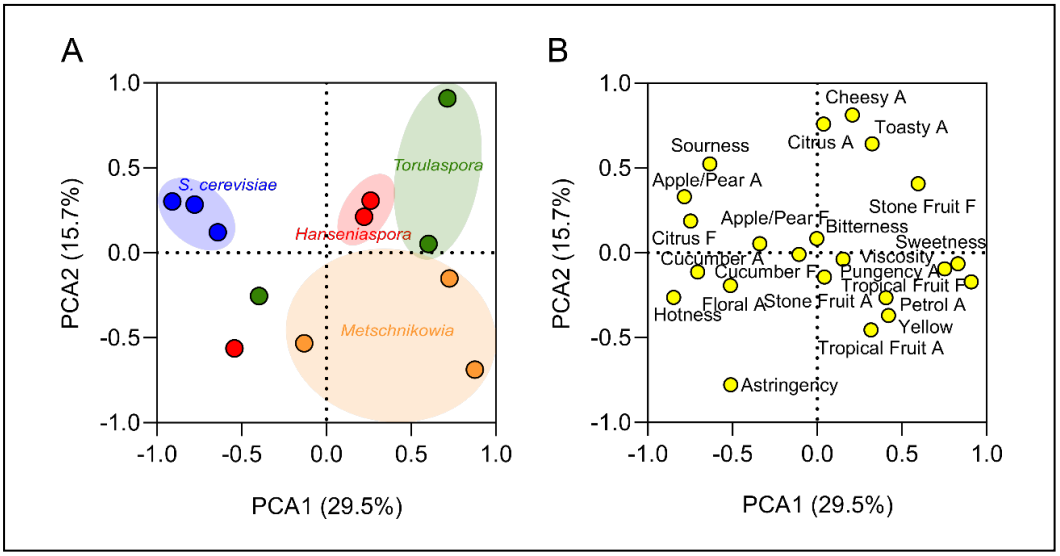
Australian Government
Wine Australia

AWRI Australian Wine Research Institute

Bioprospecting Australian microbial genetic diversity



FINAL REPORT TO WINE AUSTRALIA

Project Number: 4.4.3

Principal Investigator: **Anthony Borneman**

Research Organisation: **Australian Wine Research Institute**

Date: **September 2022**

Project title: Bioprospecting Australian microbial genetic diversity

Authors: Anthony Borneman, Cristian Varela, Cristobal Onetto

Date: September 2022

Address: The Australian Wine Research Institute, Wine Innovation Central Building, Hartley Grove, cnr Paratoo Rd, Urrbrae (Adelaide), SA 5064

Disclaimer/copyright statement:

This document has been prepared by The Australian Wine Research Institute ('the AWRI') as part of fulfilment of obligations towards the Project Agreement AWR 1701-4.4.3 and is intended to be used solely for that purpose and unless expressly provided otherwise does not constitute professional, expert or other advice.

The information contained within this document ('Information') is based upon sources, experimentation and methodology which at the time of preparing this document the AWRI believed to be reasonably reliable and the AWRI takes no responsibility for ensuring the accuracy of the Information subsequent to this date. No representation, warranty or undertaking is given or made by the AWRI as to the accuracy or reliability of any opinions, conclusions, recommendations or other information contained herein except as expressly provided within this document. No person should act or fail to act on the basis of the Information alone without prior assessment and verification of the accuracy of the Information.

To the extent permitted by law and except as expressly provided to the contrary in this document all warranties whether express, implied, statutory or otherwise, relating in any way to the Information are expressly excluded and the AWRI, its officer, employees and contractors shall not be liable (whether in contract, tort, under any statute or otherwise) for loss or damage of any kind (including direct, indirect and consequential loss and damage of business revenue, loss or profits, failure to realise expected profits or savings or other commercial or economic loss of any kind), however arising out of or in any way related to the Information, or the act, failure, omission or delay in the completion or delivery of the Information.

The AWRI acknowledges and agrees that the Information was commissioned by Wine Australia under the terms of the Project Agreement and agrees to the provision of copyright permissions as required by this Agreement.

The Information must not be used in a misleading, deceptive, defamatory or inaccurate manner or in any way that may otherwise be prejudicial to the AWRI.

Table of contents

1. Abstract 4
2. Executive summary 4
3. Acknowledgements 5
4. Background 6
5. Project aims 6
6. Methods 7
7. Results and discussion 12
8. Outcome and conclusion 42
9. Recommendations 44
10. Appendix 1: Communication 44
11. Appendix 2: Intellectual property 45
12. Appendix 3: References 45
13. Appendix 4: Staff 48
14. Appendix 5: Other supporting material (including raw data, if appropriate) 48

1. Abstract

Uninoculated (wild) wine fermentations are conducted by a consortium of wine yeast and bacteria that establish themselves either from the grape surface or from the winery environment. Wild ferments are considered to provide improved mouthfeel, aroma and flavour complexity compared to inoculated ferments, but at the expense of reliability and reproducibility. This project has applied a combination of genomic and microbiological techniques to investigate Australian wine microbiomes and how non-*Saccharomyces* yeasts and winemaking interventions can be used shape wine outcomes through altering microbial composition.

2. Executive summary

Wild ferments show a far more complex progression of microbial species than inoculated wines and, accordingly, a more complex taste and aroma profile. Differences in these resident microflora between vineyards and wineries are therefore thought to play a key role in defining regional expression of wine characteristics.

To investigate the yeast microbiomes of Australian wild fermentations, a DNA-based yeast profiling framework was established and used to investigate commercial uninoculated Chardonnay and Shiraz wines, with this work later expanded to include Pinot Noir and smaller numbers of ferment samples from other varieties.

Results were obtained from 852 wines from 34 wineries from across 12 Australian winemaking GIs and showed that wines contained a highly variable yeast microbiome that differed across regions, vintages, wineries and vineyards.

While the yeast profiling methodology used for the winery studies provided detailed information on the ferment microbiology, the extended time needed due to centralised processing and complicated sample logistics meant that results were obtained well after the completion of vintage. Other methods were therefore investigated to progress the methodology towards 'in-field' testing and a time frame that would allow for ferment intervention based upon analytical data. A method was developed that simultaneously isolated and stored the DNA of mixed microbial samples, which could be used as an alternative to current requirements for cold-chain logistics and DNA extraction. This removed a key roadblock in the transition towards in-field analysis, while also providing a means to streamline logistics for sample storage and transport to centralised locations for processing. It is now conceivable that larger wine laboratories, which can currently handle molecular diagnostics such as qPCR, could perform this technique on-site.

As wild fermentations do not involve the addition of commercial yeast, winemaker intervention is limited, and the effects of interventions on the microbiome of the wine are often poorly understood. Experiments were therefore performed to assess the impact of common and relatively easy to implement interventions - SO_2, aeration and temperature changes – on the microbial dynamics and chemical properties of wine.

Given its role as the major antimicrobial used in wine, the effect of SO_2 on both the microbiome and wine composition was assessed across four years, moving from laboratory-scale experiments to winery trials. Moderate addition of SO_2 prior to the initiation of a wild ferment resulted in a drastic and reproducible change to the yeast species present. This was accompanied by significant changes in the chemical profile of the wines, which could be associated with distinct sensory outcomes.

The effect of aeration on wild fermentation was assessed across three vintages, with initial laboratory-scale experiments also transitioning to winery trials and sensory studies. When

provided at the appropriate time, aeration had the potential to aid wild *Saccharomyces* fermentation efficiency and decrease the time taken for fermentation completion.

Finally, the effect of varying fermentation temperature was investigated in both Chardonnay and Shiraz at laboratory scale. Here, it was shown that increasing temperature allowed for higher cell numbers and faster fermentations, but this came at the expense of wine quality.

These interventions provide a means to alter flavour and aroma outcomes (through the use of SO_2) and to affect fermentation efficiency (through the judicious use of aeration). By combining the ability to monitor microbial populations through the yeast profiling techniques with the winemaker interventions that were investigated, the risk of negative wild ferment outcomes can be decreased.

In addition to yeast microbiome profiling, a collection of more than 18,000 wine yeast isolates was established from a range of wild ferment wine samples over a four year period. Species identification indicated that 35 different yeast species were represented in the collection, which provided a foundational germplasm resource that was subsequently used to identify novel non-*Saccharomyces* isolates for use as commercial starter strains and for wine yeast population genomics studies.

While this project provided information that can be used to lower the perceived risks associated with spontaneous fermentation, it is likely that the majority of wines produced in the Australian market will still make use of inoculation with commercial yeast starter cultures. To address this key market segment, commercial non-*Saccharomyces* strains can be used to provide novel and/or complex flavour profiles relative to the use of *S. cerevisiae* alone, while improving fermentation reproducibility and reliability relative to the use of wild fermentation. An iterative screening approach was used to screen a set of almost 1,300 non-*Saccharomyces* yeast strains collected during this project for desirable winemaking properties. Three candidate strains were progressed through to winery-scale fermentation and full sensory analysis and showed promising characteristics as potential commercial starter strains for increasing flavour and aroma attributes during winemaking.

This project has delivered a holistic view of the yeast and fungal microbiology that underpins wild fermentations undertaken across Australia. It has provided a methodology to provide winemakers with an ongoing means to investigate the unique biology of their fermentations, while outlining tools to alter wine characteristics and assist in fermentation efficiency. Selected non-*Saccharomyces* strains were also developed to provide options for the significant sectors of the Australian industry that wish to continue using commercial starter strains, but desire winemaking properties that go beyond those provided by standard wine yeast.

3. Acknowledgements

This work was supported by Australia's grapegrowers and winemakers through their investment body Wine Australia with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster in Adelaide, SA.
The project team would like to acknowledge all of the wineries that kindly provided samples of their wild wine fermentations over the course of the project.

Greg Nattrass, Luke Wilson and Louisa Rose of Yalumba are thanked for providing juice for performing laboratory-scale wild fermentation and for hosting winery trials for the SO_2 and aeration trials in 2020 and 2021.

The AWRI sensory team and WIC Winemaking Services are also thanked for their contributions to this project.

Isolates from Tasmania and the Torres Strait were collected in collaboration with Prof. Vlad Jiranek from the University of Adelaide. The project team would like to thank Steve Cronin, Andry Sculthorpe and the Tasmanian Aboriginal Council for allowing access to Trawtha Makuminya, and Matt Taylor and The Tasmanian Land Conservancy for allowing access to Skullbone Plains and Five Rivers—Serpentine. The project team would also like to thank Vic McGrath, the Torres Strait Regional Authority and the Erub Community Council for enabling a visit to Erub Island. Special thanks to Michael O'Brien for his inputs on the planning stages of this work and to Kapua Gutchen, elder of the Erub Community.

4. Background

Wine is a complex beverage, comprising thousands of metabolites that are produced through the action of yeasts and bacteria in fermenting grape must. When grapes are crushed and allowed to ferment naturally, a complex microbial succession of yeasts and bacteria is generally observed. In the very early stages of fermentation, aerobic and apiculate yeasts, and yeast-like fungi from genera such as *Aureobasidium*, *Rhodotorula*, *Pichia*, *Candida*, *Hanseniaspora* and *Metschnikowia*, which reside on the surface of intact grape berries or winery equipment, represent the majority of the microbiota (Fleet 2008).

However, most of these species, especially the aerobic yeasts, succumb early in the succession of the fermentation in response to falling oxygen levels and increasing ethanol concentrations. Mildly fermentative yeasts, such as *Hanseniaspora uvarum*, *Candida stellata*, *Metschnikowia pulcherrima, Torulaspora delbrueckii* and *Lachancea thermotolerans* can proliferate and survive well into the fermentation, but fall in numbers as ethanol concentrations increase, although it has been reported that *C. stellata* can survive up to 12% ethanol and complete fermentation (Beltran et al. 2002, Combina et al. 2005, Fleet 1990,Fleet et al. 1984).

Despite the vastly higher numbers of non-*Saccharomyces* yeasts early in the fermentation process, the major wine yeast, *Saccharomyces cerevisiae* is responsible for the bulk of the ethanolic fermentation. However *S. cerevisiae* is not readily isolated from intact grape berries and is therefore generally found in very low numbers at the start of fermentation (Martini et al. 1996, Mortimer and Polsinelli 1999). Regardless, due to its higher fermentative ability, growth rate and tolerance to ethanol, *S. cerevisiae* supplants the various non-*Saccharomyces* yeasts, becoming the dominant species from mid-fermentation such that an almost monoculture of this one species is established by the end of fermentation.

While traditional microbiological techniques have provided important insights into the microbial succession that occurs in spontaneous ferments, the breadth of ferments investigated and the depth at which individual species contributions could be resolved have been limited. Recent advances in culture-independent methods for species analysis, such as amplicon based phylotyping (also known as meta-barcoding) and metagenomics, provide a high-throughput means to analyse large numbers of microbiological samples at great depth (Caporaso et al. 2011). Accordingly, these techniques are now being adapted for the study of wine fermentation and several amplicon based studies have been conducted to investigate vineyard and wine microbiomes (Bokulich and Mills 2013, Bokulich et al. 2012, 2014, 2016, Pinto et al. 2015,Taylor .et al. 2014).

5. Project aims

The contribution of wild wine microorganisms promises to be a critical driver of Australian regional terroir and a means for wineries to increase profits by producing unique products with market distinction. This project aimed to leverage the analytical power of next generation genomics and high-throughput screening to advance knowledge of wild wine microbiota and how they can be exploited to aid winemaking goals.

Deliverables in this project were:

- Data available from metagenomic analysis, describing wild wine fermentations from across the spectrum of Australian winemaking regions
- Yeast and bacterial isolates sourced from wild fermentations and natural sources, deposited in the AWRI culture collection
- New strains of *Saccharomyces* and non-*Saccharomyces* yeasts characterised and available for commercial application
- Knowledge on the effects of winemaking interventions on sensory and chemical characteristics of wild fermentations available
- Methods and equipment for 'in-winery' metagenomic analysis and fermentation management assessed
- Practical advice and extension materials for winemakers.

6. Methods

6.1 Winery wild fermentation analysis

6.1.1 Sample acquisition

Wineries were supplied with pre-labelled, 50 mL falcon tubes. Approximately 40 mL of fermenting grape must was collected at four timepoints per fermentation, based upon refractometry levels (T1, directly after must was transferred to tank; T2, after a 1 °Bé drop in sugar; T3, 50% sugar remaining; T4, 10% sugar remaining). Samples were then stored frozen until collection and processing.

6.1.2 Sample processing and ITS profiling

DNA was isolated using the DNeasy PowerFood Microbial DNA Isolation Kit (Qiagen) following the manufacturer's instructions. Bead-beating was carried out using a combination of 0.1 mm and 0.5 mm zirconia/silica beads (BioSpec Products) in a Precellys Evolution homogeniser (Bertin Instruments) at 8,000 RPM for 4 x 60 sec.

A two-step PCR performed using sequences designed to amplify the fungal ITS region, while adding experiment-specific inline barcodes and appropriate adaptors for the Illumina sequencing platform (Sternes and Borneman 2016). Sequencing was performed using 2 x 300 bp chemistry (Ramaciotti Centre for Genomics), with post-sequencing processing performed as described in (Sternes and Borneman 2016).

6.1.3 LAMP ITS

Two sets of LAMP primers were designed based on the consensus of 70% of the LSU-ITS-SSU sequence of the *Saccharomycetales*.

F3_2-2
CTTTCCATCACTGTACTTGTTC
B3_2-2
TCGATGAAGAACGCAGC
FIP_2-2
CGAGTTGTTTGGGAATGCAGCTCGCTATCGGTCTCTCGCCA
BIP_2-2
TGACGCTCAAACAGGCATGCCATCGAATCTTTGAACGCACAT

F3_2-1
TGACGCTCAAACAGGCATGC
B3_2-1
AGTAAGCGCAAGTCAT
FIP_2-1
GCATCGATGAAGAACGCAGCGAAGCGTTCAAAGATTCGATGATTC

BIP_2-1
GCCTCACTAAGCCATTCAATCGGGCTTGCGTTGATTACGTCCC

All LAMP reactions on purified DNA were performed using the Warmstart LAMP kit (New England Biolabs) according to manufacturer's instructions using purified DNA.

Sequencing libraries were produced from the LAMP products using the Rapid Sequencing kit (SQK-RAD004, Oxford Nanopore) and sequenced on a MinION flowcell (R9.4.1, Oxford Nanopore) according to manufacturer's instructions.

6.1.4 Rapid prep

For alkaline lysis, 10 µL of cell suspension in YPD was mixed with 10 µL of 0.05 M NaOH and incubated at 98°C for 10 min. The reaction was allowed to cool to room temperature and then neutralised by the addition of 20 µL of 0.1 M Tris-HCL buffer (pH 7.5). The neutralised lysate was used directly in the LAMP reaction.

For sample preparation using Whatman FTA® paper (Merck), unless otherwise specified, 125 µL of sample was applied as a single spot onto an FTA card and allowed to dry at room temperature. A 2 mm disk was then removed from the card and placed in a PCR tube. The disk was washed twice with FTA purification reagent and then twice with TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). The disk was then left to dry in the PCR tube until used directly in a PCR reaction.

The mock community consisted of a mixture of *Candida* (35% by direct cell count), *Brettanomyces* (3%), *Hanseniaspora* (24%), *Metschnikowia* (10%), *S. cerevisiae* (9%) and *Torulaspora* (20%). Cultures of each strain were grown separately in YPD until stationary phase, counted and then combined in equal volumes prior to serial dilutions being performed.

6.2 Wild fermentation intervention

6.2.1 Sulfite

Commercial, high-solids Chardonnay juice (not pre-treated with SO_2 during harvesting or processing) was obtained directly after destemming and crushing from Yalumba during the 2018 and 2019 vintages. The juice was transferred to 2 L Schott bottles and then treated with either potassium metabisulfite (ACE Chemical Company) to the appropriate final total SO_2 concentration or 250 mg of reduced glutathione (GSH, Sigma-Aldrich). Each experiment was performed in triplicate. Bottles were sealed with airlocks and incubated at 18°C. Ferments were assessed at least every 24 hours by refractometry and sugar analysis, with samples taken for ITS profiling at four approximate sugar levels (T1, directly after treatment; T2, 90% sugar remaining; T3, 50% sugar remaining; T4, 10% sugar remaining) from an in-built sampling port.

