
dnaK	Chaperone protein DnaK[2]	CCAACGAGGAAGCAGATAAG	GCCAACATCGGACAAAGT	This study
gshR	Glutathione reductase[2]	CCAGCGAGTTTAGTGATAAGG	GAAATCGACGGGAAGAGATAAA	This study
cysK	Cysteine synthase A[2]	CTGATGATGGGATCAAAGGG	CCCAGGGTTCGATAGTAGAT	This study

[1]Sourced from *Saccharomyces* Genome Database (https://www.yeastgenome.org/)

[2]Sourced from UniProt database (https://www.uniprot.org/)

2.6. Statistical analysis

R version 3.6.3 was used to perform ANOVA with Tukey post-hoc tests, Kruskall-Wallis and pairwise Wilcoxon rank sum test, area under the curve (AUC) analysis and to generate graphs for AF, MLF, LAB growth, GSH and H_2S measurements. Non-parametric tests (Kruskall-Wallis and pairwise Wilcoxon rank sum test) were used for data that did not pass the Shapiro-Wilk normality test. Significant differences in relative gene expression were determined using paired t-test in GraphPad Prism 8.

3. Results and discussion

3.1. Alcoholic Fermentation and Malolactic Fermentation

Alcoholic fermentation was unaffected by co-inoculation with LAB (Table 2), as was also observed in Chapter 4 (Chapter 4: Table 4). Further to this, EC1118 and NT50 growth were unaffected by inoculation with LAB (Table 2; Figure S1, Appendix D).

Table 2: Alcoholic fermentation (AF) finishing time, AF performance (area under the curve; AUC) and yeast growth (area under the curve for live cells mL^{-1}) for co-inoculation and sequential fermentations. Values are the average of six replicates ± standard deviation. Unavailable data is indicated by "–". There were no statistical differences between yeast-alone and yeast co-inoculated with LAB.

		Co-inoculation			**Sequential inoculation**		
Yeast	**LAB**	AF finishing time (days)	AF performance (AUC)	Yeast growth (AUC, live cells mL^{-1})	AF finishing time (days)	AF performance (AUC)	Yeast growth (AUC, live cells mL^{-1})
EC1118	None	7 ± 0	10468 ± 146	$3.3 \times 10^{10} \pm 2.7 \times 10^{9}$	-	-	-
	Alpha	7 ± 0	10496 ± 336	$3.4 \times 10^{10} \pm 3.9 \times 10^{9}$	7 ± 0	10943 ± 618	$5.3 \times 10^{10} \pm 6.1 \times 10^{9}$
	VP41	7 ± 0	9995 ± 912	$2.9 \times 10^{10} \pm 2.6 \times 10^{9}$	7 ± 0	11168 ± 540	$5.6 \times 10^{10} \pm 4.0 \times 10^{9}$
NT50	None	7 ± 0	10947 ± 741	$2.7 \times 10^{10} \pm 2.6 \times 10^{9}$	-	-	-
	Alpha	7 ± 0	10991 ± 612	$3.2 \times 10^{10} \pm 3.6 \times 10^{9}$	7 ± 0	11134 ± 397	$3.6 \times 10^{10} \pm 3.4 \times 10^{9}$
	VP41	7 ± 0	10928 ± 235	$3.2 \times 10^{10} \pm 4.0 \times 10^{9}$	7 ± 0	11396 ± 512	$3.5 \times 10^{10} \pm 3.2 \times 10^{9}$

This PhD project is the first report examining gene expression during both sequential and co-inoculation of yeast and LAB to elucidate differences in gene expression within yeast and LAB based on inoculation strategy. During co-inoculation, VP41 completed MLF in 8 and 11 days with EC1118 and NT50, respectively (Figure 4A). Whereas Alpha completed MLF at 24 days co-inoculated with EC1118 and was sluggish and incomplete after 36 days when co-inoculated with NT50 (Figure 4A). The difference in MLF speed during co-inoculation corresponded with LAB growth, as Alpha did not achieve as high a concentration as VP41 (Figure 4B).

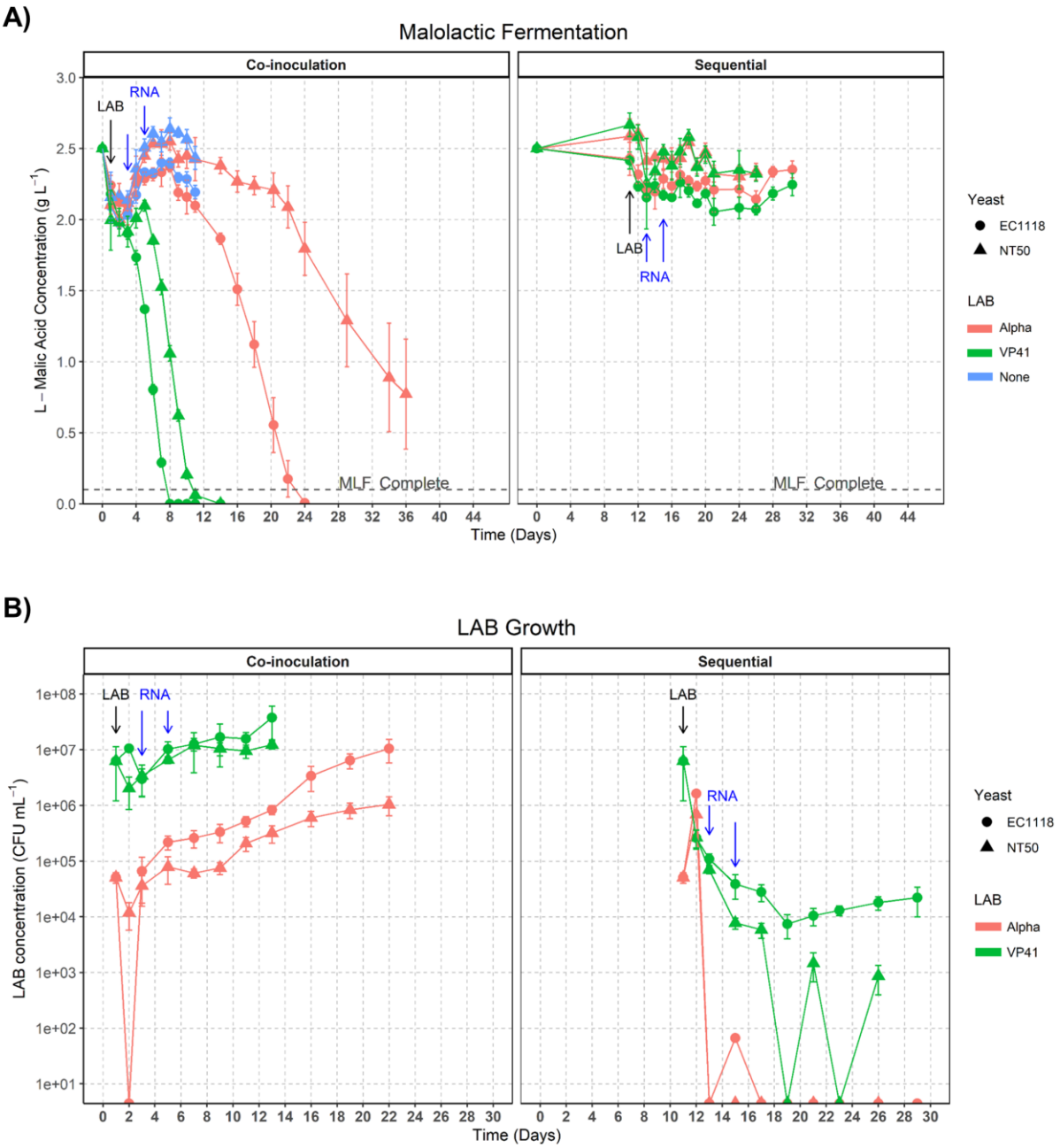

Figure 4: A) Malolactic fermentation progress for co-inoculation and sequential fermentations with EC1118 (circles) and NT50 (triangles). MLF was deemed complete when L-malic acid concentration was reduced to 0.1 g L^{-1} (horizontal dashed line). **B)** Alpha and VP41 growth (cfu mL^{-1}) over the course of co-inoculation and sequential fermentations with EC1118 and NT50 yeast. Different colours indicate the LAB strain or yeast only controls (red = Alpha; green = VP41; blue = none). LAB were inoculated 24 hours post-yeast for co-inoculation, or at Day 11 for sequential inoculation (black arrows). Samples collected for RNA extraction are indicated by blue arrows. Values are the mean of 6 biological replicates and error bars are the standard deviation.

During sequential fermentations, Alpha's demise occurred three days after inoculation, and VP41 could not sustain high enough cell density to perform MLF (Figure 4). This could not have been solely attributed to the pH of the sequential fermentations, which ranged between 3.4 and 3.5 (Table S1, Appendix D), compared to co-inoculation where pH would have also been close to 3.5 (data not available). However, in comparison to co-inoculation, LAB inoculated into sequential fermentations would have been subjected to higher ethanol concentration since AF was complete (Table 2). It is known that ethanol and pH act synergistically, negatively impacting LAB growth (Lonvaud-Funel 1995), and so this may have been the reason for the demise of LAB in sequential fermentations. Since MLF completion was different for LAB when co-inoculated with either EC1118 or NT50, it was hypothesised that there would be differences between the yeast that could be demonstrated by differences in gene regulation. In this work, the gene expression differences in sulfur related metabolic pathways were investigated.

3.2. Gene Expression

An initial trial was performed to determine what volume of sample would allow for enough RNA to perform qPCR for both yeast and LAB genes. A previous study used cell mass from 50 mL samples to obtain adequate LAB RNA after LAB ethanol exposure (Betteridge *et al.* 2018). This volume would not have been appropriate for the current work since the samples also contained yeast cells. In consideration of this, volumes of 1, 2, 5 and 10 mL were collected and used for trial RNA extractions. The only samples that had amplification of both yeast and LAB genes were from 1 mL samples. The RNA extractions performed for 2, 5 and 10 mL samples may have had inhibition due to the amount of biomass. Observationally, during the Trizol and chloroform steps, a thick layer formed between the two liquid

phases which was not seen with the 1 mL samples. Based on the above a sample volume of 1 mL was chosen for subsequent RNA extractions. Sampling time-points were chosen based on MLF and LAB growth data due to the difficulties of obtaining enough RNA from LAB during fermentation. The chosen sampling times were 48 and 96 hours post-LAB inoculation (corresponding to Days 3 and 5 in co-inoculation fermentations, and Days 13 and 15 in sequential fermentations) because VP41 and Alpha growth was increasing (Figure 4B), and there were differences in MLF progress (Figure 4A).

Co-inoculation fermentation samples were tested for both yeast and LAB gene expression, while for sequential fermentations, only LAB genes were attempted to be assessed since the yeast had all completed AF and there was a high concentration of dead yeast in the fermentation (Table S2, Appendix D). Although great care and precision were used during RNA extraction, the LAB RNA yield was too low for both sequential and co-inoculation samples, and consequently gene expression was unable to be measured. Potential reasons are discussed later.

Relative gene expression of EC1118 and NT50, co-inoculated with Alpha and VP41, were assessed at 48 and 96 hours post LAB inoculation (Table 3).

3.2.1. Relative gene expression after 48 hours of co-inoculation

After 48 hours, there were differences in relative gene expression between yeast co-inoculated with VP41 and Alpha LAB, and also between EC1118 and NT50 yeast strains (Table 3, Figure 5). Alpha and VP41 co-inoculated with EC1118 had increased expression of yeast genes *DUG1*, *DUG3*, *ECM38*, *JLP1*, *MET5, MET10*, *MMP1*, *OPT1* and *SSU1,* whereas NT50 co-inoculated with Alpha and VP41 had increased expression of *CYS3, GSH1, DUG1*, *DUG2, ECM38, OPT1, MMP1* and

SSU1 compared to their respective yeast-alone controls. (Table 3, Figure 5). These genes are related to production of cysteine from cystathionine (*CYS3*), generation of cysteine from L-cysteinylglycine (*DUG1*), conversion of GSH to L-cysteinylglycine (*DUG2*, *DUG3*, *ECM38*), conversion of sulfonates to sulfite (*JLP1*), generation of H_2S from sulfite (*MET5*, *MET10*), use of alternative sulfur sources (*MMP1*), production of γ-L-glutamyl-L-cysteine from cysteine (*GSH1*), transmembrane transport of GSH (*OPT1*) and sulfite export (*SSU1*).

Genes involved in the sulfate assimilation pathway (SAP) and H_2S liberation (*MET5*, *MET10*, *CYS3*, *CYS4, SKP2*) have been extensively studied in *S. cerevisiae* for the purposes of understanding and optimising metabolic pathway regulation, for example: reducing undesirable volatile sulfur compounds (i.e. H_2S; Huang *et al.* 2017), or increasing production of desirable sulfur-containing antioxidants (i.e. GSH; Hara *et al.* 2012). However, there lacks information about SAP regulation in response to co-inoculation with LAB or other yeast.

