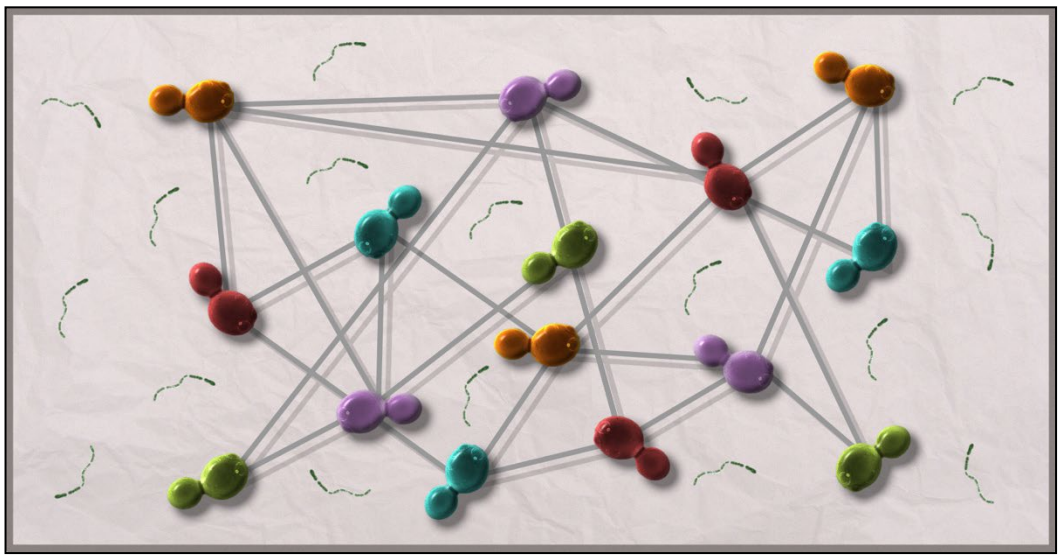
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The relationship between grape juice composition and the progress of alcoholic and malolactic fermentation



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1. Abstract

An outstanding problem in oenology is an understanding of the basis for sub-optimal fermentation of fruit whose compositional features are considered sufficient. The interaction between members of the grape microbial community has not been well considered in previous work. This project integrated yeast and bacterial research to explore the impact of oenological ecology on fermentation efficiency. Negative and positive interactions were identified between bacteria, non-*Saccharomyces* yeast and *Saccharomyces cerevisiae*. The response of microbial community members to inhibitory compounds was explored, with a particular focus on SO_2 tolerance in bacteria and SO_2 production by yeast.

2. Executive summary

Under the surface of a grape juice fermentation is a complex web of interactions between yeast, bacteria and their environment. Over the course of this project the contribution of individual microorganisms to the modification of grape fermentation composition was considered, starting with *Aureobasidium pullulans* and progressing to the effects of other non-*Saccharomyces* yeast found in fermentations. The changes imposed by these yeasts were shown to affect the behaviour of *S. cerevisiae* yeasts. Likewise, the strain-specific effects of *S. cerevisiae* on *Oenococcus oeni* were explored with a particular focus on SO_2 production. Finally, a detailed examination of the responses of *O. oeni* to SO_2 were undertaken, including approaches to their acclimatisation, optimal timing of their inoculation and an exploration of yeast-bacterial interactions during co-inoculation.

The physiological characterisation of *Aureobasidium pullulans*, one the most abundant species in freshly pressed grape juice, revealed its ability to substantially alter the juice matrix, causing depletion of zinc and iron. The results demonstrated that *A. pullulans* could compete for nutritional resources in juice and affect the fermentation performance of *S. cerevisiae*. A detailed physiological characterisation of *A. pullulans* also revealed that this organism contained enzymes capable of decreasing the concentration of galacturonic acid-based polysaccharides and hydrolysable gallotannin. It also produced enzymes capable of stimulating the release of glycosidically linked monoterpenes. These features of *A. pullulans* are all of oenological relevance.

The metabolic interactions between a wider array of non-*Saccharomyces* yeast and *S. cerevisiae* were characterised. This work demonstrated a general inhibition of *S. cerevisiae* by non-*Saccharomyces* yeast, with each interaction appearing to operate via different mechanisms. While the characterisation of these varied interactions is not yet complete, initial work suggests that at least some of them are based on a competition for nutrients. Given that many of the non-*Saccharomyces* yeast investigated in this work are increasingly available as cultures that can be inoculated into juice and must, the work has direct relevance for the management of these more complex fermentation regimes. These organisms are also members of the microbial community naturally present in freshly harvested fruit. Therefore, this research may provide insight into problematic fermentations observed in nutritionally replete fruit.

A survey of strain-specific interactions between an array of different *S. cerevisiae* yeast and select non-*Saccharomyces* yeast demonstrated that some *S. cerevisiae* strains are more competitive in mixed community environments than others. The study points toward selection criteria for yeast performance attributes that are not typically considered.

Unlike *S. cerevisiae*, bacterial fermentation can benefit from mixed community fermentations. The presence of non-*Saccharomyces* yeasts was found to stimulate MLF. These disparate observations raise the question of whether non-*Saccharomyces* yeast can be used to stimulate MLF without detrimental effects on alcoholic fermentation.

In parallel with studies of yeast-yeast interaction, the project team focused on various issues related to the production of and tolerance to SO_2. A detailed exploration of two factors known to

be inhibitory to yeast, copper and SO_2 concentration, was completed. Copper and SO_2 arguably have the largest effect on strain-dependent performance. That the mechanisms of resistance to these two inhibitory compounds interact within yeast metabolism was unforeseen and provided a deeper insight into a fundamental metabolic function of yeast, the assimilation of sulfate. The findings have important practical implications for strain development, indicating that a less forcefully driven *SSU1* gene may decrease the metabolic burden in commercial yeast strains. It is also informative concerning other fundamental features of fermentation management; that is, the production of hydrogen sulfide and SO_2 and, more broadly, nitrogen management.

The findings about the relationship between SO_2 tolerance in yeast and SO_2 production stimulated a broader analysis of the production potential of a representative set of *S. cerevisiae* wine strains. SO_2 concentrations produced by yeast were found to vary widely, with approximately half of the strains increasing SO_2 concentrations and the other half decreasing them. This knowledge provides an immediate initial selection criterion by which wine yeast can be selected as compatible partners for MLF and provides some metrics that can be used by winemakers wishing to control SO_2 concentrations in their wines.

A system for assessing the tolerance of *O. oeni* to SO_2 was developed that demonstrated there was little variation in SO_2 tolerance among *O. oeni* strains. A Chardonnay co-inoculation study was useful for understanding the features of SO_2 inhibition in a winemaking context. The question about the relative contributions of molecular SO_2 and total SO_2 to the viability loss experienced by *O. oeni,* and the inhibition of MLF remains unresolved. The work suggested that a long-held view regarding the potential for consumption of acetaldehyde-bound SO_2 by *O. oeni* may not be correct and led directly to laboratory and pilot-scale experiments investigating interactions between bacteria and SO_2-producing yeasts.

The direct effect of SO_2 production by yeast on *O. oeni* survival and MLF progress was assessed through a series of co-inoculation fermentation trials. Although most interactions between bacteria and high-SO_2-producing yeast were unsurprisingly negative, one high-SO_2-producing yeast was identified that supported maintenance of bacterial viability and efficient MLF progress. This exception to the common observation revealed acetaldehyde as a key metabolite supporting a robust relationship between yeast and bacteria. The yeast strain produced a vast transient excess of this acetaldehyde that appeared to quench the detrimental effects of SO_2 for long enough that the bacterium could survive. The work was important for two reasons; firstly, it demonstrated that *O. oeni* could become acclimatised to high total SO_2 concentrations and, secondly, that *O. oeni* is more than likely exquisitely sensitive to molecular SO_2.

An investigation was undertaken of the transcriptional changes associated with SO_2 stress in *O. oeni*. This work showed that major transcriptional changes occurred following exposure to 5 mg/L SO_2 and indicated that this compound primarily interacts with intracellular proteins, DNA and the cell envelope of *O. oeni*. Overall, *O. oeni* had the ability to counteract mild SO_2 stress through direct cellular responses. However, exposure to greater SO_2 stress caused irreparable damage. The bacterium was found to lack components that could actively and specifically counteract SO_2, such as those found in many yeasts.

3. Acknowledgements

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Australia provided analytical support. In particular, the team would like to acknowledge the efforts of Jang-Eun Lee from the Korea Food Research Institute for untargeted metabolite analysis.

The project team would also like to acknowledge Anthony Borneman for his contribution to quantitative trait loci analysis, the sensory team, including Leigh Francis, Desiree Likos, Eleanor Bilogrevic, Damian Espinase Nandorfy and the quantitative assessment panel for their help with sensory analysis of wine, Mark Solomon, Alex Schulkin, Mango Parker and Lisa Pisaniello from the AWRI for their assistance in LC-MS, GC-MS and HPLC analyses for analytical chemistry support and Stella Kassara and Keren Bindon for assistance with phenolic analysis of wine. Robyn Kievit is acknowledged for her work engineering the plasmids used in the project.

WIC Winemaking Services contributed to the preparation of grape juice and the production of wine.

4. Background

Difficulties with alcoholic and malolactic fermentation are routinely reported and can be attributed to a diverse range of causes. Poor fermentation progress can occur even in juices and wines that otherwise satisfy the usual criteria indicative of appropriate fermentation progress (e.g. YAN, Baumé and SO_2). Previous work highlighted strain-specific limitations and essential nutrient requirements, which contributed to an understanding of compositional features of juice and must that underpin fermentation performance issues related to *S. cerevisiae*.

The factors not considered in earlier work relate to the ecological environment into which *S. cerevisiae* and *O. oeni* are inoculated. Uncontrolled growth of non-target microorganisms has been reported to be inhibitory to alcoholic fermentation, either through the consumption of nutrients or the production of secondary metabolites. How other organisms within the ecological milieu of juice or must compete with, or contribute to, the functions of *S. cerevisiae* and *O. oeni* during fermentation is a particular focus of this work.

In addition to the ecological features of juice and must, the human element also requires consideration. Sulfur dioxide additions to bins and crushers are used to control pre-fermentation microbial activity, however, even moderate levels of total SO_2 can negatively affect the progress of malolactic fermentation, and larger additions can become inhibitory to even the more SO_2-resistant wine yeasts. In addition, some yeasts produce large amounts of SO_2, which can compound the effects of earlier additions. This is a particular concern as simultaneous alcoholic and malolactic fermentations are increasingly being used to more efficiently manage scheduling issues associated with post-fermentation MLF. This project explored in great detail the contribution to ferment SO_2 concentration by yeast, the timing of its production and how that yeast-derived SO_2 interacts with other products of metabolism to modulate *O. oeni* survival and MLF kinetics.

The areas of yeast and bacterial fermentation performance are interrelated, and understanding the risks and capturing opportunities of yeast-yeast and yeast-bacterial interactions required an integrated approach. Hence this project brought together two previously separate research areas, yeast and bacterial fermentation, to realise an integrated approach to studying alcoholic and malolactic fermentation performance.

5. Project aims

This project, together with others underway in the Australian grape and wine community, contributed to the following broad aim:

- Improved understanding, monitoring and management options are available for alcoholic and malolactic fermentation, and for the management of difficult fermentations.

Project deliverables were:

- Knowledge of yeast implantation efficiencies
- Knowledge of juice or wine compositional factors that give rise to yeast or bacteria strain-specific fermentation problems
- A model system for the investigation of sequential inoculation performance
- Assessment of strategies for a transformation system for *O. oeni*
- Understanding of minimum inhibitory concentrations of common wine matrix elements on strains of *O. oeni*
- Knowledge of incompatibilities between yeast and bacteria that result in poor fermentation performance
- Knowledge of genetic determinants of MLF performance
- Understanding of fermentation management practices that limit the negative impacts of co-fermentation and their wine style consequences
- Assessment of market readiness of new *O. oeni* strains sourced from other projects
- Knowledge from years 1-3 validated through feedback from industry trials
- Practical advice and extension materials for winemakers.

6. Methods

6.1 Knowledge of yeast implantation efficiencies

Copper-tolerant (AWRI 3471) and copper-sensitive (AWRI 3807 and AWRI 3470) spores were mated to produce diploid yeast that contained barcodes from each of the respective parents. Mating was undertaken by growing each parent in YPD overnight at 28°C and mixing equal volumes of each culture. Potential zygotes were isolated using a micromanipulator and were screened for mating-type by PCR. Two diploids were isolated in this way. AWRI 3811 was the product of a cross between AWRI 3471 and AWRI 3807. AWRI 3001 was the product of a cross between AWRI 3471 and AWRI 3470. Diploids were sporulated, and their copper tolerance was assessed in 100 mL cultures. Copper tolerant and sensitive spores were pooled, DNA isolated and the pooled DNA sequenced. Bulk segregant analysis was undertaken by mapping single nucleotide polymorphisms of each parent and estimating their frequency in the respective high tolerance and low tolerance pools.

CUP1 copy number was estimated by quantitative PCR using the R package qpcR (Ritz & Spiess 2008). Yeast strain fitness was estimated as described in Schmidt et al. (2019). The genes in the upper branch of the sulfur assimilation pathway were overexpressed via the replacement of their native promoters with constitutively expressed yeast promoters (Ottoz & Rudolf 2018). *SSU1* promoter replacement in the AWRI 3471 strain was undertaken using the approach of Storici and Resnick (2006) using *natMX* selective and *GIN11* counter selective markers as previously described (Varela et al. 2012). A full description of the methods can be found in the submitted manuscript by Onetto et al. (2022).

6.2 Knowledge of juice or wine compositional factors that give rise to yeast or bacteria strain-specific fermentation problems

6.2.1 Strategies to minimise SO_2 inhibition of malolactic starter cultures in white wine – starter culture acclimatisation

A preliminary study (2 L scale) was undertaken to investigate the effects of acclimatising commercial bacterial starter cultures on MLF performance in Chardonnay wine (pH 3.5, 14.2% v/v alcohol) with moderate (40 mg/L) and high (60 mg/L) concentrations of total SO_2. For each wine treatment, commercial strains of *O. oeni* (YV Select, Viniflora CH35) were evaluated using three different protocols:

(i) control (no acclimatisation)
(ii) 24 hours acclimatisation
(iii) 48 hours acclimatisation.

Further studies were undertaken to identify critical factors affecting the survival of a commercial *O. oeni* strain (YV Select) during acclimatisation, including utilisation of L-malic acid and SO_2 concentration.

6.2.2 Metabolic interactions between non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* that give rise to fermentation problems

Laboratory-scale (100 mL) fermentations were conducted using chemically defined media (CDM). Initial characterisation of the interaction state as either positive or negative, and specific mechanisms of interaction (cell-to-cell contact-dependent, nutrient competition and/or antimicrobial metabolite production) were investigated using inter-species co-cultivations and non-*Saccharomyces* culture filtrates.

Five non-*Saccharomyces* yeasts were evaluated in these experiments. *Aureobasidium pullulans*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* and *Lachancea thermotolerans*. Chemical characterisation of non-*Saccharomyces* culture filtrates was performed by HPLC, HPLC-MS, ICP-MS, LCMS, Orbitrap and enzymatic methods. A blue fluorescent protein (BFP) gene was introduced in the HO ORF of the *S. cerevisiae* strains used to allow cell counting of *S. cerevisiae* by flow cytometry in the presence of a different yeast species as previously described in Onetto et al. (2021).

6.2.3 *S. cerevisiae* strain-specific responses to competition with non-*Saccharomyces* yeast

The competitive fitness of 93 *S. cerevisiae* strains in response to five non-*Saccharomyces* yeasts (*A. pullulans*, *H. uvarum*, *M. pulcherrima*, *T. delbrueckii* and *L. thermotolerans*) individually, and a pool of all five, was evaluated using a wine yeast barcode library (WYBC) (described in Schmidt et al. 2019). Each non-*Saccharomyces* species was inoculated into 100 mL flasks for 24 h, after which 1 mL of WYBC pool was added to each flask. A control treatment without the presence of a non-*Saccharomyces* species was also included.

After three days of growth, samples were taken for DNA extraction, and 1 mL of each flask was used to inoculate another set of treatments and control flasks using a serial batch approach. Sequential inoculations were performed twice for a total of three (T1, T2 and T3) sampling time points. Genomic DNA was extracted using a Gentra Puregene Yeast/Bact kit (Qiagen, Doncaster, Victoria, Australia) and Amplicons for Illumina sequencing were generated using primers Illum_P5_S50(1-6) and Illum_P7_N70(1-12), and PCR conditions as previously described (Schmidt et al. 2019). Sequencing was performed at the Ramaciotti Centre for Genomics (UNSW Sydney, Australia). Bioinformatic and data analysis were performed as described in Onetto et al. (2021).

6.2.4 Defining the impact of non-*Saccharomyces* yeasts on sequential malolactic fermentation (MLF)

MLF performance of two *Oenococcus oeni* commercial strains (VP41 and CH35) was evaluated in laboratory scale wines fermented using five non-*Saccharomyces* yeasts (*A. pullulans*, *H. uvarum*, *M. pulcherrima*, *T. delbrueckii* and *L. thermotolerans*) and *S. cerevisiae*. Malic acid consumption was monitored enzymatically for 32 days.

6.2.5 Identifying new wild *O. oeni* strains tolerant to wine stress factors

More than 200 natural *O. oeni* strains isolated from South Australian wild ferments over the 2017-2019 vintages were phenotypically screened for tolerance to pH and ethanol stress by assessment of growth (colony size) on a semi-defined solid medium (MRS-FM) adjusted to pH 3.2, 3.4 and 3.6, and 6, 9 and 12% v/v ethanol.