Sample processing and ITS profiling were performed as indicated in Section 6.1.2.

For sensory analysis of 2021 vintage wines, a panel of nine assessors (eight female, one male) was used, with an average age of 51 years (SD=5.5) Wines were evaluated by quantitative sensory descriptive analysis methodology. All panellists were screened for their sensory acuity, regularly participated in sensory training and had extensive experience in wine sensory descriptive tasks. Over three two-hour training sessions, wines from the study were progressively used to generate and refine appropriate descriptive attributes and definitions through a consensus-based approach (Bindon et al. 2014). Wine sample evaluation was performed as described previously (Varela et al. 2020) with some modifications. Samples were presented to panellists in 30 mL aliquots in 3-digit-coded, covered, ISO standard wine glasses at 22–24°C, in isolated booths under daylight-type lighting, with randomised modified Williams Latin Square presentation order, in duplicate, generated by Compusense Cloud sensory evaluation software (Compusense Inc., Canada). All samples were expectorated. Wines were assessed in sets of three and assessors were forced to have a 60 second rest between samples

and a minimum 10 minute rest between trays. Each presentation replicate was poured from a separate bottle. The intensity of each attribute was rated using an unstructured 15 cm line scale, with indented anchor points of 'low' and 'high' placed at 10% and 90% respectively, coded from 0 to 10. Data were acquired using Compusense Cloud sensory evaluation software. Panel performance was assessed using Compusense software and R with SensomineR (sensominer.free.fr/) and FactomineR (factominer.free.fr/) packages.

6.2.2 Temperature

Commercial, high-solids Chardonnay juice (not pre-treated with SO_2 during harvesting or processing) was obtained directly after destemming and crushing from Yalumba (Barossa Valley, SA) during the 2021 vintage. Shiraz grapes were obtained from McLaren Vale, SA, which were not pre-treated with SO_2 during harvesting or processing and were crushed by WIC Winemaking Services (Adelaide, Australia) during the 2022 vintage. The composition of the Chardonnay juice was: sugar 210 g/L, yeast assimilable nitrogen (YAN) 350 mg N/L, titratable acidity (TA) 7.1 g/L and pH 3.42, whereas the composition of the Shiraz must was: sugar 240 g/L, YAN 250 mg N/L, TA 4.6 g/L and pH 3.67.

Chardonnay juice was transferred to sterile 2 L Schott bottles which were sealed with airlocks, whereas Shiraz must was transferred to 2 L plastic containers equipped with a stainless steel mesh to keep the cap submerged and sealed with airlocks. Ferments were performed in triplicate at three different temperatures 12°C, 22°C and 28°C. Ferments were continuously stirred at 250 rpm using magnetic stirrers.

Samples were taken daily for analysis until the end of fermentation (<2 g/L of residual sugar). All ferments were sampled for ITS-profiling based on sugar concentrations. In addition to profiling, samples were also plated onto WL agar (Amyl Media Pty Ltd, Australia) and L-lysine medium (Oxoid, UK) to estimate total and non-*Saccharomyces* viable cell numbers, respectively.

6.2.3 Chemical analysis

Wine density, specific gravity, pH, volatile acidity, alcohol concentration and titratable acidity were determined using a Foss WineScan FT 120 as described by the manufacturer (Foss, Denmark). The concentrations of residual sugar, glycerol, malic, tartaric and citric acids and ethanol were measured by high performance liquid chromatography (HPLC) using a Bio-Rad HPX-87H column as described previously (Varela et al. 2004). Ammonia and free α-amino acid nitrogen were determined enzymatically using the Ammonia Enzymatic Test Kit and the Primary Amino Acid Nitrogen Test Kit, respectively (Vintessential Laboratories, Australia) according to the manufacturer's instructions, and using a GalleryTM Discrete Analyser (Thermo Fisher Scientific, USA). Analysis of higher alcohols and acetate and ethyl esters was performed by Metabolomics SA using gas chromatography-mass spectrometry (GCMS) with stable isotope dilution analysis (SIDA) as previously described (Bizaj et al. 2012). Low molecular weight sulfur compounds (LMWSC) were determined by using headspace cool-on-column gas chromatography coupled with a sulfur chemiluminescence detector (HS-COC-GC-SCD) (Siebert et al. 2010).

6.3 Yeast strain isolation and identification

Serial dilutions of grape must were plated onto several different types of solid media including WL (Amyl Media Pty Ltd), L-lysine (Oxoid, UK) and malt-extract (Amyl Media Pty Ltd) agar and incubated at 20°C for 3 days. Resulting yeast colonies were transferred to WL media in 96-well format using a PIXL colony picking robotic platform (Singer Instruments, UK).

High-throughput colony ITS sequencing was performed on single isolates using primers designed to amplify the fungal ITS region, while adding experiment-specific inline barcodes and appropriate adapters for the Illumina sequencing platform as described previously (Sternes et al. 2017). Sequencing was performed using 2 x 300 bp chemistry (Ramaciotti Centre for Genomics), with post-sequencing processing performed as described in (Sternes and Borneman 2016).

6.3.1 High-throughput screening

Individual colonies identified as non-*Saccharomyces* were picked manually, arrayed in a 96-well format, which included a *S. cerevisiae* control strain (EC1118) in each plate, and kept as glycerol frozen stocks at -80°C.

Frozen stocks with arrayed colonies were pinned in duplicate on YPD agar plates using a Tecan Freedom EVO 150 robotic platform with a stainless steel 96-pin tool (Tecan Group Ltd, Switzerland) and incubated at 28°C for 3 days. Plates were then replicated onto synthetic complete (SC) agar medium (20 g/L of glucose, 1.7 g/L yeast nitrogen base without ammonium sulfate or amino acids, 5 g/L ammonium sulfate and 20 g/L agar) and incubated at 28°C for 5 days.

For ethanol tolerance, SC source-plates were replicated onto SC agar media containing 3%, 6% or 12% (v/v) ethanol. For osmotic stress, source-plates were replicated onto SC agar media containing either 20% or 40% (w/v) glucose. For copper tolerance, source-plates were replicated onto SC agar media containing 0.2 mM copper sulfate. Growth under anaerobiosis was assessed on SC agar followed by incubation in a Type B Vinyl chamber anaerobic hood (Coy Labs, Michigan, USA). Growth at low temperature was assessed by incubating SC agar plates at 12°C. Sulfite tolerance was assessed by replicating onto SC agar plates containing 4.5 g/L tartaric acid and 1 mM sodium sulfite (Naumov et al. 2013). Hydrogen sulfide (H_2S) production was assessed in SC agar media containing copper.

Unless indicated otherwise, all plates were incubated at 28°C for 5 days and then imaged (Phenobooth, Singer Instruments, UK). Digital images were used to quantify colony sizes and measure colony size (PhenoSuite v2.20.504.1, Singer Instruments, UK).

The sensitivity of isolates to environmental conditions was quantified by expressing the $\log_2$ ratio of colony size under each test condition versus the colony size on SC agar. For sulfite tolerance, the $\log_2$ ratio was obtained from the colony size in media with sodium sulfite over the colony size on SC media containing tartaric acid. H_2S production was expressed as the $\log_2$ ratio of the colony brightness in SC media over the colony brightness on media containing copper.

Five different enzyme activities were assessed: cellulase, pectinase, protease, β-lyase and β-glucosidase. Colonies from SC source plates were inoculated into 96-well plates containing 200 µL of SC liquid media and incubated at 28°C for 2 days. Cultures were then used to inoculate 96-well plates containing modified SC medium, incubated at 28°C for 3 days and assessed as follows. For cellulase activity, plates contained SC medium plus 1 g/L of carboxymethyl cellulose, with activity measured spectrophotometrically with congo red (Haft et al. 2012). For pectinase activity, plates contained SC medium plus PGA (1.5 g/L polygalacturonic acid), with activity determined spectrophotometrically with ruthenium red (Ortiz et al. 2014). Protease activity was determined spectrophotometrically with trichloroacetic acid in cultures grown in SC medium with 2.5 g/L azocasein (Lario et al. 2015). For β-glucosidase activity, source cultures were grown in SC medium with cellobiose, and β-glucosidase activity determined spectrophotometrically with p-nitrophenyl-b-D-glucopyranoside (PNPG) (Schmidt et al. 2011). β-Lyase activity was measured spectrophotometrically (310 nm) from the release of 2-mercaptothiazole by cultures grown in SC medium with TAC (1 g/L S-(2-thiazolyl)-L-cysteine) and 12 g/L yeast carbon base(Cordente et al. 2022).

All enzyme activities were expressed as the $\log_2$ ratio of the absorbance value for each activity over the absorbance value for uninoculated media.

For growth assays and enzyme assays, the differences between strains were normalised by converting $\log_2$ ratios to discrete values (scores from 1 to 4) based on the $\log_2$ ratio of a reference strain. For agar plates, the *S. cerevisiae* control strain was used as the reference, while for enzyme assays, uninoculated media was used as the reference. Positive attributes scored

higher; that is, colonies with log_2 ratios between 75 and 100% of the log_2 ratio of the *S. cerevisiae* control were given a score of 4, colonies with ratios between 50 and 75% were given a score of 3, colonies with ratios between 25 and 50% were given a score of 2, whereas colonies with ratios lower than 25% were given a score of 1. For H_2S production, colonies with log_2 ratio between 100 and 120% of the log_2 ratio of the *S. cerevisiae* control were given a score of 4, colonies with ratios between 120and 130% were given a score of 3, colonies with ratios between 130 and 150% were given a score of 2, whereas colonies with ratios higher than 150% were given a score of 1.

6.3.2 Confirmation of enzyme activities

Colonies showing either high β-glucosidase or β-lyase activities were evaluated in chemically defined grape juice (CDGJ). For β-glucosidase, colonies were inoculated in duplicate into 96-well plates containing 200 µL of CDGJ supplemented with PNPG in each well, while for β-lyase, colonies were inoculated into 96-well plates containing 200 µL of CDGJ supplemented with TAC. Plates were incubated for 3 days at 28°C with activities determined spectrophotometrically as described in Section 6.4.1.

6.3.3 Laboratory-scale fermentation screening

Selected strains were evaluated for their ability to express either β-glucosidase or β-lyase activities during wine fermentation. Chardonnay must was prepared from grapes obtained from Blewitt Springs, Clarendon Hills, SA and contained 240 g/L of glucose+fructose and 260 mg N/L of yeast assimilable nitrogen (YAN). Must was supplemented with either Gordo grape marc glycosides (0.4 mg/L) or thiol precursors (cystheine-3MH 100 ug/L, cystheine-4MMP 100 µg/L, glutathione-3MH 1.5 mg/L, glutathione-4MMP 1.5 mg/L) to determine β-glucosidase and β-lyase activity, respectively. Yeast starter cultures were grown overnight in YPD medium at 28°C with shaking (200 rpm). Cultures were then used to inoculate 100 mL of sterile grape must, diluted 1:1 with water, in 250 mL Erlenmeyer flasks. Flasks were incubated overnight at 22°C with shaking (120 rpm) under aerobic conditions and then used to inoculate grape must. Fermentations were performed in triplicate in sterile 100 mL Schott bottles equipped with airlocks and contained 80 mL of filter-sterilised grape must. Ferments were incubated at 22°C (120 rpm). After 50% of sugar was consumed, *S. cerevisiae* AWRI1631 ($1x10^6$ cells/mL) was inoculated into each bottle to ensure completion of fermentation. Samples were taken for chemical analysis after sugar was completely consumed.

6.3.4 Winemaking trials

Selected strains were evaluated for their ability to release either terpenes or thiols in sequential fermentation with *S. cerevisiae*. Fermentations were performed in triplicate using Riesling grape juice (Clare Valley, SA). Winemaking was performed by WIC Winemaking Services (Adelaide, Australia). Grapes were received and stored at 0°C for two to three days prior to crushing and destemming. After crushing, the must was homogenised, divided in 50 kg aliquots and placed in plastic fermenters. Total sulfur dioxide was adjusted to 50 mg/L by adding potassium metabisulfite (PMS). The must was then drained and pressed and the enzyme Rohavin (AB Biotek, Sydney, Australia) was added to each fermenter at a rate of 4 mL/100 L to improve juice extraction and clarification. Fermenters were cold settled at 0°C for three to five days and then racked. Clarified ferments were maintained at 15°C for 24 hours before inoculation. Ferments were sequentially inoculated with *S. cerevisiae* PDM at approximately 50% sugar consumption. Fermentations were conducted at 15°C until sugar concentration was below 3 °Bé (5.4 °Brix). Although some ferments proceeded faster than others, all ferments were kept in contact with solids for the same time. After fermentation was completed, sulfite was adjusted to 80 ppm, wines were then incubated for 48 h before cold stabilisation. Wines were then racked off and stored for a minimum of 28 days at 0°C before being filtered and bottled in 375 mL screw cap bottles. Wines were stored at 18°C for four months before analysis. Samples were taken during fermentation to determine yeast population dynamics and basic chemical composition.

6.3.5 Chemical analysis

Wine density, specific gravity, pH, volatile acidity, alcohol and titratable acidity were determined using a Foss WineScan FT 120 as described by the manufacturer (Foss, Denmark). Free and total SO_2 were measured by the aspiration method (Rankine and Pocock 1970). The concentrations of residual sugar, glycerol, malic, tartaric and citric acids and ethanol were measured by high performance liquid chromatography using a Bio-Rad HPX-87H column as described previously (Varela et al. 2004). Ammonia concentration was quantified by using the Glutamate Dehydrogenase Enzymatic Bioanalysis UV-method test (Roche, Germany). Free α-amino acid nitrogen was determined by the o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay procedure (Dukes and Butzke 1998) using a Roche Cobas FARA spectrophotometric autoanalyser (Roche, Switzerland). Analysis of higher alcohols, acetate-, and ethyl esters was performed using gas chromatography-mass spectrometry using a stable isotope dilution analysis as previously described (Bizaj et al. 2012). Analysis of free thiols, 3-MH, 3-MHA, and 4-MMP was performed using the derivatising agent 4,4'-dithiodipyridine (TCI, Asis Scientific, Australia) and LC-MS/MS as described previously (Cordente et al. 2022). Analysis of monoterpenes and C13-norisoprenoids was performed by Membrane-Assisted Solvent Extraction GC-MS as described previously (Pisaniello et al. 2022).

6.3.6 Sensory analysis

Wines were evaluated by quantitative sensory descriptive analysis methodology. A sensory panel was convened from the pool of AWRI's trained descriptive analysis panel employees, composed of twelve assessors (ten female, two male) with an average age of 48 years (SD = 7.8). All panellists were screened for their sensory acuity, regularly participated in sensory training and had extensive experience in wine sensory descriptive tasks. Over four two-hour training sessions, wines from the study were progressively used to generate and refine appropriate descriptive attributes and definitions through a consensus-based approach (Bindon et al. 2014). Wine sample evaluation was performed as described previously (Varela et al. 2020) with some modifications. Samples were presented to panellists in 30 mL aliquots in 3-digit-coded, covered, ISO standard wine glasses at 22–24°C, in isolated booths under daylight-type lighting, with randomised modified Williams Latin Square presentation order, in duplicate, generated by Compusense Cloud sensory evaluation software (Compusense Inc., Canada). All samples were expectorated. Wines were assessed in sets of three and assessors were forced to have a 60 second rest between samples and a minimum 10 minute rest between trays. Each presentation replicate was poured from a separate bottle. The intensity of each attribute was rated using an unstructured 15 cm line scale, with indented anchor points of 'low' and 'high' placed at 10% and 90% respectively coded from 0 to 10. Data were acquired using Compusense Cloud sensory evaluation software. Panel performance was assessed using Compusense software and R with SensomineR (sensominer.free.fr/) and FactomineR (factominer.free.fr/) packages.

6.3.7 Genome sequencing

Genomic DNA was extracted from liquid cultures using a QIAGEN Gentra Puregene Yeast/Bact Kit. Sequencing libraries were prepared using the Illumina Nextera library kit and barcoded and pooled in a single Miseq sequencing run using 2x300 bp chemistry (Ramaciotti Centre for Genomics).

Nucleotide variant calling and phylogenetic comparison were performed as detailed previously (Borneman et al. 2016).

7. Results and discussion

7.1 Wild fermentation analysis

7.1.1 Fungal profiling of Australian wild fermentations

To investigate the microbial populations that were present across commercial wild fermentations in Australia, a microbial survey was established that initially encompassed commercial producers of uninoculated Chardonnay and/or Shiraz wines, which was later

expanded to also include Pinot Noir and smaller numbers of ferment samples from other varieties.

Samples were collected across five vintages (2017-2021), with a series of four sampling times requested per wine fermentation (T1, directly after must was transferred to tank; T2, after a 1 °Bé drop in sugar; T3, 50% sugar remaining; T4, 10% sugar remaining). A total of 3,270 samples were processed across the five years, comprising 852 wines from 34 wineries (Table 1). Due to the inconsistencies of commercial production, samples were not able to be obtained from the same winery and/or variety across all five years.

Table 1. Wild ferment samples processed

Year	Variety	Wines
2017	Chardonnay	62
	Shiraz	51
2018	Cabernet Sauvignon	8
	Chardonnay	84
	Grenache	12
	Pinot Noir	30
	Riesling	2
	Sangiovese	4
	Shiraz	64
	Tempranillo	4
	Viognier	10
2019	Cabernet Sauvignon	8
	Chardonnay	78
	Grenache	15
	Pinot Noir	24
	Shiraz	56
	Viognier	8
2020	Cabernet Sauvignon	8
	Chardonnay	62
	Grenache	13
	Pinot Noir	20
	Shiraz	47
	Viognier	8
2021	Cabernet Sauvignon	8
	Chardonnay	60
	Grenache	14
	Pinot Noir	18
	Shiraz	60
	Tempranillo	4
	Viognier	10

Each sample was processed to isolate DNA and subjected to PCR using primer designed against the fungal ITS region (Bokulich and Mills 2013). Amplified DNA was subsequently subjected to high-throughput sequencing and post-sequencing processing to identify operational taxonomic units (OTUs). OTUs define a collection of sequence reads with similar sequence, which are then used to represent the presence of a specific species within the sample. Species identity of each OTU was inferred by comparing the sequence of the OTU with ITS sequences of fungal isolates of known identity that exist in the public domain.