Table 3: Relative expression[a] of yeast genes when co-inoculated with either Alpha or VP41 LAB. Differential gene expression was assessed at 48 and 96 hours post-LAB inoculation. Bold values indicate a significant difference in gene expression between the co-inoculated fermentations for each yeast at each time point (paired t-test, $p < 0.05$).

	48 Hours				96 Hours			
	EC1118		NT50		EC1118		NT50	
Gene	**Alpha**	**VP41**	**Alpha**	**VP41**	**Alpha**	**VP41**	**Alpha**	**VP41**
CYS3	0.0 ± 0.0	0.0 ± 0.0	**7.14 ± 0.36**	**0.0 ± 0.0**	-1.47 ± 1.01	-2.19 ± 0.78	4.36 ± 0.31	4.55 ± 0.75
CYS4	-9.53 ± 0	-9.53 ± 0	0.0 ± 0.0	0.0 ± 0.0	-1.38 ± 0.25	-0.97 ± 0.59	-1.21 ± 0.16	-0.31 ± 0.67
DUG1	1.51 ± 0.56	1.38 ± 0.27	1.91 ± 1.01	1.29 ± 0.67	1.47 ± 0.81	0.27 ± 0.77	0.4 ± 0.48	-0.13 ± 0.53
DUG2	0.0 ± 0.0	0.0 ± 0.0	**0.0 ± 0.0**	**10.21 ± 0.26**	10.98 ± 0.91	9.84 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
DUG3	**0.0 ± 0.0**	**9.84 ± 0.28**	-1.36 ± 0.2	-1.24 ± 0.86	1.69 ± 0.69	1.59 ± 0.93	-1.34 ± 0.38	-1.19 ± 0.5
ECM38	10.41 ± 0.6	9.65 ± 0.22	-0.53 ± 0.23	0.07 ± 0.26	0.6 ± 0.43	0.62 ± 0.75	0.32 ± 0.51	-0.4 ± 0.49
FYV12	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
GSH1	**-0.07 ± 1.18**	**-11.81 ± 0**	**-9.58 ± 0**	**0.15 ± 0.84**	**0.93 ± 1.18**	**-10.59 ± 0**	0.0 ± 0.0	0.0 ± 0.0
GSH2	-9.28 ± 0	-9.28 ± 0	-10.66 ± 0	-10.66 ± 0	1.04 ± 0.59	0.8 ± 0.72	0.0 ± 0.0	0.0 ± 0.0
JLP1	**10.29 ± 0.51**	**0.0 ± 0.0**	0.0 ± 0.0	0.0 ± 0.0	**1.24 ± 0.77**	**-9.51 ± 0**	0.0 ± 0.0	0.0 ± 0.0
MET5	11.32 ± 1	11.21 ± 0.68	**-2.87 ± 0.74**	**-11.79 ± 0**	1.71 ± 0.88	-0.91 ± 0.46	0.21 ± 0.67	-0.72 ± 0.47
MET10	**0.0 ± 0.0**	**6.6 ± 0.19**	-1.46 ± 0.54	-0.49 ± 0.8	2.41 ± 0.88	2.31 ± 1.64	**-0.19 ± 1.14**	**-6.56 ± 0.17**
MMP1	**0.0 ± 0.0**	**7.26 ± 0.34**	1.16 ± 1.16	-0.84 ± 0.82	4.23 ± 1.02	1.97 ± 0.48	-1.81 ± 1.08	0.23 ± 2.44
OPT1	8.34 ± 0.24	9.23 ± 0.11	0.64 ± 0.92	0.48 ± 1.23	2.55 ± 0.68	-0.17 ± 0.45	-0.51 ± 0.64	-0.26 ± 1.02
SKP2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	**1.74 ± 1.01**	**-9.45 ± 0**	0.0 ± 0.0	0.0 ± 0.0
SSU1	2.54 ± 0.36	2.18 ± 0.17	0.48 ± 1.6	-0.06 ± 1.08	1.2 ± 0.41	0.33 ± 0.39	0.8 ± 0.87	0.28 ± 1.26

[a]log2 fold changes with respect to yeast-alone fermentation controls. A positive value indicates higher gene expression than the yeast only controls, and negative value indicates lower gene expression compared to yeast only controls when yeast strains were co-inoculated with different LAB as indicated.

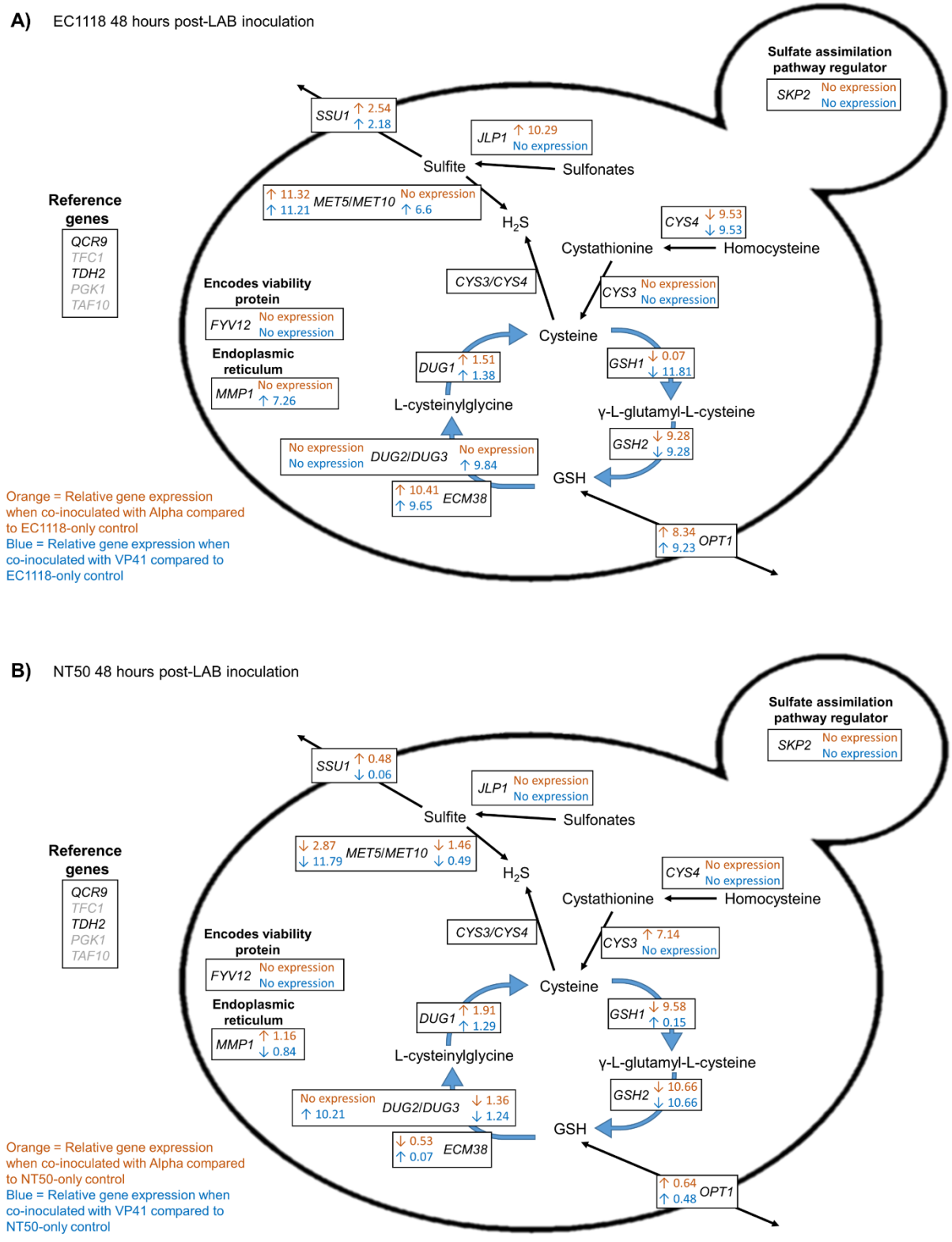

Figure 5: Relative gene expression (log2 fold-changes) 48 hours post-LAB inoculation for EC1118 (A) and NT50 (B). Relative expression was determined by calculating the ratio of log2-fold change of yeast-only control values and yeast co-inoculated with LAB values. An increase in gene expression is indicated by ↑ while a decrease is indicated by ↓. The reference genes used for normalisation are in black text, while unused reference genes are in grey.

A previously reported study has assessed *S. cerevisiae* gene regulation in response to co-inoculation with *Hanseniaspora guilliermondii* and found increased expression of *CYS3*, *CYS4*, *MET10* and *SSU1* and decreased H_2S liberation after 48 hours of fermentation (Barbosa *et al.* 2015). In consideration of the H_2S measurements, the authors reported that H_2S liberation did not correlate to their SAP expression results, since an increase in SAP gene expression theoretically should correspond to a higher H_2S concentration (Barbosa *et al.* 2015), These results differ from the findings in the current work, where EC1118 co-inoculated with Alpha and VP41 had increased expression of *MET5* (Table 3) but similar levels of H_2S liberation (Figure 6) compared to EC1118 alone. Similarly, EC1118 co-inoculated with VP41 had increased *MET10* expression relative to the yeast alone and EC1118 co-inoculated with Alpha.

CYS3 and *CYS4* had no detected or decreased gene expression for EC1118 co-inoculated with LAB compared to yeast alone. EC1118 SAP gene expression at 48 hours post LAB inoculation could hypothetically result in increased production of H_2S from sulfite and decreased cysteine production from homocysteine and cystathionine in response to co-inoculation with LAB. However, at 48 hours post-LAB inoculation, no difference was seen in H_2S measurements between yeast-only controls and yeast-co-inoculated with LAB (Figure 6).

Alternatively, NT50 co-inoculated with Alpha and VP41 had the opposite gene expression trend for SAP genes, with an increase in *CYS3*, and decrease in *MET5* and *MET10*. For NT50, this difference in gene expression could potentially have led to increased cystathionine production from homocysteine and a decrease in H_2S production from sulfite. Though at this time-point there were no differences in H_2S concentration between the yeast-only control and co-inoculations with LAB (Figure 6).

Though there were some overall differences in SAP gene expression, the ultimate impact of yeast co-inoculation with LAB on yeast sulfur compound production or utilisation could not be determined. Future work may involve the measurement of SAP related protein expression and sulfur compounds to try and determine if *S. cerevisiae* co-inoculation with different *O. oeni* does in fact lead to an overall change in sulfur metabolism.

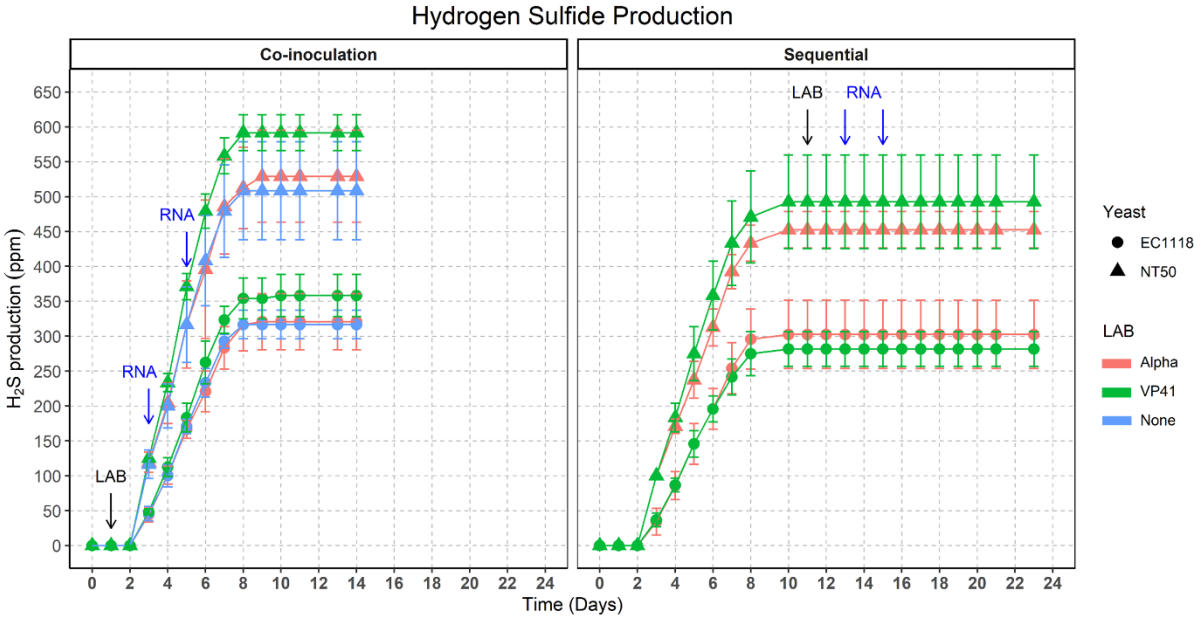

Figure 6: Cumulative hydrogen sulfide production over the duration of AF and MLF for sequential and co-inoculation fermentations. H_2S was measured daily by recording H_2S values indicated on silver nitrate H_2S detector tubes (detection range 25-1000 ppm). Shapes indicate yeast strain (circles = EC1118; triangles = NT50) and colour indicates LAB (red = Alpha; green = VP41) or yeast-only control (blue). Values are the mean of six replicates and error bars are the standard deviation.