The phenotyped *O. oeni* strains were also whole genome sequenced and assembled. Initial clustering of genetically similar strains was performed on the genome assemblies using MASH distances and a minimum average nucleotide identity (ANI) of 99%. Strains within each cluster

were then compared by mapping the reads to a representative strain and identifying single nucleotide polymorphisms (SNPs) as previously described Olm et al. (2021).

Pairwise comparisons were performed between the identified SNPs within each cluster of strains, and strains with an ANI ≥ 99.999% were considered genetically identical. Phylogenetic analysis was performed using the SNPs identified in all strains against the reference genome of *O. oeni* strain AWRI B429. In the final stage, 12 genetically distinct strains exhibiting superior pH and ethanol tolerance were selected for an assessment of MLF performance compared to a commercial reference strain by co-inoculation in Chardonnay (pH 3.3).

6.3 Assessment of strategies for a transformation system for *O. oeni*

The molecular elements required for the construction of plasmids used in attempts to transform *O. oeni* were produced by Genescript. Transformations were performed using electroporation and the Bacterial Protocol number 5 from Biorad on a GenePulser Xcell. Methods were based on those described in Spath et al. (2012). The electroporation buffer is provided in Table 1. Bacterial strains were provided by the AWRI Wine Microorganism Culture Collection.

Table 1. Buffer used for the electroporation of *O. oeni*

Electroporation component	Concentration
KH_2PO_4	5 mM
$MgCl_2{*}6H_2O$	0.2 mM*
Glycerol	10% (v/v)
Ethanol - absolute	10% (v/v)
Sucrose	0.5 M
H_2O	
pH 5.0, sterilise by filtration	

6.4 Understanding of minimum inhibitory concentrations of common wine matrix elements on strains of *O. oeni*

6.4.1 Minimum inhibitory concentrations of SO_2 on *O. oeni*

Initial studies examined the inhibition of *O. oeni* growth by acetaldehyde-bound SO_2 (0-25 mg/L) in a semi-defined wine-like medium (pH 3.5, 10% v/v ethanol) at a laboratory scale. As this method indicated bacterial growth was inhibited by low bound SO_2 concentration, an alternative was investigated in which bacteria strains were screened for SO_2 tolerance by co-fermentation with a compatible, non SO_2-producing yeast strain in Chardonnay grape juice.

Twenty phylogenetically distinct *O. oeni* strains were screened for tolerance to acetaldehyde-bound SO_2 (0-60 mg/L) following co-inoculation in Chardonnay juice (pH 3.3). The yeast strain (*S. cerevisiae* AWRI 2863) was selected based on the amount of SO_2 it produced and its compatibility with *O. oeni*. Fermentations (100 mL) at each SO_2 concentration were initiated by yeast inoculation. Pre-acclimatised *O. oeni* strains were co-inoculated into each SO_2 treatment 24 hours after the yeast. The tolerance of *O. oeni* strains to acetaldehyde-bound SO_2 was determined by assessing change in bacterial viability after five days. A final study examined the inhibition of *O. oeni* by acetaldehyde-bound and molecular SO_2 in Chardonnay grape juice.

6.5 Knowledge of incompatibilities between yeast and bacteria that result in poor fermentation performance

6.5.1 Screening wine yeast strains for SO_2 production

The SO_2 production potential of 94 genetically distinct *S. cerevisiae* wine strains, comprising isolates of commercial wine yeast preparations and other wine yeast strains from the AWRI Wine Microorganism Culture Collection was assessed at laboratory scale (100 mL) following fermentation in Chardonnay juice (initial SO_2 concentration 41 mg/L). After fermentation, wines were clarified and filtered (0.2 µm) before analysis of total SO_2.

6.5.2 The effects of yeast-derived SO_2-production on survival and MLF performance of *Oenococcus oeni* during co-fermentation in Chardonnay

Saccharomyces cerevisiae wine yeast strains with known SO_2-producing capability were assessed for their impacts on the survival and MLF performance of selected commercial *O. oeni* strains following co-inoculation in Chardonnay. Co-fermentations were conducted at both laboratory (100-500 mL) and pilot (35 L) scales, with MLF progress monitored by quantification of L-malic acid and viable bacteria concentration. The concentrations of total SO_2 and, in some cases, acetaldehyde were also determined during fermentation, from which low concentrations of free SO_2 were calculated using acetaldehyde-bisulfite equilibria [$K_d = 1.5 \times 10^{-6}$, pH 3.3]. All experiments were conducted in triplicate.

6.6 Knowledge of genetic determinants of MLF performance

6.6.1 SO_2 tolerance of *O. oeni* and the potential role of *tauE* sulfite transport gene

The effects of bound SO_2 exposure on the survival of representative *O. oeni* with (four strains) or without (four strains) the *tauE* gene were examined in a fermented model wine medium. The latter medium was prepared by fermenting chemically defined grape juice medium (CDM) with *S. cerevisiae* AWRI 1483 (D254), pH adjustment to 3.5 and 12% v/v ethanol. Two wines were prepared from this, which were either treated with SO_2 (final total SO_2 concentration of 14 mg/L) or left untreated as a control (final SO_2 concentration <4 mg/L). Survival (viable cell concentration) of *O. oeni* strains was monitored after three days of exposure to each treatment (70 mL, triplicate).

6.6.2 Transcriptomic analysis of *O. oeni* SO_2 stress response

Transcriptional analysis of the genetic elements used by *O. oeni* to counteract SO_2 stress was undertaken using an RNA sequencing (RNA-seq)-based approach. Cells of *O. oeni* AWRI B429 were cultured in a semi-defined synthetic medium (pH 3.5, 250 mL) in a continuous culture system. The transcriptomes of steady-state *O. oeni* cells were measured 30 minutes and 4 hours after exposure to two SO_2 treatments (5 and 10 mg/L). RNA-seq analysis was undertaken using Illumina NextSeq and the Nanopore long-read genome assembly of *O. oeni* for accurate transcriptome mapping.

7. Results and discussion

7.1 Knowledge of yeast implantation efficiencies

The commercial and environmental prevalence of copper and SO_2 has provided an environment where phenotypic and genotypic variance in the resistance to these two compounds has manifested in wine yeast and environmental isolates, including medical specimens (Strope et al. 2015). Freshly prepared grape juice typically contains between 0.5 and 1.5 mg/L of copper, with concentrations up to 7 mg/L (Schmidt et al. 2011) or higher, depending on copper usage in the vineyard (Provenzano et al. 2010). Free SO_2 can also be present in juice or must at concentrations greater than 20 mg/L.

Elevated concentrations of both copper and SO_2 in grape juice have been shown to have strain-dependent effects on the effectiveness of fermentation initiation (Cavazza et al. 2013, Morgan et al. 2019). Wine yeasts have different mechanisms to deal with copper and SO_2 stress. Copper resistance is delivered through copy number expansion of a metallothionine gene (*CUP1*) that regulates intracellular copper concentrations (Fogel et al. 1983). SO_2 tolerance is provided by a protein transporter *SSU1*, the overexpression of which is caused by a promoter alteration (Goto-Yamamoto et al. 1998). Despite the apparent independence of these two mechanisms, a

relationship between SO_2 tolerance and copper sensitivity in wine yeast has been noted (Schmidt et al. 2019).

The project team sought to understand the relationship between SO_2 and copper tolerance because of the potential for a direct impact of these stress resistance mechanisms on the survival of yeast strains following inoculation. To determine the contribution of *CUP1* copy number variation to copper tolerance in wine yeast, the copy number status of 94 yeast strains was determined and compared with competitive fitness scores in a copper-containing medium. The comparison identified many strains that exhibited copper sensitivity despite carrying a high number of *CUP1* copies (Figure 1). This finding is consistent with Peter et al. (2018), who found that copy number expansion explains only 44.5% of phenotypic variation in copper tolerance.

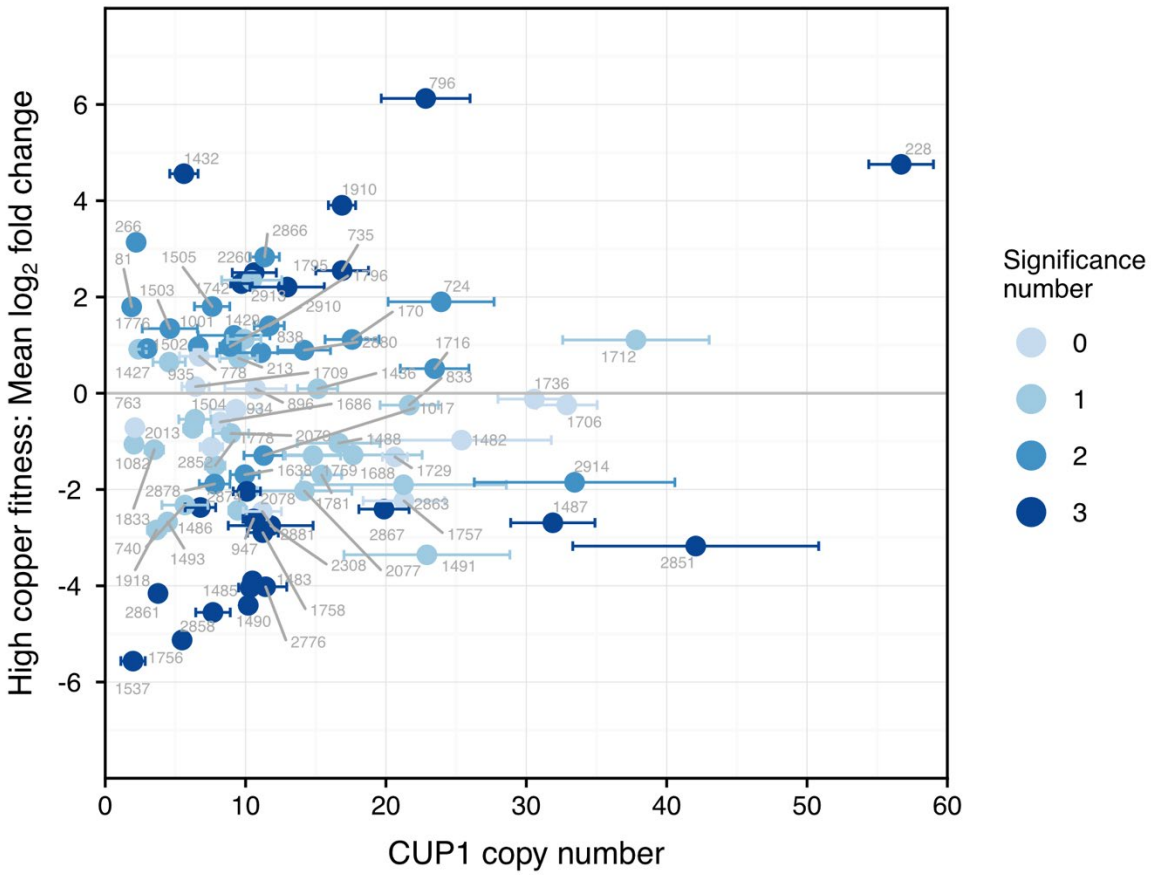


Figure 1. The relationship of yeast strain resistance to high copper concentrations with *CUP1* copy number. Points show the mean copper resistance (y-axis) and *CUP1* copy number (x-axis) of three independent experiments. Each spot gives the data for a different strain, as indicated by the associated AWRI culture collection number. Error bars show the estimated variance in *CUP1* copy number (standard deviation) as determined by qPCR.

The genetic contributions to copper sensitivity in selected strains were determined by mating strains with equivalent or differential *CUP1* copy numbers and/or fitness in high-copper media, followed by bulk segregant analysis of progeny. This work identified *SSU1*, the gene responsible for SO_2 tolerance, as a major contributing factor to copper sensitivity in wine yeast with a high *CUP1* copy number. Such yeast would otherwise have been predicted to be resistant to elevated copper concentrations.

The contribution of *SSU1* to copper sensitivity was confirmed through the reciprocal deletion of *SSU1* in a hybrid between a copper-resistant (AWRI 796) and a copper-sensitive (AWRI 1487) yeast (Figure 2). These deletion analyses showed that it is the version of *SSU1* derived from the SO_2-tolerant, copper-sensitive strain (AWRI 1487) and is the cause of copper sensitivity in the hybrid.

Transcriptomic and proteomic analysis identified a potential metabolic limitation induced by growth in high copper in SO_2-tolerant wine yeast. These analyses also revealed that copper sensitivity was not the result of an inability to produce the Cup1p metallothionine in *SSU1* overexpressing yeast.

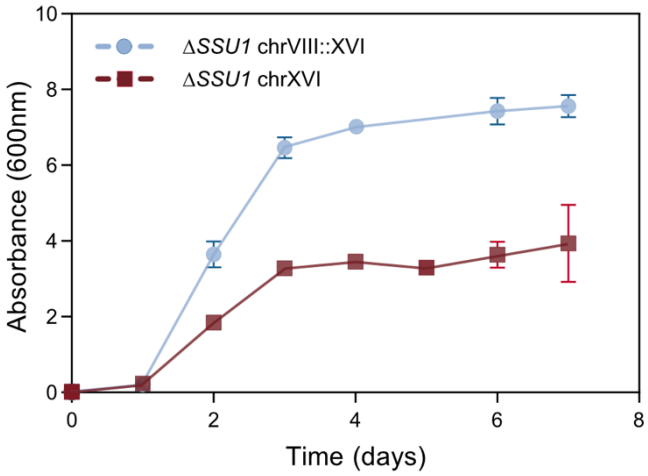


Figure 2. The effect of deletion, of each of the two *SSU1* copies, on copper tolerance of a hybrid strain produced by the mating of AWRI 796 and AWRI 1487. Lines show growth following deletion of *SSU1* on chr XVI (wild type, blue) and chrVIII::XVI (translocated, red) in 10 mg/L of copper. Error bars show standard deviation. Where error bars are not visible, they are obscured by the point, which shows the mean (n=3).

Analysis of the sulfate assimilation pathway using a series of overexpression studies suggested that *SSU1* overexpression restricts the flux of sulfur through to H_2S. However, decreased total H_2S production was not directly observed in *SSU1* overexpressing yeast. A restriction in the flux of sulfur through the assimilation pathway also suggests that these yeast strains would be more susceptible to sulfate limitation.

A comparison between the growth of *SSU1* overexpressing and control yeast strains in media with decreasing sulfate concentrations did not reveal increased sensitivity to sulfate limitation. However, the experiment did help to define a threshold concentration of sulfate below which growth was increasingly limited. When an *SSU1* overexpressing strain was evaluated in a medium in which sulfate was limited, it was found to be highly sensitive to copper, with as little as 2 mg/L suppressing growth and impeding sugar utilisation. There was evidence for an interaction between copper and sulfate concentration, which delayed growth and extended fermentation times in *SSU1* overexpressing yeast (Figure 3).

This work has important practical implications for strain development, indicating that a less forcefully driven *SSU1* gene may decrease the metabolic burden of SO_2 tolerance, leading to better performance characteristics in commercial yeast strains.

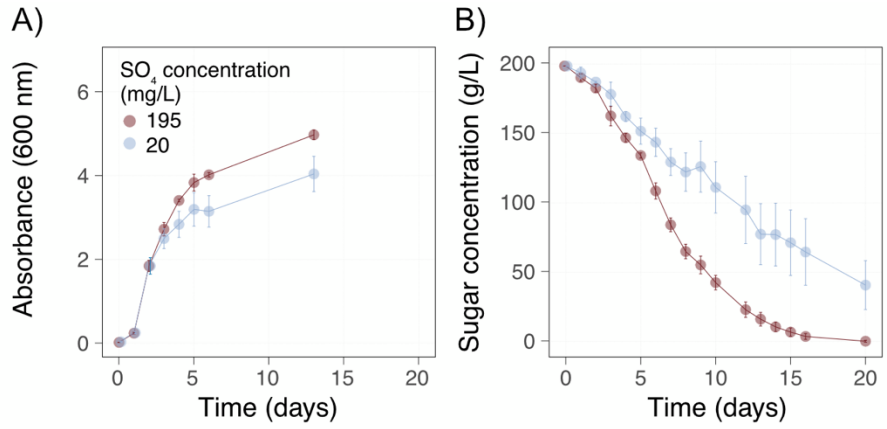


Figure 3. The combined effect of copper concentration (6 mg/L) and sulfate concentration (20 and 195 mg/L) on the growth and fermentation kinetics of the yeast strain AWRI 4052 (*SSU1* overexpressing yeast). Points show the mean of three replicates with error bars indicating standard deviation

7.2 Knowledge of juice or wine compositional factors that give rise to yeast or bacteria strain-specific fermentation problems

7.2.1 Strategies to minimise SO_2-inhibition of malolactic starter cultures in white wine – starter culture acclimatisation

Although most commercial malolactic cultures are claimed to survive direct inoculation into wine, significant viability loss can occur when wines with harsh conditions are inoculated. In particular, white and sparkling base wines can present a hostile environment for malolactic bacteria, with inhibitory factors typically including one or a combination of low pH (2.9-3.4), moderate to high concentrations of total SO_2 (up to 50-60 mg/L), lower fermentation temperature (down to 12°C) and a lower concentration of nutrients.

Of these factors, high SO_2 concentrations can be especially difficult for *O. oeni* to overcome, particularly free and molecular SO_2. Bacterial inhibition may also be associated with bound forms of SO_2, rendering SO_2 a major obstacle to the survival and performance of malolactic starter cultures. A long-standing approach to improving malolactic starter culture performance in difficult wine conditions is the implementation of an acclimatisation procedure (Zapparoli et al. 2016). However, while such procedures may improve MLF outcomes, there is a lack of knowledge concerning their efficacy, particularly regarding the alleviation of SO_2-inhibition of direct inoculation starter cultures.