Of the 3,270 samples, 2,975 provided enough data for downstream comparison (≥1000 ITS sequencing reads). The abundance of individual OTUs (species) within each sample was then determined based upon the frequency of reads for each OTU. This provided a representation of

fungal microbiome (identity and relative abundance) for each of the 3,270 samples. As most of these samples formed part of a sampling time course, it was also possible to follow the temporal changes in the microbiome across individual wines from crush to dryness.

Across all of the samples, a total of 83 fungal genera were observed to be present (≥1% relative abundance in at least one sample), with 51 of these representing greater than 5% of the total abundance in at least one sample. The level of fungal diversity was highest just after crush (sampling point T1), with 8.0±3.0 genera observed on average (≥1% abundance). *Aureobasidium* was the most prevalent genus observed across the T1 samples accounting for 35% of the total abundance across all samples (Figure 1).

The T2 and T3 timepoints were defined by a decrease in the number of genera that was concomitant with an increase in a small number of non-*Saccharomyces* genera, as well as *Saccharomyces*. *Hanseniaspora* was the main non-*Saccharomyces* genus to show significant abundance at the T2 and T3 time points, with 35% of T2 time points displaying ≥20% abundance of *Hanseniaspora*. Diversity between ferments was often observed, especially in presence/absence of specific genera.

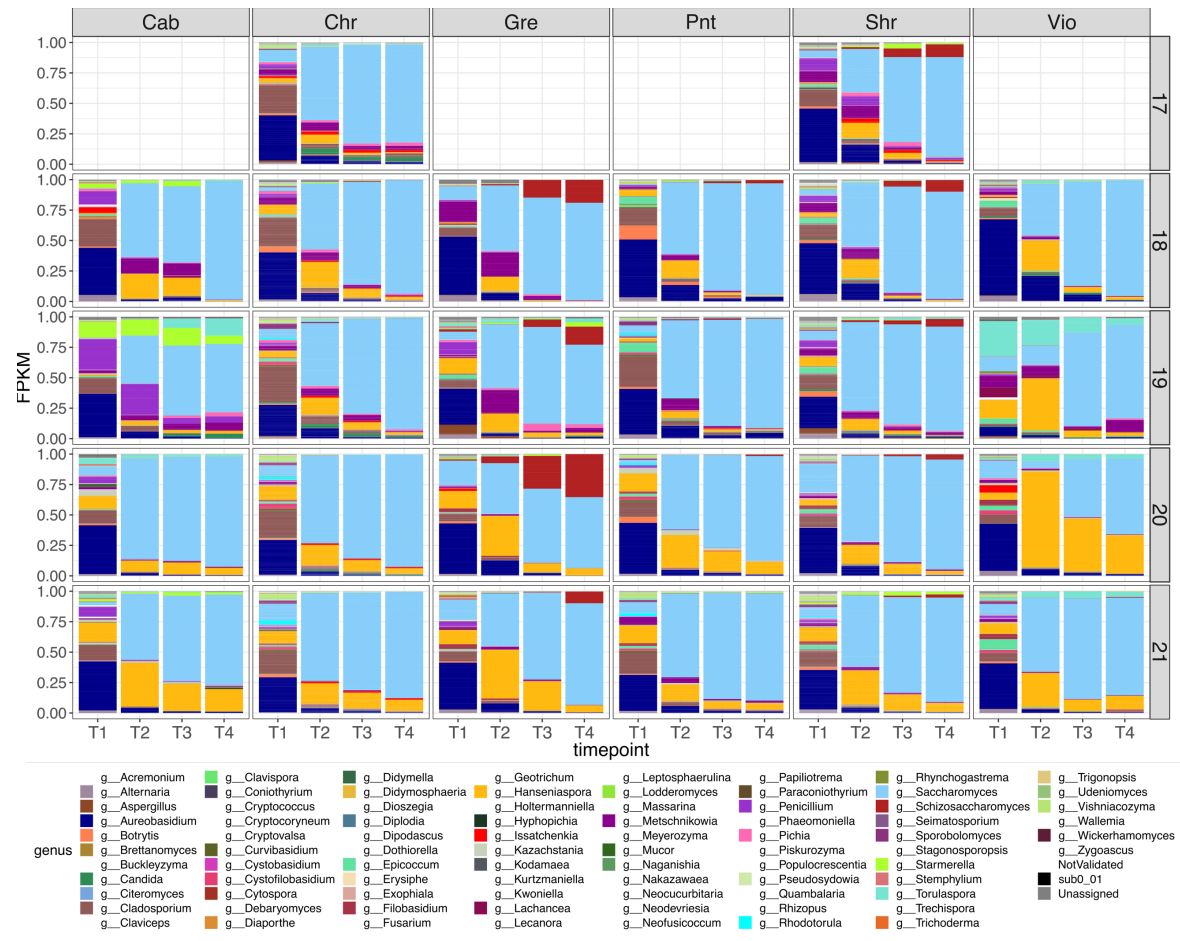


Figure 1. Fungal profiling of wild wine fermentations across six grape varieties and five vintages. OTUs are grouped to the genus level and all genera that are present at ≥5% abundance are shaded according to the legend below the graphs. Abbreviations; Cab, Cabernet Sauvignon; Chr, Chardonnay; Gre, Grenache; Pnt, Pinot Noir; Shr, Shiraz; Vio, Viognier.

Metschnikowia (7.8% of ferments at ≥20% abundance), *Pichia* (syn *Issatchenkia*) (2.3%) *Candida* (1.2%), *Torulaspora* (1.0%), and *Starmerella* (0.8%) and *Schizosaccharomyces* (0.8%) were also observed in specific T2 ferments. This same set of species were also most commonly observed in the T3 ferments, although their overall abundance was reduced compared to T2, with the exception of *Schizosaccharomyces*, which increased to being present at ≥20% abundance in 3% of ferments and *Saccharomyces* which increased from 79.9% to 96.9% between T2 and T3.

By the end of fermentation (T4) diversity decreased significantly when compared to T1 with only 2.3±1.5 genera observed on average per sample (≥1% abundance). In these late ferment samples, *Saccharomyces* dominated (≥90% abundance) in 67% of fermentations and represented 87% of the total abundance across all samples (Figure 1).

The only other genus that displayed ≥90% abundance in any T4 sample was *Schizosaccharomyces*, which was observed at this high level in five different Shiraz ferments (from the same McLaren Vale winery) across three different vintages. In this specific winery, *Schizosaccharomyces* appears to be a key component of the late-ferment microbiota, as it was seen at ≥40% abundance in 100% of their Grenache ferments and 55% of their Shiraz at the T4 timepoint (Figure 1).

7.1.2 In-winery metagenomic analysis

The techniques described above allow for in-depth investigation of ferment microbiology. However, due to the centralised nature of the methodology and equipment that is required, results are generally not obtained in a timeframe that is compatible with providing targeted fermentation intervention based upon the results. Investigations were therefore initiated to transition the yeast profiling workflow to provide near real-time data on the microbial composition of ferments, which would enable informed interventions to be made.

Loop-mediated Isothermal Amplification (LAMP) is an alternative methodology to PCR. Like PCR, LAMP is able to amplify sections of DNA based upon the design of specific primer sequences. However, unlike PCR, which requires complex thermal cycling and works best with purified DNA samples, LAMP requires incubation at a single temperature and can produce robust target amplification from crude cell lysates.

It was reasoned that a crude cell-lysis protocol combined with LAMP could provide a potential replacement for the DNA isolation and PCR that was used for the laboratory-based fungal profiling work reported in Sections 7.1.1 and 7.2. This would enable these key steps to be transitioned to the winery.

The PCR protocol for fungal profiling makes use of two primers that were designed to target two highly conserved regions of DNA that flank the highly variable ITS1 region (Bokulich and Mills 2013). The ITS PCR protocol therefore works across fungal species but produces a unique DNA-barcode for each species that enables the identification of fungal species within a mixed sample.

While PCR requires the design of two primers, which can be highly flexible in their length and distance from each other, LAMP requires the design of at least four primers (ideally including two additional 'loop' primers for maximum efficiency), with far more restrictions on the sequence and arrangement. The applicability of using ITS LAMPseq for in-field determination of fungal community structures was explored by designing primers based on a LSU-ITS-SSU consensus sequence comprising 70% of the *Saccharomycetales*. While two sets of primers were able to be designed, due to sequence constraints surrounding the ITS, both lacked the loop primers and were limited to a basic four-primer arrangement.

Both primer sets were tested against purified DNA from *S. cerevisiae* S288c, *S. cerevisiae* AWRI 796, *T. delbrueckii* AWRI 2845, *L. thermotolerans* AWRI 2009 and *B. bruxellensis* AWRI 2804, as well against a combination of all five strains, with two reaction times trialled (30 min and 1 hour). A low level of amplification was detected with one set of primers, but only after one hour of amplification. Amplification products using this primer set were then subjected to Oxford nanopore sequencing. Following sequencing it was confirmed that the amplification observed in all samples was due to non-specific primer amplifications and did not contain the target amplicon.

The limitations based upon designing a suitable set of LAMP primers against a multi-species target did not prove to be viable as a replacement for PCR-based ITS amplification. While the LAMP protocol was not deemed viable, methodologies were explored that provided crude cell lysates or DNA that were compatible with the ITS protocol.

Alkaline lysis was first tested as a rapid-DNA preparation methodology. This involved treating pelleted wine samples (containing microbial cells) with concentrated sodium hydroxide to lyse the cellular material and then neutralising samples prior to their use directly in the PCR reaction. The alkaline lysis protocol was applied to a mock mixed yeast community and compared against the standard methodology of DNA isolation. While alkaline lysis was able to provide a DNA template suitable for PCR amplification, the method displayed significant species-specific bias, with *Metschnikowia* being over-represented and other yeast species showing lower representation. This effect precluded the use of alkaline lysis as a simple preparatory technique for mixed-species samples as relative-abundance ratios would not reflect true proportions of species.

Whatman FTA paper provides another methodology for the preparation (and archiving) of crude DNA samples from biological specimens. FTA paper was therefore assessed as an in-field DNA extraction and room-temperature archiving methodology for yeast profiling. The ability for the FTA process to determine the proportions of a fungal mock community was evaluated at a range of cell concentrations and compared to current DNA extraction processes (Figure 2). The FTA process was shown to be highly reproducible and to detect all members of the mock community. When compared to the current DNA extraction process, the FTA showed a slightly different abundance profile and slightly reduced sensitivity.

While further experimentation will be required to optimise these aspects of the process, the FTA methodology has significant promise as a field-deployable system for microbial DNA purification. Furthermore, the ability for the FTA paper to stabilise DNA samples at room temperature provides the means to remove cold-chain logistics from centralised sample processing models such as those used in Section 7.1.1, which require that samples be stored frozen during vintage and then shipped on dry ice.

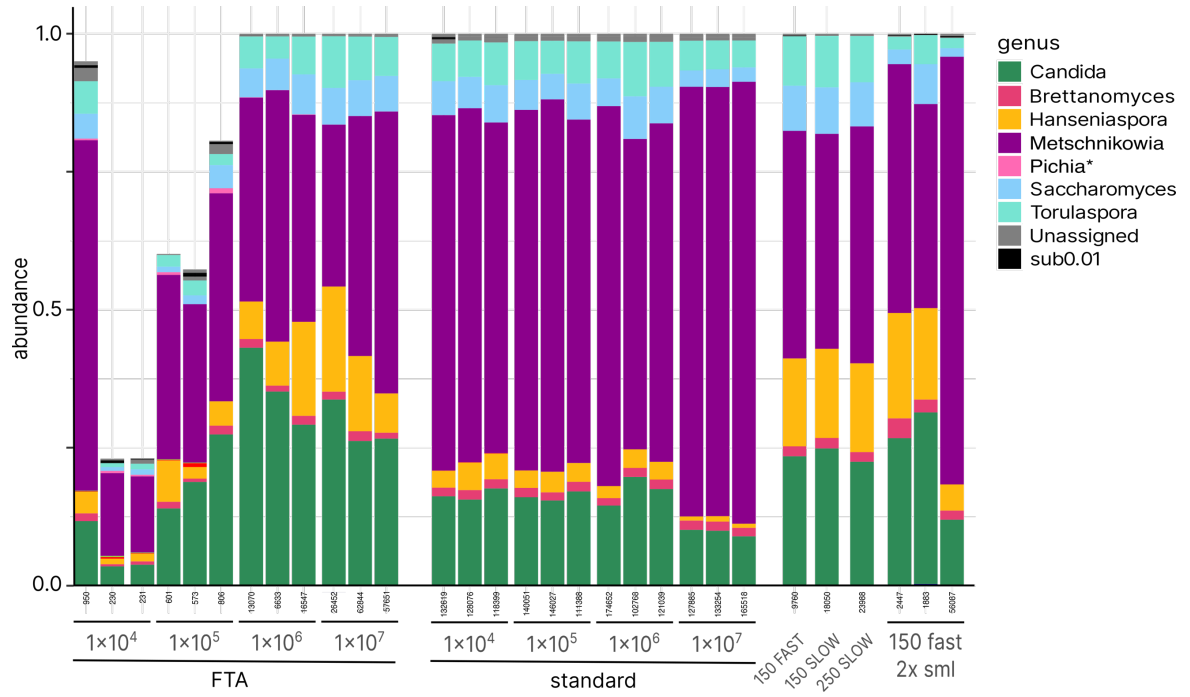


Figure 2. Application of FTA paper for rapid DNA extraction from fungal mock communities. Four different concentrations of fungal mock community were either processed using Whatman FTA reagents (FTA) or kit-based DNA extraction (standard) and subjected to PCR-based fungal profiling. FTA performance was also assessed using the $1x10^7$ cell concentration by varying sample application speed (fast or slow), volume (150 μL or 250 μL) or using a single large paper punch or two small punches (2x sml).

7.2 Wild fermentation intervention

Vineyard geography, environment and management practices, including harvest timing, juice/must processing and fermentation conditions can all affect yeast population dynamics during wine fermentation. Providing winemakers with the ability to predictably tailor the microbiome of a wild ferment through considered use of winemaking interventions could allow for specific wine characteristics to be attained while preserving the role of wild yeast.

Three different winemaking interventions were assessed for their impact on the yeast microbiome of wild ferments during this project: the addition of sulfite (SO_2), aeration and alterations in ferment temperature.

7.2.1 Sulfite

Of the processing conditions that are readily modulated by winemakers, the addition of the antimicrobial agent SO_2 represents the most broadly available intervention practice. Previous microbiological studies have shown that species (and strains) of the major wine yeasts can respond differently to the application of SO_2. Typically, commercial strains of *S. cerevisiae* display high tolerance to SO_2, while 'wild' yeasts display lower tolerances and are therefore thought to be broadly selected against through the application of moderate amounts of SO_2 prior to the start of fermentation (Andorrà et al. 2008, Morgan et al. 2017, 2019).

To address the effect of SO_2 addition on the yeast microbiota during uninoculated Chardonnay fermentations, ITS profiling was used to assess population dynamics during ferments performed across two successive vintages using a range of pre-ferment SO_2 levels. This was combined with chemical analysis of the resulting wines to determine if any observable changes in population structure could be correlated with alterations in the organoleptic qualities of the wine.

In vintage 2018, grape juice was treated with one of five different concentrations of total SO_2 (0, 40, 60, 80 and 100 mg/L). However, in addition to the antimicrobial effect of SO_2, it also represents a powerful antioxidant. To attempt to differentiate between the antimicrobial and antioxidant effects of SO_2 addition, an alternate antioxidant, glutathione (GSH, 250 mg/L), was also assessed for its effects on yeast community structure.

The addition of GSH did not affect the duration of fermentation; however, SO_2 had a significant impact on the length of time required for the fermentation to reach completion, with two of the 100 mg/mL treatments requiring five to seven days longer than the control ferments (26-day fermentation) and one of the 100 mg/mL treatments becoming stuck with 13g/L of residual sugar.

Across the 18 samples from 2018 (six treatments in triplicate), a total of 26 fungal genera were detected that exceeded 0.01% of the total abundance in at least one sample (Figure 3). The highest level of fungal diversity was observed at the T1 timepoint, where *Hanseniaspora*, *Metschnikowia*, *Saccharomyces* and *Torulaspora* dominated the fermentations from T2 through T4, accounting for over 95% of the total ITS reads.

As seen for the fermentation kinetics, GSH addition did not affect the overall population structure relative to the control samples; however, the addition of SO_2 had a significant, but differential effect on the four main genera observed across the samples (Figure 3). *Metschnikowia* displayed the highest sensitivity to SO_2, with 40 mg/L completely inhibiting the growth of this genus by the T2 timepoint. *Torulaspora* was shown to have a higher abundance at 40 mg/L relative to 0 mg/L; however, this genus was progressively inhibited by higher concentrations of SO_2 in a gradient from 60 mg/L through to 100 mg/L, at which point it was completely inhibited at timepoint T2. *Hanseniaspora* and *Saccharomyces* were both shown to be tolerant across all the tested SO_2 concentrations, with *Hanseniaspora* increasing in its total proportion relative to the other genera as the concentration of SO_2 was increased.