Unlike the results for SAP genes, genes involved in the GSH-cysteine cycle had similar gene expression for both EC1118 and NT50 (Figure 5, Table 3). EC1118 co-inoculated with both LAB strains and NT50 co-inoculated with Alpha had decreased expression of *GSH1* and *GSH2* compared to the yeast-only controls. NT50 co-inoculated with VP41 differed, with increased *GSH1* expression, and decreased *GSH2* expression.

DUG1 expression increased in both EC1118 and NT50 co-inoculated with VP41 and Alpha, while *DUG3* expression increased in EC1118 co-inoculated with VP41 and decreased in NT50 co-inoculated with Alpha and VP41. *DUG2* was not expressed in either EC1118 or NT50 co-inoculated with Alpha, or EC1118 co-inoculated with VP41, while it had increased expression for NT50 co-inoculated with VP41. Additionally, *ECM38* expression differed substantially between yeast strains, with EC1118 co-inoculated with LAB having up to 10-fold increase in expression, while NT50 co-inoculated with LAB had a negligible increase or decrease compared to the yeast-only control. The general trend for both EC1118 and NT50 was a decrease in expression for genes involved in utilising cysteine to generate GSH (*GSH1*, *GSH2*), and an increase in genes related to production of cysteine from GSH (*DUG1*, *DUG2*, *DUG3*, *ECM38*; Figure 5).

NT50 co-inoculated with VP41 had a clear increase in *DUG2* expression and decrease in *DUG3* in comparison to all EC1118 fermentations and the NT50 yeast-only control, however, this was unable to be correlated with GSH concentration since there was no difference in GSH concentration between NT50 co-inoculated with LAB and NT50 alone (Figure 7).

At 48 hours post-LAB inoculation, amino acid content had decreased to between 0.5 - 4.4 mg L^{-1} (Table S3, Appendix D). Amino acid depletion, in particular

cysteine, would drive metabolism toward cysteine production, potentially leading to an increase in genes related to cysteine production from GSH (i.e. *DUG1*, *DUG2*, *DUG3*, *ECM38*), and decrease in genes related to cysteine incorporation into GSH (i.e. *GSH1*, *GSH2*). The increase in gene expression related to cysteine synthesis for yeast co-inoculated with LAB may therefore be a result of competition for nutrients. Yeast fermenting alone would be able to scavenge all the available nutrients from the medium, while yeast co-inoculated with LAB would need to compete for resources (Bartle *et al.* 2019a).

In addition to differences in SAP and glutathione pathway regulation, at 48 hours post-LAB inoculation, EC1118 and NT50 in co-inoculated fermentations also differed in *OPT1* and *SSU1* gene expression compared to their corresponding yeast-only controls. EC1118 co-inoculation with VP41 and Alpha had increased gene expression for both *OPT1* and *SSU1* compared to yeast only control, as well as higher fold expression than NT50 co-inoculated with LAB. An increase in *OPT1* gene expression could result in an increase in Opt1p, thereby increasing GSH transport into or out of the cell.

Considering that genes related to GSH conversion to cysteine increased, it is plausible that *OPT1* gene expression and subsequent Opt1p protein increased for GSH import rather than export at this time point. Higher expression of *OPT1* in EC1118 compared to NT50 could also be related to the availability of GSH, since EC1118 also had higher concentration of GSH compared to NT50, therefore having a greater opportunity to import more GSH (Figure 7). It is important to note that only whole sample GSH was measured, and not intracellular versus extracellular concentration.

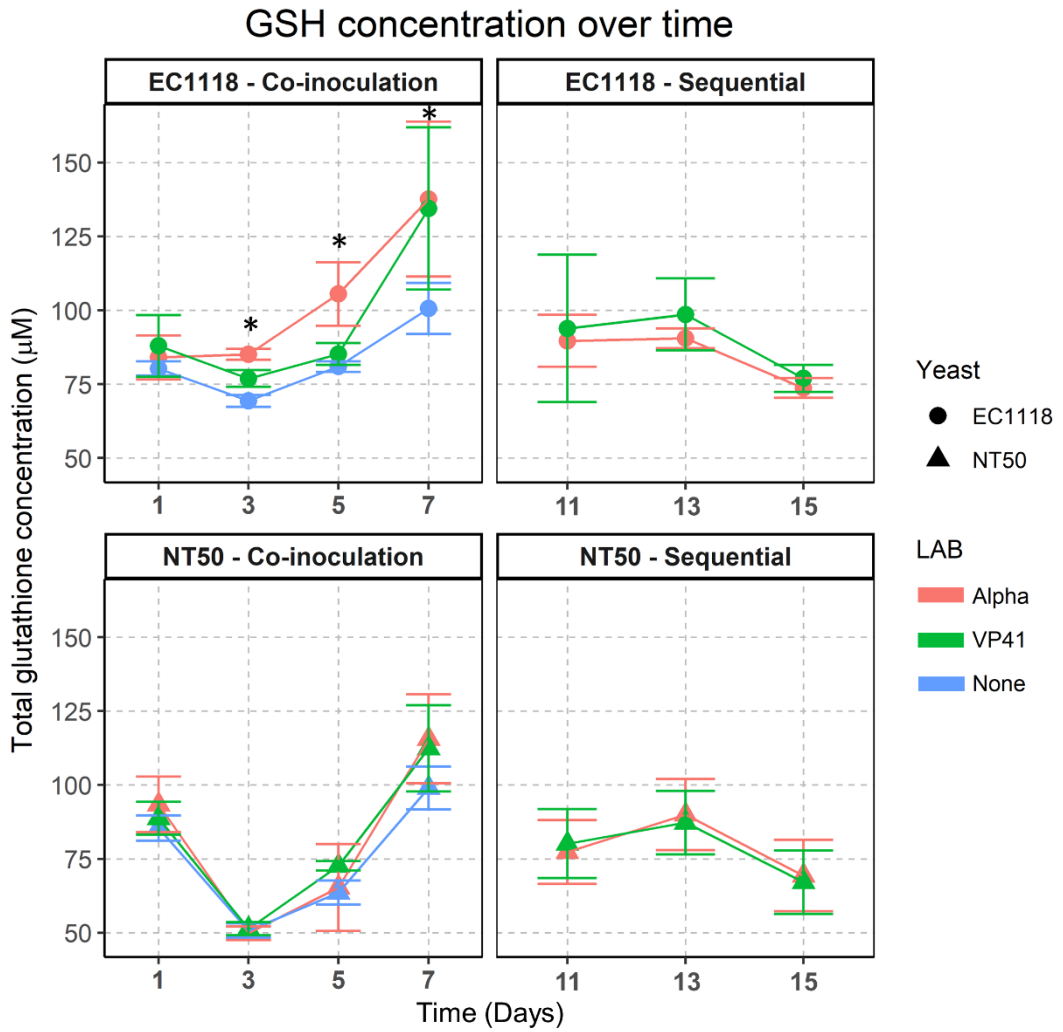

Figure 7: Total glutathione (GSH) concentration measured at days 1, 3, 5 and 7 for co-inoculated fermentations, and days 11, 13 and 15 for sequentially inoculated fermentations. Total glutathione was measured by enzymatic assay. Shapes indicate yeast strain (circles = EC1118; triangles = NT50) and colour indicates LAB (red = Alpha; green = VP41) or yeast-only control (blue). Values are the mean of six replicates and error bars are the standard deviation. *****Significant differences between LAB strains and/or yeast-alone control (Kruskall-Wallis and pairwise Wilcoxon rank sum test, $p < 0.005$)

Similarly to *OPT1*, *SSU1* expression differed between yeast. Expression of *SSU1* in *S. cerevisiae* in response to sulfite stress (50 mg L^{-1}) has been reported to vary depending on yeast strain (Nardi *et al.* 2010). Ssu1p, encoded by *SSU1*, is responsible for sulfite efflux, which is a detoxification system within yeast. However, in this work no sulfite was added to the starting juice, and therefore any sulfite produced by the yeast may have induced *SSU1* expression. EC1118 co-inoculated with LAB had higher fold-expression of *SSU1* than NT50-LAB co-inoculations (Table 3, Figure 5), which may be due to yeast strain genetic differences, as reported by Nardi *et al.* (2010).

For EC1118 in particular, the increase in *SSU1* expression in co-inoculations with LAB compared to yeast-only controls suggests that co-inoculation may increase the yeasts response to sulfite as well as increased expulsion of sulfite into the medium. However, this would need to be confirmed by determination of Ssu1p synthesis and SO_2 measurement over the course of co-inoculation. From this work, it is not known whether *SSU1* expression influenced MLF. Though results in Chapter 5 suggest that *SSU1* expression may be an important contributor to LAB MLF performance, as yeast *SSU1* haploinsufficiency enabled faster MLF by LAB. Overall, more research into this phenomenon is required in order to fully understand the role of LAB co-inoculation on yeast differential *SSU1* expression.

Yeast can utilise alternative sulfur sources, such as sulfonates, if they are experiencing sulfur starvation, though *S. cerevisiae* does not grow well on sulfonates alone (Linder 2012). Upregulation of *JLP1* and *MMP1* has been associated with utilisation of alternate sulfur sources by *S. cerevisiae*, specifically including increased expression after 3 and 7 days of VIN13 co-inoculated with *O. oeni* S6 (Rossouw, du Toit & Bauer 2012). It was hypothesised that this could be due to yeast and LAB competition for sulfur sources, but there was a lack of

information about LAB sulfur requirements (Rossouw, du Toit & Bauer 2012). Although VIN13 had increased *JLP1* and *MMP1* expression at all time-points measured, this was not the case for EC1118 and NT50. Alpha and VP41 co-inoculation with EC1118 and NT50 resulted in differing expression of *JLP1* and *MMP1* (Table 3, Figure 5). However, this is not surprising since yeast strains respond differently to external changes, and in the case of NT50, the strain is a *S. cerevisiae* x *S. kudriavzevii* hybrid and has differences in metabolic regulation compared to *S. cerevisiae* (Combina *et al.* 2012). Even though the differences in *JLP1* and *MMP1* are intriguing, there still is a lack of information about LAB sulfur requirements and so the actual reason for *JLP1* and *MMP1* differential expression during co-inoculation is unclear. This result also re-iterates the strain specificity of yeast-LAB compatibility, and how it is influenced even at the level of gene expression.

3.2.2. Relative gene expression after 96 hours of co-inoculation

Relative gene expression after 96 hours of co-inoculation differed from gene expression at 48 hours for both EC1118 and NT50 (Table 3, Figure 5 and 8). For EC1118, the relative expression for many genes increased or decreased less than 2-fold compared to the yeast-only control. The exception to this was increased expression of *DUG2* and *MMP1*, and either increased or decreased expression of *SKP2*, *GSH1* and *JLP1* with differential expression being influenced by which LAB the yeast was co-inoculated with. NT50, on the other hand, had larger differences in gene expression for *CYS3*, *CYS4*, *GSH1*, *GSH2*, *DUG2* and *MET5* compared to the 48 hour time point (Figure 5).

For NT50, the relative expression of many genes compared to yeast-alone at the 96-hour time-point were not substantial, with only *CYS3* and *MET10* having a greater than 2-fold increase and decrease in differential expression, respectively.

At this point in the fermentation, GSH concentration in EC1118 had increased more for co-inoculations with Alpha than VP41 and yeast-alone, which may aid in explaining the difference in *OPT1* expression between EC1118 fermentations with different LAB. The GSH concentration in NT50 fermentations were similar (Figure 7), and differential gene expression for *OPT1* was not significantly different between co-inoculations with different LAB, or substantially different from the yeast-only control.

Interestingly, there was no significant difference in *MET5, MET10, CYS3* and *CYS4* relative expression between EC1118 co-inoculations (Table 3, Figure 8), despite there being a difference in H_2S concentration (Figure 6). This also occurred for NT50 co-inoculations, where no significant difference was found for *CYS3*, *CYS4* and *MET5* but VP41 co-inoculations had higher H_2S. There was a significant difference in *MET10* expression (Table 3), though the expression was significantly decreased for VP41 co-inoculations, the opposite of what was expected. Though this inverse relationship was observed by Barbosa *et al.* (2015) as discussed earlier.

