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New and Improved Yeast and Bacterial Starter Cultures-Novel Attributes, Process Efficiency and Wine Distinctiveness

FINAL REPORT to WINE AUSTRALIA

Project Number: **UA 1803 - 2.1**

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Research Organisation: **University of Adelaide**

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Novel Attributes, Process Efficiency and Wine Distinctiveness

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Abbreviations

CDGJM	Chemically Defined Grape Juice Medium
DAP	Diammonium Phosphate (Ammonium Dibasic Phosphate)
DE	Directed Evolution
FAN	Free Amino acid Nitrogen
FCDGJM	Fermented Chemically Defined Grape Juice Medium
FEG	Fermentation Essential Genes
FSO_2	Free sulfur dioxide
TSO_2	Total sulfur dioxide
LYDL	Lab Yeast Deletion Library
LAB	Lactic acid bacteria
MFCA	Medium Chain Fatty Acids
MLF	Malolactic fermentation
MRS	De Man, Rogosa and Sharpe medium
MRSAJ	MRS with Apple Juice
NGS	Next Generation Sequencing
ORF	Open Reading Frame
PMS	Potassium metabisulfite
RCDGJM	Red CDGJM
RFCDGJM	Red Fermented CDGJM
SD	Standard deviation
SNP	Single Nucleotide Polymorphism
WYDL	Wine Yeast Deletion Library
WGS	Whole Genome Sequencing

Abstract

This project continued our research to develop an improved microbial toolkit of yeast and bacteria strains with desirable fermentation traits and wine aroma attributes. Methods investigated included directed evolution and CRISPR Cas9 gene editing tools. A Systems Biology approach was used, to provide a sound understanding of the genetic basis of improved characteristics. The key outputs were a diverse collection of microbial strains, mainly comprising non-*Saccharomyces* yeast species and lactic acid bacteria. These were isolated from the environment, industry and scientific collections. We have also advanced the understanding of what are considered as new risks to winemaking i.e. the use of juice dilution and the presence of bacteriophage. We found that juice dilution reduced fermentation duration by up to 28% when used within legal limits, and also results in many changes to the volatile profile of finished wines, which are not fully repaired through nutritional supplementation. Bacteriophage were found in almost all samples analysed and we have preliminary evidence suggesting that under certain conditions, purified phage can delay malic acid utilisation by lactic acid bacteria.

The outcomes of this project have the potential to generate many benefits for winemakers, primarily by decreasing the risk of problem fermentations.

Executive Summary

The use of yeast and bacteria, specifically selected for desirable traits leading to reliable and predictable primary and secondary fermentation remains the most common method to ensure beneficial fermentation outcomes in new-world industrial winemaking. Even though many commercial strains are available in the market, feedback from the Wine Australia Research Advisory Committee, as well as surveyed winemakers and suppliers during this project, is that issues with fermentations still exist that are directly related to microbial failure. This is also exacerbated by changes in the technical requirements of microbes, such as being able to perform even when the composition of grape musts may vary due to changes in viticultural practices and climate change. The core objective of this project was to improve the wine microbial toolkit available by providing strains and information regarding methods for improvement and use. This included extending our previous research on the use of directed evolution for strain improvement as well as investigating potential new or untested microbial issues such as the use of juice dilution and the impact of bacteriophage. We specifically targeted industry requirements and our approach was holistic, whereby the techniques used to isolate and/or improve novel microbes incorporated a Systems Biology approach to provide a sound understanding of the genetic basis of improved characteristics. It further allowed us to mine for other genetic characteristics that may be of use for future strain improvement. We expect that reliable fermentation outcomes will translate into further improvements to sustainable and economical wine production with enhanced or consistent wine quality.

The key Outputs of this project were a diverse collection of microbial strains, mainly comprising non-*Saccharomyces* species (yeast) and lactic acid bacteria. These were isolated from the environment, industry and scientific collections. The yeast and some of the bacterial strains were classified to species level and then screened for traits