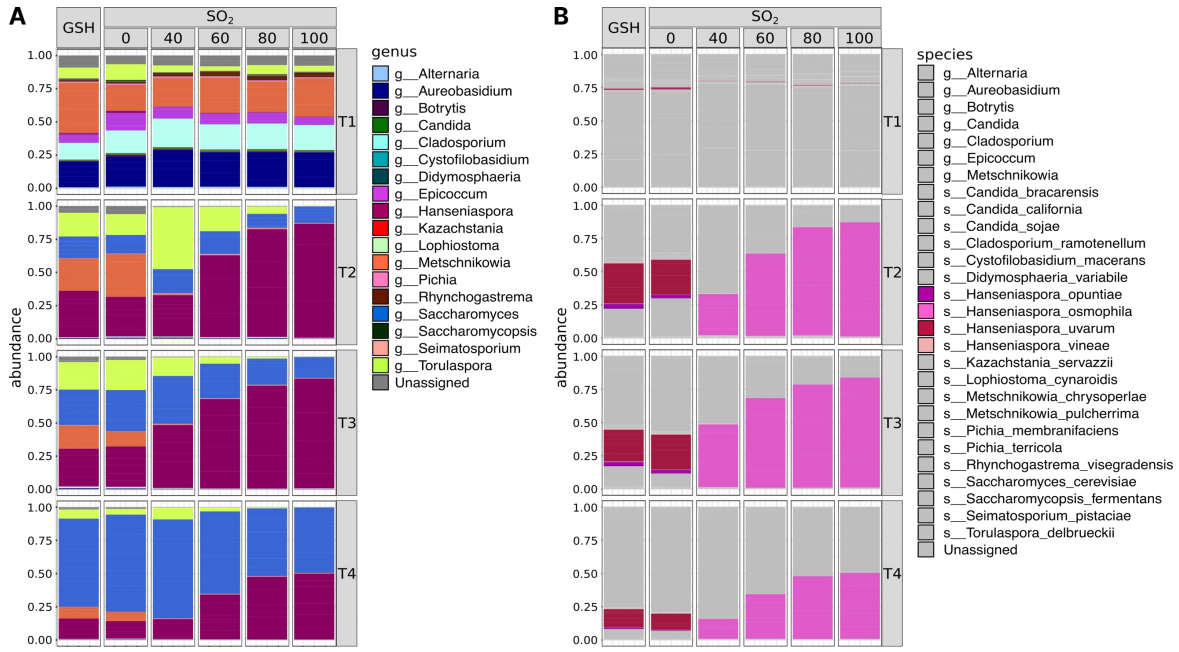


Figure 3. ITS profiling analysis of the response of *Hanseniaspora* spp. to SO_2 addition. Vintage 2018 Chardonnay juice was treated with increasing concentrations of total SO_2 (g/L) or glutathione (GSH) as an alternate antioxidant. Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilisation; T3, 50% sugar utilisation; T4, 90% sugar utilisation) and subjected to ITS profiling. **(A)** Genus level grouping. Only genera that exceeded 0.1% abundance in at least one sample are shown. **(B)** Species-level grouping, highlighting *Hanseniaspora* spp. Only species that exceeded 0.1% abundance in at least one sample are shown. For those OTU where a species-level designation was not possible, the genus-level taxonomic classification of the OTU was used. Only members of the genus *Hanseniaspora* are coloured.

As fungal ITS sequencing generally affords the ability to define OTUs to the species level, the genus-level counts were partitioned into species-level units to determine the effect of SO_2 concentration on the abundance of individual species. There were 29 species that exceeded 0.1% of the total abundance in any sample, with the genus *Hanseniaspora* displaying the highest number of individual species (n=4). While the addition of SO_2 was shown to increase the overall abundance of *Hanseniaspora* at genus level, there was a far more complex response profile when species designations were considered (Figure 3). Rather than a general increase in all species of *Hanseniaspora*, two species, *H. uvarum* and *H. opuntiae* were the dominant species when SO_2 was absent (GSH and SO_2-0 mg/L treatments). However, the addition of 40 mg/L of SO_2 resulted in a drastic shift in the species composition such that *H. osmophila* was the sole representative of this genus at 40 mg/L of SO_2. The relative abundance of this species increased substantially as SO_2 levels were raised, producing the overall increase in *Hanseniaspora* that was observed at the genus level.

A second set of fermentations was established in the subsequent year (2019) using a finer set of SO_2 treatment intervals (0, 10, 20 and 40 mg/L). Consistent with observations from the 2018 vintage, SO_2 addition affected fermentation kinetics, particularly for the 40 mg/L treatments. The 2019 ferments displayed a different overall yeast diversity compared to the 2018 samples, with a lack of OTUs that could be assigned to *Metschnikowia* and prominent contributions from OTUs assigned to *Candida* spp., which increased over the range of SO_2 concentration used, and *Kazachstania* spp. that were present at up to 20 mg/L of SO_2 (Figure 4). However, when the species level contributions were investigated, there were clear similarities between the two

vintages in the dynamics of the OTUs assigned to the genus *Hanseniaspora*. As also seen in 2018, *Hanseniaspora* was represented by the greatest number of species designations in 2019. The 2019 ferments also displayed a clear species shift that was associated with the use of SO_2, with the proliferation of *H. uvarum*, *H. opuntiae* and *H. vineae* all being inhibited by SO_2 in a concentration-dependent manner, while the proportion of *H. osmophila* was shown to be enhanced by the addition of 40 mg/L of SO_2.

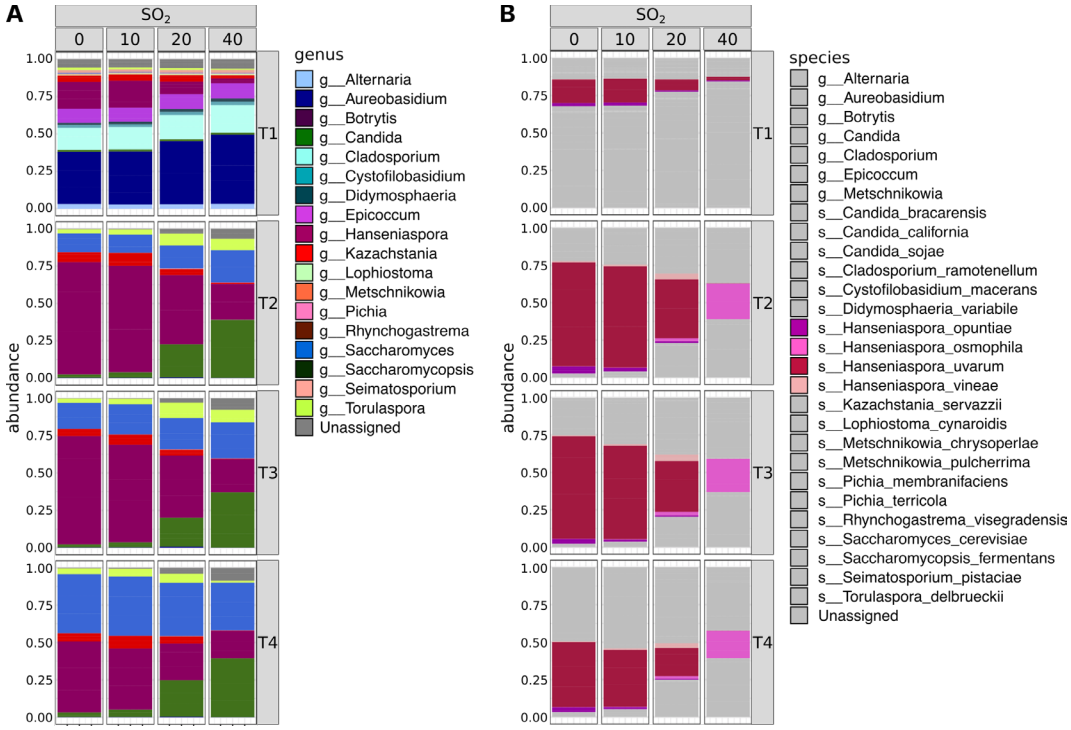


Figure 4. ITS profiling analysis of community response to SO_2 addition. Vintage 2019 Chardonnay juice was treated with increasing concentrations of total SO_2 (g/L). Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilisation; T3, 50% sugar utilisation; T4, 90% sugar utilisation) and subjected to ITS profiling. **(A)** Genus level grouping. Only genera that exceeded 0.1% abundance in at least one sample are shown. **(B)** Species-level grouping, highlighting *Hanseniaspora* spp. Only species that exceeded 0.1% abundance in at least one sample are shown. For those OTU where a species-level designation was not possible, the genus-level taxonomic classification of the OTU was used. Only members of the genus *Hanseniaspora* are coloured.

Although much is known regarding the molecular basis of SO_2 tolerance in *S. cerevisiae*, where the sulfite efflux pump, *SSU1*, provides the main mode of resistance (Divol et al. 2012, Park and Bakalinsky 2000), very little information is available on the main genetic determinants of SO_2 resistance in non-*Saccharomyces* species, although *SSU1* has been suggested to have a major role in *Brettanomyces bruxellensis* (Varela et al. 2019). However, recent comparative genomic studies of *Hanseniaspora* spp. have shown that there is a clear differentiation of this genus into two well defined phylogenetic clades, in which one of the differentiating factors is a homolog of *SSU1*, which is absent in the large clade containing *H. uvarum*, but present in the clade containing *H. osmophila* and *H. vineae* (Cadez et al. 2003, Seixas et al. 2019, Sternes et al. 2016).

While this may explain the different response to SO_2 of *H. osmophila* versus *H. uvarum*, there are likely many other factors that affect the response of a specific species, for example *T. delbrueckii*. This species, which also possesses an *SSU1* homolog and displayed increased abundance at up to 40 ppm SO_2, is less tolerant at higher SO_2 concentrations than *H. osmophila*. Likewise, *H. vineae*, which was present at levels similar to *H. osmophila* in the control ferments and which

contains an *SSU1* homolog, did not increase in abundance in response to increases in the concentrations of SO_2.

Given the significant effect of SO_2 addition on microbial community structure, it was of interest to understand whether these changes were associated with changes to the chemical composition of the wine. This was assessed through an analysis of volatile yeast metabolites known to contribute to the aromatic profile of wine. Of the 39 aroma compounds analysed, 18 displayed a significant difference (ANOVA, $P \leq 0.001$) in concentration across one of the SO_2 regimes in either 2018 or 2019. Of these, ten analytes displayed more than a 1.5-fold decrease in at least one of the SO_2 treatments, while seven displayed an increase of the same magnitude (Figure 5). Three analytes, 2-methylpropanol (decreasing in response to SO_2) and 2-phenylethyl acetate and hexyl acetate (increasing), displayed the same effect across the 2018 and 2019 vintages.

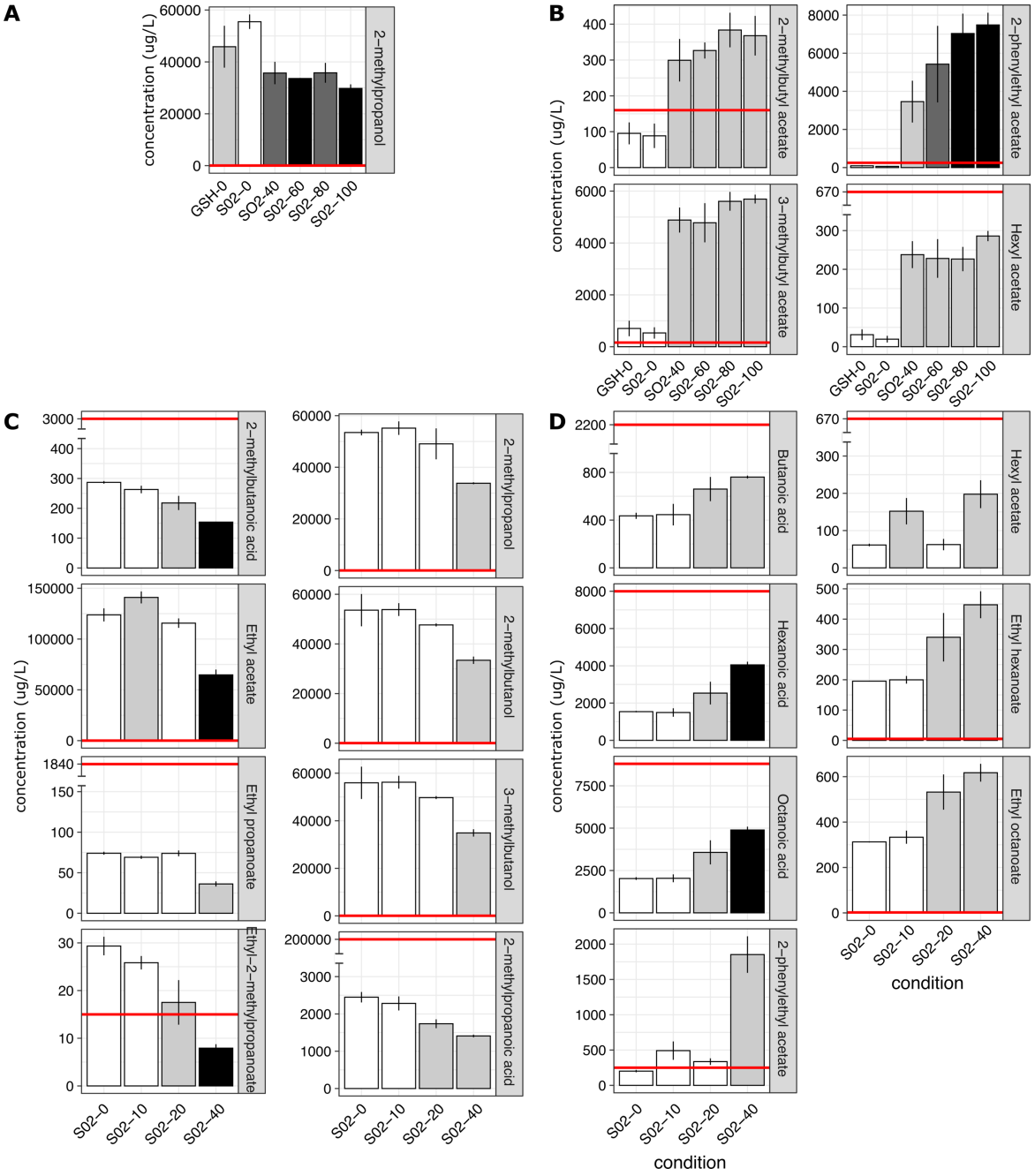


Figure 5. Concentrations differences in aroma compounds due to the addition of SO_2.
(A) Analytes with significantly reduced (ANOVA, p £ 0.001) concentrations in 2018.
(B) Analytes with significantly increased (ANOVA, p £ 0.001) concentrations in 2018.
(C) Analytes with significantly decreased (ANOVA, p £ 0.001) concentrations in 2019.
(D) Analytes with significantly increased (ANOVA, p £ 0.001) concentrations in 2018. Individual bars are shaded according to their significance group and the estimated aroma threshold is indicated in red.

In all cases, there was a significant effect at 40 mg/L SO_2. Furthermore, in most situations in which a significant difference in analyte concentration was observed across multiple SO_2 regimes, there was a correlation between SO_2 concentration and the magnitude of change. The largest change in analyte concentration was observed for the desirable aroma compound 2-phenylethyl-acetate, with the 100 mg/L SO_2 treatment in 2018 displaying over two orders of magnitude more of this metabolite than the control, and the 2019 40 mg/L SO_2 treatment having over 9 times as much 2-phenylethyl-acetate as the control. More generally, higher SO_2 concentrations resulted in decreases in short chain acetates and higher alcohols and increases

in 6-carbon and 8-carbon esters and acids. There was no effect of SO_2 concentration on low molecular weight volatile sulfur compound production.

Given the microbiological shift that was observed, it is likely that this change in ester production is due to the increasing prevalence of *H. osmophila* in these ferments with higher SO_2 levels. This is supported by published data from fermentations established with purified non-*Saccharomyces* strains, in which mixtures of *H. osmophila* and *S. cerevisiae* (90:10 ratio) were shown to produce higher concentrations of acetate esters (with the exception of isoamyl acetate) and concentrations of 2-phenylethyl-acetate almost 10-fold greater than those observed with *S. cerevisiae* alone (Viana et al. 2008, 2009).

In vintage 2020 and 2021, industry trials were established to confirm the effect of SO_2 on the microbial composition of Chardonnay wild ferments under commercial conditions. Ferments were conducted at Yalumba and consisted of duplicate samples treated with 35 mg/L of SO_2 (in addition to control ferments) at either 1,000-L or 1,500-L scale. Samples were taken at 12 hr intervals for ITS profiling.

As was observed in the laboratory-scale experiments, the addition of 35 mg/L of SO_2 was sufficient to alter the species composition of the ferments, with a change from *H. uvarum* to *H. osmophila* observed after approximately 48 hrs of fermentation (Figure 6).

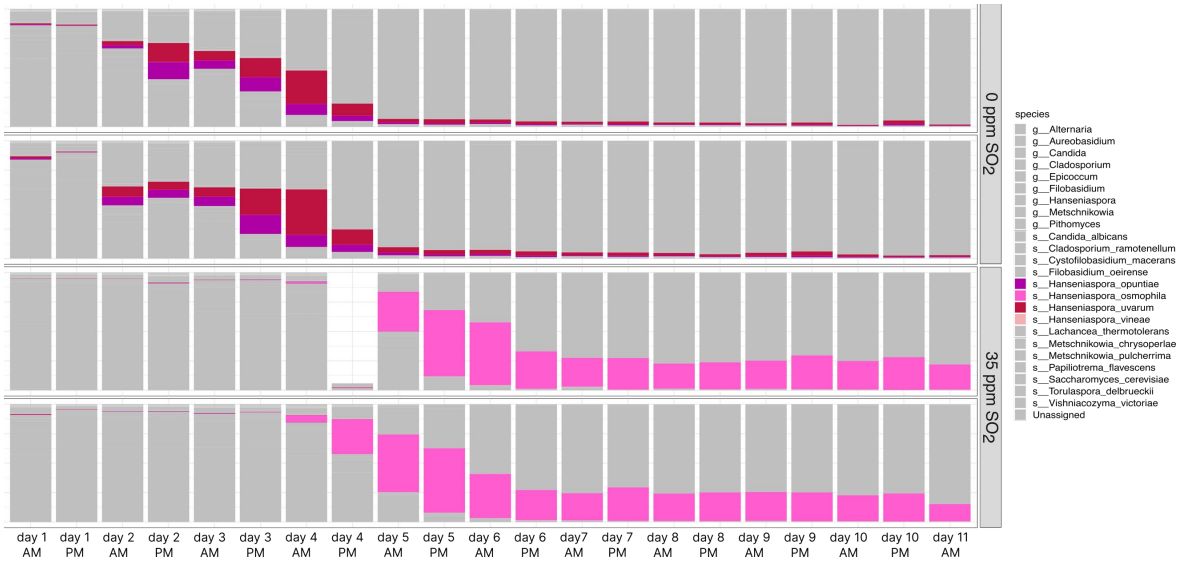


Figure 6. ITS profiling analysis of community response to SO_2 addition at winery scale. Vintage 2020 Chardonnay juice was treated with either 0 ppm or 35 ppm of total SO_2 (g/L) in duplicate fermenters. Ferment samples were taken at four timepoints (T1, at crush; T2, 10% sugar utilisation; T3, 50% sugar utilisation; T4, 90% sugar utilisation) and subjected to ITS profiling. Species-level grouping is shown, highlighting *Hanseniaspora* spp. Only species that exceeded 0.1% abundance in at least one sample are shown. For those OTU where a species-level designation was not possible, the genus-level taxonomic classification of the OTU was used. Only members of the genus *Hanseniaspora* are coloured.