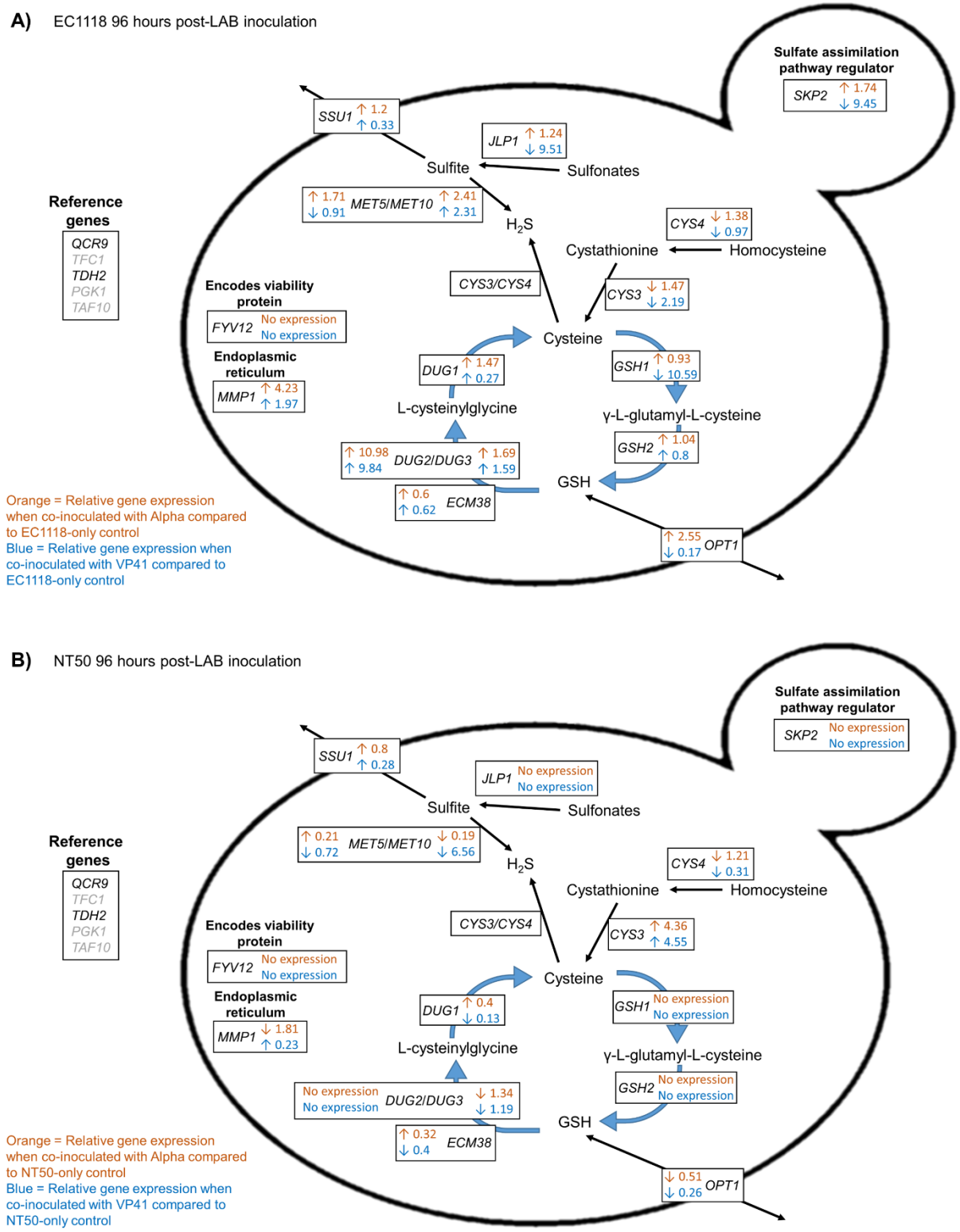

Figure 8: Relative gene expression (log2 fold-changes) 96 hours post-LAB inoculation for EC1118 (A) and NT50 (B). Relative expression was determined by calculating the ratio of log2-fold change of yeast-only control values and yeast co-inoculated with LAB values. An increase in gene expression is indicated by ↑ while a decrease is indicated by ↓. The reference genes used for normalisation are in black text, while unused reference genes are in grey.

Unlike *MET5*, *MET10*, *CYS3*, and *CYS4*, the protein encoded by *SKP2* is not involved in direct synthesis of compounds, but encodes Skp2p: a regulator of Met14p that contributes to SO_2 and H_2S production (Noble, Sanchez & Blondin 2015; Yoshida *et al.* 2011). *SKP2* expression and the encoded Skp2p degrades Met14p which subsequently decreases H_2S production (Huang *et al.* 2017). There was a significant difference in *SKP2* expression at 96 hours for EC1118 co-inoculated with Alpha and VP41, which correlated with levels of H_2S in the fermentations. The substantial decrease in *SKP2* expression for EC1118 co-inoculated with VP41 compared to yeast-only control and Alpha co-inoculations coincides with the increased amount of H_2S produced in the VP41 co-inoculation fermentations. With this work being the first report of paired H_2S measurement and *SKP2* expression during yeast-LAB co-inoculations, it is unable to be elucidated whether *SKP2* expression is directly influenced by co-inoculation. However, the evidence here suggests that LAB co-inoculation with yeast could alter SAP and H_2S related gene regulation, and subsequent production of compounds.

Lastly, in this work there was no measurable expression of *FYV12* at either time point. *FYV12* was chosen for analysis because it was reported that *FYV12* relative expression increased within VIN13 when co-inoculated with *O. oeni* S6 (Rossouw, du Toit & Bauer 2012). *FYV12* expression has been reported to increase in response to killer toxin exposure (Pagé *et al.* 2003). Rossouw, du Toit & Bauer (2012) concluded that *O. oeni* may have produced compounds that elicit a similar response to killer toxin within yeast, though no data was available to confirm the theory. In the present study, there was no measurable expression of *FYV12*, though EC1118 has been verified as killer factor active (Lallemand 2016).

3.3. Gene Expression Complications

Though input RNA was standardised prior to cDNA synthesis, samples containing mixed yeast and LAB RNA had variable expression for yeast reference genes. This was a consequence of inability to distinguish yeast and LAB RNA from each other, and hence standardisation of total RNA led to variable amounts of yeast RNA.

Unfortunately the amount of LAB RNA in the samples was too little to be tested for gene expression. This was unexpected since the samples used for the trial work were also collected 48 hours post-LAB inoculation had successful *ldhD* gene expression.

After performing DNase treatment, the small amount of LAB gDNA left in the sample had similar Ct values to the cDNA for reference genes. For this reason, LAB gene expression results could not be trusted. Hence we concluded that the protocol for extracting RNA from mixed samples requires further optimisation. Previous studies have obtained LAB gene expression results from mixed samples, as reported for *Streptococcus thermophilus* co-cultured with *Lactobacillus bulgaricus* (Sieuwerts *et al.* 2010) *and S. cerevisiae* co-cultured with *Lactobacillus delbrueckii subsp bulgaricus* (Mendes *et al.* 2013). Though these works were able to obtain adequate RNA and subsequent gene expression results for LAB, the species used (i.e. *Lb bulgaricus* and *Lb. delbrueckii*) typically grow to higher density compared to *O. oeni*. Additionally, the growth medium used was not as harsh as wine. Considering both of these factors, the approach needed to obtain adequate RNA from *O. oeni* during juice fermentation requires optimisation that is not otherwise required for other LAB.

One way around the issues of trying to obtain adequate LAB RNA and subsequent cDNA from mixed samples would be the use of RNA-seq, where cDNA synthesis is

not required. Though, yeast and LAB samples require rRNA depletion prior to RNA-seq so that these highly abundant sequences do not interfere with genomic gene expression results. Although RNA-seq is a good option to reduce processing steps for gene expression studies, it is costly and generates large amounts of data that require bioinformatics expertise for analysis. A conceivably less expensive alternative transcriptomics approach would be the use of chromatin immunoprecipitation assay (ChIP)-seq (Johnson *et al.* 2007). At the time of the Rossouw, du Toit & Bauer (2012) study, ChIP-seq was unavailable for *O. oeni*. However, since then genomes for *O. oeni* strains have been fully sequenced, giving rise to the potential for use of ChIP-seq for *S. cerevisiae*-*O. oeni* co-inoculation transcriptome studies.

4. Conclusions

This work was undertaken to further our knowledge of how yeast-LAB co-inoculation may influence transcriptional regulation of genes involved in sulfur metabolism. Gene expression was able to be analysed for yeast, but for LAB the RNA quantity and quality was inadequate. Despite the difficulty involved with trying to extract RNA from a mixed yeast-LAB sample, this work has provided useful insight into yeast sulfur pathway gene regulation in response to co-inoculation with different *O. oeni* strains. Overall gene expression differed greatly between the two time points, 48 and 96 hours post-LAB inoculation, and therefore future studies may incorporate more time-points to gain a clearer temporal progression of yeast SAP regulation.

It was revealed that both EC1118 and NT50 sulfur-related gene expression differed from each other, as well as when each yeast was co-inoculated with Alpha or VP41.

There were also differences in metabolite production, where EC1118 increased production of glutathione when co-inoculated with LAB, while NT50 showed no differences. A particularly intriguing finding was the lack of correlation between H_2S production and measured gene expression of *CYS3*, *CYS4*, *MET5* and *MET10*.

To be able to further understand sulfur metabolism in yeast and LAB during co-inoculation, future work should include analysis of additional genes from the sulfate assimilation pathway and genes that influence H_2S regulation. Additionally, optimising RNA extraction from yeast-LAB mixed samples will lead to results that enable in depth analysis of both *Saccharomyces* and *O. oeni* transcriptional regulation. In conclusion, this work has provided a starting point for future investigation into yeast and LAB sulfur metabolism and its potential influence on yeast-LAB compatibility during co-inoculation.

Chapter 7

Conclusion and future directions

In recent years, vintage compression has caused great stress on the Australian winemaking industry (Petrie 2016). The need for fast, efficient and successful fermentation is apparent, as completion of fermentations of early-ripening grape varieties (e.g. Chardonnay and Pinot Noir) enables smoother transition to fermentations of later-ripening grape varieties (e.g. Cabernet Sauvignon and Shiraz). Efficient and fast fermentation could enable reduced overall storage costs of grapes and lessened labour requirements. A solution to increasing overall fermentation speed is the implementation of co-inoculation: allowing yeast and lactic acid bacteria (LAB) to simultaneously complete alcoholic (AF) and malolactic fermentation (MLF). However, selection of yeast and LAB can be difficult due to the inability to accurately predict compatibility between strains.

Compatibility between yeast and LAB can be affected by a number of factors, including metabolite production and utilisation by yeast and LAB, such as ethanol, fatty acids and bacteriocins, and nutrient uptake, as well as physical interactions such as mixed-species biofilms and co-aggregation. Review of the literature (Chapter 1) surrounding yeast-LAB co-inoculation and compatibility revealed that there was a need to generate a more comprehensive list of compatible yeast and LAB strains, and to gain a greater understanding of what drives yeast-LAB compatibility. Hence, at the beginning of this dissertation, an overarching aim was presented: to gain a greater understanding of factors that influence compatibility between yeast and LAB in a winemaking context.

The work in this thesis focused on interactions between the two most common microbial species used in winemaking: *Saccharomyces cerevisiae* and *Oenococcus*

oeni. Initial work, presented in Chapter 3, led to the production of a compatibility table for eight commercial yeast strains that were co-inoculated with nine commercial LAB in a synthetic red juice. Compatibility between the different yeast and LAB was strain specific, leading to a ranked list of the top four compatible and bottom four incompatible yeast-LAB pairs. These eight pairs were further assessed for compatibility in Shiraz juice (Chapter 4), with a larger amount of data collection. Fermentation parameters (i.e. yeast and LAB growth, AF and MLF progress) and the utilisation and/or production of a range of compounds, both volatile and non-volatile, were measured. The work revealed a potential role of sulfur compounds, other than SO_2, that could influence yeast-LAB compatibility. Additionally, for the first time, a QTL approach was used to identify yeast genotypic differences that may influence LAB MLF performance during co-inoculation (Chapter 5). To follow-on from the findings in Chapter 5, a quantitative PCR experiment was designed to measure changes in yeast and LAB sulfur-pathway gene expression in response to co-inoculation (Chapter 6). The main conclusions from this thesis (Chapters 3-6) are summarised below:

1. AF and MLF kinetics alone were unable to be used as predictors for yeast-LAB compatibility in a synthetic juice. Though, synthetic juice is useful as a repeatable medium to study initial yeast-LAB compatibility for a large number of strains (Chapter 3).

2. Yeast-LAB compatibility is partially reliant on the juice type. Co-inoculating pairs chosen from the initial screen in red synthetic juice led to a switch in compatibility outcome for four of the eight pairs tested in Shiraz juice. In addition, measuring a range of compounds and parameters revealed a noticeable difference in esters, succinic acid and H_2S production. This was

the first time H_2S has been measured over the course of co-inoculation fermentation, demonstrating how yeast co-inoculation with some LAB strains resulted in an increase in H_2S production (Chapter 4).

3. QTL analysis further demonstrated that yeast genetic background can significantly affect LAB ability to complete MLF during co-inoculation. In particular, *S. cerevisiae SSU1* haploinsufficiency can enable faster MLF completion by SB3 *O. oeni* during co-inoculation in Shiraz juice. *SSU1* encodes Ssu1p which is involved in sulfite export. This work therefore demonstrated another way in which sulfur may have a role in yeast-LAB interactions and compatibility (Chapter 5).

4. The potential for sulfur to be much more involved in yeast-LAB interactions and compatibility is becoming clearer. Gene expression within *S. cerevisiae* strain EC1118 and *S. cerevisiae* x *S. kudriavzevii* strain NT50 varied between yeast-only fermentations, and yeast co-inoculated with LAB. The differences in *CYS3, CYS4, MET5, MET10, GSH1, GSH2, ECM38, DUG1, DUG2* and *DUG3* gene expression between yeast-only controls and yeast co-inoculated with LAB provides some intriguing insight into yeast sulfur pathway regulation in response to co-inoculation with different LAB strains. However there was difficulty in extraction of high yield and high quality RNA from the mixed yeast-LAB fermentations, and more work is required to optimise the RNA extraction process (Chapter 6).