This work demonstrated that MLF outcomes can be significantly improved under certain difficult wine conditions by acclimatising commercial malolactic starter cultures. For Chardonnay wine containing 40 mg/L total SO_2, direct inoculation of the bacterial starter cultures without acclimatisation resulted in >1,000-fold loss in viable bacteria within seven days and no degradation of L-malic acid after 47 days. Similar strain-dependent results occurred following inoculation of 24-hour acclimatised starter cultures. However, when a 48-hour acclimatisation procedure was coupled with sequential inoculation, comparatively minimal (<10-fold) loss in bacterial viability was observed. For one strain (YV Select), the bacterial population recovered sufficiently to complete MLF within 45 days (Figure 4). In contrast, inoculation of Chardonnay wine containing 60 mg/L total SO_2 caused rapid and complete death of bacteria. No L-malic acid degradation was observed in these ferments, irrespective of bacterial strain and acclimatisation procedure applied.

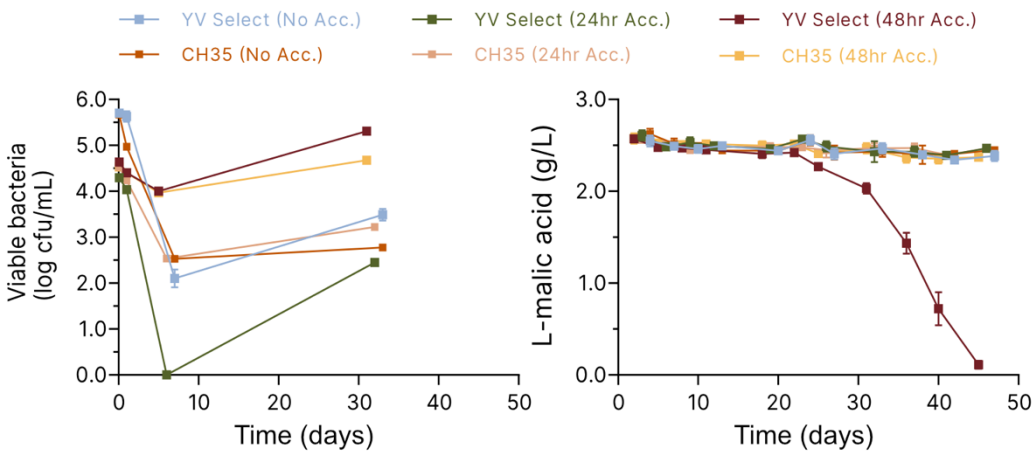


Figure 4. Effect of starter culture acclimatisation (0, 24 and 48 hours) on survival and MLF by *O. oeni* strains YV Select and CH35 after sequential inoculation in Chardonnay wine (pH 3.47, 14.2% v/v alcohol, 40 mg/L total SO_2). Symbols show mean ± sd of three replicates.

While this trial demonstrated a positive impact of acclimatisation on MLF performance, significant losses (≥10-fold) in bacterial viability were also observed during the acclimatisation procedure. The factors contributing to this loss were investigated. Initial work revealed that a model wine system could be used to replicate such loss in bacterial starter culture viability during acclimatisation and, importantly, demonstrated that final acetaldehyde-bound SO_2 concentrations of up to 25-30 mg/L had minimal impact on viability loss during this period.

Subsequent experiments demonstrated that during acclimatisation, the bacterial starter culture consumed all L-malic acid within 1 hour. Importantly, the exhaustion of L-malic acid as an energy source directly contributed to the decline in cell viability during acclimatisation in wine. *O. oeni* could only survive for a few hours without L-malic acid supplementation. These data were used to develop additional acclimatisation procedures in an attempt to improve MLF induction in higher SO_2 wines. However, regardless of the acclimatisation regime, the effective adaptation of strain YV select to 50 mg/L total SO_2 was not achieved.

7.2.2 Metabolic interactions between non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* that give rise to fermentation problems

Co-inoculation of commercial non-*Saccharomyces* yeasts with *S. cerevisiae* is a common practice, despite the limited knowledge regarding potential microbial interactions and their impact on fermentation performance. Complex microbial interactions can also be observed during the early stages of uninoculated ferments. The complexity of these microbial communities poses a challenge for the investigation of microbial interactions, and consequently, this is a largely unexplored area in the field of wine science.

Due to these challenges, a simplified dual-species approach was taken during this project to lay the foundations for the characterisation of the interactions between non-*Saccharomyces* species and *S. cerevisiae*. The non-*Saccharomyces* species investigated were selected based on their use as commercial starter cultures and their reported natural abundance in grape juice. *Aureobasidium pullulans* is one the most abundant species in freshly pressed grape juice (Sternes et al. 2017, Onetto et al. 2020) and therefore, initial experiments focused on characterising the effect of this species on the growth and fermentation kinetics of *S. cerevisiae*.

The growth of *S. cerevisiae* was evaluated in a CDM filtrate exposed to *A. pullulans* for 48 hours (Figure 5A). A clear growth arrest compared to the original media was observed when inoculating *S. cerevisiae*, indicating a nutritional and/or antimicrobial mode of competition. Individual addition of nutritional components to the *A. pullulans* filtrate showed a restoration of growth after the addition of trace elements. ICP-MS metal analyses revealed a large decrease in

the concentration of zinc and iron in the *Aureobasidium*-exposed medium, explaining the observed growth arrest.

To investigate whether the initial proliferation of *A. pullulans* could affect the growth of *S. cerevisiae* in a winemaking scenario, two different CDM, containing the lowest and highest concentrations of iron and zinc previously reported for Chardonnay grape juice (Schmidt et al. 2011) were tested. *A. pullulans* was inoculated prior to *S. cerevisiae* for 48 hours, and fermentation kinetics and cell viability were monitored (Figure 5B and C). Minor differences in fermentation kinetics were observed in the high Fe/Zn CDM. However, an apparent delay in fermentation, accompanied by a significantly lower viable cell count, was observed in the low Fe/Zn CDM treatment (Figure 5B and 1C).

Overall, the results indicate that *A. pullulans* will compete for nutritional resources in juice. This competition can result in minor effects on fermentation performance in juices with poor elemental nutrient concentrations. *A. pullulans*-based biological fungicides are commercially available to control *Botrytis* infections. Further research will be required to determine the impact of this application on the fermentation performance of *S. cerevisiae*. Additional information regarding the interaction of *A. pullulans* with *S. cerevisiae* and the physiology of this species is available in Onetto et al. (2020).

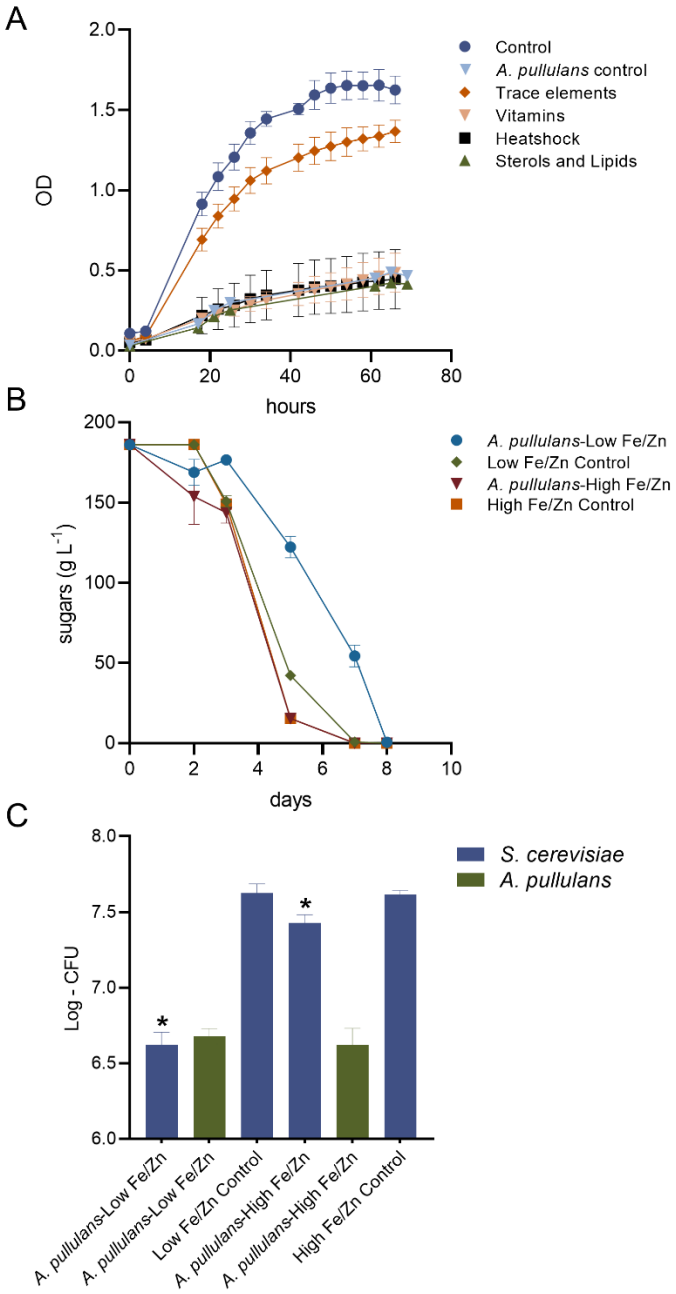


Figure 5. A. Growth of *Saccharomyces cerevisiae* inoculated in CDM filtrate exposed to *A. pullulans* for 48 hours. The filtrate was supplemented with trace elements, vitamins, sterols, lipids, and heat shocked prior to inoculation. B. Sugar consumption by *S. cerevisiae* sequentially inoculated 48 hours after *A. pullulans* in a low and high Fe and Zn CDM. C. Viable cell counts for Low and High Fe/Zn treatments 24 hours after inoculation with *S. cerevisiae*. All symbols and columns show the mean (A; n = 4, B and C; n =3) with error bars indicating the standard deviation. Significantly different values ($p < 0.05$) from the corresponding control are indicated with an asterisk.

This project sought to characterise the metabolic interactions of four additional non-*Saccharomyces* species using dual-species co-cultivations and non-*Saccharomyces* culture filtrates. Three of these species, *M. pulcherrima*, *T. delbrueckii* and *L. thermotolerans*, are already available as commercial starter cultures.

The experimental work showed differences in the mechanism of interaction between non-*Saccharomyces* species, with delays in fermentation completion observed in nearly all treatments (Figure 6). For *T. delbrueckii* (Figure 6A), *H. uvarum* (Figure 6C) and *L. thermotolerans* (Figure 6D), delayed fermentations under sequential inoculation were

exacerbated in the filtrates, suggesting a nutritional mode of competition. Conversely, *M. pulcherrima* antagonism was only observed when in direct contact with *S. cerevisiae* (Figure 6B).

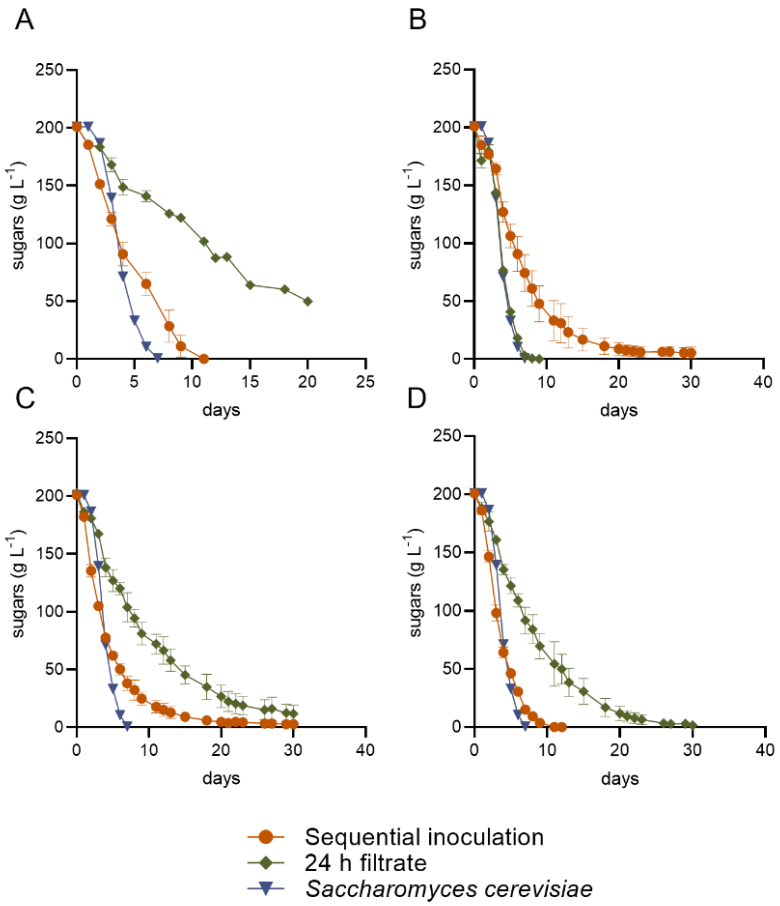


Figure 6. Sugar consumption kinetics of CDM inoculated with (A) *T. delbrueckii*, (B) *M. pulcherrima*, (C) *H. uvarum* and (D) L. *thermotolerans* for 24 hours prior to inoculation with *S. cerevisiae*. For each non-*Saccharomyces* treatment, a 24-hour non-*Saccharomyces* filtrate was also inoculated with *S. cerevisiae*. The blue label corresponds to the *S. cerevisiae* control without the presence of a non-*Saccharomyces* species. Symbols show mean ± sd (n=3).

Further characterisation of the filtrates was performed to identify potential nutritional deficiencies and the production of novel and antimicrobial metabolites. Untargeted metabolite analyses of the non-*Saccharomyces* filtrates showed striking differences in the metabolome of these species (Figure 7). Additionally, large differences in the abundance of individual metabolites compared to the original media were also observed (Figure 8), suggesting differences in physiology and potential species-specific modes of interaction.

Previously published work has suggested that *S. cerevisiae* inhibition by some of these species is related to the depletion of specific amino acids (Prior et al. 2019). Nitrogen availability analysis of the non-*Saccharomyces* filtrates showed near complete concentrations of α-amino nitrogen and NH_3 (Table 2). However, determination of the individual amino acid concentrations in these samples showed complete depletion of lysine in all samples, as well as complete depletion of methionine in the *T. delbrueckii* and *H. uvarum* treatments (Table 3).

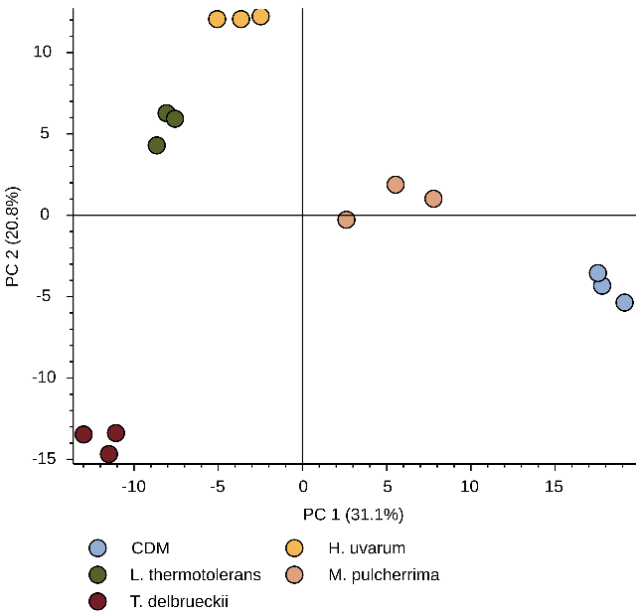


Figure 7. Principal component analyses of all identified metabolites in the non-polar fraction of the 24-hour non-*Saccharomyces* filtrates. Dots correspond to the analysis of individual replicates.

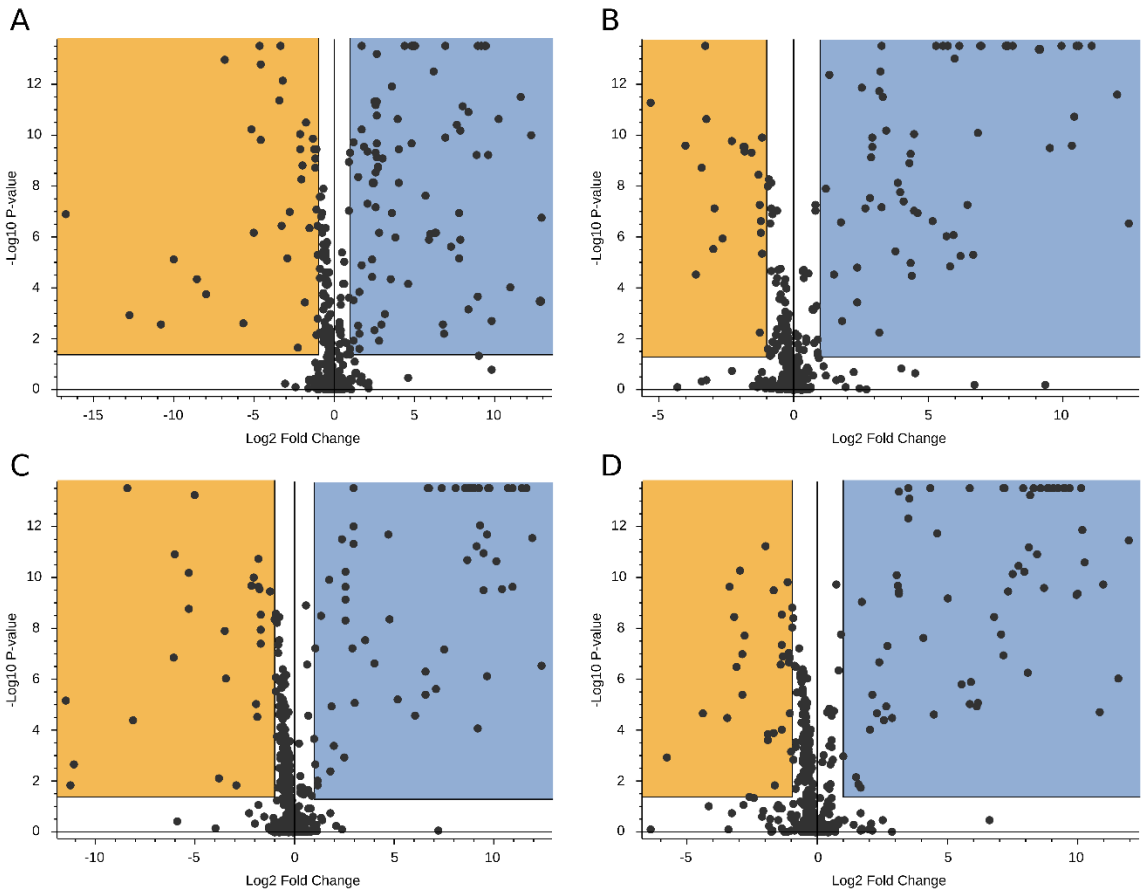


Figure 8. Differential analyses of non-polar metabolites between the 24-hour non-*Saccharomyces* filtrates and the control CDM. Points located within the blue and yellow boxes represent compounds with a $1 < \log_2$ Fold Change < -1 and a P-value < 0.05 for the comparison between (A) *T. delbrueckii*, (B) *M. pulcherrima*, (C) *H. uvarum* and (D) *L. thermotolerans* filtrate sample and the control CDM.