Chemical analysis indicated that similar alterations to the chemical profile of laboratory wines were observed under commercial conditions, with increases in acetate esters due to the addition of SO_2.

Sensory analysis was performed on wines produced in 2021 as part of combined experiment that examined the effect of either SO_2 or aeration (reported in Project 3.2.1) on wild ferment

outcomes. The six wines (three treatments [control, SO_2 and oxygen] x two fermentation replicates) were presented to assessors four times, in a Williams Latin Square random block design.

An ANOVA assessing the effect of the three treatments showed statistical evidence that nine sensory attributes, yellow (colour intensity), citrus aroma, tropical fruit aroma, stone fruit aroma, apple/pear aroma, floral aroma, nail polish remover aroma, drain aroma, and apple/pear flavour differed between the treatments. The SO_2 treated wines were generally rated highest for the fruity attributes mentioned above, and lowest for drain aroma. The control wines were rated similarly to the SO_2-treated wines but were scored at lower aroma intensities. The aeration treatment wines were rated lowest for the intensity of all fruit attributes and were rated highly for drain aroma and nail polish remover aroma.

The PCA for the replicate wines (Figure 7) was created using the nine attributes that were supported by statistical evidence as differentiating the three treatments. Principal components 1 and 2 explained 78.5% and 14.9% of the variance in the data respectively. Beyond the second principal component, eigenvalues were below 1 and explained little variability in the data. The mean sensory profiles of each treatment are shown in the radar plot in Figure 7 which provides more detail and the ability to assess significant differences among the treatments.

Principal component 1 had positive loadings for the attributes tropical fruit aroma, apple/pear

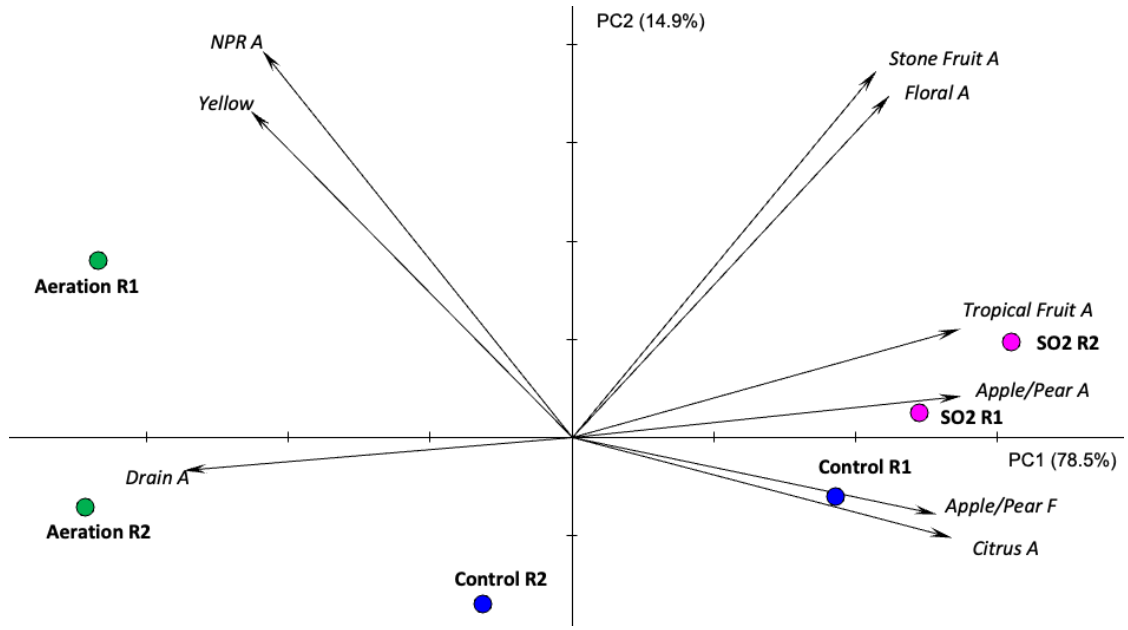


Figure 7. Principal component analysis biplot of significant ($p < 0.05$) sensory attributes for the treatment fermentation replicates for the Chardonnay wines

aroma, apple/pear flavour, and citrus aroma. Treatment replicates to the far right of the PCA (control replicate 1 and both of the SO_2 treatment replicates) were rated highly in these attributes. Conversely, drain aroma was negatively loaded along PC1, with both aeration treatment replicates being rated highly in this attribute. The vertical separation of the replicates was determined by the intensity of stone fruit aroma, floral aroma, nail polish remover aroma and yellow (colour intensity). Aeration replicate 1 was found to be rated highest for yellow (colour intensity) and nail polish remover aroma; however, most of the separation observed was along PC1. The fermentation replicates show a small amount of intra-treatment variation, with the control replicate wines having somewhat more variability than the aeration and SO_2 treatment replicates.

The mean sensory profiles of each treatment can be visualised in the radar plot presented in Figure 8. The sensory profiles were similar across most attributes for the control and SO_2 treatments, with the SO_2-treated wines having a higher intensity of the fruity attributes citrus aroma, tropical fruit aroma, stone fruit aroma, apple/pear aroma and floral aroma, while also being rated lowest for drain aroma. The aeration wines were rated high in the intensity of nail polish remover aroma and drain aroma, as well as being rated low for the fruit attributes.

7.2.2 Oxygen

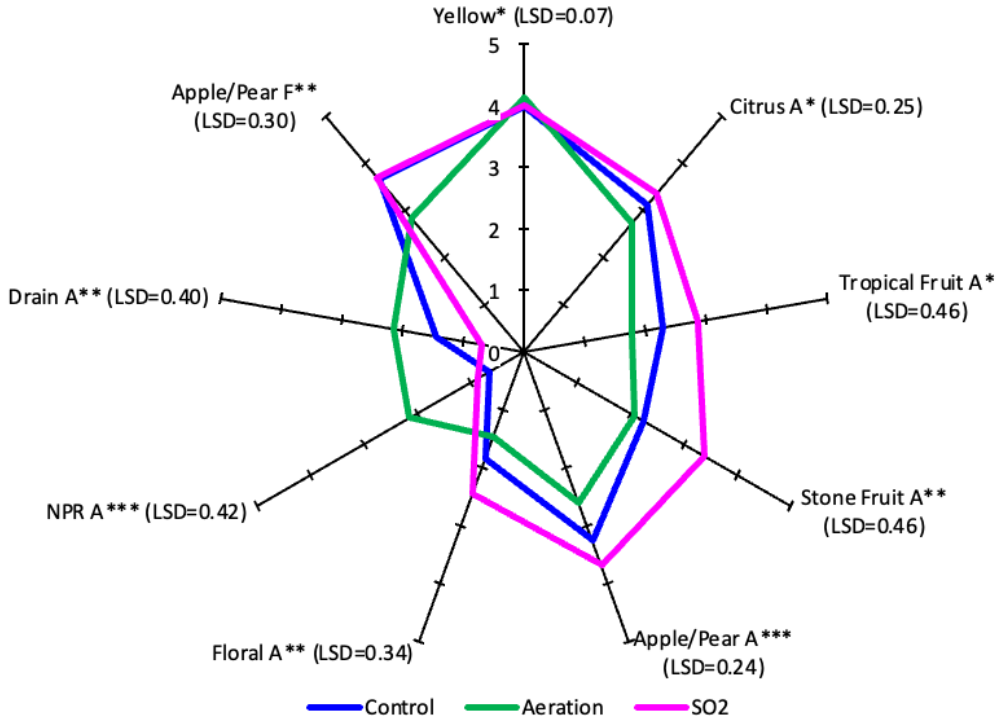


Figure 8. Mean attribute intensity scores for the significant attributes. Significant (*$P < 0.05$). LSD values at 95% confidence level were included for the significant attributes ($P < 0.05$) for the three treatments. A: Aroma, F: Flavour.

Alterations in microbial composition due to the addition of oxygen in white wine were performed in collaboration with Project 3.1.5. Details on the results of those experiments can be found in the Final Report for that project.

7.2.3 Temperature

Fermentation kinetics for wild ferments in both Chardonnay and Shiraz varied according to temperature, with lower temperatures leading to longer fermentation time in both wines (Figure 9 A and C). Wines fermented at 12°C were especially affected, taking 36 and 35 days to reach dryness in Chardonnay and Shiraz, respectively, as opposed to the 22°C (13 d/10 d) and 28°C (6 d/7 d) ferments.

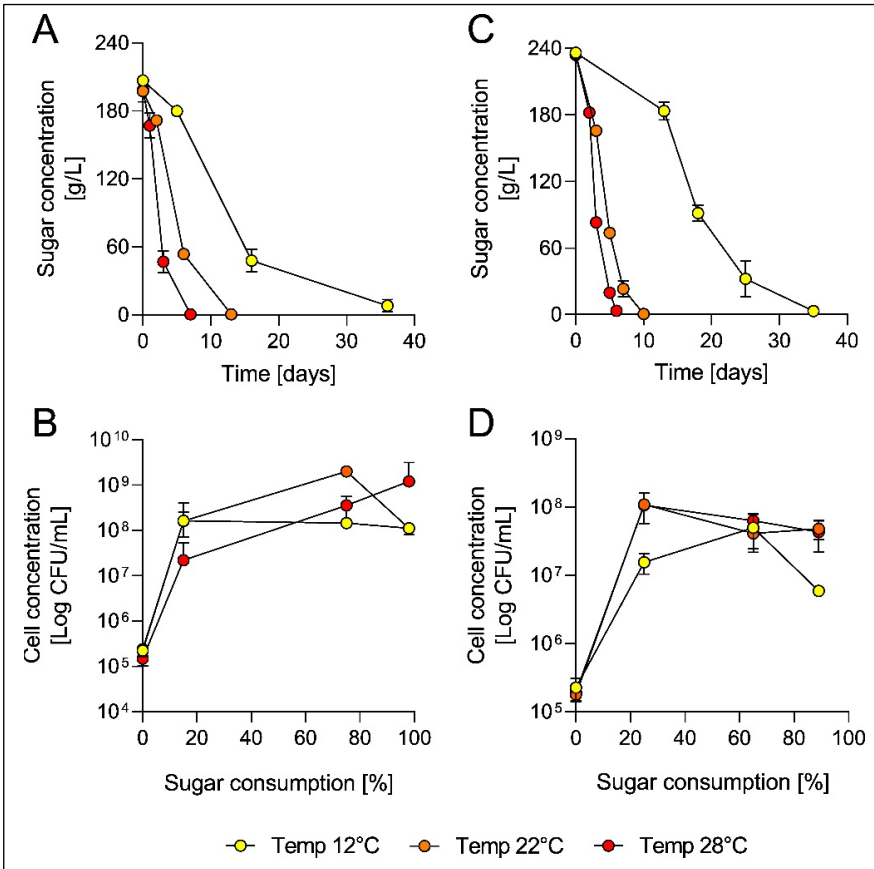


Figure 9. Variation in wild fermentation kinetics and total yeast counts according to temperature. (A) Chardonnay fermentation kinetics (B) Chardonnay cell counts (C) Shiraz fermentation kinetics (D) Shiraz cell counts

Total cell counts also differed according to fermentation temperature (Figure 9 B and D), with cell counts from the 12°C ferments being significantly lower than either the 22°C or 28°C ferments.

Microbial composition varied in both Chardonnay and Shiraz wild ferments, according to fermentation stage and temperature (Figure 10). Although several genera were found before fermentation, the genera *Hanseniaspora*, *Saccharomyces* and *Torulaspora* dominated fermentation in Chardonnay. At similar time points, higher relative abundance of *Hanseniaspora* species was found at lower temperatures.

Whereas *Saccharomyces*, *Lachancea* and *Starmerella* abundances were higher at 28°C for equivalent fermentation stages. *Torulaspora* abundance was lower at 28°C than in other conditions, particularly at the last sampling point. In Shiraz, the genera *Saccharomyces*, *Metschnikowia*, *Hanseniaspora* and *Starmerella* dominated fermentation. The relative abundance for *Saccharomyces*, *Starmerella* and *Pichia* increased with temperature. While *Metschnikowia* relative abundance was highest at 12°C, *Hanseniaspora* abundance was highest at 22°C.

Temperature altered the volatile composition of wines from both varieties. The total concentration of esters decreased in wines from both varieties with increasing temperatures (Figure 11), mainly driven by a reduced concentration of ethyl esters. In Chardonnay the concentration of higher alcohols was greatest in wines fermented at 22°C and 28°C, while in Shiraz this concentration was highest in wines fermented at 22°C. The total concentration of volatile acids was lowest in wines fermented at 12°C for both varieties. In conclusion, fermentation temperature can influence microbial structure and shape wine volatile composition. Although higher temperatures can be an attractive strategy to speed up

fermentation, they can negatively affect wine flavour and aroma lowering ester production and increasing higher alcohols.

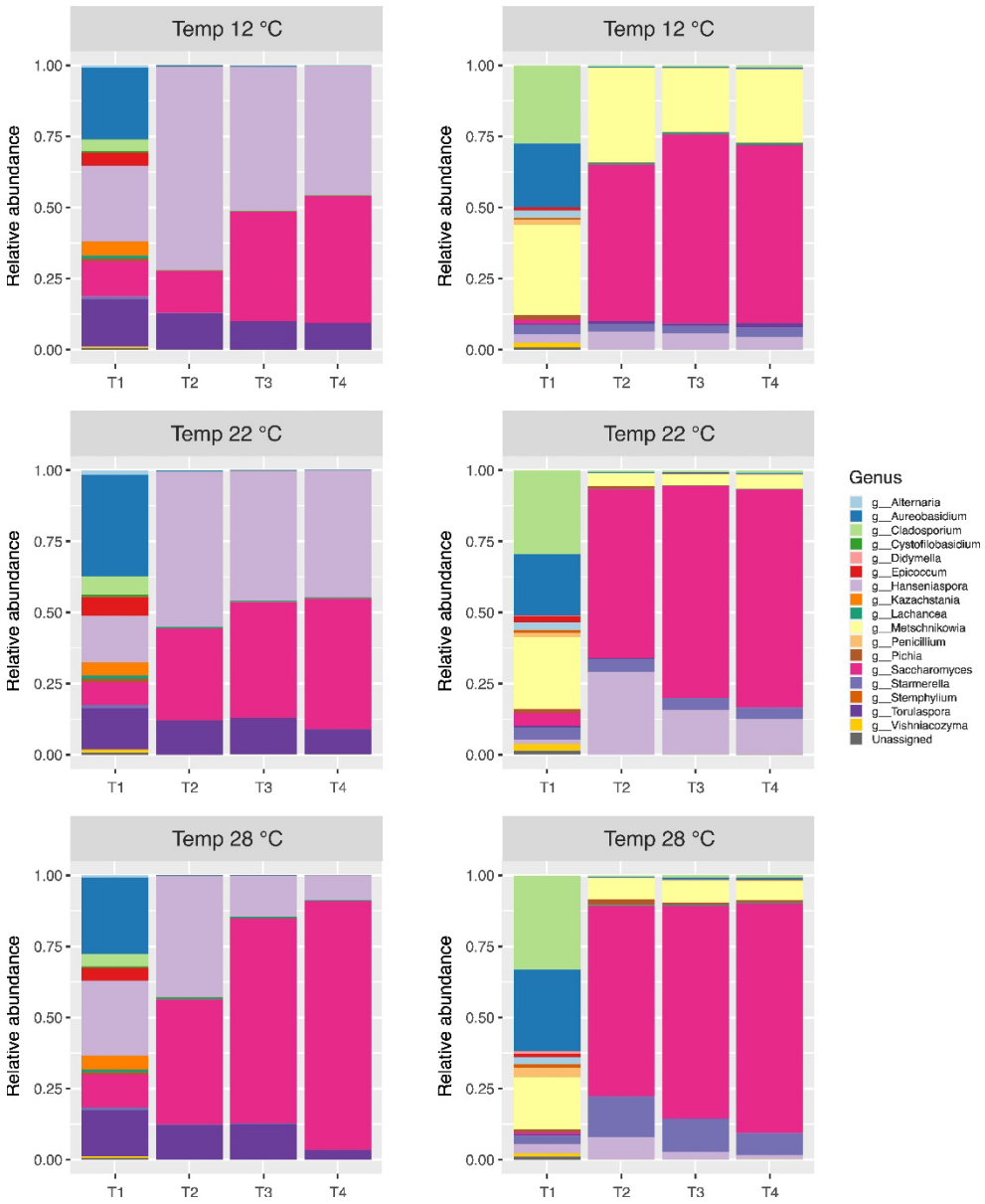


Figure 10. Variation in microbial composition in wild fermentations. Ferments were performed in Chardonnay (left) and Shiraz (right) at 12°C, 22°C or 28°C.