Future directions

The work presented here has generated a range of research directions that would benefit from follow-up. These research directions are proposed as follows:

1. **Reassessing yeast-LAB compatibility in different juice types**

   It was demonstrated in Chapters 3 and 4 that changing the fermentation medium resulted in a change in compatibility status for some yeast-LAB pairs. Future work with the strains used in this dissertation could involve the use of other common red juice types (i.e. Cabernet Sauvignon, Merlot, Grenache, or red blends) and Chardonnay, where MLF is also commonly executed. Every juice type would need metabolite and amino acid characterisation to enable more in-depth analysis of the impact of variety on yeast-LAB compatibility. Comparison of the composition of each juice type could lead to greater understanding of how juice composition may ultimately favour or hinder yeast-LAB compatibility. This dataset would be of great use to winemakers, as it could support decisions of which strains to choose for which juice types.

2. **Surveying a broader range of commercial yeast and LAB strains**

   There are hundreds of commercial yeast and LAB strains available for winemakers to use. Now, with the availability of a high-throughput fermentation system such as the Tee-bot used in this study, researchers have the ability to investigate up to 92 yeast-LAB co-inoculation combinations in triplicate, alongside yeast-alone controls, all at the same time. Though small scale, this initial screening process could be used for

hundreds of combinations of yeast-LAB pairs in different juice types, enabling the generation of initial compatibility information for an even larger list of yeast and LAB strains.

3. Commercial-scale wine fermentations

It is also important to follow-up the experiments performed here with commercial scale fermentations. After understanding how strain dependent yeast-LAB compatibility can be, it is useful to see how yeast and LAB perform together in an environment that is a larger volume, non-sterile and also may contain grape solids (as is common for red winemaking). Performing a commercial study would enable collation of data that could be used to determine whether any modifications of small-scale approaches could enable better comparison to commercial scale winemaking. Commercial trials could also be used to further study the role of sulfur compounds in an industrial context, possibly leading the way to new monitoring practices of LAB to ensure MLF success.

4. Developing a pre-treatment method to be able to implement flow cytometry for LAB monitoring

Monitoring LAB growth in real-time would aid winemakers and wine researchers by reducing the time and cost of determining LAB viability during fermentation. Flow cytometry relies on single cells or single particles passing through a light source, and the resultant light scatter giving a measurable value that can be plotted visually. Currently, for *O. oeni* and *Lb. plantarum* the formation of chains impacts the reliability of flow cytometry methods. Work on other chaining bacteria has been performed,

but utilises sonication to break chains apart prior to flow cytometry analysis (Bitoun *et al.* 2012; Culp *et al.* 2011). Sonication can damage cells, leading to unreliable viability determination. Though, for wine LAB, sonication prior to flow cytometry may provide more accurate results than colony counting, since colonies may also arise from a chain of cells. Alternatively to sonication, use of machines that enable microscopy of cells that have passed through the flow cytometer may be of great value, if economically viable.

5. Monitoring a larger number of sulfur compounds over the course of fermentation

This work has begun to uncover the role of sulfur compounds, other than SO_2, in yeast-LAB compatibility. It would be beneficial to measure sulfur compounds, including cysteine, methionine, H_2S, glutathione and polysulfides over the duration of AF and MLF to try and elucidate the impact they have on strain compatibility. It would also be useful to determine which and what concentration of sulfur compounds *O. oeni* benefits from. This would be invaluable to winemakers, since information in regards to the sulfur nutritional requirements of *O. oeni* and other wine LAB are currently lacking.

6. Utilising another QTL library to support the findings in this work

In order to further understand the impact of *SSU1* efficiency and the translocation XV-t-XVI on LAB MLF performance, the M2xF15 QTL library could be used for SB3 co-inoculations. The M2xF15 yeast QTL library has been used to study genotypes/phenotypes related to H_2S production

(Huang, Roncoroni & Gardner 2014) and AF performance in wine (Peltier *et la.* 2018b). Additionally, *SSU1* hemizygote strains used in this work showed similar phenotype to SBxGN *SSU1* hemizygote strains. Conducting a QTL analysis with M2xF15 may also reveal other QTLs that could influence yeast-LAB compatibility, since under comparable fermentation conditions, M2xF15 yeast progeny shared only 2 out of 36 AF-related QTLs with SBxGN (Peltier *et al.* 2018b). This study was looking for the interaction effect between environment (i.e. juice type), genotype and fermentation parameters (i.e. AF progress and yeast growth). A similar methodology could be applied to investigate the interaction effect between juice type, yeast genotype, co-inoculation and compatibility with LAB.

7. Exploring *SSU1* and Ssu1p in relation to co-inoculation and MLF outcomes

To gain greater insight into how *SSU1* expression in *S. cerevisiae* strains can impact MLF by LAB during co-inoculation, *SSU1* gene expression should be measured over the course of fermentation. In addition to *SSU1* gene expression, levels of Ssu1p should also be measured. Considering the differences in gene expression that have already been reported for wild-type strains and those with translocations (Goto-Yamamoto et al. 1998; Park & Bakalinsky 2000), it would be useful to know if Ssu1p protein levels also differ between industrially relevant strains. Further to this, understanding commercial yeast Ssu1p efficiency could give winemakers a way to naturally control fermentation by choosing strains with low Ssu1p efficiency for co-inoculation, or high Ssu1p efficiency for biological control.

8. Using alternative sequencing approaches to better define changes in sulfur related gene regulation within yeast and LAB

Prior to this work, transcriptomic experiments have been performed to identify changes in *S. cerevisiae* gene expression when co-inoculated with either LAB (Russouw *et al.* 2012) or other yeast (Barbosa *et al.* 2015; Tondini *et al.* 2019). However, for the single study that used a transcriptomic approach for *S. cerevisiae-O. oeni* co-inoculations (Russouw *et al.* 2012), the approach required a known genome for each of the species involved. At that time, the *O. oeni* genome was unknown and therefore ChIP-seq was unable to be used for *O. oeni* gene analysis. Since then, multiple *O. oeni* genomes have been sequenced, and therefore the possibility of using ChIP-seq has become available. Considering the potential role of sulfur compounds in LAB growth and MLF performance, assessing *O. oeni* transcriptional regulation in response to co-inoculation with *S. cerevisiae* would start to broaden our understanding of *O. oeni* sulfur requirements.

In conclusion, the work presented in this dissertation demonstrates the complexity of *S. cerevisiae-O. oeni* interactions during co-inoculation fermentation. Compatibility of yeast and LAB strains is dependent on juice type, yeast and LAB strain, confounded by the negative impacts of low pH and increasing ethanol. This work has extended the current knowledge of the role of sulfur compounds in yeast-LAB compatibility, and opened up many avenues for future research on yeast-LAB compatibility during winemaking, leading to a better understanding of yeast-LAB relationships. The work here can be utilised by winemakers to make more informed decisions on the yeast and LAB they choose for fermentation. Gaining an understanding of the role of sulfur compounds will greatly benefit

winemakers in the future, allowing for specific choices based on the desired end result.

Appendix A

Supplementary information for Chapter 3

Table S1: Flow cytometer settings for Guava Easycyte 12HT (Millipore)

Parameter	**Value**
Forward scatter (FSC)	11.81
Side scatter (SSC)	1
Yellow-Blue	3.36
Threshold	FSC; 1000

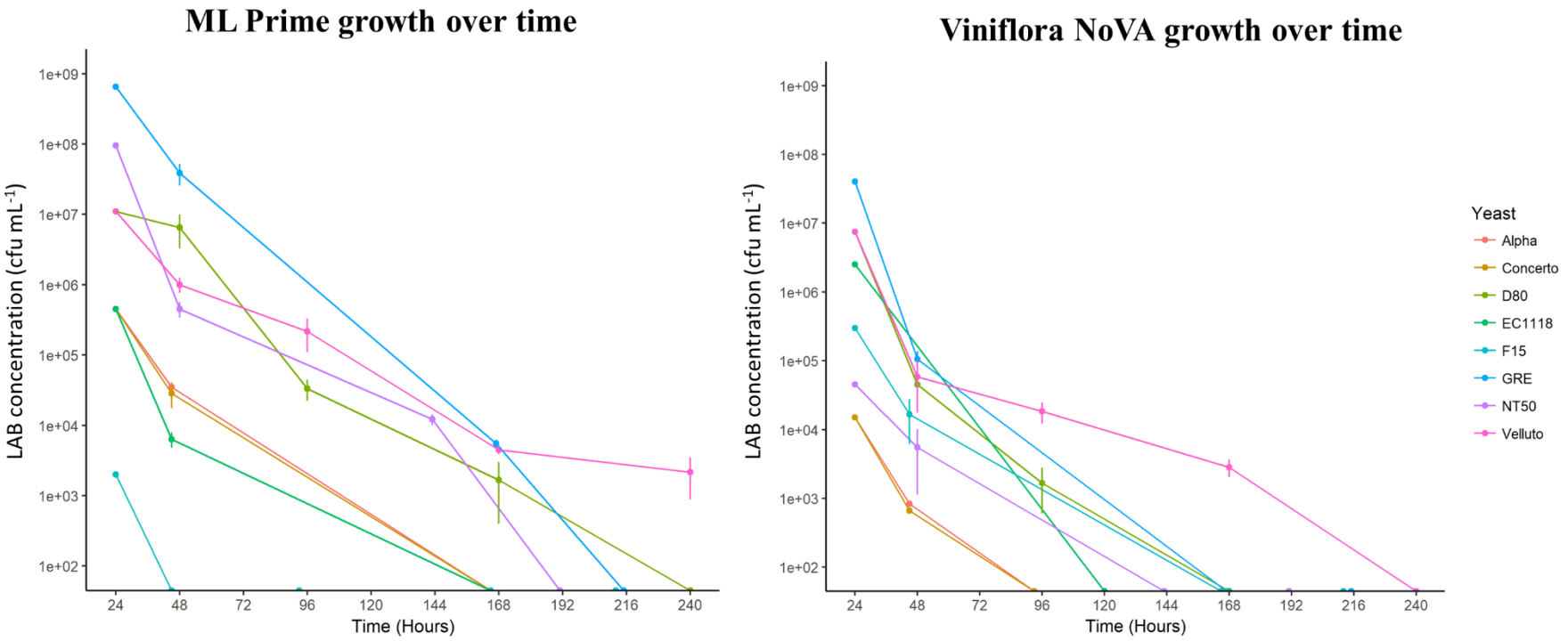

Figure S1: *Lb. plantarum* strains Prime and NoVA growth (colony forming units mL^{-1}) over the course of fermentation. Colours indicate the yeast strain the LAB was co-inoculated with. Each point is the mean of triplicates and error bars represent standard deviation.

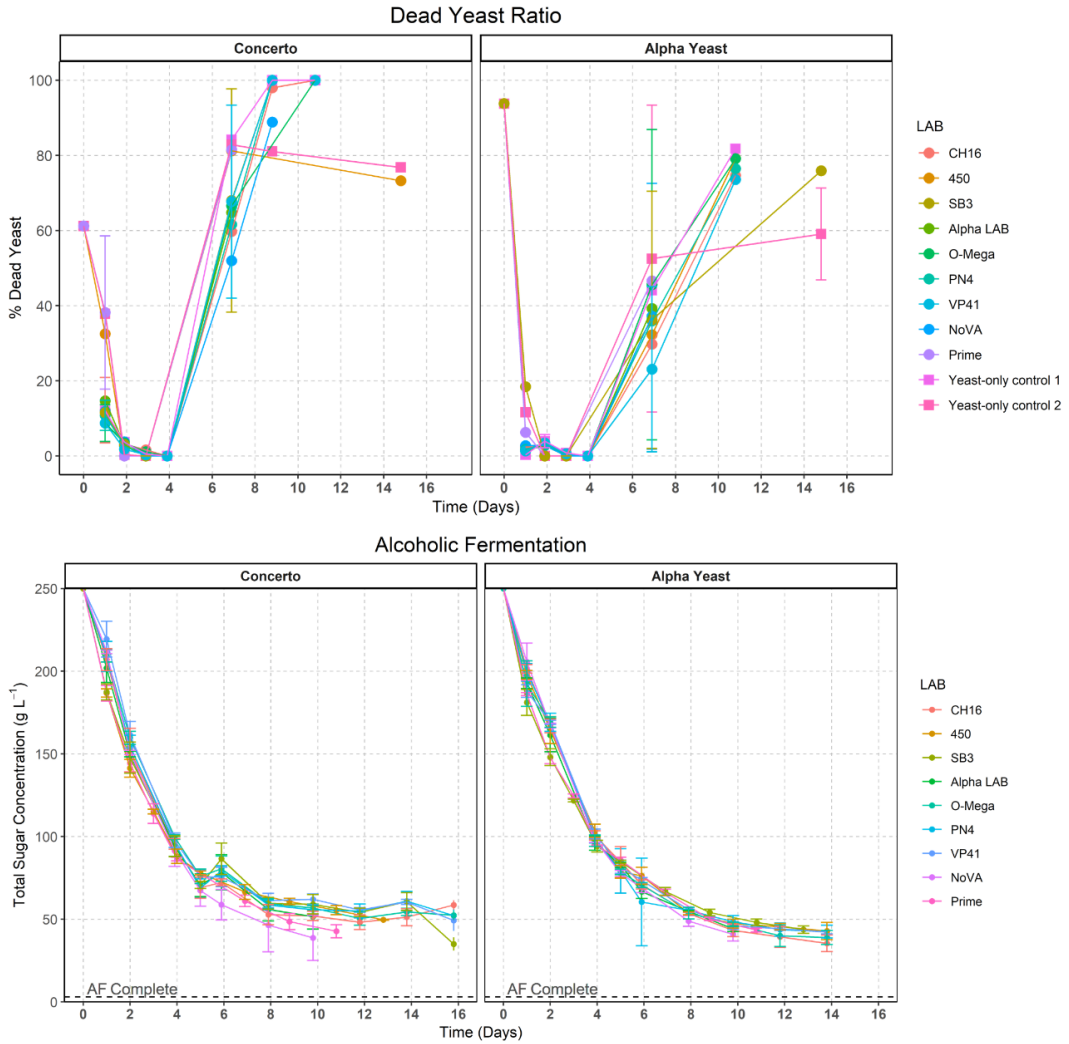

Figure S2: Comparison of dead yeast percentage and alcoholic fermentation progress for non-*Saccharomyces* strains Concerto and Alpha Yeast with each LAB. Plots are separated by yeast strain used for co-inoculation, and colours indicate the LAB strain conducting MLF. The dashed line specifies 3 g L^{-1} total sugar which is the end point for AF.