considered to be desirable to the wine industry. Where required, methodology for these screens was designed and improved. We analysed strains for fermentation reliability, including (for bacteria) malolactic fermentation in the presence of high SO_2, the enzymatic activities of protease, lipase, β-glucosidase as well as the ability to utilise the spoilage compound acetic acid and also for antimicrobial activity against the spoilage organism *Brettanomyces bruxellensis.* Strains with these capabilities were identified in each of these categories. The most promising strains were analysed in small industrial scale fermentations where finished wines were then examined by sensory panels and for volatile chemical profiles. Here the highlights included four non-*Saccharomyces* strains from the species *Debaryomyces hansenii, Aureobasidium pullulans, Kazachstania servazzii and Kazachstania aerobia,* each contributing interesting floral profiles to wine aroma, as well as one *Saccharomyces cerevisiae* (C7H_B4) improved for fermentation reliability using directed evolution and found to also contribute to desirable wine aroma. We also sequenced the genomes of six strains evolved through directed evolution and the two *Kazachstania sp.* of interest and isolated from the environment. Comparison of genetic differences of these to our Fermentation Relevant Yeast Gene Database (also expanded in this project) advanced knowledge of what the causal genetic modifications may be for certain phenotypes of interest in a wine context. We then tested some of these hypotheses and advanced the methodology of CRISPR Cas9 gene editing in wine yeast through construction and analysis of the performance of six proof-of-concept engineered strains in the most commonly used commercial wine yeast strain in Australia (Lalvin EC1118®). Most helpfully, this also included using a technique that under current Australian legislation would see the modified strain being classified as non-GMO.

We have also advanced the understanding of what are considered as new risks to winemaking; the use of juice dilution and the presence of bacteriophage. Here we found that juice dilution does indeed reduce fermentation duration, by up to 28% when used within legal limits, and also results in many changes to the volatile profile of finished wines, often not repaired through nutritional supplementation. Bacteriophage were found in almost all samples analysed and we have preliminary evidence suggesting that under certain conditions, purified phage can delay malic acid utilisation by lactic acid bacteria. Since this project has included many lines of research, there have also been a number of other novel findings not originally proposed as Outputs. Wherever possible and appropriate, we have attempted to capture any such findings. One example is that as part of attempting to understand the mechanisms behind SO_2 tolerance of lactic acid bacteria, we also discovered that one of the key influences is the availability of methionine during alcoholic fermentation. A lack of this amino acid results in elevated SO_2 production by yeast, which in turn, inhibits malolactic fermentation by lactic acid bacteria.

Through this project we have managed to deliver many advances to the field of wine microbiology and our findings have been disseminated in many formal publications and presentations:

- 14 scientific journal articles (2 more in draft form)
- Two technical articles
- One technical brochure
- 13 conference presentations
- Seven accepted theses (with a further two PhD students completing shortly)

- Lectures to undergraduate and postgraduate Oenology/Viticulture and Biotechnology students

Background

Reliable and predictable fermentations are key to quality winemaking practices and as such, research in this field is vital to the improvement, refinement and adaptability to the ever-changing landscape of the Australian wine industry. Here we refer to reliability in terms of trouble-free completion and predictability in terms of fermentation duration, minimal yeast/bacterial supplement needs and appropriate production of desirable and undesirable metabolites. As we report here, feedback from winemakers is that they continue to experience fermentation issues likely due to microbial short- comings and that they request improved starter cultures to reduce this occurrence. Furthermore, since consumer demand for wine styles is ever-evolving, a toolkit of microbes with a broad array of capabilities is required for the Australian wine industry to remain competitive internationally.

Due to the ever-changing landscape, the challenge of achieving reliable and predictable fermentations is only increasing. On one hand, winemakers are seeking to reduce the use of additives such as antimicrobials, stabilisation agents and nutrients or are undertaking fermentation under more extreme conditions for stylistic reasons, such as at lower temperatures, thereby expecting more from 'wanted/useful' microbes. On the other hand, more 'difficult' juices (e.g. those higher in sugar, lower in nutrients, disease affected/carrying higher loads of spoilage microbes/requiring more SO_2), are being thrust upon winemakers by climate extremes.

Expansion of the microbial toolkit available to industry, through both yeast and bacteria and how best to use them, underpinned by knowledge about genes and gene variants essential for specific fermentation scenarios, offers a solution to the challenges faced by today's wine industry. Reduction of the risk of problem fermentations will result in improved profit margins, not only via preservation of about $30 million in wine (a conservative estimation (at 1%) of the Australian wine ruined annually), but also in significant reductions in production costs associated with the management of the ~10% of fermentations reported as problematic. Benefits also extend to the remaining ~90% of fermentations not necessarily reported as problematic, thereby giving further cost reductions. Predictable, robust and more reliable (shorter) fermentations will require fewer inputs (energy, additives, labour, etc) and result in less wastage with the salvage of poorer quality fruit, thereby reducing overall environmental impact. Greater profitability and sustainability will assist the wine industry to maintain its critical employment role, particularly in regional areas. This will be achieved by enhancing profitability and expanding markets domestically (through new products), as well as helping provide sought-after, ideally high value, wine to emergent overseas markets.