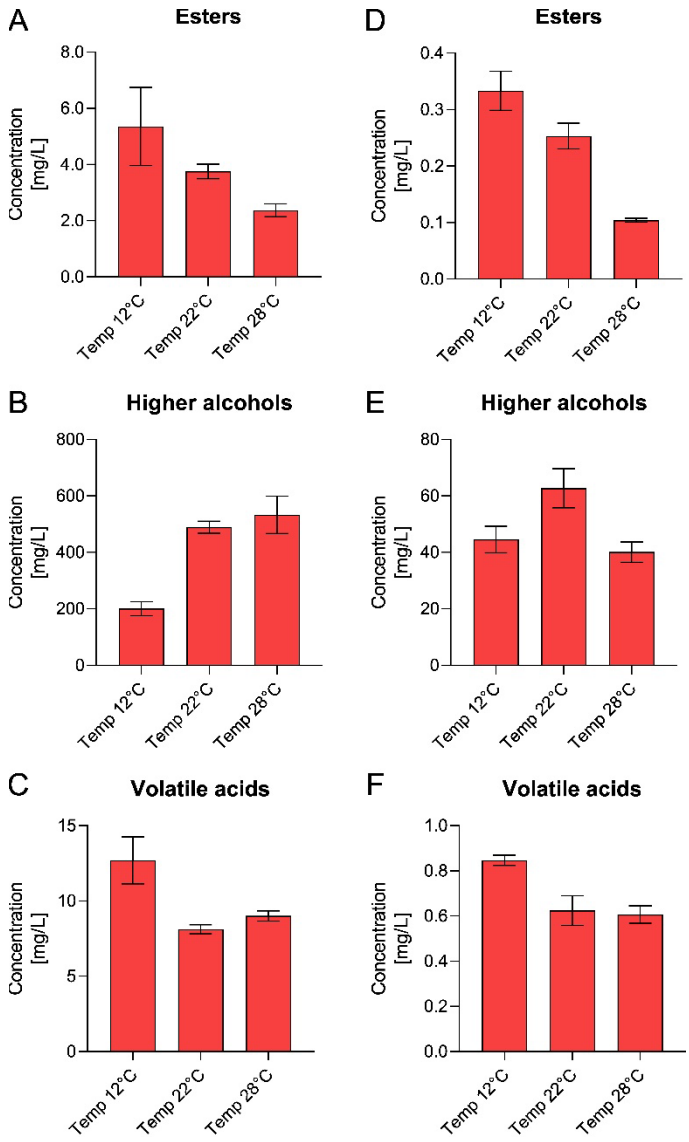


Figure 11. Variation in volatile composition of wild fermentations according to temperature (A-C) Chardonnay (D-F) Shiraz

7.3 Yeast isolation and screening

7.3.1 Wild yeast isolation and identification

A collection of Australian wild wine yeast isolates was established from a range of fresh ferment samples across vintages 2017-2021. Yeasts were only isolated from uninoculated ferments and, while generally focusing on the beginning of fermentation, were obtained throughout the fermentation process.

Across the five vintages, over 18,000 individual isolates were collected and stored in 96-well arrays (Table 2). High-throughput colony-based ITS sequencing was used to identify each isolate, while also screening for potential mixed-cultures (Figure 12).

Table 2. Yeast isolates obtained by year

Year	Number Isolates
2017	1,968
2018	6,721
2019	2,409
2020	1,734
2021	5,500

Isolates were obtained across at least 35 species (Table 3), with *H. uvarum* (28.9% total isolates) representing the most numerous non-*Saccharomyces* yeast species isolated and *S. cerevisiae* comprising 59.3% of the strains. Frequency of strain isolation therefore reflects the results of the fungal profiling work, with *S. cerevisiae* and *Hanseniaspora* comprising the most abundant yeasts across the wild ferment samples.

This collection of strains provided a foundational dataset that was subsequently used to identify novel non-*Saccharomyces* isolates for use as commercial starter strains (Section 7.3.2) and for wine yeast population genomics studies, focusing on either *S. cerevisiae* (Section 7.3.3) or *Hanseniaspora* (Section 7.3.4).

In addition to wine isolates, work performed in collaboration with the University of Adelaide isolated yeasts from two different geographical locations in Australia, the Central Plateau in Tasmania (1,029 isolates) and Erub Island in the Torres Strait (71 isolates).

Table 3 Presumptive genus/species identification

Presumptive ITS ID	**Count**
Aureobasidium	7
Candida	21
Candida albicans	2
Candida bracarensis	12
Candida glabrata	9
Cystofilobasidium capitatum	24
Cystofilobasidium macerans	3
Debaryomycetaceae	11
Filobasidium	15
Filobasidium oeirense	2
Guehomyces pullulans	1
Hanseniaspora	1,448
Hanseniaspora guilliermondii	79
Hanseniaspora opuntiae	190
Hanseniaspora osmophila	29
Hanseniaspora uvarum	1,263
Hanseniaspora valbyensis	89
Hanseniaspora vineae	12
Hyphopichia burtonii	1
Kazachstania aerobia	4
Kazachstania servazzii	34
Lachancea thermotolerans	3
Metschnikowia	444
Metschnikowia chrysoperlae	22
Metschnikowia pulcherrima	91
Metschnikowia sinensis	5
Meyerozyma	3
Papiliotrema flavescens	2
Pichia kudriavzevii	1
Pichia terricola	2
Quambalaria cyanescens	1
Saccharomyces cerevisiae	6,408
Saccharomycodes ludwigii	8
Torulaspora delbrueckii	262
Vishniacozyma carnescens	16

7.3.2 Non-*Saccharomyces* screening and strain development

A total of 1,291 different non-*Saccharomyces* isolates, comprising thirteen different genera were selected from strains isolated from uninoculated wine ferments during 2014-2019, including many isolated as part of the activities presented in Section 7.3.1 (Table 4). Strains were arrayed in 96-well format and screened for growth under different environmental conditions as well as being evaluated for the production of key enzyme activities (Figure 12).

Table 4. Non-*Saccharomyces* isolates screened

Genus	Number of isolates
Aureobasidium	4
Candida	79
Cystofilobasidium	3
Filobasidium	1
Hanseniaspora	637
Hyphopichia	1
Kazachstania	6
Lachancea	4
Metschnikowia	321
Pichia	2
Rhodotorula	6
Torulaspora	219
Wickerhamomyces	8
Total	**1,291**

Colony phenotype scores were used to create a heatmap with hierarchal clustering (Figure 12). Three main clusters were identified. Isolates in the first cluster included several *T. delbrueckii* and *Metschnikowia* species and some *Candida* and *Hanseniaspora* species. Numerous isolates in this cluster were not very tolerant to ethanol, SO_2 and anaerobic conditions, whereas many showed protease and/or β-lyase activities. A second cluster included mainly *T. delbrueckii* and some *Candida* species. Most of these isolates were tolerant to all the environmental conditions evaluated, including an ethanol concentration of 12%, but did not show many enzyme activities. The third and largest cluster included most of the *Hanseniaspora* isolates and several *Metschnikowia* species. Isolates in this cluster were sensitive to ethanol, many were also sensitive to osmotic stress, copper and anaerobic conditions but also showed a range of enzyme activities including protease, β-lyase and/or β-glucosidase activities.

Screening results were used to select isolates with the potential to affect wine fermentation outcomes, with isolates that showed tolerance to 6% ethanol, 20% glucose and SO_2, while displaying low H_2S production (screening scores ≥2), shortlisted for further consideration. From these shortlisted isolates, those displaying high scores for β-glucosidase and/or β-lyase activity were selected for further characterisation. For β-glucosidase, 25 isolates were selected, while 121 isolates were chosen that displayed for β-lyase activity. The 146 selected isolates were then evaluated for either β-glucosidase or β-lyase activity in chemically defined grape juice.

Nine isolates showed β-glucosidase activity in chemically defined grape juice, including five *Wickerhamomyces anomalus* isolates and single isolates of *Hanseniaspora*, *Pichia terricola*, *Cystofilobasidium capitatum*, and *Rhodotorula*.



Figure 12. Hierarchical clustering of non-*Saccharomyces* wine isolates. Isolates were screened for their tolerance to common stress conditions and enzyme activities relevant to wine production. Results were converted to discrete scores with the ability to grow under different stress conditions, the presence of enzyme activities and low H_2S production considered positive attributes that were given high values.

Sixty-nine isolates showed β-lyase activity in chemically defined grape juice. As a further screen, these isolates were then evaluated specifically for thiol production, with 10 isolates showing significant thiol production (four *T. delbrueckii*, three *Metschnikowia*, two *H. valbyensis* and one *H. osmophila*).

All 19 isolates that passed the secondary testing (nine with β-glucosidase and ten with β-lyase), were then evaluated in Chardonnay juice (in sequential inoculation with *S. cerevisiae*) supplemented with either glycosides or thiol precursors. In juice supplemented with glycosides, one strain (Hanseniaspora_2014_P4_A9) produced significantly more monoterpenes, with high concentrations of linalool, α-terpiniol, β-citronellol and β-damascenone observed. In the absence of observable faults, as the sole glycosidase-releasing candidate, Hanseniaspora_2014_P4_A9 was selected for study at winery scale.

In juice supplemented with thiol precursors, three *Metschnikowia* strains produced higher concentration of thiols (3MH plus 3-MHA); however, two strains also produced very high concentrations of ethyl acetate. Metschnikowia_2017_P4_G11 was therefore selected for further characterisation at winery scale.

Although the four *T. delbrueckii* isolates evaluated did not release the expected high concentrations of thiol in the Chardonnay must, they were shown to produce high concentrations of uncommon ethyl esters. These included ethyl propanoate, ethyl-2-methyl propanoate, ethyl-2-methyl butanoate and ethyl-3-methyl butanoate. Only one of these isolates, T. delbrueckii_2018_P2_B8, produced acceptable concentrations of ethyl acetate and was selected for study at winery scale.

Three isolates, Hanseniaspora_2014_P4_A9, Metschnikowia_2017_P4_G11 and T. delbrueckii_2018_P2_B8 which produced increased concentration of monoterpenes, thiols and uncommon ethyl esters, respectively, were progressed through the winery trials. Riesling was chosen as the most appropriate grape variety to evaluate both β-glucosidase and β-lyase activities, as it is documented to naturally contain both monoterpene and thiol precursors at appreciable levels. As for the laboratory-scale ferments, a sequential inoculation strategy was employed to ensure compatibility with *S. cerevisiae* and that ferments would progress to dryness to allow for sensory evaluation.

While the *S. cerevisiae* control completed fermentation in 10 days, non-*Saccharomyces* ferments finished fermentation between 13-15 days. All wines had comparable residual sugar (1.0-1.5 g/L) and ethanol concentrations (approximately 11.2 % v/v). Volatile acidity was slightly higher in non-*Saccharomyces* wines compared to control *S. cerevisiae* wines (0.5 g/L vs 0.4 g/L). Non-*Saccharomyces* wines had higher ethyl acetate concentration than control wines (55 mg/L vs 40 mg/L). Wines produced with *Hanseniaspora* had slightly higher linalool concentrations, while wines fermented with *Metschnikowia* and *Torulaspora* displayed higher concentrations of thiols. Wines produced with *Torulaspora* and *S. cerevisiae* alone had higher concentrations of ethyl-2-methyl propanoate, total esters and total volatile acids. *S. cerevisiae* control wines displayed increased higher alcohol concentrations than the non-*Saccharomyces* wines. These results indicate that the three selected non-*Saccharomyces* strains were able to produce increased concentrations of volatile compounds associated with their selected enzyme activities. However, the modest concentrations of thiols and monoterpenes suggested that the Riesling juice that was used in the trial was a compromise that did not achieve the maximum differences that would have been possible had different juices been selected that would specifically suit a single activity.

The overall volatile composition differed largely between wines, with PCA showing clustering according to yeast species (Figure 13). *S. cerevisiae* wines clustered separately from the other wines to the right of the PCA plot and were associated with 2- and 3-methyl butanoic acids, ethyl propanoate and ethyl-3-methyl butanoate. Wines produced with *Hanseniaspora* clustered in the upper left quadrant and were associated with linalool, terpinolene and geraniol. Wines produced with *Metschnikowia* and *Torulaspora* grouped closely together and were associated with 3MH, benzyl mercaptan and butanol.

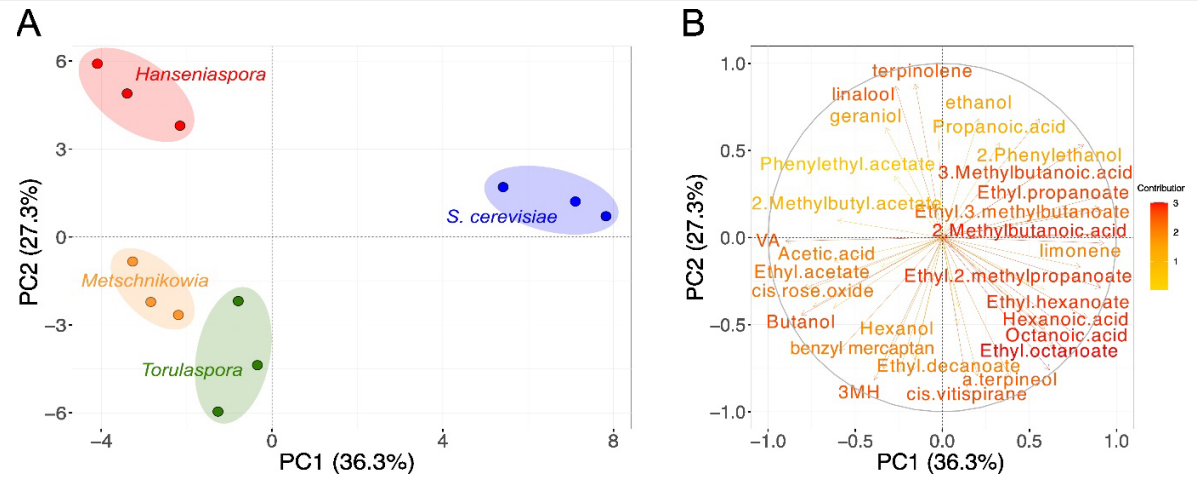


Figure 13. Principal component analysis for volatile compounds in Riesling wines sequentially fermented with selected non-*Saccharomyces* isolates and *S. cerevisiae*. (A) Scores and (B) loadings. Loading colours indicate the relative contribution of individual compounds to the PCA.

Sensory analysis showed that in general, all wines tended to be high in sourness, with no evident aroma differences but showing some flavour differences. Two attributes, colour intensity and viscosity mouthfeel differed significantly among treatments (Figure 14) and some evidence indicated that the attributes sweetness, citrus flavour and tropical fruit flavour also varied. Although all wines showed similar residual sugar, non-*Saccharomyces* wines were rated highest for viscosity and sweetness. Wines produced with *Metschnikowia* were rated highest for tropical fruit flavour, while *S. cerevisiae* wines were highest for citrus flavour.

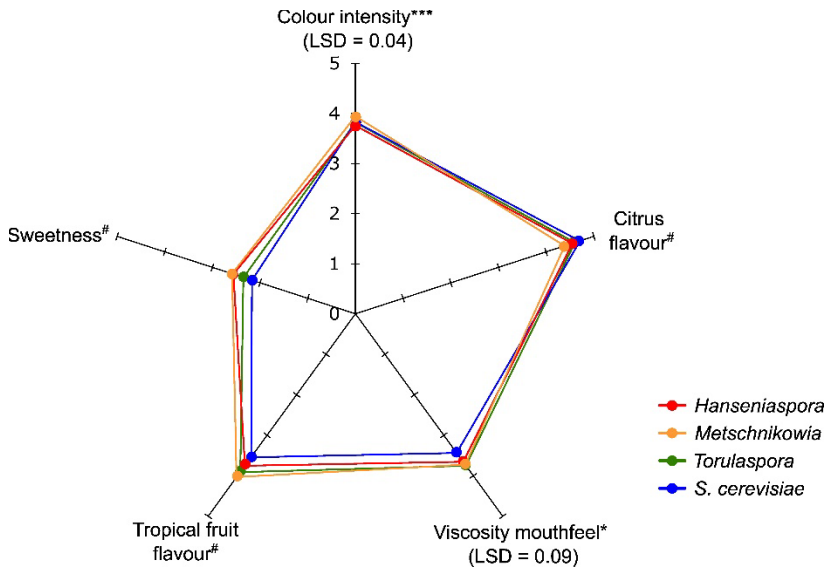


Figure 14. Sensory analysis of non-*Saccharomyces* wines. Mean attribute intensity scores for significant (*P < 0.05) and approaching significance attributes (# P < 0.10). LSD values at 95% confidence level included for the significant attributes (P < 0.05) for the four treatments.

Principal component analysis using all sensory attributes showed varied clustering among treatments (Figure 15). While individual replicates for *S. cerevisiae* wines clustered close together, replicates for non-*Saccharomyces* wines showed some variation. *Metschnikowia* wines were located in the lower right quadrant of the PCA plot and were associated with tropical fruit aroma, tropical fruit flavour, stone fruit aroma and yellow colour intensity. Two replicates for *Hanseniaspora* grouped close to the middle of the plot and were associated with bitterness and cucumber flavour, while one replicate was located in the lower left quadrant. Similarly, two replicates for *Torulaspora* were located in the upper right quadrant and were linked to stone fruit flavour, and cheesy and toasty aromas, while one replicate was located in the lower left quadrant. These results suggest that only some of the differences observed in volatile composition translated to differences in the aroma and flavour of these Riesling wines. Nevertheless, non-*Saccharomyces* wines were associated with sensory profiles that differed from those of *S. cerevisiae* wines. Thus, the non-*Saccharomyces* strains evaluated represent another tool to shape wine flavour and aroma.

It is likely that other grape varieties, which have increased monoterpene and/or thiol precursors, may be better than Riesling at showcasing the sensorial differences observed for the selected non-*Saccharomyces* strains. Thus, it is recommended to evaluate the three non-*Saccharomyces* strains in additional grape varieties to fully characterise their potential to shape wine style.