Table 2. Yeast available nitrogen concentration (mg/L) of CDM after 24 hours of inoculation with non-*Saccharomyces* species

Treatment	α-amino nitrogen	NH_3	YAN
CDM	176	155	303
T. delbrueckii	148	137	261
M. pulcherrima	157	153	282
H. uvarum	148	129	255
L. thermotolerans	152	145	271

Table 3. Amino acid concentration (mg/L) in CDM after 24 hours of inoculation with non-*Saccharomyces* species. Data is mean of three replicates.

Amino acid	*T. delbrueckii*	*M. pulcherrima*	*H. uvarum*	*L. thermotolerans*	CDM
Alanine	176.8	209.5	198.1	212.9	206
Arginine	400.2	452.5	410.4	395.9	452
Asparagine	4.7	6.2	4.8	5.7	6.4
Aspartic acid	36.2	54.2	41.1	33	52.3
Cysteine	9.2	11.7	10.8	8.9	10.6
Glutamic acid	102.4	96.3	105.3	108.4	119
Glutamine	104.9	124.2	147.7	163.2	194
Glycine	7.7	8.6	9.1	10.8	8.5
Histidine	10.8	12.9	10.5	12.2	16.8
Isoleucine	19.1	15.1	14.5	19.2	23.4
Leucine	17.1	12.4	8.4	11.1	23.5
Lysine	0	0	0	0	5.8
Methionine	0	0.5	0	0.3	1.9
Phenylalanine	13.2	10.4	10	9.2	15.7
Proline	656.2	686.2	670.1	677.1	674
Serine	75.1	99.4	78.4	95.5	105
Threonine	87.3	114.4	80.5	90.8	119
Tryptophan	1.6	4.4	2.4	3.2	6.2
Tyrosine	5.5	5.4	5.6	6.8	6.4
Valine	38.1	37.7	32.9	39.8	42.3

7.2.3 *S. cerevisiae* strain-specific responses to competition with non-*Saccharomyces* yeast

The inter-yeast interaction experiments described above confirmed that the antagonism/competition of non-*Saccharomyces* species against *S. cerevisiae* can affect fermentation performance. These experiments were performed using a single strain of *S. cerevisiae,* and it is unclear whether these negative interactions are strain-specific. Characterisation of strain-specific responses to co-inoculation would enable the identification of specific *S. cerevisiae* strain/non-*Saccharomyces* combinations that minimise the negative impacts of sequential inoculation on fermentation performance.

The strain-specific responses of *S. cerevisiae* to competition with non-*Saccharomyces* species using a genomically barcoded library of 93 *S. cerevisiae* strains (Schmidt et al. 2019) were

investigated using a serial-batch experimental approach. Observations made in serial-batch experiments were confirmed using individual *S. cerevisiae* strains.

Serial-batch experiments are conducted by taking a sample from a ferment after a defined period, in this case three days, and using it to inoculate another ferment. This approach allows diversification in population structure based on the fitness of individuals within that population. Sugar concentrations were measured in the sample used to inoculate the next batch as an indication of the overall capacity of the *S. cerevisiae* population to conduct fermentation of media that had been conditioned by exposure to different non-*Saccharomyces* yeast. These samples were designated T1, T2 and T3 in Table 4. A differential effect of the individual non-*Saccharomyces* species on the residual sugar concentration of mixed *S. cerevisiae* cultures at day 3 of each serial batch was observed (Table 4). Ferments containing *A. pullulans* and *M. pulcherrima* had the highest residual sugar concentration after three days of fermentation time (Table 4). Relative to the control condition, all mixed cultures had higher residual sugar concentrations at T2 and T3.

Competitive growth experiments showed the presence of strain-specific fitness responses to the non-*Saccharomyces* treatments (Figure 9) that varied depending on the non-*Saccharomyces* species present. To confirm these results, eight *S. cerevisiae* strains that showed either a strongly positive or negative fitness response to the presence of two non-*Saccharomyces* species were individually assessed in dual-species fermentation experiments (Figure 10).

In both non-*Saccharomyces* treatments, *S. cerevisiae* strains that showed a positive and negative $\log_2$ fold-change in competitive experiments correlated with faster and slower sugar consumption rates, respectively (Figure 10A and B). The correlation between the two experimental outcomes confirms that there is *S. cerevisiae* strain-dependent variation in response to a non-*Saccharomyces* species.

The results obtained in this project indicate that strain selection will influence fermentation performance when using non-*Saccharomyces* species. Therefore, appropriate strain/yeast combinations are required to optimise fermentation. Additional information regarding this set of experiments is available in Onetto et al. (2021).

Table 4. Residual sugar concentration (g/L) at three days post inoculation in each of three serial-batch fermentations (T1, T2 and T3). All data points correspond to the mean of three replicates ± sd.

Treatment	**T1**	**T2**	**T3**
A. pullulans	159 ± 5	175 ± 25	145 ± 20
H. uvarum	73 ± 3	90 ± 4	81 ± 2
M. pulcherrima	126 ± 2	141 ± 8	128 ± 5
T. delbrueckii	92 ± 8	121 ± 4	93 ± 8
L. thermotolerans	84 ± 4	97 ± 5	71 ± 4
WYBC control	101 ± 3	83 ± 1	69 ± 7

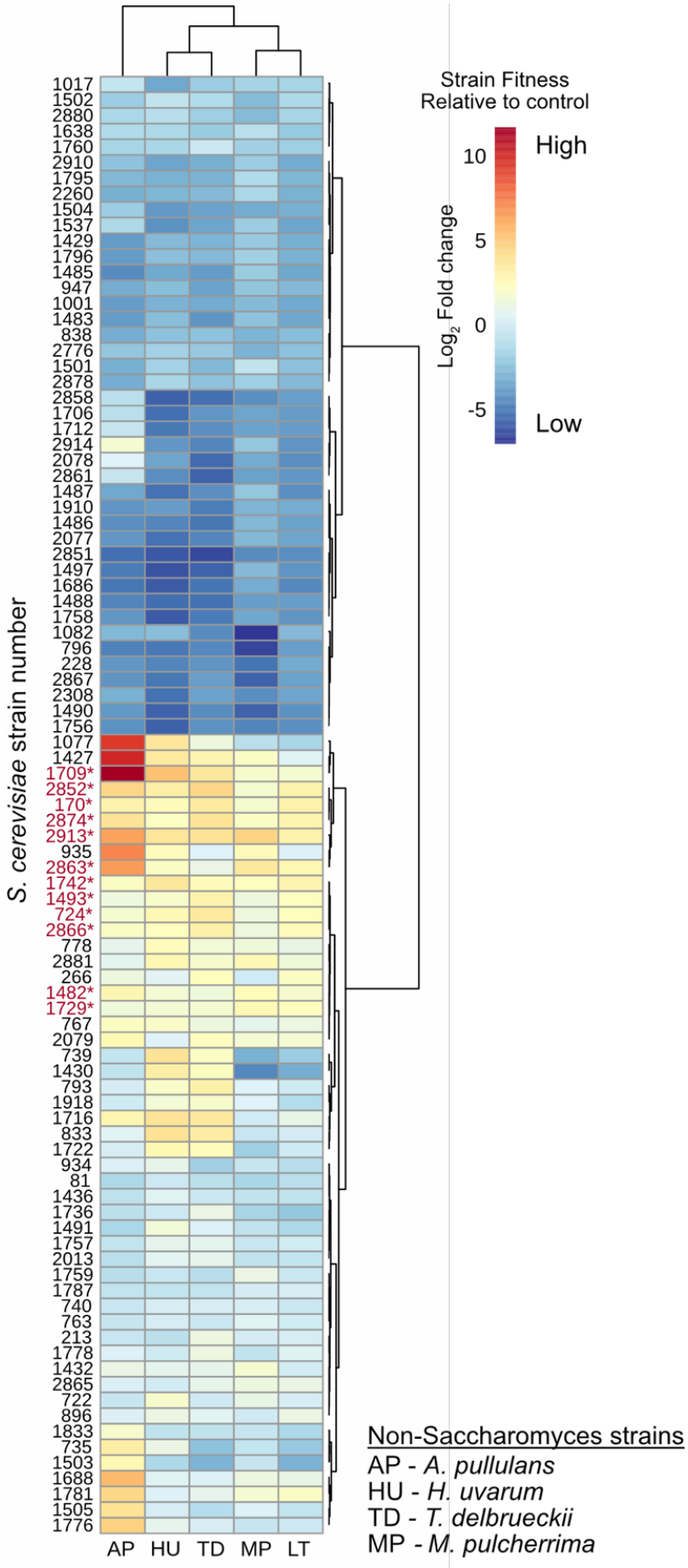


Figure 9. *S. cerevisiae* fitness measured as $\log_2$ Fold Change (FC) relative to control conditions in the presence of a non-*Saccharomyces* species. Data are clustered across fitness and treatments. Strains with an asterisk (*) showed a $\log_2$ FC ≥ 1 across all treatments. AP: *A. pullulans*, HU: *H. uvarum*, TD: *T. delbrueckii*, MP: *M. pulcherrima* and LT*: L. thermotolerans*.

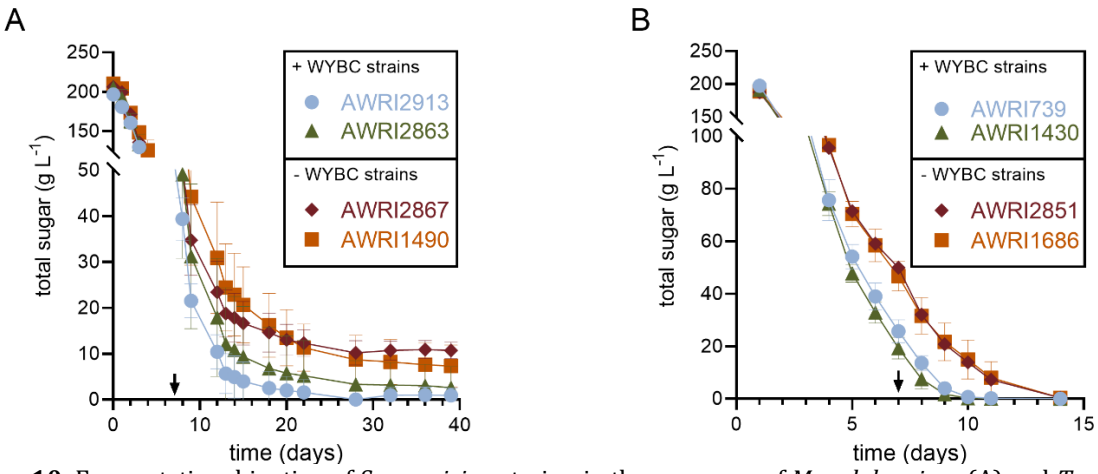


Figure 10. Fermentation kinetics of *S. cerevisiae* strains in the presence of *M. pulcherrima* (A) and *T. delbrueckii* (B). + and - WBYC strains refer to strains that showed a positive or negative $\log_2$ FC in the WYBC library competitive fitness experiments for the specific non-*Saccharomyces* treatment (Figure 9). The arrows indicate the time point when all *S. cerevisiae* controls finished fermentation. Data points correspond to the mean of three replicates ± sd.

7.2.4 Defining the effect of non-*Saccharomyces* yeasts on sequential malolactic fermentation (MLF).

The effects of non-*Saccharomyces* yeast are unlikely to be confined to alcoholic fermentation. With increasing use of co-inoculation strategies the changes in juice and must composition induced by non-*Saccharomyces* yeast also have the potential to affect malolactic fermentation.

MLF performance of two commercial *O. oeni* strains was evaluated following primary fermentation using a combination of five non-*Saccharomyces* species and *S. cerevisiae.* Malic acid consumption kinetics showed clear strain-dependent beneficial effects of these non-*Saccharomyces* species on MLF performance (Figure 11).

MLF using the strain VP41 was faster in ferments containing non-*Saccharomyces* species relative to the control condition (Figure 11A). On the contrary, wines inoculated with *O. oeni* strain CH35 showed slower MLF rates and no MLF progression in the *L. thermotolerans* treatment and *S. cerevisiae* control. Although preliminary, the results obtained during this series of experiments suggest that these non-*Saccharomyces* species benefit MLF when *O. oeni* is sequentially inoculated.

Previous studies have shown variability in the effects of non-*Saccharomyces* species on *O. oeni* strains (Balmaseda et al. 2021). Additional research is required to understand the underlying mechanisms of MLF stimulation and enable an appropriate non-*Saccharomyces* species/*O. oeni* strain combination to be identified.

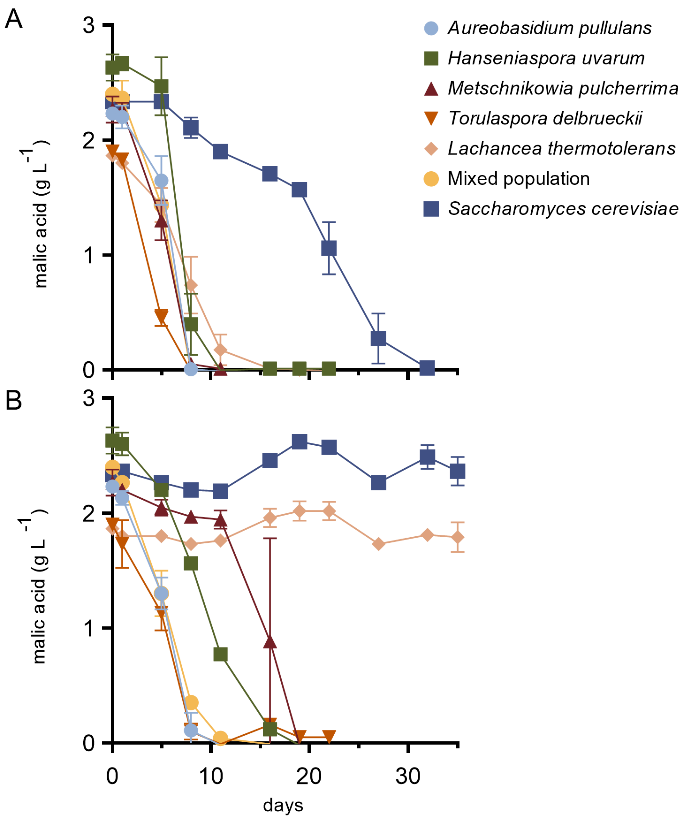


Figure 11. Malolactic fermentation kinetics of *O. oeni* strains (A) VP41 and (B) CH35 inoculated in wines fermented using five non-*Saccharomyces* and *S. cerevisiae*. Data points correspond to the mean of three replicates ± sd.

7.2.5 Identifying new wild *O. oeni* strains tolerant to wine stress factors

In a continued effort to identify naturally occurring *O. oeni* strains with potential for future commercial use, more than 200 natural *O. oeni* strains isolated from South Australian wild ferments over the 2017-2019 vintages were subjected to several stages of screening for robustness, genetic diversity and promising oenological traits. From the initial screening of the main cohort of strains by assessment of growth response to pH (pH 3.2-3.6) and ethanol (6-12% v/v) stress and subsequent genomic dereplication, a phenotypically and genetically diverse subset of 39 representative strains was identified (Figure 12).

Twelve genetically distinct strains exhibiting superior pH and ethanol tolerance were selected to assess MLF performance by co-inoculation in Chardonnay (Figure 12). From this final screening, two *O. oeni* isolates were identified with favourable MLF performance characteristics and similar impacts on alcoholic fermentation. Acetic acid and diacetyl production by these strains was similar to a commercial reference strain (Table 5). They also did not possess genes associated with the formation of the biogenic amines histamine, tyramine or putrescine and had enzymatic capacity for citric acid metabolism (Table 6). Collectively, the robust MLF performance and distinct genomic traits of these new *O. oeni* strains suggest their potential for commercialisation.

Table 5. Fermentation performance and impact on wine chemical composition of two selected South Australian wild *O. oeni* strains (B1351 and B1371) after co-fermentation with *S. cerevisiae* AWRI 2863 in

Chardonnay. Strain B429 is a commercially available comparison. Data are the mean (SD) of three replicate fermentations.

Strain	Time (days) to complete fermentation		Final wine analysis			
	Malolactic	Alcoholic	Glu + Fru (g/L)	L-malic acid (g/L)	Acetic acid (g/L)	Diacetyl (mg/L)
AWRI B429	15	21 (6)	1.5 (0.4)	<0.2	0.29 (0.08)	0.3 (0.4)
AWRI B1351	15	19 (4)	1.2 (0.6)	<0.2	0.29 (0.09)	0.2 (0.3)
AWRI B1371	15	20 (7)	1.5 (1.0)	<0.2	0.25 (0.08)	0.1 (0.1)

Table 6. Genes associated with biogenic amine formation and citric acid utilisation of two selected South Australian wild *O. oeni* strains (B1351 and B1371) after co-fermentation with *S. cerevisiae* AWRI 2863 in Chardonnay. Strain B429 is a commercially available comparison. Data are the mean (SD) of three replicate fermentations.