The core objective of this project was to improve the wine microbial toolkit available for fermentation, by providing the industry with robust and tailored yeast and bacteria specifically targeted to address industry requirements (as per advice from the Research Advisory Committee and industry practitioners). Our approach was a holistic one whereby techniques used by us previously to produce novel microbes, have been integrated into Systems Biology, resulting in a better understanding of the genetic basis for improvements and enabled 'mining' for genetic modifications specific to a selection

of desirable traits.

Various techniques were employed to achieve this, including isolation from the natural environment, directed evolution, gene modification via CRISPR Cas9 technologies for proof-of-concept strains, combined with screening programs to isolate novel yeast and bacterial strains targeting traits identified as important to Australian winemakers. In particular, non-*Saccharomyces* yeasts were targeted because of their inherently diverse traits, some of which are already suited to winemaking. Genetic strain characterisation was undertaken in parallel with strain isolation and development to provide an increased understanding of the basis for key traits, which we used to guide further improvement efforts.

The targets of desirable traits have focussed on those called for by industry including, greater reliability, more flavour complexity, easier processing of grapes to wine with fewer inoculations, fewer additives, or fewer stabilisation steps.

As from the original proposal, the initial objectives for target strains and traits initially included:

1. *Saccharomyces* strains that achieve quicker dominance and fermentation speed under today's challenging conditions e.g. those arising from dilution of high sugar juice, wherein previously unseen nutrient imbalances may complicate fermentation success and flavour profile.
2. Non-*Saccharomyces* yeast with distinct flavour impacts, useful enzymatic activities (e.g. pectinases, proteases, lipases), or the ability to bioacidify wine or suppress spoilage yeast.
3. Non-*Saccharomyces* yeast with the ability to persist longer and impact wine composition more significantly. Ideally such strains would not require a *Saccharomyces* starter to complete.
4. *Saccharomyces* and/or non-*Saccharomyces* with greater SO_2 tolerance and/or production to ensure a selective advantage over spoilage organisms and for 'natural' wine preservation, respectively.
5. Lactic acid bacterial (LAB) strains better suited to inoculation at the start of fermentation (with yeast) through increased SO_2 tolerance, osmotolerance and competitiveness.
6. LAB with increased tolerance to under-investigated causes for MLF failure such as those linked to micronutrients, inhibitors or bacteriophage.

Since the main objective of this work was to provide strains and further knowledge of their use in winemaking, we also undertook a comprehensive review of the currently available commercial strains, the technology behind how they were produced as well as new and upcoming technologies that may be useful. This was to ensure our objectives were the most appropriate for the current market. Further to this we also surveyed winemakers and commercial microbial supply companies to document their current strategies regarding the use of microbes in winemaking and sought their opinions about future requirements.

This project has resulted in many publications, and as such, work already reported in the literature will only be briefly summarised here. Please refer to the original publications for further detail. For convenience a copy of each of these publications is also included as an Appendix to this report.

Project Aims and Performance Targets

The Outputs and Performance Targets outlined in the approved application 'New and Improved Yeast and Bacterial Starter Cultures-Novel Attributes, Process Efficiency and Wine Distinctiveness' (UA 1803 - 2.1) for a four year duration are shown below. The original target dates are shown. Since a number of the Aims in the approved Application were overlapping and spanned across years, we have grouped similar items together into six Aims to simplify reporting. We have highlighted how the original Aims and Outputs fit within these six main Aims by notating original aims with main Aims in **BOLD** in the table below.