Table S2: Residual glucose and fructose concentrations for non-*Saccharomyces* yeast strains Concerto and Alpha Yeast.

Yeast	**Bacteria**	**Residual glucose (g L^{-1} ± SD)**	**Residual fructose (g L^{-1} ± SD)**	**Ratio of residual glucose:fructose (± SD)**
Concerto	CH16	17.0 ± 1.3	41.6 ± 2.2	1:2.5 (± 0.2)
	450	13.1 ± 0.5	36.5 ± 0.5	1:2.8 (± 0.1)
	SB3	6.7 ± 1.8	28.3 ± 2.1	1:4.3 (± 0.8)
	Alpha LAB	14.3 ± 4.8	37.3 ± 2.7	1:2.8 (± 0.9)
	O-Mega	14.8 ± 1.1	37.5 ± 2.5	1:2.5 (± 0.2)
	PN4	14.5 ± 2.5	37.7 ± 4.5	1:2.6 (± 0.2)
	VP41	12.9 ± 2.4	36.4 ± 3.9	1:2.9 (± 0.2)
	NoVA	9.3 ± 4.7	29.4 ± 9.0	1:3.5 (± 1.1)
	Prime	11.0 ± 2.3	31.6 ± 2.0	1:2.9 (± 0.5)
	None	10.1 ± 2.7	30.9 ± 6.7	1:3.1 (± 0.4)
Alpha Yeast	CH16	6.9 ± 2.3	28.4 ± 2.8	1:4.4 (± 1.2)
	450	9.4 ± 1.4	33.4 ± 3.9	1:3.6 (± 0.1)
	SB3	11.4 ± 0.9	32.3 ± 1.4	1:2.8 (± 0.1)
	Alpha LAB	11.5 ± 0.5	32.8 ± 1.5	1:2.9 (± 0.2)
	O-Mega	8.2 ± 1.4	30.8 ± 3.1	1:3.8 (± 0.4)
	PN4	8.6 ± 1.2	33.8 ± 2.7	1:4.0 (± 0.3)
	VP41	8.8 ± 0.4	33.4 ± 1.3	1:3.8 (± 0.3)
	NoVA	9.8 ± 1.4	31.0 ± 2.8	1:3.2 (± 0.3)
	Prime	11.2 ± 0.5	32.2 ± 0.9	1:2.9 (± 0.1)
	None	8.6 ± 0.9	30.1 ± 2.6	1:3.5 (± 0.5)

Table S3: AF completion times for yeast-only controls. Fermentations were completed over multiple batches leading to two control measures for each yeast.

Yeast	Control for indicated LAB co-inoculation	AF finishing time (days ± SD)	Significantly different from other pairs?	Rank for completion (fastest to slowest)
EC1118	450, Alpha, CH16, NoVA, SB3, VP41, PN4	5 (± 0)	Yeast only control was faster than all pairs	**2**
	O-Mega, Prime	7 (± 0)	EC1118 paired with Prime was faster than yeast only control	
D80	Prime, Alpha, VP41, O-Mega, SB3, 450, CH16, NoVA	9 (± 0.3)	No	**4**
	PN4	10 (± 0.3)	No	
GRE	450, Alpha	8 (± 0.7)	No	**3**
	CH16, NoVA, O-Mega, PN4, Prime, VP41, SB3	8 (± 0)	No	
NT50	450, CH16, NoVA, O-Mega, PN4, Prime, SB3	5 (± 0)	No	**1**
	Alpha, VP41	5 (± 0.3)	No	
F15	Alpha, CH16, SB3, VP41, 450, NoVA	11 (± 0)	No	**5**
	O-Mega, PN4, Prime	10 (± 0)	No	
Velluto	450, Alpha, CH16, NoVA, O-Mega, Prime, SB3, VP41	13 (± 0)	No	**6**
	PN4	14 (± 0)	No	

Table S4: LAB inoculation rates across fermentations with each yeast. Non-significant differences between co-inoculation with specified yeast are listed by row in the significance column.

LAB	Range in inoculation concentration cfu mL^{-1}	Significance (Tukey post-hoc $p < 0.005$)
CH16	2×10^5 - 2×10^9	EC1118, F15 [ns]
450	3.5×10^6 - 2.15×10^9	Concerto, F15, GRE [ns]
		D80, Velluto [ns]
SB3	2×10^4 - 1.5×10^9	Alpha Yeast, F15 [ns]
		D80, Velluto [ns]
Alpha LAB	8×10^4 - 1.95×10^9	Alpha Yeast, Concerto, EC1118, F15, GRE [ns]
		D80, Velluto [ns]
O-Mega	3×10^4 - 8×10^7	Alpha Yeast, Concerto, F15 [ns]
		D80, EC1118, Velluto [ns]
PN4	1.5×10^4 - 1×10^9	Alpha Yeast, Concerto, F15 [ns]
		GRE, Velluto [ns]
VP41	5.5×10^5 - 3×10^9	Alpha Yeast, Concerto [ns]
		D80, Velluto [ns]
NoVA	1.5×10^4 - 4×10^7	Alpha, Concerto, F15 [ns]
		D80, Velluto [ns]
Prime	2×10^3 - 6.5×10^8	Alpha, Concerto, EC1118, F15 [ns]
		D80, Velluto [ns]

*ns = not significant between those yeasts

Appendix B

Supplementary Information for Chapter 4

Table S1: Mixed standard used for GC-MS analysis of Shiraz juice samples. 50 µL of standard was added to each vial prior to analysis.

Compound used for standard	**Concentration in mixed standard (mg L^{-1})**
d8-Ethyl acetate	0.197
d16-Octanal	0.164
d7-Benzyl alcohol	1.04
d17-2-Ethyl hexanol	0.133
Z-3-Hepten-1-ol	3.4
d11-Hexanoic acid	3.72
2-n-Hexyl furan	0.027

Table S2: Mixed standard used for GC-MS analysis of fermentation samples. 10 µL of standard was added to each vial prior to analysis.

Compound used for standard	**Concentration in mixed standard (mg L^{-1})**
d13-Hexanol	920
d11-Hexanoic acid	930
d16-Octanal	82.1
d5-Ethyl nonanoate	9.2
d3-Linalool	1.73

Table S3: Average reduction in LAB cell numbers after inoculation and prior to population density recovery. The values are the mean of triplicates. These values were ranked from largest to smallest decrease to allow easier comparison with compatibility. The percentage of population decrease was also calculated.

Yeast	**Bacteria**	**Reduction in viability (cfu mL^{-1})**	**Percentage population decrease**	**Decrease ranked largest to smallest (8-1)**	**Compatibility in Shiraz juice**
D80	PN4	9.95×10^6	99.48	3	Compatible
	O-Mega	6.00×10^8	99.98	8	Compatible
EC1118	Alpha	1.99×10^6	99.65	2	Compatible
	CH16	2.10×10^7	100	5	Compatible
NT50	Alpha	1.99×10^6	99.52	1	Incompatible
	CH16	2.10×10^7	100	4	Incompatible
	VP41	1.35×10^8	99.91	7	Compatible
Velluto	VP41	1.35×10^8	99.85	6	Compatible

Table S4: Amino acids measured by HPLC after AF and MLF. Concentrations are displayed as mean values (mg L^{-1}) of triplicates ± standard deviation. No value is shown for amino acids that were undetectable at time of measurement. Table continues on next page.

		Time-point	β-Alanine	γ-aminobutyric acid (G-Aba)	Alanine	Arginine	Asparagine	Aspartic acid	Glutamine
Yeast	**Bacteria**	Juice	0.6 ± 0.1	402.8 ± 9.8	38.2 ± 1.2	1073.7 ± 78.5	1.9 ± 0.2	12.5 ± 0.3	71.4 ± 3.5
D80	None	After AF				4.1 ± 0.1	0.7 ± 0.4		
	O-Mega	After AF			1.1 ± 0.3	4.5 ± 0.3	1.1 ± 0.2		
		After MLF			0.9 ± 0.4	2.7 ± 0.1	0.5 ± 0.2		
	PN4	After AF				3.8 ± 0.3	0.9 ± 0.1		
		After MLF		2.9 ± 0.7	5.4 ± 1.3	15.1 ± 2.9	1.9 ± 0.1		
EC1118	None	After AF				5.4 ± 0.3	1.7 ± 0.9		
	Alpha	After AF				5.1 ± 0.3	0.7 ± 0.1		
		After MLF		67.4 ± 11.5	7.5 ± 0	38.3 ± 1.9	8.7 ± 1.2	5.7 ± 0.2	
	CH16	After AF				5.1 ± 0.2	1.1 ± 0.3		
		After MLF		61.7 ± 6.6	8.7 ± 1.7	27.7 ± 23	9.9 ± 1.5	6.1 ± 0.4	
NT50	None	After AF				4.6 ± 0.2	0.6 ± 0.3		
	Alpha	After AF				4.4 ± 0.4	0.9 ± 0.3		
	CH16	After AF				4 ± 0.5	0.6 ± 0.3		
	VP41	After AF				4.4 ± 0.1	0.5 ± 0		
		After MLF		1.5 ± 0.5	1.6 ± 0.5	10.8 ± 0.7	4.1 ± 0.6		
Velluto	None	After AF			1 ± 0.2	11.7 ± 0.6	1.1 ± 0.2		
	VP41	After AF			0.9 ± 0.3	11.5 ± 0.7	1.3 ± 0.4		
		After MLF		2.6 ± 0.5	8.6 ± 0.3	35.3 ± 0.3	3.3 ± 0.4		

Table S4: Continued; amino acids measured by HPLC after AF and MLF. Concentrations are displayed as mean values (mg L^{-1}) of triplicates ± standard deviation. No value is shown for amino acids that were undetectable at time of measurement. Table continues on next page.

		Time-point	Glutamic acid	Glycine	Histidine	Isoleucine	Leucine	Lysine	Methionine
Yeast	**Bacteria**	Juice	19.9 ± 3.7	2.5 ± 0.2	42.1 ± 3.3	13.6 ± 0.7	30.8 ± 1.1	10.9 ± 0.1	
D80	None	After AF		1 ± 0.1					
	O-Mega	After AF		1.1 ± 0.2					
		After MLF		0.4 ± 0.3					
	PN4	After AF		0.9 ± 0.4					
		After MLF	7.2 ± 0.8	2.6 ± 0.4				4.2 ± 0.9	
EC1118	None	After AF		0.9 ± 0.3					
	Alpha	After AF		0.7 ± 0.3					
		After MLF	24.1 ± 2.6	3.6 ± 0.3	3.2 ± 0.3		7 ± 0.1	14.3 ± 0.9	6.2 ± 0.1
	CH16	After AF		0.8 ± 0.3					
		After MLF	23 ± 1.9	3.8 ± 0.5	3.3 ± 0.7		7.8 ± 1.1	15.6 ± 1.4	6.4 ± 0.9
NT50	None	After AF		0.4 ± 0.2					
	Alpha	After AF		0.5 ± 0.2					
	CH16	After AF		0.6 ± 0.1					
	VP41	After AF		0.5 ± 0.2					
		After MLF		1.5 ± 0.3				3.7 ± 0.3	
Velluto	None	After AF		1.5 ± 0.1					
	VP41	After AF		1.6 ± 0.2					
		After MLF	9.8 ± 0.1	3.5 ± 0.4	3.3 ± 0.1		5.7 ± 0.1	11.7 ± 0.6	5.9 ± 0.5

Table S4: Continued; amino acids measured by HPLC after AF and MLF. Concentrations are displayed as mean values (mg L^{-1}) of triplicates ± standard deviation. No value is shown for amino acids that were undetectable at time of measurement.