Strain	Presence/absence of genes				
	Histamine	Tyramine	Putrescine		Citrate
	hdc	tdc	odc	agdi	
AWRI B429	-	-	-	-	+
AWRI B1351	-	-	-	-	+
AWRI B1371	-	-	-	-	+
AWRI B429	-	-	-	-	+

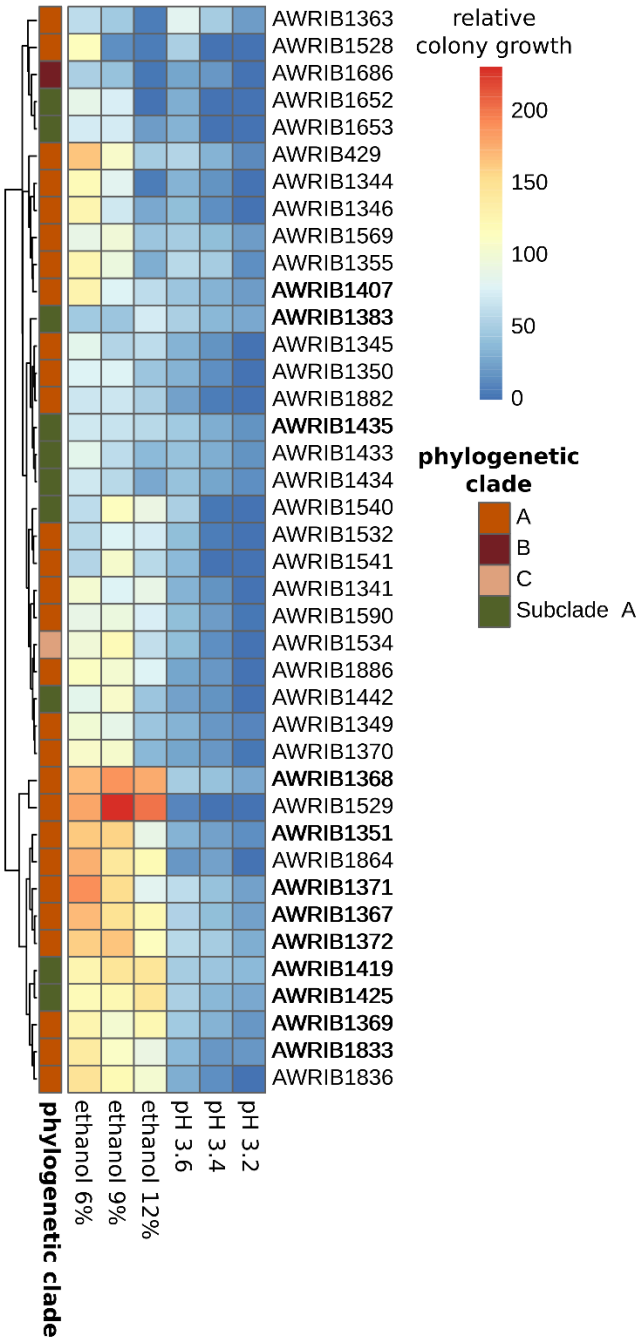


Figure 12. Relative colony growth and corresponding phylogenetic clade of 39 genetically distinct *O. oeni* natural isolates. Rows are clustered based on relative colony growth for all conditions. Phylogenetic clade classifications are based on previously described clades for this species (Sternes & Borneman 2016, Lorentzen et al. 2019). Relative growth was estimated using the colony size in the control condition for each treatment (0% ethanol and pH 5.0). Isolates in bold were individually assessed through co-inoculation in Chardonnay juice.

7.3 Assessment of strategies for a transformation system for *O. oeni*.

There are a limited number of reports detailing the transformation of *Oenococcus oeni* (Assad-García et al. 2008, Eom et al. 2012, Darsonval et al. 2018). Contained within these reports was the promise that the genetic basis of various performance attributes of *O. oeni* could be tested directly and that the functionality of the extensive pan-genome of *O. oeni* (Sternes & Borneman 2016) could become available for functional analysis in the native host and the environment in which it has an important functional role. Acknowledging the difficulty of the task but encouraged by the above reports, the project team attempted to repeat the transformation of this organism.

Bacterial strain selection was an important consideration at the outset of this work. Plasmid incompatibility, the inability of two plasmids to co-exist in the same host, is a well-characterised phenomenon that influences plasmid maintenance and stability (Helinski 2022). Therefore, bacterial strains were sought that did not harbour native plasmids. Strains were screened using primers designed to detect features of a native plasmid identified in AWRI B429; The Replication Initiator Protein A (RepA), a transcriptional regulator (TetR) and an NADH:flavin oxidoreductase (Oye) (Table 7). Four strains were identified that did not contain any detectable plasmid elements, AWRI B343, AWRI B787, AWRI B875 and AWRI B1062.

Table 7. Presence of plasmids in strains of *O. oeni* as determined by PCR-based detection of typical plasmid features Protein A (RepA), transcriptional regulator (TetR) and NADH:flavin oxidoreductase (Oye). + indicates that the feature was detected in the specified strain.

Strain ID	Accession	Rep A	TetR	Oye
AWRI B324	SAMN04297040	-	+	-
AWRI B343	SAMN04297056	-	-	-
AWRI B419	pOENI-1	+	+	+
AWRI B429		-	+	+
AWRI B435	SAMN04297071	+	-	+
AWRI B661	SAMN04297102	-	-	
AWRI B663	SAMN04297103	-	-	
AWRI B787	SAMN04297112	-	-	-
AWRI B791	SAMN04297113	+	-	+
AWRI B864	SAMN04297123	+	-	+
AWRI B875	SAMN04297128	-	-	-
AWRI B1062	SAMN04297004	-	-	-

A second important consideration is the natural competence of the selected strain. Natural competence is the ability to actively transport environmental DNA fragments across the cell envelope and into the cytoplasm (Krüger & Stingl 2011). Some elements of the natural competence system may also facilitate artificial transformation, particularly elements of the ComG system that bring environmental DNA into proximity with the cytoplasmic membrane. Of the strains screened here, one strain had a complete set of natural competence genes (ComE and ComG)(Sternes & Borneman 2016) and lacked a detectable native plasmid. That strain, selected for subsequent transformation attempts, was AWRI B787.

Finally, poor transformation efficiencies have been reported due to high levels of non-specific cell-wall-associated endonucleases in various bacterial genera (Pyne et al. 2013). These nucleases can degrade non-host DNA, thereby reducing its concentration prior to or following transformation, leading to transformation recalcitrance. *O. oeni* nuclease activity was evaluated by incubating crude lysates with 1 µg of purified plasmid DNA and a universal restriction enzyme buffer as described by Pyne et al (2013). The purified plasmid appeared to be stable after a 2.5-hour incubation at 37°C, indicating the absence of nucleases detrimental to the transformation of *O. oeni*.

Bacterial strains were further characterised by their resistance to antibiotic selection systems typically used in molecular microbiology. Earlier reports of transformation used erythromycin as a selectable marker. This was naturally the starting point for the project team's investigations. In addition to erythromycin, the antibiotics chloramphenicol, ampicillin and nourseothricin were also evaluated as potential selectable markers.

Surprisingly, given its previously reported use in *O. oeni* transformations, *O. oeni* had a strong tendency to generate spontaneous resistance to erythromycin. Chloramphenicol and nourseothricin were effective at suppressing the growth of undiluted *O. oeni* at 12.5 µg/L and 25 µg/L, respectively.

A series of plasmids was designed for the attempts to transform *O. oeni*. Plasmids are small circular double-stranded DNA molecules capable of replicating themselves in a host organism. A functional plasmid typically has two key features: a means of selection (discussed above), and an origin of replication (Ori). The origin of replication can vary depending on the host organism. The Ori used in many molecular biology plasmid tools is a truncated version of one found in a plasmid (Col) of the gram-negative bacterium *E. coli*. The typical mechanism of replication initiation in gram-negative bacteria differs from that in gram-positive bacteria such as *O. oeni* (Helinski 2022). Plasmid incompatibility (discussed above) is also a feature that, at least in part, has its origins in molecular features of the various origins of replication. These ideas shaped the direction of the various plasmid designs used in the attempts to transform *O. oeni.*

A series of plasmids was constructed, initially based on the classic pUC plasmids (Vieira & Messing 1982). An additional antibiotic marker (erythromycin) under the control of a putative *O. oeni* promoter was ligated into the plasmid pUC57. A synthetically manufactured *O. oeni* origin of replication, identified from whole genome sequence data, was also integrated into the plasmid to create pCJ_OO (Table 8). Despite containing two origins of replication, the plasmid replicated with high efficiency in *E. coli* and conferred both ampicillin and erythromycin resistance. The plasmid conferred erythromycin resistance to *E. coli* despite being driven by a predicted *O. oeni* promoter, demonstrating the basic functionality of the new plasmid. pCJ_Oo provided the opportunity to test two selectable markers (ampicillin and erythromycin) and an alternative origin of replication in *O. oeni*.

Table 8. Plasmids constructed during the project with key features listed

Plasmid id	**Source**	**Marker 1 P_Ec**	**Marker 2 p_Oo**	**Marker3 P_Oo**	**Ori**
pCJ_Oo	pUC57	amp	eryth		ColE1 and Oo1
pRK_Oo	pCJ_Oo	amp	eryth	GFP	
pRK_Oo1	pRK_Oo	-	eryth	GFP	ColE1 and Oo1
pRK_Oo2	pRK_Oo1	-	chlor	GFP	ColE1 and Oo1
pRK_Oo3		-	amp	GFP	ColE1 and Oo1
pRep256_sfGFP	pRK_Oo	-	chlor	sfGFP	ColE1 and rep256

Electrotransformation, based on the method described by Assad-Garcia et al. (2008) was used to attempt to introduce the plasmid pCJ_Oo into *O. oeni* using ethanol as a membrane fluidising agent to facilitate DNA transfer into the cell. Several erythromycin-resistant colonies were isolated, but all had spontaneously gained resistance to the antibiotic and did not contain a detectable plasmid.

Green fluorescent protein (GFP) was introduced into the plasmid pCJ_Oo to act as an independent reporter for the presence of the plasmid within antibiotic-resistant cells. The inclusion of this gene, under the control of an additional *O. oeni* promoter, resulted in a very large plasmid (pRK_Oo – 6,602 base pairs). It is known that transformation efficiency is inversely proportional to plasmid size. Therefore, the ampicillin marker was removed from pRK_Oo, creating pRK_Oo1 with reduced size (5,681 bp). The erythromycin selectable marker was replaced with chloramphenicol and ampicillin to produce pRK_Oo2 and pRK_Oo3, respectively. In all cases, the transformation of the plasmids into *E. coli* provided resistance to the antibiotic and generated fluorescent bacteria.

Electroporation of the plasmids into *O. oeni* generated antibiotic-resistant bacteria at frequencies similar to that observed when water was used instead of a plasmid. None of the antibiotic-resistant isolates exhibited fluorescence. The number of antibiotic-resistant colonies did not increase relative to the concentration of plasmid used in the transformation (100 – 6,000 ng). The only increase in the number of antibiotic-resistant colonies observed occurred when the chloramphenicol concentration was reduced from 12.5 µg/L to 8 µg/L.

To this point, an assessment of the plasmids and transformation attempts stimulated a review of all the elements used. The inability to transform *O. oeni* could be accounted for by any of the following plasmid features:

- Inefficient or non-functional origin of replication
- Inefficient or non-functional promoter elements
- Poorly performing terminator sequences resulting in interference with the expression of adjacent genes.

The putative *O. oeni* origin of replication used in the pCJ and pRK series of plasmids did not contain a ParA like protein that can help with portioning of plasmids to daughter cells. Although still likely to be a functional theta-like origin of replication, it was reasoned that perhaps a complete Ori was necessary for plasmid maintenance in *O. oeni*. A minimal origin of replication (688 bp) from *L. plantarum* was identified by Darsonval et al. (2016) that did not utilise a replication protein (p256). The small size of this Ori enabled a substantial reduction in plasmid size when it was used to replace Oo1 in pRK_Oo.

Typical bacterial promoter sequences include motifs positioned at specific sites relative to the transcription start site (TSS). Each nucleotide in a promoter consensus sequence represents the most frequently occurring nucleotide, however, few promoter sequences match the exact consensus sequence. Secondary properties of promoters may also influence transcription. These properties include: stability, curvature, and bendability of DNA in promoter regions (Kanhere & Bansal 2005). The genome of the *O. oeni* strain PSU1 was searched for sequence motifs that matched the optimal signature of a bacterial promoter. Seven potential promoters were identified with the following features:

- Contained Shine-Dalgarno Sequence (SDS) (AAGGAGGT) within 12 bp of an ATG
- Contained Pribnow box (-10 hexamer) TANAAT (minimum 4/6 bases required)
- Contained -35 element TTGACA (4/6 bases required)
- -10 and -35 elements were approximately 15-20 bases apart
- Region did not overlap coding sequence (was an intergenic element)

The selected putative promoter element (PSU-1 promoter 7) was synthesised and inserted upstream of a chloramphenicol resistance gene. Within the plasmid, the chloramphenicol resistance gene did not have its own terminator element; its terminator was shared with GFP. A candidate replacement terminator was selected (BBa_B0015, www.parts.igem.org) which contained two forward terminators, minimising the potential for interference from adjacent genes.

Finally, an alternative to GFP was identified (sfGFP) (Pédelacq et al. 2005) with improved folding kinetics and stability. sfGFP was used to replace the GFP in the original pRK_Oo plasmid. The wholly redesigned plasmid (pRep256_sfGFP) was only 3,242 bp in size. pRep256_sfGFP replicated well in *E. coli,* and fluorescence from sfGFP was bright. Numerous attempts to transform pRep256_sfGFP into either *O. oeni* or *L. plantarum* were unsuccessful.

In the absence of a clear path forward with additional options for the modification of either plasmid or transformation protocol, this aspect of the project was not pursued further.

7.4 Understanding of minimum inhibitory concentrations of common wine matrix elements on strains of *O. oeni*

7.4.1 Minimum inhibitory concentrations of SO_2 on *O. oeni*

It is well recognised that *O. oeni* has high sensitivity to SO_2. Molecular SO_2 concentrations >0.3 mg/L are suggested as lethal for most wine lactic acid bacteria (S.Krieger-Weber et al. 2015). However, accurate and reliable data regarding the tolerance of *O. oeni* to the different forms of SO_2 in wine is lacking. The limited availability of information is likely due to a range of factors, particularly those relating to the complex chemical nature of SO_2, including pH-dependent equilibria, reactivity with other wine components and resultant proportions of the different forms of SO_2 occurring in the wine matrix.

A further complication arises from the chemical reactivity of SO_2 with oxidised juice matrix elements, causing inherent difficulties in maintaining reproducible and consistent concentrations of SO_2 in laboratory-scale media. Since free/molecular SO_2 concentrations inhibitory towards *O. oeni* are below the detection limit of most conventional analytical methods (<2-3 mg/L), there is little reference to the sensitivity of malolactic starter cultures to this form of SO_2.

Wine lactic acid bacteria also exhibit apparent sensitivity to bound SO_2 (Larsen et al. 2003, Wells & Osborne 2011) and, in practice, >50 mg/L bound SO_2 is generally considered inhibitory for bacterial growth and MLF induction in wine (Fugelsang 1997). Nevertheless, the inhibitory properties of bound SO_2 are highly dependent upon wine matrix composition and should only serve as a guide. To this end, this work endeavoured to better understand bacterial inhibition by bound and free forms of SO_2 in wine, using both co-inoculation and sequential inoculation strategies.

The resistance of two *O. oeni* strains (AWRI B706, AWRI B334) to acetaldehyde-bound SO_2 was evaluated using a sequential inoculation strategy in a semi-defined model wine test medium (pH 3.5, 10% v/v ethanol). Both strains exhibited similar and comparatively low resistance to acetaldehyde-bound SO_2, with inhibitory and bactericidal effects observed at 5 mg/L and ≥15 mg/L bound SO_2, respectively (Figure 13). Further testing of one strain (AWRI B706) revealed limited alleviation of SO_2 toxicity under conditions of anaerobiosis or with excess acetaldehyde. Further study is required to determine which factors seen in wine conditions may induce greater SO_2 resistance in certain strains of *O. oeni*.

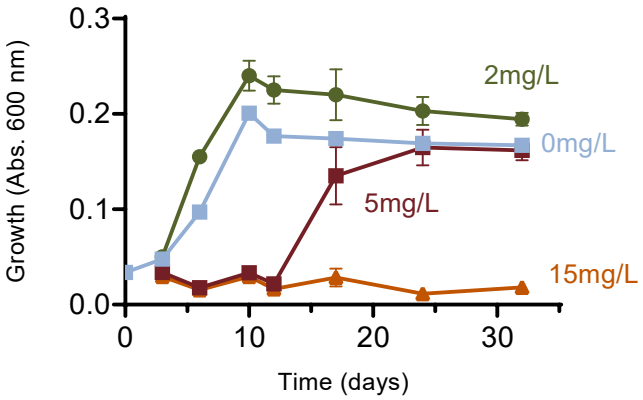


Figure 13. Growth inhibition of *O. oeni* AWRI B706 by acetaldehyde-bound SO_2 in semi-synthetic test wine (pH3.5, 10%v/v ethanol, 22°C).