Objectives were combined into six main Aims to align with Outputs and Performance targets and thus reporting:

1. Yeast or bacteria with novel individual traits or combinations of traits
2. Information on the impacts of the new strains on wine composition, processing and stability. Recommendations on the use of new strains (must composition and microbial integrity and wine style
3. Basis for microbial enhancement determined by genome, RNA sequencing and/or QTL analysis, as appropriate
4. Knowledge on the efficacy of various strain improvement methods
5. Role of bacteriophage agents in stuck or sluggish MLF defined and quantified from a number of fermentations and wineries
6. Dissemination of new knowledge to Industry

Outputs and Performance Targets July 2017-June 2018

Aim (Year 3/ Year 5 deliverable)	Outputs	Activities	Target date
AIM 1: Yeast or bacteria with novel individual traits or combinations of traits	1A. A collection of candidate yeasts and bacteria with diverse properties for use as starting material for development through the remainder of the project. 1B. Establishment of laboratory methods and initiation of screening of the microbial collection.	a) In addition to isolates already in our microbial collection, initiate sourcing of specific strains highlighted in the literature. b) Methods will be researched and tested to examine traits such as production of SO_2, acidification, and activities of polysaccharides, proteases, lipases and pectinases.	30/6/2018

AIM 2: Information on the impacts of the new strains on wine composition, processing and stability	1C. Data on the fermentation performance of the most promising 1 or 2 DE yeast strains from UA 1302 to complete strain evaluation. 1D. Data on the adverse impacts of dilution of high sugar-content juices on nutrient balance and therefore yeast and LAB fermentation reliability.	c) Evolved yeast strains selected previously for rapid and reliable fermentation will be evaluated in juice trials at the UA winery (50 kg red must fermentations in triplicate). Such volumes will allow provision of sensory profiles via triangle tests, preference tests and RATA (to be conducted in July-August 2018). d) Fermentation trials of juice from grapes picked well beyond desirable maturity (high sugar-content) after dilution and with varying nutrient supplementation.	30/6/2018

Outputs and Performance Targets July 2018-June 2019

Aim (Year 3/ Year 5 deliverable)	Outputs	Activities	Target date
AIM 1: Yeast or bacteria with novel individual traits or combinations of traits	2A. Data on the definition of starting conditions with appropriate stresses to be applied to LAB in directed evolution (DE) experiments. 2B. Yeast and bacterial strains identified with the potential to eliminate addition of additives or processing and data demonstrating relative efficacy of strains exhibiting such traits.	a) New targets for DE will be defined based on background work or emerging industry priorities. Initial targets will include increased tolerance to low pH and high SO_2. In each case, minimum inhibitory concentrations (MIC) will be established and the diversity of the starters increased through EMS mutagenesis pre DE. b-1) Microbial collection will be tested to examine traits such as production of SO_2, polysaccharides, acidification, proteases, lipases and pectinases. b-2) Proof-of-concept GM yeast engineered for improved desirable fermentation capabilities utilising modern molecular biology such as CRISPR/Cas9.	30/6/2019 30/6/2019 30/6/2019
AIM 2: Information on the impacts of the new strains on wine composition, processing and stability	2C. Data on the sensory impact of the most promising 1 or 2 DE yeast strains from UA 1302 to complete strain evaluation and publication.	c) Sensory profiles of evolved yeast strains selected previously for rapid and reliable fermentation will be undertaken via triangle tests, preference tests and RATA. If funding allows, this will also include analysis of 20L white juice fermentations.	31/12/2018
AIM 2: Recommendations on the use of the new strains (i.e. must composition and microbial integrity, wine style, etc).	2D. Fact sheets (advice to winemakers) based on detailed characterisation of risks arising from dilution of high-sugar juices.	d) Analysis of fermentation performance of yeast and bacteria matched with analysis of volatile and/or non-volatile composition of wines from diluted juices. Specific risks and impacts to be evaluated include nutrient and vitamin dilution/imbalances and use of mains (chlorinated) water. Fact sheets generated.	30/6/2019
AIM 6: Disseminated findings (peer-reviewed, trade articles, technical notes, conference /seminar presentations	2E. 1 x peer reviewed publication and 1 x technical publication reporting promising 1 or 2 DE yeast strains from UA 1302		31/12/2019