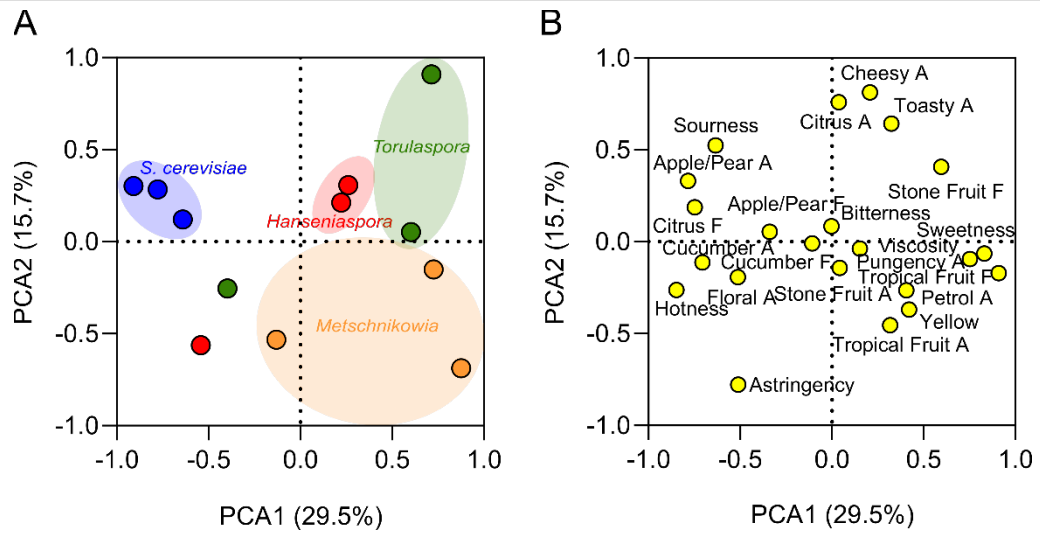


Figure 15. Principal component analysis for all sensory attributes in Riesling wines. Wines were sequentially fermented with selected non-*Saccharomyces* isolates and *S. cerevisiae*. (A) Scores and (B) loadings.

7.3.3 *Saccharomyces* population genomics

Saccharomyces species and nominally *S. cerevisiae*, are the main species responsible for the bulk conversion of sugar to ethanol during inoculated wine fermentation. As shown in Section 7.1, ITS profiling of the collection of wild fermentation samples clearly showed that this remains the case for these uninoculated fermentations. However, as ITS profiling is unable to identify isolates beyond the species level, these analyses are not able to ascertain the strain identity of the *S. cerevisiae* within the wild ferment. Anecdotal concerns exist that uninoculated fermentations are undertaken by commercial strains that have inadvertently contaminated the fermentation.

To determine the strain-level identity of the *S. cerevisiae* isolates present in wild ferments, a selection of isolates that were presumptively identified as *S. cerevisiae* by ITS sequencing were subjected to whole-genome sequencing and phylogenetic analysis along with sequences of commercial wine yeasts and other isolates from both wine and non-wine sources (Borneman et al. 2016). The wild ferment isolates spanned four different vintages and seven different wineries and were isolated from both red and white wines.

Previous phylogenetic analysis of *S. cerevisiae* determined that the isolates from wine and grapes (regardless of their geographic source) form a genetically related clade with environmental isolates from Europe (Borneman and Pretorius 2015). This study supported that finding, with all of the wild isolates grouping within the wine/grape clade (Figure 16).

Analysis of variation within the wine clade demonstrated that most of the fermentations contained more than one strain of *S. cerevisiae*. However clonal isolates were commonly found to be conserved across both wines from the same winery, but also between years, suggesting that resident strains of *S. cerevisiae* can persist between vintages.

While instances of commercial clones were detected in some wild ferments, this was relatively rare, with approximately 5% of isolates obtained from the selected wild fermentations matching a sequenced commercial clone. While the Pris de Mousse (PdM) group of strains represents the most commonly used commercial yeast globally, there were only two detectable instances of wild ferment-sourced *S. cerevisiae* strains matching any of the members of this group. Furthermore, the wines from which these commercial matches were obtained were also

populated by multiple different isolates, indicating that the PdM commercial contaminants did not dominate the fermentation ecosystem.

This work demonstrates that the strains of *S. cerevisiae* that are found in uninoculated ferments are generally not genetically identical to known commercial starter strains. However, these wild isolates displayed a genetic signature that was consistent with a European 'wine' origin and it is likely that these strains are formed through sexual-recombination between commercial (and possibly wild, non-wine) strains.

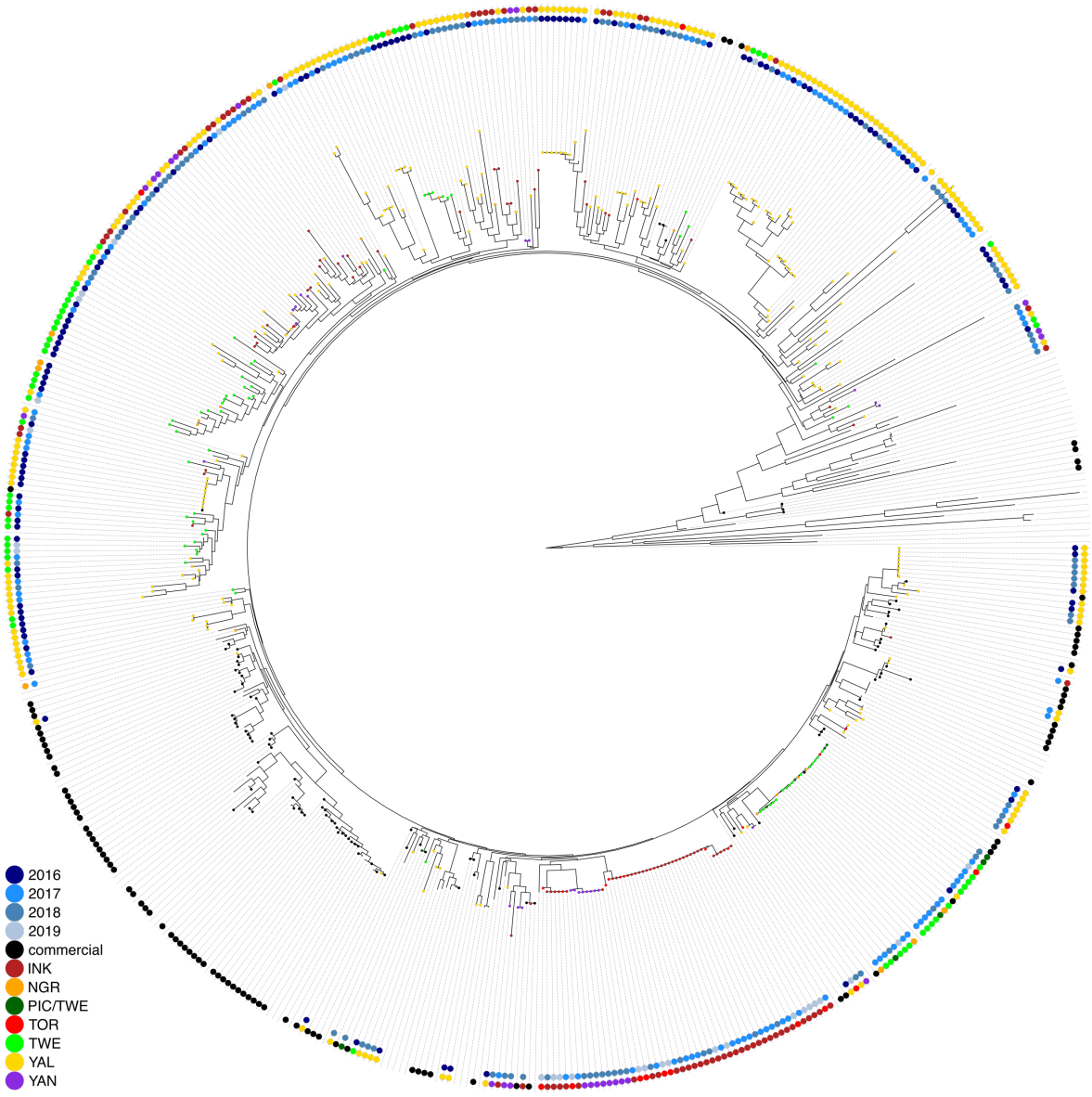


Figure 16. Whole-genome phylogeny of *S. cerevisiae* isolates from wild wine fermentations. Nodes are shaded according to source and year of isolation.

7.3.4 *Hanseniaspora* population genomics

Outside of *S. cerevisiae*, the genus *Hanseniaspora* was the next most frequently isolated yeast from the 2017-2021 isolate set. Apart from a small number of isolates (Sternes et al. 2016), little is known regarding the species diversity that is encountered within wild ferments.

To investigate this, 167 isolates with a presumptive ITS identification of *Hanseniaspora spp*. were subjected to whole genome sequencing and comparative genomics (Figure 18). Phylogenetic analysis was able to group all but four of the isolates into clades containing a known reference species. Eight different species were represented, with *H. uvarum* being the most frequent.

Four isolates (yellow nodes in Figure 17) were not able to be assigned to a species based upon the phylogenetic analysis. Two isolates were found to be basal to the *H. uvarum* clade, while the remaining two were positioned basal to the *H. guilliermondii* clade.

Further comparative genomic analysis determined that these four isolates were in fact interspecific hybrids between two different *Hanseniaspora* species. These isolates are therefore similar to hybrids encountered in wine strains of *Saccharomyces*, such as Vin7 (Borneman et al. 2012) or lager yeasts (Dunn and Sherlock 2008, Nakao et al. 2009). Given the usefulness of other hybrid species in fermentative processes, the winemaking characteristics of these hybrids will be of interest.

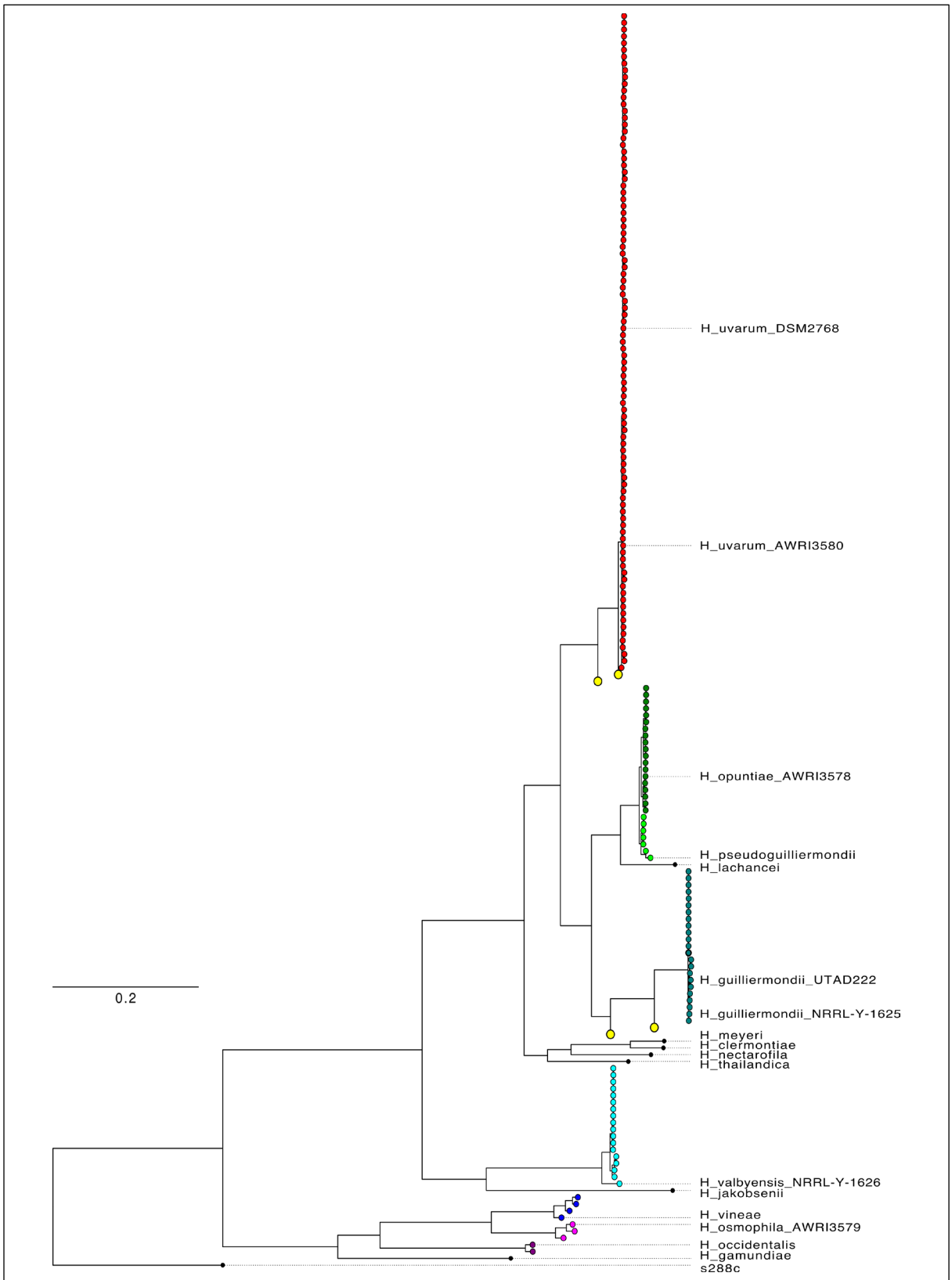


Figure 17. Whole-genome based phylogenetic analysis of *Hanseniaspora* isolates. Species references are labelled, with wild isolates coloured according to species clades. Yellow-nodes represent wild isolates that fall outside of current species groups.

7.3.5 *Aureobasidium* population genomics

Eight presumptive *Aureobasidium pullulans* isolates that were obtained from the wild grape must samples and which were taxonomically confirmed through ITS sequencing were selected for whole-genome sequencing (Table 5). While classified as *Aureobasidium*, a distinct colony morphology was observed in three isolates.

Long-read sequencing using the MinION platform allowed near chromosome-scale assembly of each of the eight isolates, with the majority of the assembled contigs being terminated by telomeric repeat sequences.

Table 5. *Aureobasidium* genome statistics

Isolate	Source	Species	Genome size (Mb)
AWRI4229	Shiraz, Adelaide Hills, 2017	*A. pullulans*	30.9
AWRI4230	Shiraz, Adelaide Hills, 2017	*A. pullulans*	29.8
AWRI4231	Shiraz, Adelaide Hills, 2017	*A. pullulans*	31.0
AWRI4232	Shiraz, Adelaide Hills, 2017	*A. pullulans*	30.1
AWRI4233	Chardonnay, Adelaide Hills, 2017	*A. mustum*	27.2
AWRI4619	Shiraz, McLaren Vale, 2018	*A. vineae*	25.9
AWRI4620	Shiraz, McLaren Vale, 2018	*A. uvarum*	25.6
AWRI4621	Shiraz, McLaren Vale, 2018	*A. pullulans*	30.1

Five of the *de novo* assembled genomes where highly similar to a previous genome assembly of *A. pullulans* strain EXF-150 (Gostinčar et al. 2014). However, three isolates (AWRI 4233, AWRI 4619 and AWRI 4620) produced smaller genome assemblies with fewer predicted proteins.

Comparative genomic analysis was performed that investigated protein coding potential, phylogenetic association, average nucleotide identity and genomic synteny difference across the isolates, in addition to several reference genomes.

Based on the comparative genomics results, isolates AWRI 4229-AWRI 4232 and AWRI 4621 are proposed to be taxonomically classified as *A. pullulans*. In addition, there is evidence to propose that isolates AWRI 4233, AWRI 4619 and AWRI 4620 represent three newly discovered *Aureobasidium* species.

Aureobasidium mustum Onetto, Schmidt, Roach & Borneman sp. nov.
MycoBank: 836845
Holotype: AWRI 4233

Aureobasidium vineae Onetto, Schmidt, Roach & Borneman sp. nov
MycoBank: 836849
Holotype: AWRI 4619

Aureobasidium uvarum Onetto, Schmidt, Roach & Borneman sp. nov
MycoBank: 836846
Holotype: AWRI 4620

8. Outcome and conclusion

8.1 Performance against planned outputs

8.1.1 Data available from metagenomic analysis, describing wild wine fermentations from across the spectrum of Australian winemaking regions

To investigate the microbial populations that were present across commercial wild fermentations in Australia, a microbial survey was established that initially encompassed commercial producers of uninoculated Chardonnay and Shiraz wines, which was later expanded to also include Pinot Noir and smaller numbers of ferment samples from other varieties.

Samples were collected across all five vintages for which this project was funded (2017-2021). A total of 3,270 samples were processed across the five years, comprising 852 wines from 34 wineries. Wine regions from across six Australian States were represented (Granite Belt, Hunter Valley, Canberra District, Geelong, Adelaide Hills, Barossa Valley, Clare Valley, Coonawarra, Langhorne Creek, McLaren Vale, Margaret River, Tasmania).

A total of 83 fungal genera were observed at significant levels, with fungal diversity highest just after crush. Yeast microbiomes were shown to be highly variable and were influenced by region, vintage, winery and the vineyard from which fruit was sourced.

8.1.2 Yeast and bacterial isolates sourced from wild fermentations and natural sources, deposited in the AWRI culture collection

A collection of Australian wild wine yeast isolates was established from a range of fresh ferment samples across vintages 2017-2021. A variety of media, including those that preferentially selected for non-*Saccharomyces* yeasts, were employed to maximise the yeast diversity. Over 18,000 individual isolates were collected and identified by high-throughput ITS, with isolates obtained across at least 35 species.

Microbial isolates were obtained from two different geographical locations in Australia, the Central Plateau in Tasmania (1,029 isolates) and Erub Island in the Torres Strait (71 isolates).

8.1.3 New strains of *Saccharomyces* and non-*Saccharomyces* yeasts characterised and available for commercial application

Almost 1,300 non-*Saccharomyces* strains were screened for five enzymatic activities important for winemaking, while also being resistant to the common winemaking stresses of ethanol, high sugar, copper, low temperature, anaerobiosis and producing low H_2S.

Three strains were finally selected and were used to inoculate winery-scale trials in Riesling juice. This was followed with full chemical analysis and sensory, which showed that the strains had the potential to improve desirable characteristics during wine fermentation and are now available for progression through to possible commercialisation.

Saccharomyces isolates from this project were screened for desirable winemaking properties in collaboration with Project 3.2.1.

8.1.4 Knowledge on the effects of winemaking interventions on sensory and chemical characteristics of wild fermentations available

Experiments were performed that assessed the impact of SO_2, oxygen and temperature on the microbial dynamics and chemical properties of wine.

Given its role as the major antimicrobial compound that is added to wine, the effect of SO_2 was assessed across four successive vintages in Chardonnay wine (yeast profiling and chemical analysis), with initial laboratory-scale experiments transitioning to winery trials in the final two

vintages. Moderate SO_2 addition (35 ppm total SO_2) was shown to produce a drastic and reproducible change in the species composition of *Hanseniaspora* yeasts present in the ferment. This was accompanied by significant changes in the chemical profile of the wines. Sensory analysis performed on the final-year wines showed that these microbial and chemical changes led to sensorially distinct wines.