		Time-point	Phenylalanine	Proline	Serine	Threonine	Tyrosine	Valine	Hydroxyproline
Yeast	Bacteria	Juice	20.9 ± 1.4	2998.4 ± 366.9	32.6 ± 0.5	60 ± 1.5	24.6 ± 2.1	32.3 ± 1.4	11.9 ± 0.1
D80	None	After AF		2916.9 ± 195.2					12 ± 0.1
	O-Mega	After AF		3207.4 ± 256.6					13.5 ± 1.5
		After MLF		3073.3 ± 67.8					12 ± 0.4
	PN4	After AF		2977.9 ± 419.5					12.2 ± 0.8
		After MLF		3216 ± 472.2					12.2 ± 0.6
EC1118	None	After AF		2587 ± 546					12.5 ± 0.7
	Alpha	After AF		2517.1 ± 91.8					12 ± 0.2
		After MLF	5.3 ± 0.3	3472.3 ± 245		1.1 ± 0.9	2.8 ± 0.1	1.9 ± 0.2	12.3 ± 0.6
	CH16	After AF		2956.2 ± 111					13.1 ± 0.8
		After MLF	6 ± 0.7	3777.9 ± 675.1		1.1 ± 0.3	3 ± 0.5	2.2 ± 0.3	13.1 ± 1.2
NT50	None	After AF		2735.6 ± 78.6					11.7 ± 0.2
	Alpha	After AF		2740.1 ± 192.1					11.7 ± 0.6
	CH16	After AF		2478.5 ± 111.3					11.7 ± 0.3
	VP41	After AF		2647.8 ± 163.7					11.7 ± 0.3
		After MLF		3181.2 ± 257.7					12.3 ± 0.5
Velluto	None	After AF		3317.1 ± 176.4					11.7 ± 0.7
	VP41	After AF		3636.3 ± 465.6					12.3 ± 0.6
		After MLF	4.5 ± 0.2	3686.2 ± 162.3		0.5 ± 0.5	2.1 ± 0.6	1.5 ± 0.1	12.7 ± 0.1

Table S5: L-malic acid concentration at the end of the experiment for yeast-only fermentations. Values are mean concentration (g L^{-1}) for triplicates ± standard deviation.

Yeast	**L-malic acid (g L^{-1})**
D80	2.0 ± 0.0
EC1118	1.9 ± 0.0
NT50	1.9 ± 0.1
Velluto	1.5 ± 0.0

Table S7: Compounds measured by GC-MS for samples taken at experiment completion. Concentrations are displayed as mean values (mg L^{-1}) of triplicates ± standard deviation.

Yeast	Bacteria	1-hexanol	2-methylbutanoic acid	3-(methylthio)-1-propanol	Benzyl alcohol	Ethyl acetate	Ethyl octanoate
D80	None	4028 ± 89	762 ± 7	7271 ± 94	499 ± 32	7120 ± 383	236 ± 9
	PN4	3976 ± 73	1187 ± 129	7362 ± 877	468 ± 30	8651 ± 1615	238 ± 61
	OMEGA	3999 ± 22	749 ± 11	6852 ± 535	537 ± 44	7335 ± 150	235 ± 5
EC1118	None	3652 ± 222	807 ± 229	5827 ± 1582	511 ± 27	7069 ± 1023	228 ± 39
	Alpha	3616 ± 93	883 ± 31	7285 ± 981	519 ± 65	9745 ± 221	292 ± 16
	CH16	3560 ± 44	863 ± 20	6776 ± 316	484 ± 25	9361 ± 1038	329 ± 24
NT50	None	3579 ± 78	765 ± 4	5338 ± 259	481 ± 30	8025 ± 1117	433 ± 88
	Alpha	3747 ± 124	909 ± 46	5628 ± 1133	422 ± 40	8312 ± 918	465 ± 22
	CH16	3816 ± 137	1040 ± 35	6306 ± 368	484 ± 26	8508 ± 384	455 ± 31
	VP41	3782 ± 125	979 ± 32	5910 ± 320	460 ± 32	8918 ± 862	438 ± 19
Velluto	None	3723 ± 67	1167 ± 84	3689 ± 345	468 ± 10	8659 ± 663	276 ± 24
	VP41	3629 ± 89	1080 ± 30	3676 ± 283	491 ± 20	6682 ± 655	332 ± 19

Appendix C

Supplementary information for Chapter 5

Table S1: Parameters measured during the QTL mapping experiment for yeast alone and yeast co-inoculated with SB3. Pop BN yeast are labelled with the prefix "CM" followed by an ID number. Parameters for alcoholic fermentation have an AF suffix, malolactic fermentation parameters have an MLF suffix. Prefixes tend, t35, t50 and t80 are time to complete AF and time to complete 35%, 50% or 80% of AF, respectively. Prefix s50-80 is the slope value for points between t50 and t80. Pct_malic_AF and Pct_malic_co are the percentage of L-malic acid consumption (positive %) or production (negative %), in comparison to the starting L-malic acid concentration of 2.5 gL^{-1}, assessed at the end of the experiment. Malic_acid_LAB_consumed is the concentration of L-malic acid estimated to have been consumed solely by SB3 at the end of the experiment. Yeast alone values are the mean of duplicates and yeast-co-inoculated with SB3 are the mean of triplicates. Unmet parameters are designated "–".

	Measures for yeast alone							Measures for yeast co-inoculated with SB3							
Yeast	**Residual L-malic acid (g L)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**Pct_malic_AF (%)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**tend-MLF (hours)**	**Pct_malic_co (%)**	**malic_acid_LAB_consumed (g L)**
SB	2	109	29	38	60	-2.8	19.3	110	25	34	55	-3	407	100.0	1.9
GN	2.5	202	23	35	67	-2	-0.1	202	24	36	66	-2.1	–	18.5	0.4
BN	2.5	122	27	36	61	-2.5	2.0	122	24	33	56	-2.7	397	100.0	2.4
SBxGN	2.4	118	25	34	59	-2.5	5.7	121	22	32	57	-2.5	420	100.0	2.3
CM108	2.6	149	26	37	64	-2.3	-3.9	148	25	35	61	-2.5	–	15.1	0.5
CM144	2	168	29	41	71	-2.1	18.8	169	25	36	64	-2.3	323	100.0	1.9
CM145	2.1	141	26	37	64	-2.3	15.5	140	24	34	60	-2.5	295	100.0	2.0
CM149	2.7	209	28	41	72	-2	-8.0	193	25	37	67	-2.1	241	100.0	2.6
CM170	2.2	116	26	36	62	-2.4	12.0	117	24	34	58	-2.6	206	100.0	2.1
CM177	2	149	27	39	68	-2.2	18.7	148	24	35	61	-2.4	243	100.0	1.9
CM193	2.4	135	25	37	64	-2.3	4.9	137	23	33	59	-2.4	370	100.0	2.3
CM194	2.4	159	26	38	68	-2.2	5.5	162	25	36	63	-2.4	–	56.6	1.2
CM195	2.2	141	28	40	67	-2.3	13.2	139	24	36	62	-2.4	–	36.6	0.6
CM197	1.9	199	30	44	76	-2	22.4	176	25	36	64	-2.3	237	100.0	1.8

	Measures for yeast alone							Measures for yeast co-inoculated with SB3							
Yeast	**Residual L-malic acid (g L)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**Pct_malic_AF (%)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**tend-MLF (hours)**	**Pct_malic_co (%)**	**malic_acid_LAB_consumed (g L)**
CM198	2.3	150	26	38	65	-2.4	6.0	163	24	35	61	-2.4	–	15.9	0.2
CM199	2.5	137	28	39	66	-2.4	-1.6	139	24	35	62	-2.4	424	93.7	2.3
CM201	2.3	124	24	33	56	-2.7	8.3	125	23	31	49	-3.5	–	29.0	0.5
CM203	1.8	177	31	44	72	-2.2	27.9	170	25	37	68	-2	353	100.0	1.7
CM204	1.6	142	28	39	67	-2.3	35.8	142	25	37	64	-2.3	204	100.0	1.5
CM205	1.8	–	33	48	91	-1.5	28.5	168	23	34	62	-2.3	–	48.3	0.4
CM208	2.3	123	27	36	62	-2.5	6.7	122	24	33	58	-2.5	–	19.4	0.3
CM209	2.6	146	25	36	62	-2.4	-4.1	152	24	35	62	-2.4	–	63.5	1.6
CM210	2.5	138	27	39	66	-2.3	-0.2	140	23	34	61	-2.4	–	55.6	1.3
CM211	2.1	117	26	35	56	-2.9	17.4	117	23	31	52	-3.1	–	71.9	1.3
CM212	2.1	150	28	40	68	-2.3	16.4	153	25	36	63	-2.3	–	35.0	0.4
CM213	2.8	163	29	42	73	-2.1	-10.1	172	25	36	64	-2.3	–	19.3	0.7
CM214	1.8	134	28	39	64	-2.6	26.6	129	23	33	58	-2.5	408	98.0	1.7
CM215	1.7	125	28	37	59	-2.8	31.2	121	25	33	55	-3	292	100.0	1.6
CM217	1.9	157	28	41	70	-2.2	25.5	156	26	37	66	-2.2	322	100.0	1.8
CM219	2	140	25	36	63	-2.4	21.3	139	24	35	61	-2.5	–	30.8	0.2
CM220	1.6	140	26	37	63	-2.5	37.3	137	23	33	58	-2.5	213	100.0	1.5
CM221	2	116	28	37	62	-2.6	19.3	113	23	33	58	-2.5	320	100.0	1.9
CM222	2.3	–	44	139	–	–	6.6	–	28	49	–	–	–	18.0	0.3
CM223	2.2	163	27	39	68	-2.2	10.4	164	24	35	62	-2.3	–	22.1	0.3
CM224	2.3	136	28	40	66	-2.4	9.7	131	24	35	61	-2.5	390	100.0	2.2
CM225	1.9	153	26	37	64	-2.4	24.8	155	24	35	62	-2.4	–	36.0	0.2
CM226	2.4	130	28	39	66	-2.4	5.6	132	25	36	62	-2.4	409	97.7	2.2
CM228	2.3	134	25	36	62	-2.4	6.9	128	21	31	56	-2.6	402	100.0	2.2
CM230	2.3	132	25	36	62	-2.5	6.9	130	25	35	60	-2.6	400	100.0	2.2

	Measures for yeast alone							Measures for yeast co-inoculated with SB3							
Yeast	**Residual L-malic acid (g L)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**Pct_malic_AF (%)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**tend-MLF (hours)**	**Pct_malic_co (%)**	**malic_acid_LAB_consumed (g L)**
CM231	1.8	140	29	41	68	-2.3	29.3	139	24	35	61	-2.4	–	43.1	0.3
CM232	2.2	142	29	42	70	-2.3	11.8	145	26	37	64	-2.3	–	34.9	0.5
CM233	1.9	113	23	32	54	-2.9	22.1	112	27	35	53	-3.5	292	100.0	1.8
CM234	2.2	151	27	38	66	-2.3	11.2	150	23	34	61	-2.4	–	21.2	0.2
CM235	2.2	–	28	40	73	-2	11.4	206	25	37	67	-2.1	–	49.8	0.9
CM236	2	142	25	36	62	-2.4	21.4	141	22	33	59	-2.4	191	100.0	1.9
CM237	1.7	144	26	38	65	-2.3	31.4	144	21	31	57	-2.5	410	100.0	1.6
CM238	1.7	121	26	35	57	-2.9	31.8	120	22	32	55	-2.7	245	100.0	1.6
CM239	1.7	142	29	40	68	-2.3	30.8	139	25	36	62	-2.4	–	47.4	0.4
CM24	1.9	116	23	32	58	-2.5	22.9	112	24	33	54	-3	341	100.0	1.8
CM240	1.9	135	27	38	64	-2.4	24.0	133	26	37	61	-2.6	404	100.0	1.8
CM241	2.7	140	27	39	68	-2.2	-7.8	137	25	36	63	-2.3	–	18.5	0.6
CM242	2.3	129	24	35	60	-2.5	8.6	128	21	31	56	-2.5	403	100.0	2.2
CM243	2.1	128	26	37	62	-2.5	15.8	123	23	33	58	-2.5	–	36.8	0.5
CM244	2.2	144	26	38	66	-2.3	13.0	144	20	30	54	-2.6	373	100.0	2.1
CM245	1.4	136	26	37	64	-2.3	44.0	135	27	37	62	-2.6	183	100.0	1.3
CM246	2.5	117	27	36	60	-2.7	1.6	115	22	31	55	-2.7	–	28.6	0.6
CM248	1.8	115	27	35	56	-3.1	26.6	113	22	32	53	-2.9	265	100.0	1.7
CM249	2.2	128	25	36	62	-2.5	11.2	129	25	35	59	-2.6	290	100.0	2.1
CM250	2.4	142	28	39	65	-2.5	2.6	141	24	35	61	-2.4	–	17.0	0.3
CM251	2.5	130	25	36	63	-2.4	0.6	129	21	31	55	-2.6	–	30.8	0.7
CM252	1.9	127	26	36	62	-2.5	25.0	123	23	33	58	-2.6	–	34.2	0.2
CM253	1.8	127	27	38	64	-2.5	26.9	126	24	34	60	-2.5	–	37.0	0.2
CM254	2.5	–	34	57	–	–	-1.3	–	27	43	177	-0.5	158	100.0	2.4
CM255	1.8	132	27	38	65	-2.4	27.5	129	22	33	58	-2.5	–	95.7	1.6