Outputs and Performance Targets July 2019-2020

Aim (Year 2 / Year 4 deliverables)	Outputs	Activities	Target date
AIM 2: At least 2-3 new yeast strains generated and evaluated for the wine industry by a combination of methodologies.	3A. 2 new yeast or LAB strains with desirable attributes and data on their evaluation available for the wine industry Information on the impacts of the new strains on wine composition, processing and stability	Strain performance will be evaluated in comparison to current industry best practice for each target trait under laboratory and pilot-scale (>20 L) conditions.	30/06/2020
AIM 3: Data compiled from 'omics experiments and/or QTL analyses will provide a better understanding of the genetic basis for successful fermentations of improved strains	3B. Information on which yeast genes play a role in successful wine fermentation	Data compiled from 'omics experiments In-house databases related to fermentation attributes will be updated	30/06/2020
AIM 2: Risks will be identified and recommendations made to mitigate undesirable fermentation dynamics of diluted juices.	3C. Information on the impacts of juice dilution of high-sugar juices	Results from high-throughput evaluation of diluted juices (with addition of nutrient also examined to be compiled and conclusions reported either as a fact sheet, poster, journal article or news article for WA website.	30/06/2020
AIM 5: Role of bacteriophage agents in stuck or sluggish MLF defined and quantified from a number of fermentations and wineries.	3D. Determination of the importance of bacteriophage in inhibiting MLF*	Develop PCR method to detect bacteriophage (oenophage) in Oenococcus oeni*. Identify phage types by DNA extraction from isolated phage and restriction digestion*. Mimic sluggish/stuck MLF by transfection of O. oeni with purified oenophage*.	30/06/2020
AIM 1: Alternative strategies for control of Brettanomyces sp. investigated	3E. Information on antagonistic properties of new yeast strains	Develop methods to detect potential inhibitory agents* Develop inhibition assays between potential antagonistic yeast and *Brettanomyces**	30/06/2020

AIM 6: 1-3 draft manuscripts, including for industry articles, prepared detailing improved strains.	3F. 1 x peer reviewed publication and 1 x technical publication reporting novel desirable fermentation attributes discovered in microbial culture collection screening.	Preparation of publications.	30/06/2020

Outputs and Performance Targets July 2020-2021

Aim (Year 4 deliverables)	Outputs	Activities	Target date
AIM 2: Information on the best performing yeast/LAB strains derived from 25 L and larger trials. Fermentations undertaken in a range of juices over multiple vintages will be compared to current industry best practices. Improvements will be quantified in order to communicate the expected range of efficacy to Industry.	4A. Data on the fermentation performance of the best yeast/LAB strains (≥20L) in different juices.	Liaise with starter culture suppliers and industry for winery trials (2020/21/22 vintage).	30/06/2021
		Conduct further vintage (2020/21) trials (if necessary) with most promising strains.	30/06/2021
AIM 4: Knowledge on the efficacy of various strain improvement methods. As we expect new technologies to be utilised for strain improvement, we will identify shortcomings (if any) and benefits of these as applied in the wine microbiology space.	4B. Survey of current and up-coming strain improvement technologies. Provide wine industry relevant examples (WA funded projects) Dissemination of findings as peer or industry magazine article, web blog or conference seminar	Review literature on current and upcoming methods for strain improvement, including legislation. Survey (online) winemakers and yeast supply companies as to number of strains per improvement methodology and uptake by industry, compare this to 2019 AWRI industry practices survey. Analyse WA funded projects (UA and AWRI) as to success of strategies implemented.	30/06/2021

AIM 3: Basis for microbial enhancement determined by genome, RNA sequencing and/or QTL analysis, as appropriate. Latest gene technologies coupled with bioinformatic processing tools will be used to decipher how desirable strain properties are conferred	4C. Yeast and bacterial databases of genetic changes that putatively confer improved phenotypes Improved metabolic models of key microbial metabolic pathways Experimental design determined for further strain improvement	Databases of genetic variations of yeast or bacteria leading to improved phenotypes will be built. This will encompass those identified from sequencing and/or QTL analysis of novel isolates and the fermentome (expanded in here), as well as from literature and other available yeast databases. Interrogation of databases will be undertaken to build and improve on key microbial metabolic pathway models directly related to chosen microbial enhancement(s). Key candidate yeast mutants will be designed to both test pathway models as well as to maximise any possible further contribution to strain improvement(s).	30/06/2021
AIM 5: Data on the presence of novel inhibitors or bacteriophages in wineries with failed MLF	4D. Validation as to whether bacteriophage can cause failed MLF	Survey commercial preparations and samples from representative, problematic and successful MLFs for presence of phage	30/06/2021
AIM 6: At least 5 peer-reviewed or industry publications on improved strains and knowledge gained on genetic basis of strain improvement	4E. 3 x peer reviewed publications and 2 x technical publications reporting on generation of improved strains and their oenological attributes	Prepare publications	30/06/2021
AIM 2: 3-6 yeast and/or LAB strains with enhanced or distinct properties offered to starter culture companies for evaluation for commercialisation. To be offered on a rolling basis over the project time frame as the strains are generated and appropriately characterised	4F. Up to 4 new yeast or LAB strains with desirable attributes and data on their evaluation available for the wine industry	Provide data on strain evaluations under lab scale (<20L) conditions	30/06/2021
		Offer strains to yeast/bacterial suppliers for evaluation prior to commercialisation	30/06/2021