A large proportion (> 75%) of the twenty phylogenetically distinct *O. oeni* strains screened for tolerance to acetaldehyde-bound SO_2 (0-60 mg/L) following co-inoculation in Chardonnay demonstrated that up to 60 mg/L acetaldehyde-bound SO_2 can be tolerated. A smaller proportion (25%) of strains exhibited variable sensitivity to initial acetaldehyde-bound SO_2 at concentrations of 20-40 mg/L. Some strains showed sensitivity to wine fermentation conditions other than SO_2 or were affected by both (Table 9, Figure 14).

Table 9. Response of 20 *O. oeni* strains to increasing initial concentrations of acetaldehyde-bound SO_2 following co-inoculation in Chardonnay grape juice. Bacteria strains were considered sensitive to SO_2 treatments when viable cell concentration was reduced by >0.5 log cfu/mL after five days compared to the non-treated control.

Acetaldehyde-bound SO_2 (mg/L)	Number of strains	
	Sensitive	Tolerant
20	0	20 (100%)
40	2 (10%)	18 (90%)
60	4 (20%)	16 (80%)

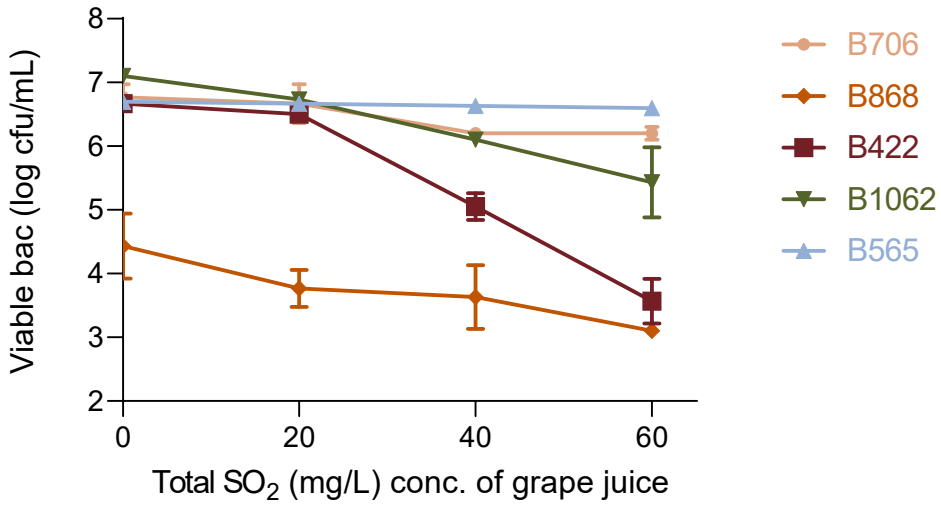


Figure 14. Viable cell concentrations of representative *O. oeni* strains exhibiting sensitive and tolerant responses to increasing initial concentrations of acetaldehyde-bound SO_2 following co-inoculation in Chardonnay grape juice (pH 3.3). Data show the average viable bacteria concentration of each SO_2 treatment five days after co-inoculation with *S. cerevisiae* AWRI 2863 (n=3).

Although the co-fermentation methodology provided relevant information relating to SO_2 tolerance of *O. oeni* under wine conditions, further investigation revealed that the concentrations of acetaldehyde-bound and molecular SO_2 present during the early stages of fermentation can be highly dynamic. Consequently, this technique could not be relied upon to accurately determine the respective inhibitory concentrations of these parameters in wine.

In a final study, the response of *O. oeni* to comparatively stable concentrations of acetaldehyde-bound SO_2 and calculated molecular SO_2 was examined in grape juice. In this assay, it was demonstrated that *O. oeni* VP41 was highly sensitive (>1,000-fold reduction in viable cell concentration) to molecular SO_2 concentrations of approximately 8 µg/L and 13 µg/L (Figure 15). Further study is required to accurately determine the minimum inhibitory concentrations of molecular (and also bound) SO_2 on *O. oeni* and the ability of this species to acclimatise to increasing concentrations of SO_2.

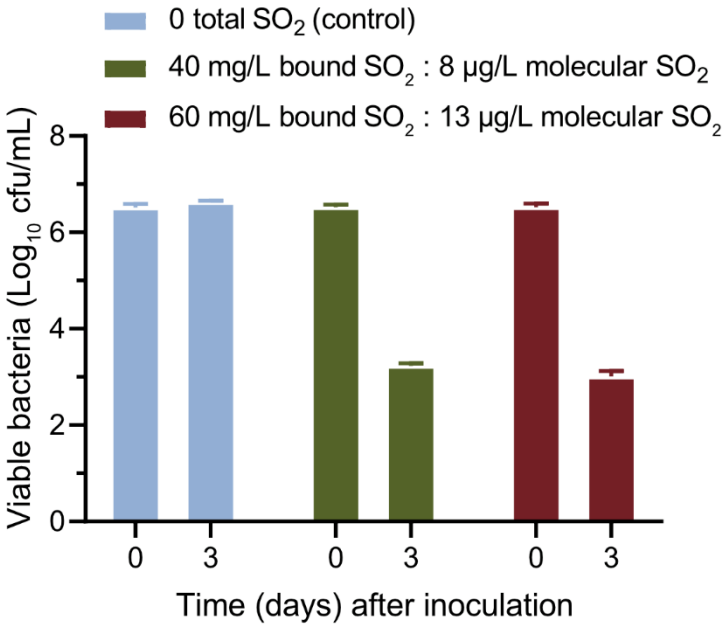


Figure 15. Effects of increasing concentrations of calculated molecular SO_2 and acetaldehyde-bound SO_2 on viable cell concentration of *O. oeni* VP41 in Chardonnay grape juice (pH 3.3). Data show average viable bacteria concentration after three days exposure to each SO_2 treatment in grape juice.

7.5 Knowledge of incompatibilities between yeast and bacteria that result in poor fermentation performance

7.5.1 Yeast strain SO_2 production potential

Of the wide range of interactions that can occur between wine yeast and malolactic bacteria strains, the potential for bacterial inhibition arising from the production of SO_2 by wine yeast is well recognised. As a by-product of sulfate assimilation, the production of SO_2 by *Saccharomyces cerevisiae* strains is generally low (10-30 mg/L), although some strains can produce more than 100 mg/L SO_2. Moreover, some yeast can consume exogenous SO_2 and for low SO_2-producing strains, its presence can repress SO_2 formation.

Given the importance of SO_2 as a potent inhibitor of malolactic starter cultures, the SO_2 production potential of representative *S. cerevisiae* wine strains was investigated. The study of the SO_2 production potential of 94 genetically distinct *S. cerevisiae* wine strains showed that the final total SO_2 concentrations produced by the yeast strains (20-212 mg/L) varied widely (>10-fold), with approximately half of the strains either increasing or decreasing the initial SO_2 concentration (Figure 16). The 90th and 10th percentiles of SO_2 concentrations were 63 mg/L (22 mg/L more than initial concentration) and 29 mg/L (12 mg/L less than initial concentration), respectively. Such strain-dependent variation in SO_2 production implies these wine yeasts may also display similar variability in SO_2-mediated inhibition of *O. oeni*.

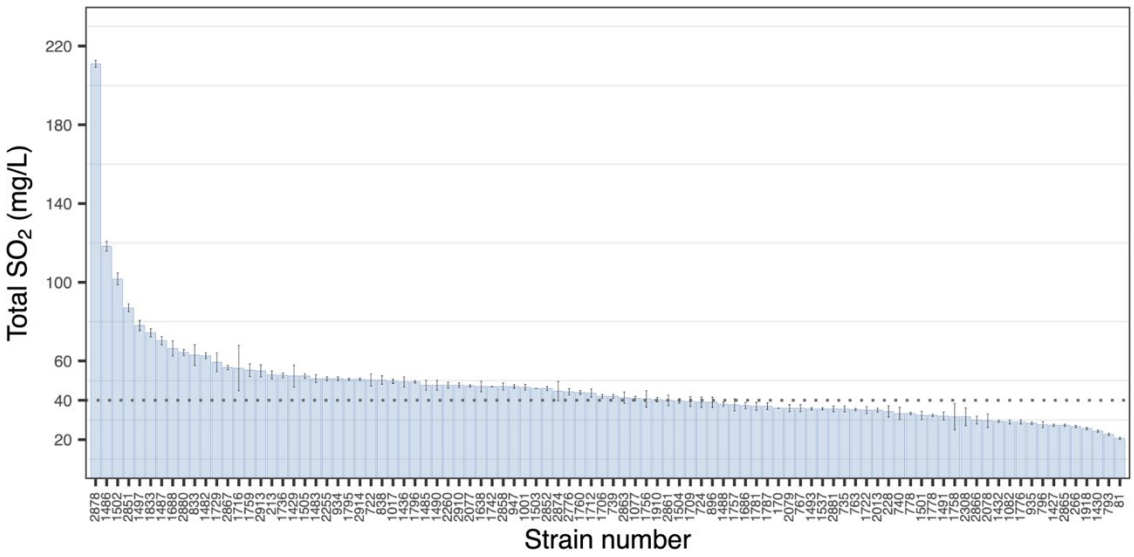


Figure 16. Total SO_2 concentration in Chardonnay wine after fermentation with different strains of *S. cerevisiae*, identified by their AWRI culture collection number on the y-axis. The dotted line indicates the total SO_2 concentration of the Chardonnay juice prior to fermentation. Blue bars show mean total SO_2 concentration, with error bars showing standard deviation (n = 3).

A study of representative low- and high-SO_2 producing yeast strains revealed that most SO_2 was produced within the first five days of fermentation (Figure 17), suggesting that yeast-derived SO_2 production could also significantly affect survival of malolactic starter cultures following co-inoculation with *O. oeni*.

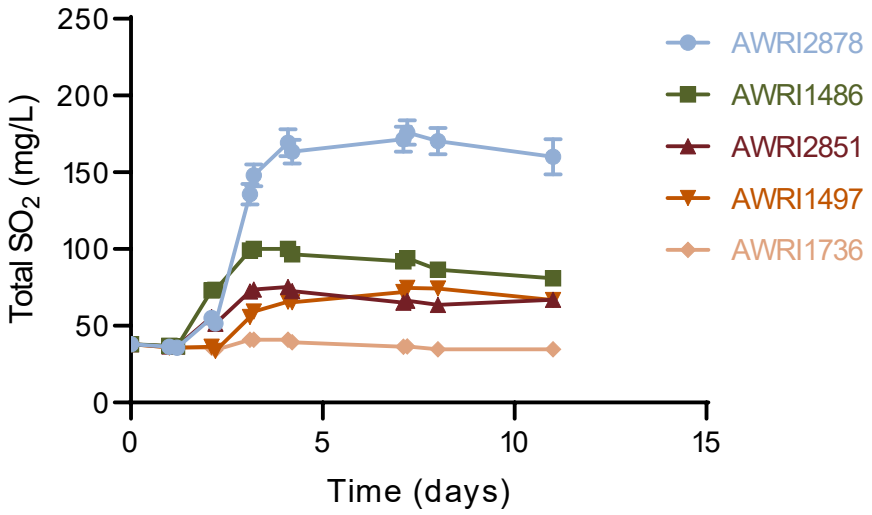


Figure 17. Kinetics of total SO_2 formation by different *S. cerevisiae* strains during fermentation in Chardonnay. Data are mean total SO_2 concentration with error bars showing standard deviation (n = 3).

7.5.2 Yeast strain SO_2 and acetaldehyde production - effects on *O. oeni* survival during co-fermentation

Five representative low- and high-SO_2-producing yeast strains were assessed for their impacts on the survival and MLF performance of a commercial *O. oeni* strain (YV Select) following co-inoculation in Chardonnay. Despite the wide-ranging impacts of these yeasts on *O. oeni* survival (Figure 18), this variation was not consistently related to the production of SO_2 or other major wine components such as alcohol, highlighting the potential impact of other yeast-derived metabolites on bacterial survival, including medium chain peptides and inhibitory peptides (Carrete et al. 2002). However, an important observation was the surprising and contrasting impacts of two SO_2-producing yeasts strains, AWRI 1497 (T73) and AWRI 2880 (NT50), on *O. oeni* survival. The former induced rapid death of bacteria and substantially delayed MLF, while the latter supported bacterial survival and efficient MLF.

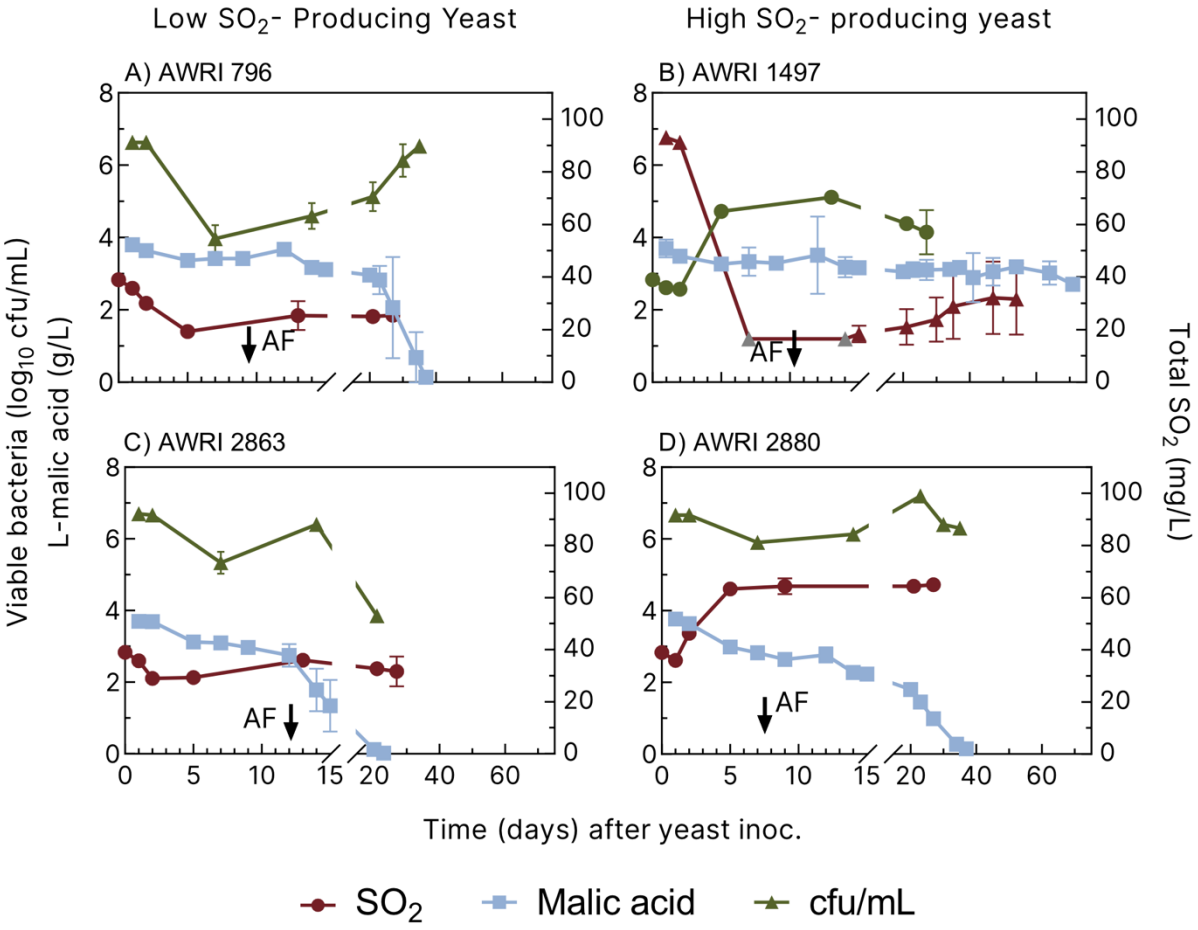


Figure 18. Effects of non-SO_2-producing *S. cerevisiae* strains (a) AWRI 796, (c) AWRI 2863, (and SO_2-producing *S. cerevisiae* strains (b) AWRI 1497 and (d) AWRI 2880 on bacterial survival and MLF by *O. oeni* YV Select following co-inoculation in Chardonnay juice (530 mL fermentations). Figures show concentrations of viable bacteria (green triangles), L-malic acid (blue squares) and total SO_2 (red circles) for respective yeast-bacteria combinations. *O. oeni* YV Select was inoculated 24 hours after inoculation of each yeast strain. Arrows indicate the completion of alcoholic fermentation. Data points for viable bacteria concentrations below the LOD (1.3 log cfu/mL) are coloured grey. Data are the average and standard deviation of three replicate fermentations.

Further investigation into the nature of these different impacts on *O. oeni* focused on the potential role of acetaldehyde, which is rapidly produced to a peak concentration during the early stages of fermentation. Differences in peak acetaldehyde formation between *S. cerevisiae* strains AWRI 2880 (NT50) (>100 mg/L) and AWRI 1497 (T73) (64 mg/L) (Figure 19) were confirmed. Consequently, the corresponding molar ratios of acetaldehyde:SO_2 in early fermentation were much higher for AWRI 2880 than AWRI 1497, suggesting that critical differences in free SO_2 concentration may also occur.

However, since the limits of detection of conventional methods of SO_2 analysis are typically 2-3 mg/L, the low concentrations of free SO_2 anticipated at this early fermentation stage were calculated from total SO_2 and acetaldehyde concentration, using acetaldehyde-bisulfite equilibria. Significantly, these results indicated that *O. oeni* was exposed to comparatively higher concentrations of molecular SO_2 in the first five days of co-fermentation with *S. cerevisiae* AWRI 1497 (T73) (4-15 µg/L), in which significant loss of bacterial viability and delayed MLF occurred, than with *S. cerevisiae* AWRI 2880 (NT50) (1-6 µg/L).