	Measures for yeast alone							Measures for yeast co-inoculated with SB3							
Yeast	**Residual L-malic acid (g L)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**Pct_malic_AF (%)**	**tend-AF (hours)**	**t35-AF (hours)**	**t50-AF (hours)**	**t80-AF (hours)**	**s50-80-AF**	**tend-MLF (hours)**	**Pct_malic_co (%)**	**malic_acid_LAB_consumed (g L)**
CM39	1.9	153	28	40	70	-2.1	25.7	165	24	35	65	-2.2	373	100.0	1.8
CM56	2.1	165	27	39	69	-2.1	17.5	168	24	35	63	-2.2	408	100.0	2.0
CM59	1.7	152	28	40	70	-2.2	30.3	159	25	37	65	-2.2	384	98.4	1.6
CM77	2.1	163	27	39	68	-2.2	17.8	166	24	35	62	-2.3	–	27.4	0.2
CM84	1.8	125	25	35	60	-2.6	26.9	122	23	32	56	-2.7	237	100.0	1.7
CM92	2.7	148	27	39	68	-2.2	-8.9	152	24	35	63	-2.3	–	35.3	1.1
CM94	2.4	138	28	40	68	-2.3	3.8	135	25	36	62	-2.4	365	100.0	2.3

Table S2: ANOVA results for SBxGN, M2xF15 and hemizygote fermentations. P-value and variance are displayed for the individual tests for *SSU1* allele (SB, GN or both: SB/GN, and M2, F15 or both: M2/F15), presence of bacteria (None or SB3), and additive allele and bacteria. Post-hoc analysis was used to determine groups (designated by the letters) for allele and bacteria variables. Measures tested were tend-AF, t35-AF and s50-80-AF which represent time to complete AF, time to complete 35% of AF and slope value for points between t50-AF and t80-AF, respectively.

	SBxGN	**tend-AF**	**t35-AF**	**s50-80-AF**
***p*-value**	Allele	0.001	0.432	0
	Bacteria	0.042	0.047	0.254
	Allele:Bacteria	0.383	0.906	0.14
Variance observed	Allele	61	10	77
	Bacteria	11	26	2
	Allele:Bacteria	4	1	6
Post-hoc group: Allele	GN	a	a	a
	GN/SB	b	a	b
	SB	b	a	b
Post-hoc group: Bacteria	None	b	b	a
	SB3	a	a	a

	M2xF15	**tend-AF**	**t35-AF**	**s50-80-AF**
***p*-value**	Allele	0.09	0.065	0
	Bacteria	0.172	0.156	0.177
	Allele:Bacteria	0.566	0.639	0.746
Variance observed	Allele	38	31	79
	Bacteria	13	10	3
	Allele:Bacteria	2	4	1
Post-hoc group: Allele	F15	a	a	b
	M2	a	a	a
	M2/F15	a	a	b
Post-hoc group: Bacteria	None	a	a	a
	SB3	a	a	a

Table S3: ANOVA results for SBxGN, M2xF15 and hemizygote fermentations. P-value and variance are displayed for the individual tests for allele (translocation: *SSU1*-t; wild-type: *SSU1*-wt; or both: *SSU1*-t/wt), presence of bacteria (None or SB3), yeast background (SBxGN or M2xF15), additive allele and bacteria, or additive allele and yeast background. Post-hoc analysis was used to determine groups (designated by the letters) for allele, bacteria and yeast background variables. Measures tested were tend-AF and s50-80-AF which are time to complete AF and slope value for points between t50-AF and t80-AF, respectively.

		tend-AF	**s50-80-AF**
***p*-value**	Allele	0.006	0
	Bacteria	0.002	0.395
	Yeast background	0.019	0.527
	Translocation:Bacteria	0.186	0.325
	Translocation:Yeast background	0	0
Variance observed	Allele	16	17
	Bacteria	15	1
	Yeast background	8	0
	Translocation:Bacteria	4	2
	Translocation:Yeast background	31	62
Post-hoc group: Allele	*SSU1*-t	a	b
	SSU1-t/wt	ab	b
	SSU1-wt	b	a
Post-hoc group: Bacteria	None	b	a
	SB3	a	a
Post-hoc group: Yeast background	M2xF15	a	a
	SBxGN	b	a

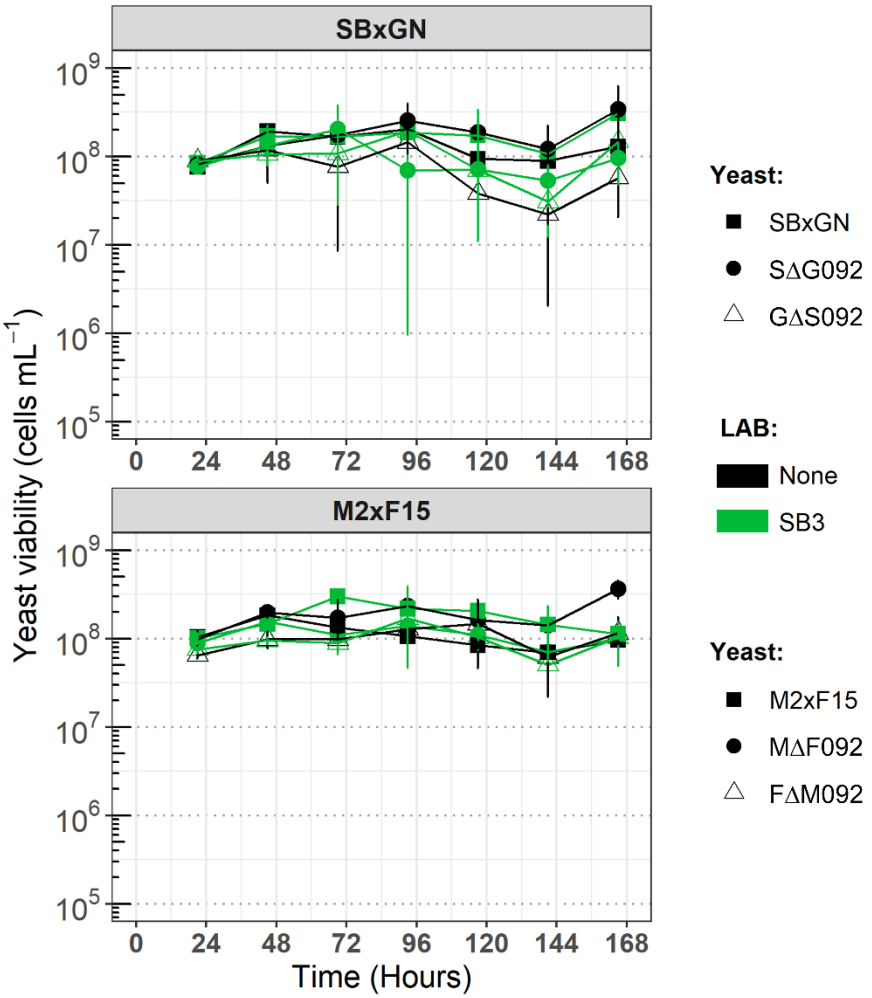

Figure S1: Yeast viability (cells mL^{-1}) measured over the course of fermentation. Colour indicate yeast alone (black) or yeast co-inoculated with SB3 (green). Shapes indicate yeast strains: SBxGN or M2xF15 (filled square), SΔG092 or MΔF092 (filled circle), GΔS092 or FΔM092 (empty triangle). Values are the mean of triplicates and error bars are the standard deviation.

Appendix D

Supplementary information for Chapter 6

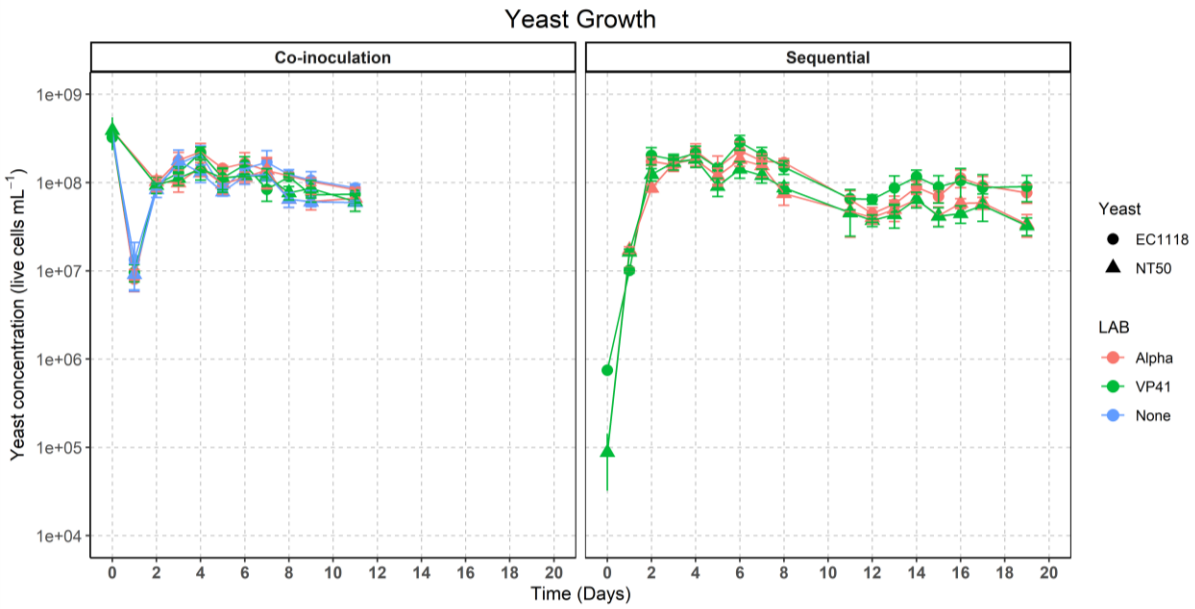

Figure S1: EC1118 (circles) and NT50 (triangles) growth (cells mL^{-1}) over the course of co-inoculation and sequential fermentations with Alpha and VP41 LAB. Different colours indicates different LAB strains (red= Alpha, green = VP41) or yeast only controls (blue). Values are the mean of six replicates and error bars are the standard deviation.

Table S1: pH measured at the conclusion of the experiment. Values are the mean of six replicates ± standard deviation.

Inoculation strategy	Yeast	LAB	pH
Sequential	EC1118	Alpha	3.4 ± 0
	EC1118	VP41	3.5 ± 0
	NT50	Alpha	3.4 ± 0
	NT50	VP41	3.4 ± 0
Co-inoculation	EC1118	None	3.4 ± 0
	EC1118	Alpha	3.6 ± 0
	EC1118	VP41	3.6 ± 0
	NT50	None	3.4 ± 0
	NT50	Alpha	3.6 ± 0
	NT50	VP41	3.6 ± 0

Table S2: Concentration (cells mL^{-1}) of dead yeast in sequential fermentations at days 13 and 15, corresponding to RNA sampling points 48 and 96 hours post LAB inoculation. Values are the mean of six replicates ± standard deviation.

Yeast	LAB	48 hours Dead yeast (mg L^{-1})	96 hours Dead yeast (mg L^{-1})
EC1118	Alpha	$3.7 \times 10^7 \pm 5.52 \times 10^6$	$5.3 \times 10^7 \pm 1.05 \times 10^7$
EC1118	VP41	$5.4 \times 10^7 \pm 2.44 \times 10^7$	$5.9 \times 10^7 \pm 2.07 \times 10^7$
NT50	Alpha	$3.8 \times 10^7 \pm 9.23 \times 10^6$	$3.9 \times 10^7 \pm 9.14 \times 10^6$
NT50	VP41	$3.4 \times 10^7 \pm 7.67 \times 10^6$	$4.3 \times 10^7 \pm 1.15 \times 10^7$

Table S3: Nitrogen* concentration measured at 48 and 96 hours post-LAB inoculation in EC1118 and NT50 co-inoculation fermentations. Values are the mean of six replicates ± standard deviation.

Yeast	LAB	48 hours Nitrogen* (mg L^{-1})	96 hours Nitrogen* (mg L^{-1})
EC1118	None	4.4 ± 0.9	10.7 ± 1.3
EC1118	Alpha	4.4 ± 1	11.9 ± 1.9
EC1118	VP41	4.1 ± 0.6	11.1 ± 1.6
NT50	None	0.5 ± 0.9	10 ± 0.8
NT50	Alpha	2.3 ± 1.7	9.2 ± 1.6
NT50	VP41	0.9 ± 0.7	9.6 ± 1.8

*Free amino nitrogen excluding proline and ammonia

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