In conjunction with Project 3.1.5, the effect of oxygen addition was assessed on Chardonnay wine (yeast profiling and chemical analysis) across three vintages, with initial laboratory-scale experiments transitioning to winery trials in the final vintage, accompanied by sensory studies. When provided at the appropriate time, aeration has the potential to aid wild *Saccharomyces* fermentation efficiency and to decrease the time taken for fermentation completion. Stuck wild ferments are a significant risk for many winemakers and protocols to decrease this possibility (while speeding up ferment completion) are expected to provide significant assistance to winemakers performing wild fermentation.

Finally, the effect of varying fermentation temperature was investigated in both Chardonnay and Shiraz at laboratory scale. Here, it was shown that increasing temperature allowed for higher cell numbers and increased fermentation kinetics, but this came at the expense of wine quality.

8.1.5 Methods and equipment for 'in-winery' metagenomic analysis and fermentation management assessed

LAMP was investigated as a means to enable yeast profiling at the winery. LAMP primers were designed against conserved areas of the fungal ITS region in an attempt to perform 'multi-species' LAMP reactions that could be used in lieu of the current methodology of DNA extraction and PCR prior to sequencing. However, LAMP was not effective on the ITS region.

In-field DNA extraction represented a roadblock to both full in-field profiling analysis, but also in simplifying current logistics that surround centralised processing procedures, as currently ferment samples must be shipped frozen (on dry-ice) for processing. Use of Whatman FTA paper was shown to provide a simple method of simultaneously isolating and storing the DNA of mixed microbial samples that could then be processed directly via PCR and sequencing. This removed one step in the transition towards in-field analysis, while also providing a means to streamline the logistics around sample storage and transport to centralised locations for processing.

8.1.6 Practical advice and extension materials for winemakers

Extension material concerning the analysis of wine fermentation and the use of non-*Saccharomyces* yeasts has been developed and presented as part of the AWRI roadshow seminar series.

Workshops were also held at the 17th and 18th AWITC conferences in 2019 and 2022, dealing with wild ferments, interventions and the use of non-*Saccharomyces* yeasts.

8.2 Practical implications and benefits

This project has provided insight into the microbiology that is underpinning wild fermentation across Australia. It has demonstrated the highly variable nature of this microbiology, at the regional, winery and ferment level. Understanding the microbiome of their wine is of great interest to winemakers who depend on wild fermentation and this project has directly provided these insights to over 30 wineries, while developing methodologies that can now be considered for deployment either through a centralised service, or with future development, as an in-winery analytical framework. Large-scale association of microbiome and chemical data on fermentation products could be used by winemakers to correlate microbiomes with distinct ferment outcomes (positive or negative). By providing the means to gather these data in an actionable timeframe, interventions could be implemented to steer the observed yeast community towards an advantageous outcome.

The use of wild fermentation can provide improved sensory attributes compared to inoculated wines, including mouthfeel, aroma and flavour complexity and integration of flavours. However, wild fermentation is often viewed as carrying a higher risk of poor fermentation outcomes (organoleptic qualities and/or efficiency) and spoilage. The interventions that have been addressed in this study provide a means to alter flavour and aroma outcomes (through the use of SO_2) and to affect fermentation efficiency (through the judicious use of aeration). By combining microbial monitoring with intervention, risks surrounding negative wild ferment outcomes can be decreased.

While this project provides findings that can be used to lower the risks associated with spontaneous fermentation, it is likely that the majority of wines produced in the Australian market will continue to make use of inoculation with commercial yeast starter cultures. To address this key market segment, commercial non-*Saccharomyces* strains can be used to provide novel and/or complex flavour profiles relative to the use of *S. cerevisiae* alone, while improving fermentation reproducibility and reliability relative to the use of wild fermentation. In fact, in 2016-2017 the International Organization of Vine and Wine (OIV) revised the international codex of oenological practices and included a monography of non-*Saccharomyces* yeasts highlighting the important role of these yeasts in bridging the gap between inoculated and wild winemaking.

This project has assembled a foundational dataset of non-*Saccharomyces* yeast strains for future use in bioprospecting studies. The utility of this resource was highlighted through its use to define three non-*Saccharomyces* strains that show promise as potential commercial starters with the ability to enhance aroma and flavour properties relative to the use of *S. cerevisiae* alone.

9. Recommendations

The development of in-field testing applications would offer a significant benefit as it would provide for a shorter analytical lead time that would allow for interventions to be made during fermentation. A testing framework based upon the fungal ITS region also has the potential to provide rapid identification of fungi responsible for contamination outside of primary fermentation.

At a minimum, further confirmation work should be performed on the use of the Whatman FTA paper as a means to provide samples to a centralised testing facility that forgoes the need for cold-chain logistics and would allow for standard courier delivery of archived fermentation samples.

The results of the SO_2, aeration and temperature interventions have provided insights into methods that can be used by winemakers wishing to perform wild fermentation to alter fermentation aroma and efficiency. The use of aeration and SO_2 has been demonstrated at winery scale, although this has been limited to Chardonnay juice. Further partnerships with wineries should be sought to test these effects more broadly.

The non-*Saccharomyces* strains that progressed through to winery trials showed promise as potential commercial starter strains. Partnership(s) with commercial yeast suppliers should be sought to perform additional vintage trials with these strains and to gauge commercial interest in licensing these yeasts for use by the Australian industry.

10. Appendix 1: Communication

10.1 Communication of outcomes

Information has been extended to industry stakeholders through roadshow presentations and workshops and presentations at the 17th and 18th Australian Wine Industry Technical

Conferences in 2019 and 2022. Details of all presentations given are published in the AWRI Annual Report, accessible from: https://www.awri.com.au/about_the_awri/annual-reports/

10.2 Peer-reviewed articles

Cuijvers, K., Van Den Heuvel, S., Varela, C., Rullo, M., Solomon, M., Schmidt, S., Borneman, A. 2020. Alterations in yeast species composition of uninoculated wine ferments by the addition of sulphur dioxide. *Fermentation* 6: 62..

Onetto, C.A., Schmidt, S.A., Roach, M.J., Borneman, A.R. 2020. Comparative genome analysis proposes three new *Aureobasidium* species isolated from grape juice. *FEMS Yeast Res.* 20: foaa052.

Varela, C., Sundstrom, J., Cuijvers, K., Jiranek, V., Borneman, A. 2020. Discovering the indigenous microbial communities associated with the natural fermentation of sap from the cider gum *Eucalyptus gunnii*. *Sci. Rep.* 10: 14716.

Varela, C., Cuijvers, K., Van Den Heuvel, S., Rullo, M., Solomon, M., Borneman, A., Schmidt, S. 2021. Effect of aeration on yeast community structure and volatile composition in uninoculated Chardonnay wines. *Fermentation* 7: 97.

Villarreal, P., Villarroel, C.A., O'Donnell, S., Agier, N., Quintero-Galvis, J.F., Peña, T.A., Nespolo, R.F., Fischer, G., Varela, C., Cubillos, F.A. 2022. Late Pleistocene-dated divergence between South Hemisphere populations of the non-conventional yeast *L. cidri*. *Environ. Microbiol.* doi.org/10.1111/1462-2920.16103.

11. Appendix 2: Intellectual property

A large collection of yeast isolates was obtained as part of this project. Ownership of the strains obtained from Tasmania and Erub Island in the Torres Strait is shared with the University of Adelaide and, respectively, The Tasmanian Aboriginal Council, The Tasmanian Land Conservancy, Torres Strait Regional Authority and Erub Community Council.

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. Three non-*Saccharomyces* strains that progressed through to winery trials showed promise as potential commercial starter strains.

12. Appendix 3: References

Andorrà, I., Landi, S., Mas, A., Guillamón, J.M., Esteve-Zarzoso, B. 2008. Effect of oenological practices on microbial populations using culture-independent techniques. *Food Microbiol*. 25: 849–856.

Beltran, G., Torija, M.J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J.M., Rozès, N., Mas, A. 2002. Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst. Appl. Microbiol*. 25: 287–293.

Bindon, K., Holt, H., Williamson, P.O., Varela, C., Herderich, M., Francis, I.L. 2014. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. *Food Chem.* 154: 90–101.

Bizaj, E., Cordente, A.G., Bellon, J.R., Raspor, P., Curtin, C.D., Pretorius, I.S. 2012. A breeding strategy to harness flavor diversity of *Saccharomyces* interspecific hybrids and minimize hydrogen sulfide production. *FEMS Yeast Res.* 12: 456–465.

Bokulich, N.A., Mills, D.A. 2013. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Appl*. *Environ*. *Microbiol*. 79: 2519–2526.

Bokulich, N.A., Joseph, C.M.L., Allen, G., Benson, A.K., Mills, D.A. 2012. Next-generation sequencing reveals significant bacterial diversity of botrytized wine. *PLoS ONE* 7: e36357.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A. 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *PNAS* 111: E139–E148.

Bokulich, N.A., Collins, T.S., Masarweh, C., Allen, G., Heymann, H., Ebeler, S.E.,Mills, D.A. 2016. Associations among wine grape microbiome, metabolome, and fermentation behavior suggest microbial contribution to regional wine characteristics. *MBio* 7: e00631-16.

Borneman, A.R., Pretorius, I.S. 2015. Genomic insights into the *Saccharomyces sensu stricto* complex. *Genetics* 199: 281–291.

Borneman, A.R., Desany, B.A., Riches, D., Affourtit, J.P., Forgan, A.H., Pretorius, I.S., Egholm, M., Chambers, P.J. 2012. The genome sequence of the wine yeast VIN7 reveals an allotriploid hybrid genome with *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* origins. *FEMS Yeast Res*. 12: 88–96.

Borneman, A.R., Forgan, A.H., Kolouchova, R., Fraser, J.A.,Schmidt, S.A. 2016. Whole genome comparison reveals high levels of inbreeding and strain redundancy across the spectrum of commercial wine strains of *Saccharomyces cerevisiae*. *G3 6(4): 957-971.*

Cadez, N., Poot, G.A., Raspor, P., Smith, M.T. 2003. *Hanseniaspora meyeri* sp. nov., *Hanseniaspora clermontiae* sp. nov*., Hanseniaspora lachancei* sp. nov. and *Hanseniaspora opuntiae* sp. nov., novel apiculate yeast species. *Int. J. Syst. Evol. Microbiol.* 53: 1671–1680.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *PNAS* 108 Suppl 1: 4516–4522.

Combina, M., Elía, A., Mercado, L., Catania, C., Ganga, A., Martinez, C. 2005. Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *Int. J. Food Microbiol.* 99: 237–243.

Cordente, A.G., Curtin, C.D., Solomon, M., Kulcsar, A.C., Watson, F., Pisaniello, L., Schmidt, S.A., Espinase Nandorfy, D. 2022. Modulation of volatile thiol release during fermentation of red musts by wine yeast. *Processes* 10: 502.

Divol, B., du Toit, M., Duckitt, E. 2012. Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. *Appl. Microbiol. Biotechnol.* 95: 601–613.

Dukes, B.C., Butzke, C.E. 1998. Rapid determination of primary amino acids in grape juice using an o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. *Am. J. Enol. Vitic*. 49: 125–134.

Dunn, B., Sherlock, G. 2008. Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome Res.* 18: 1610–1623.

Fleet, G.H. 1990. Growth of yeasts during wine fermentations. *J. Wine Res.* 1: 211–223.

Fleet, G.H. 2008. Wine yeasts for the future. *FEMS Yeast Res.* 8: 979–995.

Fleet, G.H., Lafon-Lafourcade, S., Ribéreau-Gayon, P. 1984. Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Appl. Environ. Microbiol.* 48: 1034–1038.

Gostinčar, C., Ohm, R.A., Kogej, T., Sonjak, S., Turk, M., Zajc, J., Zalar, P., Grube, M., Sun, H., Han, J., et al. 2014. Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. *BMC Genomics* 15, 549.

Haft, R.J.F., Gardner, J.G., Keating, D.H. 2012. Quantitative colorimetric measurement of cellulose degradation under microbial culture conditions. *Appl. Microbiol. Biotechnol.* 94: 223–229.

Lario, L.D., Chaud, L., Almeida, M. das G., Converti, A., Durães Sette, L., Pessoa, A. 2015. Production, purification, and characterization of an extracellular acid protease from the marine Antarctic yeast *Rhodotorula mucilaginosa* L7. *Fungal Biol.* 119: 1129–1136.

Martini, A., Ciani, M., Scorzetti, G. 1996. Direct enumeration and isolation of wine yeasts from grape surfaces. *Am. J. Enol. Vitic*. 47: 435–440.

Morgan, S.C., Scholl, C.M., Benson, N.L., Stone, M.L., Durall, D.M. 2017. Sulfur dioxide addition at crush alters *Saccharomyces cerevisiae* strain composition in spontaneous fermentations at two Canadian wineries. *Int. J. Food Microbiol*. 244: 96–102.

Morgan, S.C., Tantikachornkiat, M., Scholl, C.M., Benson, N.L., Cliff, M.A., Durall, D.M. 2019. The effect of sulfur dioxide addition at crush on the fungal and bacterial communities and the sensory attributes of Pinot Gris wines. *Int. J. Food Microbiol.* 290: 1–14.

Mortimer, R., Polsinelli, M. 1999. On the origins of wine yeast. *Res. Microbiol.* 150: 199–204.

Nakao, Y., Kanamori, T., Itoh, T., Kodama, Y., Rainieri, S., Nakamura, N., Shimonaga, T., Hattori, M., Ashikari, T. 2009. Genome sequence of the lager brewing yeast, an interspecies hybrid. *DNA Res.* 16: 115–129.

Naumov, G.I., Naumova, E.S., Marynenko, N.N., Korkhola, M. 2013. Reidentification of the chromosomal *CUP1*-translocations in wine yeasts *Saccharomyces cerevisiae*. *Mikrobiologiia* 82: 203–211.

Ortiz, G.E., Guitart, M.E., Albertó, E., Fernández Lahore, H.M., Blasco, M. 2014. Microplate assay for endo-polygalacturonase activity determination based on ruthenium red method. *Analyt. Biochem.* 454: 33–35.

Park, H., Bakalinsky, A.T. 2000. *SSU1* mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* 16: 881–888.

Pinto, C., Pinho, D., Cardoso, R., Custódio, V., Fernandes, J., Sousa, S., Pinheiro, M., Egas, C., Gomes, A.C. 2015. Wine fermentation microbiome: a landscape from different Portuguese wine appellations. *Front. Microbiol.* 6: 905.

Pisaniello, L., Watson, F., Siebert, T., Francis, L., Hixson, J.L. 2022. The varietal influence of flavour precursors from grape marc on monoterpene and C13-norisoprenoid profiles in wine as determined by membrane-assisted solvent extraction (MASE) GC-MS. *Molecules* 27: 2046.

Rankine, B., Pocock, K. 1970. Alkalimetric determination of sulphur dioxide in wine. A*ust. Wine Brew. Spirit Rev.* 88: 40.

Schmidt, S., Rainieri, S., Witte, S., Matern, U., Martens, S. 2011. Identification of a *Saccharomyces cerevisiae* glucosidase that hydrolyzes flavonoid glucosides. *Appl. Environ. Microbiol.* 77: 1751–1757.

Seixas, I., Barbosa, C., Mendes-Faia, A., Güldener, U., Tenreiro, R., Mendes-Ferreira, A., Mira, N.P. 2019. Genome sequence of the non-conventional wine yeast *Hanseniaspora guilliermondii* UTAD222 unveils relevant traits of this species and of the *Hanseniaspora* genus in the context of wine fermentation. *DNA Res*. 26: 67–83.

Siebert, T.E., Solomon, M.R., Pollnitz, A.P., Jeffery, D.W. 2010. Selective determination of volatile sulfur compounds in wine by gas chromatography with sulfur chemiluminescence detection. *J. Agric. Food Chem.* 58: 9454–9462.

Sternes, P.R., Borneman, A.R. 2016. Consensus pan-genome assembly of the specialised wine bacterium *Oenococcus oeni*. *BMC Genomics* 17: 308.

Sternes, P.R., Lee, D., Kutyna, D.R., Borneman, A.R. 2016. Genome sequences of three species of *Hanseniaspora* isolated from spontaneous wine fermentations. *Genome Announc* 4: doi.org/10.1128/genomeA.01287-16.

Sternes, P.R., Lee, D., Kutyna, D.R., Borneman, A.R. 2017. A combined meta-barcoding and shotgun metagenomic analysis of spontaneous wine fermentation. *GigaScience*: 6(7): 1-10.

Taylor, M.W., Tsai, P., Anfang, N., Ross, H.A., Goddard, M.R. 2014. Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* 16: 2848–2858.

Varela, C., Pizarro, F., Agosin, E. 2004. Biomass content governs fermentation rate in nitrogen-deficient wine musts. *Appl. Environ. Microbiol.* 70: 3392–3400.

Varela, C., Bartel, C., Roach, M., Borneman, A., Curtin, C. 2019. *Brettanomyces bruxellensis SSU1* haplotypes confer different levels of sulfite tolerance when expressed in a *Saccharomyces cerevisiae SSU1* null mutant. *Appl. Environ. Microbiol.* 85 (4): e02429-18.

Varela, C., Bartel, C., Nandorfy, D.E., Borneman, A., Schmidt, S., Curtin, C. 2020. Identification of flocculant wine yeast strains with improved filtration-related phenotypes through application of high-throughput sedimentation rate assays. *Sci. Rep.* 10: 2738.

Viana, F., Gil, J.V., Genovés, S., Vallés, S., Manzanares, P. 2008. Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol.* 25: 778–785.

Viana, F., Gil, J.V., Vallés, S.,Manzanares, P. (2009. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol*. 135: 68–74.

13.Appendix 4: Staff

Dr Anthony Borneman
Dr Cristian Varela
Dr Cristobal Onetto
Ms Kathleen Cuijvers
Mr Steven Van Den Heuvel
Ms Laura Hale

14.Appendix 5: Other supporting material (including raw data, if appropriate)

Not applicable