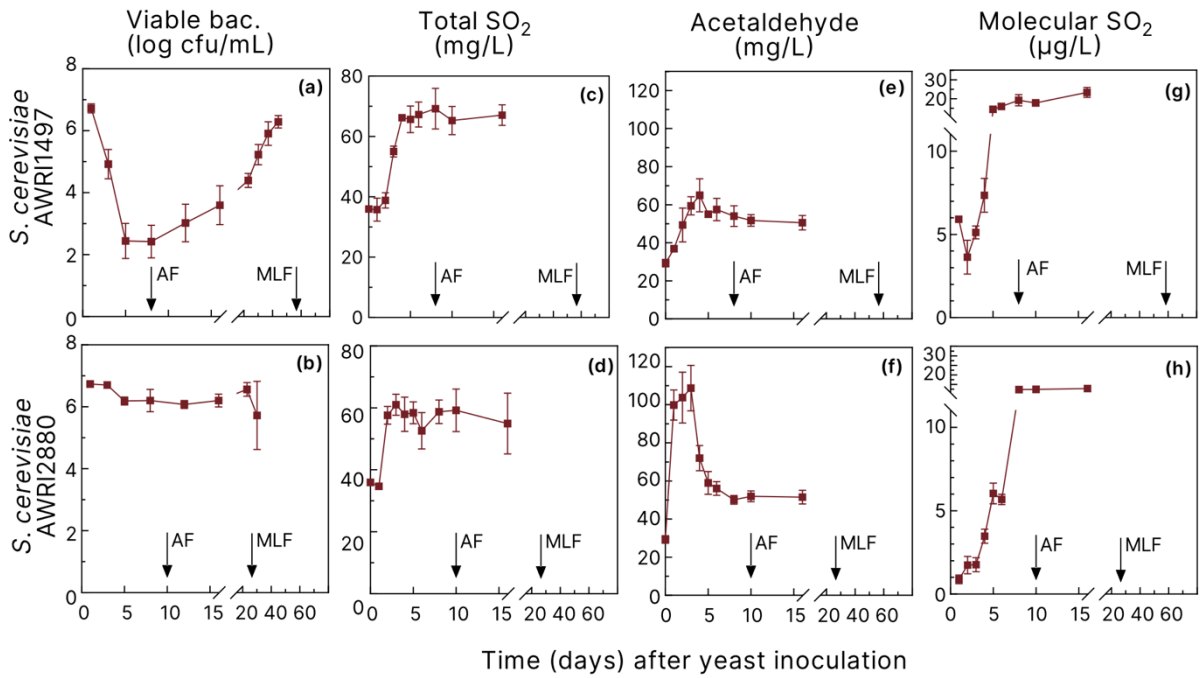


Figure 19. Effects of SO_2 and acetaldehyde producing capabilities of *S. cerevisiae* AWRI 1497 and AWRI 2880 on the survival and conduct of MLF by *O. oeni* VP41 following co-inoculation in Chardonnay juice. Figures show concentrations of viable bacteria (a, b), total SO_2 (c, d), acetaldehyde (e, f) and calculated molecular SO_2 (g, h) for respective yeast-bacteria combinations. Arrows indicate the completion of alcoholic fermentation (AF) and malolactic fermentation (MLF). Data are the average and standard deviation of nine replicate fermentations. For molecular SO_2, the combined error is shown.

These findings were confirmed in a pilot-scale study with NT50 and another SO_2-producing yeast strain, *S. cerevisiae* Actiflore Rosé (Figure 20). Importantly, this work showed a direct association between the survival of *O. oeni* and the molar ratio of acetaldehyde:SO_2 in the early stages of alcoholic fermentation and that bacterial survival and adaptation to SO_2 following co-inoculation were regulated by a correspondingly low concentration range of molecular SO_2. *Saccharomyces cerevisiae* strains with known SO_2-producing ability were further tested for early, peak acetaldehyde formation in Chardonnay, revealing considerable variation in this ability and corresponding calculated concentrations of free/molecular SO_2. This suggests that both SO_2 and early acetaldehyde production by yeast strains are important factors in determining potential SO_2 inhibition of malolactic bacteria, particularly during co-inoculation.

Notably, the pilot-scale co-fermentation study also demonstrated contrasting effects of the high- and low-SO_2-producing yeast strains on wine sensory impacts associated with *O. oeni*. In

particular, sensory assessment (Napping) revealed that the NT50 co-inoculated wines had the highest citation frequency for butter aroma and flavours. In support, the diacetyl concentration of NT50 co-inoculated wines (4.9±0.6 mg/L) was also notably greater than in those co-inoculated with either Actiflore Rosé (1.0±0.1 mg/L), AWRI 796 (<0.1 mg/L) and CY3079 (<0.1 mg/L). Further research is required to comprehensively explore the influence of co-fermentation yeast strain on the metabolic and sensory impacts of *O. oeni*.

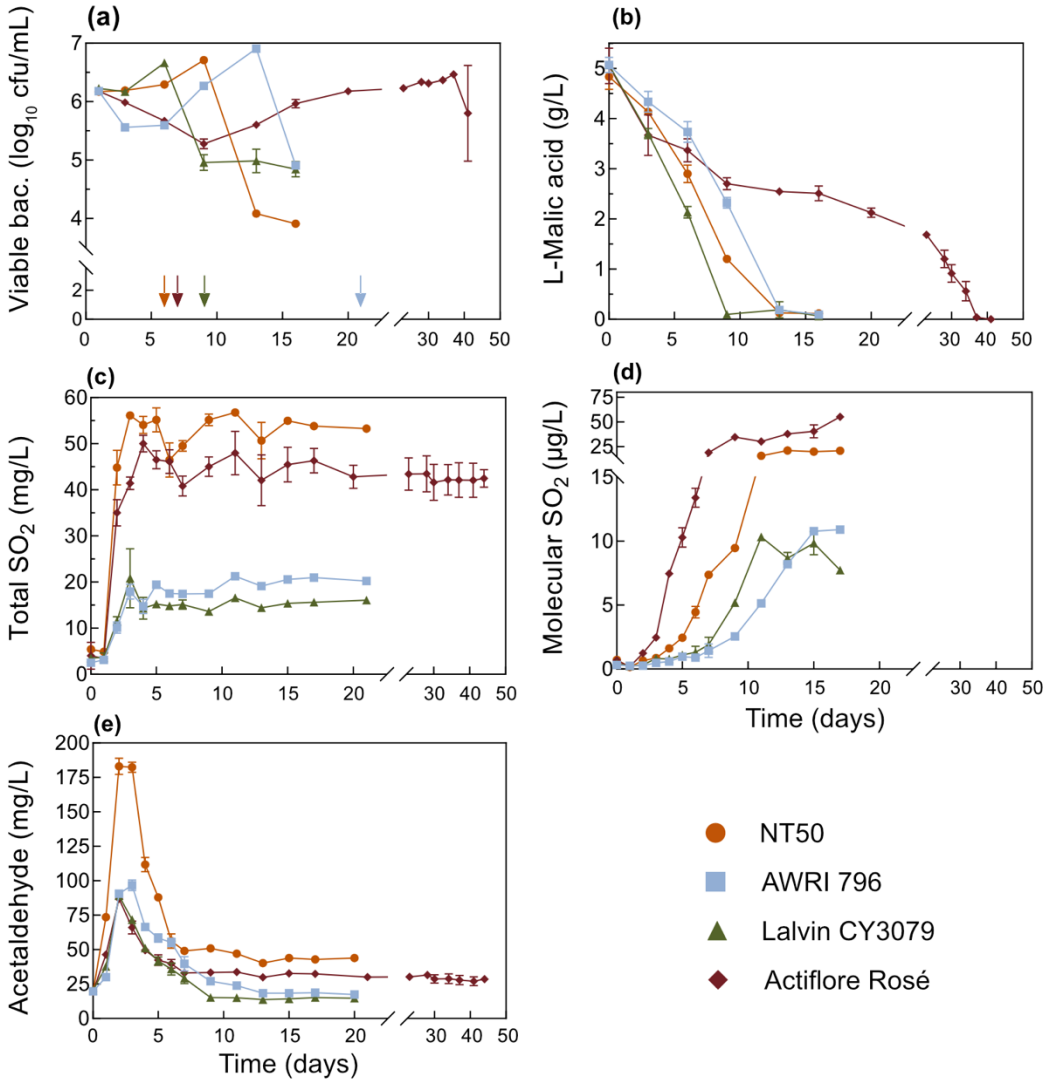


Figure 20. Effects of acetaldehyde and SO_2 producing capabilities of *S. cerevisiae* strains AWRI 796 (blue squares), Lalvin CY3079 (green triangles), Actiflore Rosé (dark red diamonds) and NT50 (orange circles) on the growth and conduct of MLF by *O. oeni* VP41 following co-inoculation in Chardonnay. *O. oeni* VP41 was inoculated 24 hours after inoculation of each yeast strain. Figures show concentrations of viable bacteria (a), L-malic acid (b), total SO_2 (c), calculated molecular SO_2 (d) and acetaldehyde (e). Arrows indicate the completion of alcoholic fermentation by respective yeast strains. Data are the average and standard deviation of three replicate fermentations (35 L).

7.5.3 Inhibition of wine yeast by malolactic bacteria

Antagonistic activity of malolactic bacteria towards wine yeast may sometimes occur, resulting in stuck or sluggish fermentation. Such antagonism has previously been observed in laboratory ferments co-inoculated with a commercial malolactic starter culture, whereby the alcoholic fermentation became stuck at 50 g/L residual sugar. However, subsequent attempts to replicate this observation in ferments of Chardonnay, Riesling, Sauvignon Blanc and Pinot Gris were unsuccessful, suggesting that the phenomenon is highly dependent on an otherwise unknown interaction or condition.

In another case, during co-fermentation with *S. cerevisiae* in Chardonnay, certain natural *O. oeni* strains caused ferments to become stuck at approximately 20 g/L residual sugar. Although the precise nature of this inhibition is unknown, the causal strains were predominantly from a specific phylogenetic group (Subclade A) within the *O. oeni* genome. This suggests that the potential for *O. oeni* to cause an inhibitory interaction with wine yeast is also a strain-dependent trait.

7.6 Knowledge of genetic determinants of MLF performance

7.6.1 SO_2 tolerance of *O. oeni* and the potential role of *tauE* sulfite transport gene

Certain *O. oeni* strains possess plasmid-related genes that encode the TauE protein, which is involved in the transport of sulfites and other sulfur-containing compounds across the cell membrane (Favier et al. 2012). As the TauE protein may provide a mechanism of SO_2 removal, its presence could improve SO_2 resistance in *O. oeni*. The effects of SO_2 (14 mg/L) exposure on the survival of representative *O. oeni* with (four strains) or without (four strains) the *tauE* gene in the model wine medium are shown in Figure 21. There was no evidence ($P<0.05$) that the presence or absence of *tauE*+ or *tauE*- in *O. oeni* contributed to a reduction in viable cell concentration after exposure to 14 mg/L total SO_2. The apparent lack of any physiological benefit of *tauE* in conferring SO_2 resistance in *O. oeni* suggested the involvement of other genetic determinants in SO_2 tolerance.

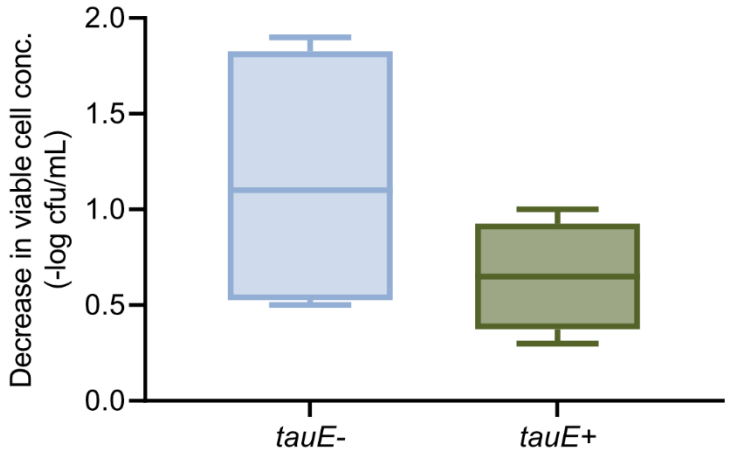


Figure 21. Effect of SO_2 (14 mg/L) on reduction in viable cell concentration of representative *O. oeni* strains with (+) or without (-) tauE in fermented model wine medium

7.6.2 Transcriptomic analysis of *O. oeni* SO_2 stress response

A critical factor underpinning the improvement of malolactic starter culture performance and use is obtaining fundamental knowledge of the physiological changes generated in *O. oeni* by wine stress factors. However, while most research efforts have focused on the biochemical response of *O. oeni* to acid and ethanol stress, knowledge concerning SO_2 stress is less defined (Bech-Terkilsen et al. 2020). The only reported toxic effect of SO_2 is the inhibition of (F_1F_o) H^+-ATPases (Carrete et al. 2002), with the molecular chaperone Hsp20 also suggested to have a role in response to SO_2 by this bacterium (Guzzo et al. 1998).

This project investigated the transcriptional changes associated with SO_2 stress in *O. oeni*. A critical part of this study was the development of a continuous culture system, in which steady-state cells of *O. oeni* AWRI B429 were established in a semi-defined medium to investigate the SO_2-specific effects at wine-like pH (Figure 22). This approach has been shown to limit the appearance of transcriptional signatures related to growth stage or growth rate, and bring increased focus to treatment effects (Daran-Lapujade et al. 2007, Knijnenburg et al. 2009).

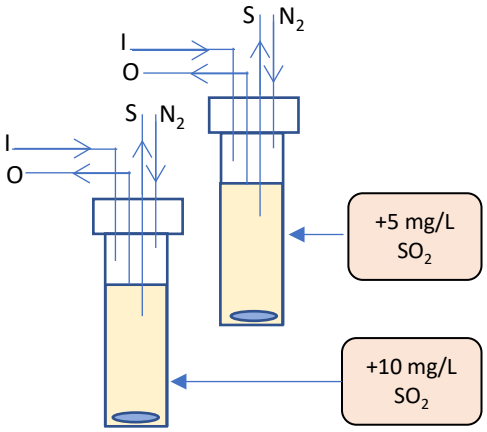


Figure 22. Diagram of chemostat culture system for investigation of SO_2 stress response of *O. oeni* AWRI B429 in semi-defined medium (pH 3.5, 22°C, anaerobic). Steady-state cultures were dosed with 5 and 10 mg/L SO_2 and sampled at specific time points for RNA-seq analysis, determination of bacterial viability and other chemical analyses. I, media inlet; O, effluent outlet; S, sample port; N_2, nitrogen gas inlet.

Differential gene expression analysis revealed major transcriptional changes following exposure to 5 mg/L SO_2 and indicated that this compound primarily interacts with intracellular proteins, DNA and the cell envelope of *O. oeni*. The molecular chaperone HSP20, which has a demonstrated function in the heat, ethanol and acid stress response, was highly upregulated, confirming its further role in the SO_2 stress response of *O. oeni* (Table 10). In addition, the 5 mg/L SO_2 treatment caused minor disruption to bacterial viability and slight modulations in the metabolism of carbohydrate (fructose), lactic and acetic acids.

Other physiological changes associated with the transcriptome relating to carbohydrate metabolism, nutrient uptake and energy suggest that, collectively, mild SO_2 stress (low-SO_2 treatment) induced remodelling of carbon metabolism, enabling rapid restoration of growth. In contrast, treatment with 10 mg/L SO_2 resulted in the absence of transcriptional stress response and a substantial (>100-fold) reduction in cell viability after 24 hours, suggesting that remodelling of metabolic flux did not occur. Overall, the data indicate that *O. oeni* does have the ability to counteract mild SO_2 stress. However, exposure to greater SO_2 stress causes irreparable cell damage, most likely through oxidative mechanisms linked to the reaction of SO_2 with intracellular proteins. A complete description of this work can be found in Onetto et al. (2021).

Table 10. Summary of main differentially expressed genes after exposure of *O. oeni* AWRI B429 to SO_2, grouped by functional category

Gene ID	Functional annotation	5mg/L SO_2[a,b]		10mg/L SO_2 [a,b]	
		30min	4h	30min	4h
Protein and DNA damage					
GGILMNFH_00272	Hsp20/alpha crystallin family protein: Small heat shock protein	0.57	6.55	nd	0.68
GGILMNFH_00467	nrdH; Glutaredoxin	1.02	1.30	nd	1.17
GGILMNFH_01815	trxA3; thioredoxin	nd	1.44	nd	nd
GGILMNFH_00629	clpE; ATP-dependent Clp protease ATP-binding subunit	nd	1.68	nd	0.51
GGILMNFH_00478	clpP; ATP-dependent Clp protease, protease subunit	0.65	1.24	nd	0.99
GGILMNFH_00327	uvrB; exonuclease ABC subunit B	nd	1.19	nd	nd
Malic acid metabolism					
GGILMNFH_01672	mleP; malate permease	0.52	1.14	0.37	0.91
GGILMNFH_01673	mleA; malolactic enzyme	0.45	1.15	0.31	0.84
Cell envelope and division					
GGILMNFH_01941	tagU; peptidoglycan teichoic acid transferase	-1.56	-3.37	-0.98	-2.71
GGILMNFH_00641	ltaS; lipoteichoic acid synthase	-0.51	-1.21	-0.53	-1.10
GGILMNFH_01614	glycosyltransferase eps cluster 2	nd	-1.31	nd	nd
GGILMNFH_01615	putative glycosyltransferase eps cluster 2	nd	-1.14	nd	nd
GGILMNFH_01619	putative glycosyltransferase eps cluster 2	-0.42	-1.13	nd	nd
GGILMNFH_01625	capsular polysaccharide biosynthesis protein	nd	-1.68	nd	nd
GGILMNFH_01854	glycosyltransferase eps cluster 1	nd	-1.07	nd	nd
GGILMNFH_01858	putative glycosyltransferase eps cluster 1	-0.31	-1.01	nd	nd
GGILMNFH_01229	FemA; peptidoglycan bridge formation glycyltransferase	nd	1.35	nd	nd
GGILMNFH_01550	rfbB; dTDP-glucose 4,6-dehydratase	nd	1.24	nd	0.71
GGILMNFH_01551	rfbC; dTDP-4-dehydrorhamnose 3,5-epimerase	nd	1.55	nd	0.72
GGILMNFH_01552	rfbA; glucose-1-phosphate thymidylyltransferase	nd	1.92	nd	nd

[a] Changes in gene expression are represented as $\log_2$ fold change ($\log_2$ FC) between the time point and control condition before SO_2 addition.
[b] Treatments in which gene expression changes show an adjusted *P* value of >.005 are represented as not detected (nd).