AIM 6: At least one student supervised to undertake related parallel work and submitting their thesis.	4G. Submission of MSc/PhD thesis on parallel work which coincide with 1 or 2 of the project aims	Supervise MSc/PhD student(s) towards degree completion	30/06/2021
		Prepare thesis, publications, etc	30/06/2021

Additional Outputs Jan-Jun 2022

Related WA Output(s)	**Outputs**	**Activities**	**Target date**
A. Data on the fermentation performance of the best yeast /LAB strains (≥20L) in juice. F. Up to 4 new yeast strains with desirable attributes and data on their evaluation available for the wine industry	H. Data on the fermentation performance of the best yeast /LAB strains (≥20L) in juice, for example: -antimicrobial (Brettanomyces inhibitory) yeasts -acetic acid reducing yeasts -SO_2 tolerant LAB -multi-stressor tolerant LAB	Liaise with starter culture suppliers in- house trials	30/6/2023
		Conduct further vintage (2022) trials with most promising strains.	30/6/2023
B. Survey of current and up-coming strain improvement technologies. Provide wine industry relevant examples (WA funded projects) C. Yeast databases of genetic changes that putatively confer improved phenotypes Experimental design proposed for future strain improvement	Experimental design(s) proposed for future strain improvement I. Up to 2 new yeast strains engineered using CRISPR technology	Data on fermentation performance of modified yeast at <20L scale	30/6/2023

D. Validation as to whether bacteriophage can cause failed MLF	J. Complete delayed output: Validation as to whether bacteriophage can cause failed MLF	Survey commercial preparations and samples from representative, problematic and successful MLFs for presence of phage	30/6/2023
At least 2 peer-reviewed or industry publications on improved strains and knowledge gained on genetic basis of strain improvement and bacteriophage incidence in wine	K. 2 x publications	Prepare publications	30/6/2023

Two consecutive extensions to the project were approved in order to complete work on UA 1803 - 2.1 target/outcomes, to complete work from previous projects, as well as continue to provide supervision of HDR students in the final stages of their PhD candidatures. A further extension to submit the Final Report was granted given the CI's departure from the university.

AIM 1: Yeast or bacteria with novel individual traits or combinations of traits

Contributors

Postdoctoral fellows: Drs Michelle Walker, Jin Zhang, Jennie Gardner, Krista Sumby, and Joanna Sundstrom, Mr Nick van Holst and Professor Vladimir Jiranek. The following students also contributed, Ms Mandy Lin, Ms Yingxin Liu, Ms Emily Hocking, Yan Berkovich, Mr Yiming Huo, Ms Chitra Nair and Carla Virdis.

Background

The objective of this Aim was to isolate, purify, characterise and improve isolates of *Saccharomyces cerevisiae*, non-*Saccharomyces* and lactic acid bacteria (LAB) including *Oenococcus oeni* and *Lactobacillus plantarum* for more efficient and reliable alcoholic and malolactic fermentation in juice and wine. This was undertaken using a multipronged approach. Initially we assembled a collection of diverse yeast and bacteria from a number of sources, these being environmental (wine and non-wine), other beverages (beer, kombucha) and publicly available culture collections. Sugar-rich environmental niches were purposefully chosen to sample as we suspected that these would harbour microbes already well adapted to high sugar environments and thus likely suitable for wine fermentations. After purification of clonal isolates, the species of each was identified and isolates were then screened for novel and/or useful capabilities; for instance, their growth and fermentative capacity in high sugar media, and specific enzymatic (i.e. high beta-glucosidase activity) and antimicrobial activities such as the growth inhibition of the common wine spoilage organism *Brettanomyces bruxellensis*. To enable screening of the high number of isolates housed in our collection we also designed specific methodology allowing efficient high throughput analysis.