8. Outcome and conclusion

8.1 Performance against planned outputs

8.1.1 Knowledge of yeast implantation efficiencies

A detailed exploration of two factors known to be inhibitory to yeast (copper and SO_2 concentration) was completed. Copper and SO_2 arguably have the largest effect on strain-dependent performance. That the mechanisms of resistance to these two inhibitory compounds interact the way they do within yeast metabolism was unforeseen and provided a deeper insight into a fundamental metabolic function of yeast, the assimilation of sulfate. The findings have important practical implications for strain development, indicating that a less forcefully driven *SSU1* gene may decrease the metabolic burden in commercial yeast strains. They are also informative regarding other fundamental features of fermentation management, including the production of hydrogen sulfide and SO_2 and, more broadly, nitrogen management. SO_2 production by yeast is addressed in more detail in a subsequent section.

8.1.2 Knowledge of juice or wine compositional factors that give rise to yeast or bacteria strain-specific fermentation problems

The physiological characterisation of *A. pullulans*, one the most abundant species in freshly pressed grape juice, revealed its ability to substantially alter the juice matrix, causing depletion of zinc and iron. The results demonstrated that *A. pullulans* could compete for nutritional resources in juice and influence the fermentation performance of *S. cerevisiae*. A detailed physiological characterisation of *A. pullulans* also revealed that this organism contained enzymes capable of decreasing the concentration of galacturonic acid-based polysaccharides and hydrolysable gallotannin. Enzymes potentially capable of stimulating the release of glycosidically linked monoterpenes were also present. These features of *A. pullulans* are all of oenological relevance.

Characterisation of the metabolic interactions between a wider array of non-*Saccharomyces* yeast and *S. cerevisiae* demonstrated general inhibition of fermentation performance, with each interaction appearing to operate via different mechanisms. While the characterisation of these varied interactions is not yet complete, initial work suggests that at least some of them are based on competition for nutrients. Given that many of the non-*Saccharomyces* yeast investigated in this work are increasingly available as cultures that can be inoculated into juice and must, the work has direct relevance for the management of these more complex fermentation regimes. That these organisms are also members of the microbial community naturally present in freshly harvested fruit may provide some insight into problematic ferments in otherwise nutritionally replete fruit.

A survey of strain-specific interactions between an array of different *S. cerevisiae* yeast and non-*Saccharomyces* yeast demonstrated that some *S. cerevisiae* strains are more competitive in mixed community environments than others. The study points toward selection criteria for yeast performance attributes that are not typically considered.

Unlike *S. cerevisiae*, bacterial fermentation can benefit from mixed community fermentations. The presence of non-*Saccharomyces* yeasts stimulated MLF. These disparate observations raise the question of whether non-*Saccharomyces* yeast can be used to stimulate MLF without detrimental effects on alcoholic fermentation.

8.1.3 A model system for the investigation of sequential inoculation performance

During the project, several model systems were generated to enable the characterisation of *O. oeni* under different conditions. The effect of SO_2 on the transcriptional response of *O. oeni* was characterised using a continuous culture system with a defined medium. The development of the continuous culture system enabled the transcriptional signature of SO_2 stress to be isolated from transcriptional changes that can occur due to differences in growth rate or medium composition.

A system for assessing the tolerance of *O. oeni* to SO_2 in model ferments was developed. Through careful yeast strain selection and the use of equimolar acetaldehyde and SO_2 concentrations, the team demonstrated minimal variation in SO_2 tolerance among *O. oeni* strains. The work suggested that a long-held view that *O. oeni* can consume acetaldehyde-bound SO_2 may not be correct and led directly to laboratory and pilot-scale experiments investigating interactions between SO_2-producing yeasts and bacteria.

8.1.4 Assessment of strategies for a transformation system for *O. oeni*

A comprehensive assessment of transformation strategies for *O. oeni* was undertaken, beginning with target strain genetic evaluations, propensity toward spontaneous development of resistance to antibiotics and an assessment of the potential to degrade exogenous DNA. A design, test and redesign strategy was implemented to construct plasmid vectors using various modular elements across an iterative series. Despite this effort, the project team ultimately could not confirm the stable transformation of *O. oeni*. While this outcome was disappointing, the project team is convinced that additional effort in this area would be no more fruitful than what has already been achieved.

8.1.5 Understanding of minimum inhibitory concentrations of common wine matrix elements on strains of *O. oeni*

The project team focused its efforts on the characterisation of the minimum inhibitory concentration (MIC) of SO_2. Two different model matrices were used to determine the MIC of SO_2 in *O. oeni* because the inhibitory properties of bound SO_2 are highly dependent upon wine matrix composition. Not surprisingly, these two systems gave different results. The surprise was the scale of the difference.

In a purely defined media system, 5 mg/L acetaldehyde-bound SO_2 delayed growth, and 15 mg/L prevented the growth of *O. oeni*, while in a Chardonnay co-inoculated system designed to mimic winemaking practice, *O. oeni* could survive exposure to 60 mg/L of acetaldehyde-bound SO_2.

This work is incomplete in terms of delivering an understanding of minimum inhibitory concentrations of SO_2. Although both systems probed the MIC with acetaldehyde-bound SO_2, there were important differences between them, meaning that the derived information from the two systems is useful in different contexts. The defined medium system was useful for informing the design of experiments exploring the transcriptional response to SO_2. The Chardonnay co-inoculation study was useful for understanding the features of SO_2 inhibition in a winemaking context. The question about the relative contributions of molecular SO_2 and total SO_2 to the viability loss experienced by *O. oeni* and the inhibition of MLF remains unresolved.

8.1.6 Knowledge of incompatibilities between yeast and bacteria that result in poor fermentation performance

Given that SO_2 is a potent inhibitor of malolactic bacteria, the project team characterised the SO_2 production potential of representative *S. cerevisiae* wine strains. SO_2 concentrations produced by yeast (20-212 mg/L) varied widely (>10-fold), with approximately half of the strains either increasing or decreasing the initial SO_2 concentration. This knowledge provides an immediate initial selection criterion by which wine yeast can be selected as compatible partners for MLF.

The direct effect of SO_2 production by yeast on *O. oeni* survival and MLF progress was assessed through a series of co-inoculation fermentation trials. Although most interactions between bacteria and high-SO_2-producing yeast were negative, one high-SO_2-producing yeast was identified that supported maintenance of bacterial viability and efficient MLF progress. This exception to the common observation helped reveal a deeper understanding of the yeast-bacterial relationship involving acetaldehyde. The yeast strain produced a vast transient excess of this metabolite that appeared to quench the detrimental effects of SO_2 for long enough that the bacterium could survive. The work was important for two reasons; firstly, it demonstrated

that *O. oeni* could become acclimatised to high total SO_2 concentrations and, secondly, that *O. oeni* is more than likely exquisitely sensitive to the molecular form of SO_2.

During the characterisation of environmental isolates of *O. oeni,* certain strains were found to cause ferments to become stuck at approximately 20 g/L of residual sugar. While the precise nature of this inhibition is unknown, the observation demonstrated that inhibitory interactions of *O. oeni* with wine yeast might also be a strain-dependent trait.

8.1.7 Knowledge of genetic determinants of MLF performance

An investigation was undertaken of the transcriptional changes associated with SO_2 stress in *O. oeni*. This work showed that major transcriptional changes occurred following exposure to 5 mg/L SO_2 and indicated that this compound primarily interacts with intracellular proteins, DNA and the cell envelope of *O. oeni*. Overall, *O. oeni* had the ability to counteract mild SO_2 stress through direct cellular responses. However, exposure to greater SO_2 stress caused irreparable damage. The bacterium was found to lack specific active machinery to counteract SO_2, such as those found in many yeasts.

8.2 Could changing the methodology/technology have improved the outcome?

Some aspects of the project were heavily reliant on DNA synthesis for the construction of molecular-genetic components. Difficulties were experienced in the accurate manufacture of these components, leading to delays in specific project milestones and limiting the degree to which the potential experimental space could be adequately explored. That not all DNA sequences can be manufactured efficiently was under-appreciated by the project team at the project outset. More effective methods for synthesising non-ideal DNA molecules would have expedited some project deliverables.

8.3 Practical implications

There are several features of this project with direct practical use. Knowledge of the amount of SO_2 produced by yeast has already been incorporated into educational material. An under-appreciated feature of wine yeast physiology is that yeast can produce more than 60 mg/L or consume more than 10 mg/L of SO_2. That this is tied inextricably to SO_2 tolerance in yeast and that strains with high SO_2 tolerance relinquish resilience in other aspects of their metabolism helps to build a more nuanced picture of yeast strain performance attributes.

Knowledge of strain-dependent *S. cerevisiae* – non-*Saccharomyces* compatibility, or lack thereof, lays the foundations of a resource to guide the implementation of more complex germplasm applications in winemaking. An improved understanding of the nutritional requirements of different non-*Saccharomyces* species and the effects they can have on the composition of the juice matrix will lead to fewer fermentation issues.

A more detailed understanding of the effects of SO_2 on *O. oeni*, their response to the timing and extent of SO_2 production by yeast and the dependence of that response on the transient amount of acetaldehyde that yeast produce all help to refine strategies for co-inoculated MLF. As a result of these investigations, our advice to industry about the optimal time for inoculating *O. oeni* in co-inoculated ferments has changed. It is now recommended that the time delay for co-inoculation of *O. oeni* following *S. cerevisiae* inoculation is not greater than 24 hours.

8.4 Benefits to industry

The AWRI's extensive experience in consulting and advising winemakers on the rescue of suboptimal fermentation during vintage has made it abundantly clear that the prediction of suboptimal fermentations is extremely poor beyond the knowledge of sugar and nitrogen impacts. An emerging understanding of the interaction between *S. cerevisiae* yeast strain and other members of the fermentation ecosystem, including intentionally inoculated non-*Saccharomyces* yeast and *O. oeni* strains, has highlighted known and identified previously unknown predictive factors of fermentation performance. This understanding has contributed to the knowledge now available to winemakers to better manage fermentation.

9. Recommendations

This project has demonstrated the uniqueness of a variety of microbial interactions. Future work should focus on identifying the mechanisms underpinning competition/antagonism between these non-*Saccharomyces* species and *S. cerevisiae*. Knowing the compositional basis of negative interactions will allow winemakers to derive the benefits of inoculating with non-*Saccharomyces* species without sacrificing fermentation timeframes or risking potential stuck ferments.

The benefits of non-*Saccharomyces* species for MLF need to be validated extensively in different juices, especially due to the difference responses observed between *O. oeni* strains. Selected natural *O. oeni* isolates need to be evaluated in a broader range of fermentation conditions to assess their general resilience, determine whether they have any attributes beyond their ability to consume malic acid, and determine their usefulness relative to already available commercial strains.

O. oeni strains that were found to induce stuck ferments in co-inoculated scenarios deserve closer scrutiny. These isolates could provide a window into a potential cause of stuck fermentation issues in sets of wines for which current explanations are unsatisfactory.

10. Communication of project outcomes

Outcomes and knowledge generated during the progress of this project were communicated to peers through peer-reviewed publications and conference presentations. Information was extended to industry stakeholders through articles in trade journals, presentations in the AWRI roadshow program and webinars. These are listed in detail below.

10.1 Presentations delivered during the conduct of the project

Schmidt, S.A., Cowey, M. 2022. Fermentation 101: Getting back to basics with fermentation management. 18th AWITC workshop, 26 June 2022.

Schmidt, S.A. 2019. Why filter? And managing risk when you don't. Winemaker Symposium: 'Don't EF it up', Enzymes and Filtration. Wine Tasmania, Hobart, Australia, 5 November 2019

Schmidt, S.A. 2018. Supporting humanity's oldest profession in the genomics era. Waite in the Spotlight, Waite Campus, University of Adelaide, South Australia, 7 December 2018.

Schmidt, S.A. 2018. Spoilt for choice: Towards a comprehensive understanding of yeast traits to facilitate strain selection. ASVO Seminar – Fermentation: Converting research to reality, Charles Hawker Conference Centre, University of Adelaide, 27 September 2018.

Schmidt, S.A. 2017. Survival of the fittest: The fight for dominance in grape juice and the genetics underpinning yeast strain performance. 7th Australasian Conference on Yeast Products and Discovery, Federation University, Ballarat, Victoria, 29 November – 1 December 2017

Onetto, C.A, Costello, P, Kolouchova R, Jordans C, McCarthy, J., Schmidt, S.A. 2021. Transcriptomic analysis of *Oenococcus oeni* SO_2 stress response. Crush Grape and Wine Science Symposium 2021.

Schmidt, S.A. 2019. Causes and management of slow and stuck fermentations. AWRI roadshow seminar, McLaren Vale, 14 November 2019.

Schmidt, S.A. 2019. Nutrient management in a DAP limited world. Accolade Technical meeting, 15 February 2022

Schmidt, S.A. 2019. Causes and management of slow and stuck fermentations. AWRI roadshow seminar, Stanthorpe, 10 May 2022.

Schmidt, S.A. 2019. Causes and management of slow and stuck fermentations. AWRI roadshow seminar, Hunter Valley, 1 June 2022.

Schmidt, S.A. 2019. Causes and management of slow and stuck fermentations AWRI roadshow seminar, Mudgee, 2 June 2022.

Costello, P. Malolactic fermentation – Research Roundup. Wine Tasmania Winemaker Symposium, 16 October 2018.

Costello, P. 2018. Malolactic starter culture performance in difficult wine conditions – can it be improved? Crush 2018 Grape and Wine Science Symposium.

Costello, P. 2022 Malolactic fermentation – limitations and opportunities. Workshop presentation (Fermentation 101). 18th AWITC workshop, 26 June 2022.

10.2 Posters presented during the conduct of the project

Costello, P. Rullo, M, Jordans, C., Kolouchova, R., Schmidt, S. 2019.MLF and SO_2 stress – what are the real limits for malolactic bacteria? 17th AWITC, July 2019.

Costello, P., Kolouchova, R., McCarthy, J., Jordans, C., Schmidt, S. 2022. SO_2-production potential and early, transient acetaldehyde formation by *Saccharomyces. cerevisiae* - implications for *Oenococcus oeni* co-fermentation. 18th AWITC, June 2022.

Costello, P., Kolouchova, R., McCarthy, J., Jordans, C., Schmidt, S. 2022. Malolactic starter performance during co-fermentation with SO_2-producing yeast: early, transient acetaldehyde formation a key regulator. 18th AWITC, June 2022.

Onetto, C.A, Borneman, A.R., Schmidt, S.A. 2019. *Aureobasidium pullulans*: How much is known about its effect on grape juice composition? 17th AWITC, July 2019

Onetto, C., Costello, P., Kolouchova, R., Jordans, C., McCarthy, J., Schmidt, S. 2022. *Oenococcus oeni* and SO_2 stress: transcriptomic analysis reveals how this species responds to SO_2. 18th AWITC, June 2022.

Onetto, C.A, Borneman, A.R., Schmidt, S.A. 2022. Strain-specific responses of *Saccharomyces cerevisiae* to competition by non-*Saccharomyces* yeasts. 18th AWITC, June 2022.

10.3 Articles and fact sheets written or published during the conduct of the project

Costello, P. Achieving successful malolactic fermentation (AWRI fact sheet). Available from: https://www.awri.com.au/wp-content/uploads/2011/06/Malolactic-fermentation.pdf

Costello, P. Malolactic fermentation in red wine (AWRI fact sheet). Available from: https://www.awri.com.au/wp-content/uploads/2020/09/MLF-in-red-wine.pdf

Costello, P., Jordans, C., Schmidt, S., Herderich, M., Johnson, D. 2019. Trials and tribulations of MLF: can timing of inoculation and MLF nutrients help? *Wine Vitic. J.* 34: 38–41.

Costello, P.J., Kolouchova, R., McCarthy, J.M., Schmidt, S.A. 2022. Early acetaldehyde formation by sulfur dioxide-producing *Saccharomyces cerevisiae* strains: a key regulator of *Oenococcus oeni* survival during co-fermentation in Chardonnay. *Article drafted and ready for submission to peer-reviewed journal.*

Onetto, C.A., Borneman, A.R., Schmidt, S.A. 2021. Strain-specific responses by *Saccharomyces cerevisiae* to competition by non-*Saccharomyces* yeasts. *Fermentation.* 7(3): 165.

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Schmidt, S.A., Kolouchova, R., Forgan, A.H., Borneman, A.R. 2019. Evaluation of *Saccharomyces cerevisiae* wine yeast competitive fitness in enologically relevant environments by barcode sequencing. *G3 Genes Genomes Genetics* 10(2): 591–603.

11. Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in publications remains with the publisher. No inventions or patentable discoveries

have been identified from the work. No Program IP has been commercialised at the time of writing this report.

This project has generated knowledge, know-how and data on the ecological drivers of yeast and bacterial fermentation performance and the genetics underpinning their resilience. This public knowledge has been, and will continue to be, disseminated to stakeholders through a variety of channels.

11.1 *Oenococcus oeni* strains isolated and characterised with commercial potential

A large number of environmental isolates of *O. oeni* were physiologically characterised during the project. Two were identified as having commercial potential. Those two strains were:

- *O. oeni* AWRI B1351
- *O. oeni* AWRI B1371

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14. Other supporting material (including raw data, if appropriate)

Not applicable