To maximise diversity of the microbial collection, directed evolution was also undertaken, both in yeast and bacteria. With yeast, we further evolved and analysed strains from previous research work (UA 1302) in an effort to further improve the performance and potential industrial application. In bacteria, we attempted to evolve a strain of *Oenococcus oeni* from Lallemand (B9). This strain was reported to impart desirable aromatic properties but lack robust sulfite tolerance. In this project we attempted to improve this capability. To achieve this, determination of starting conditions, such as investigation of minimum inhibitory concentrations of stressors was required.

Improvement of lactic acid bacteria (LAB) was also attempted using both classical microbial isolation techniques and mutagenesis of strains from both our in-house collection and the industrial strain B9.

Within Aim 1, we also designed and constructed proof-of-concept strains with enhanced fermentation capabilities using CRISPR Cas9 technologies. This work was supported by data from Aim 3 where we used a Systems Biology approach to determine suitable gene targets.

Summary of Outcomes

352 yeast and 241 bacteria isolates were isolated from the environment or sourced from other collections to assemble a microbial collection for use in this project. This resource is highly valuable, especially due to its diversity, with more than 40 yeast species included, and thus its potential to include strains of commercial relevance. The bacterial collection is still in the process of being fully classified but is suspected to also be diverse, as screening has revealed a wide range of phenotypes. Since we targeted sugar rich niches to source organisms, this collection's value also lies in its specificity to applications in high sugar beverage fermentations, since these organisms are likely to be well adapted to their sugar rich native environment. Within this collection we searched for organisms with novel and desirable properties directly applicable to winemaking. Yeast were screened for capabilities such as the specific enzymatic activities, lipases, proteolytic and β-glucosidases, as well as antimicrobial (inhibition of *Brettanomyces bruxellensis*) capacity and the ability to reduce the undesirable compound acetic acid from wine.

Strains of particular interest were a *Debaryomyces hansenii* and an *Aureobasidium pullulans* with high β-glucosidases activities, isolates of *Kazachstania aerobia*, *K. servazzii*, *Aureobasidium pullulans* and *Metschnikowia guilliermondii* with high protease activity and a *Debaryomyces hansenii,* a *Hanseniaspora occidentalis* and two *Lachancea thermotolerans* with the ability to utilise acetic acid. Ten yeast capable of inhibiting *Brettanomyces* growth on solid medium were also identified, with one, an *L. thermotolerans,* also able to briefly (24 hrs) delay growth in liquid cultures.

Bacterial work began with an investigation to determine whether directed evolution could be used to improve B9, an LAB of particular interest to industry, to enable robust MLF in the presence of sulfur dioxide, as this is well known to be a limitation with this and other current commercial cultures. Ultimately this was problematic and instead we focused on identification of isolates from our collection that were already MLF efficient in the presence of sulfur dioxide. Two isolates have been found so far that are capable of efficient fermentation in the presence of 60 mg /L SO_2 in small scale fermentations. Research on these is ongoing. Work also extended to a better understanding of sulfur sources required during alcoholic fermentation to ensure SO_2 produced by yeast was minimised and thus allowed robust MLF. Under conditions investigated here, we found that supply of at least 50 mg/L of methionine was crucial to suppress yeast-derived SO_2 and as such the availability of this amino acid should be considered during winemaking. Additional enzymatic activity targets were being pursued by Ms Carla Virdis and included lipases, proteolytic and β-glucosidases. This work was not completed as Ms Virdis left the project to pursue another career path.

Importantly, methodologies used to undertake these analyses were refined, specifically for these purposes, and have significantly contributed to the successful outcomes of this project. Each are reported here and were disseminated to the research community through peer reviewed publications.

Since we intentionally used a multipronged approach to supply microbes with

enhanced microbial capacities, we also constructed genetically modified yeast strains as a proof-of-concept for how modification of certain metabolic pathways could lead to desirable outcomes. The following mutants with their novel capacities were generated using CRISPR Cas9 technology: EC1118 *ecm33* and EC1118 *ser1* had modified fermentation efficiency; EC1118 *pro1 put2* resulted in up to five-fold more proline accumulation; EC1118 *sul1* and EC1118 *sul2* reduced hydrogen sulfide production by up to 92% with no impact on sulfur dioxide production. Finally, EC1118 *can1*, designed to reduce the production of ethyl carbamate, and shown to have the same fermentation duration as the parent, requires further investigation in terms of the impact on ethyl carbamate. Importantly, this particular mutant was constructed without the use of a repair template and as such is designated as non-GMO under the current Australian legislation.

Outputs and Performance Targets: AIM 1

Output 1A: A collection of candidate yeasts and bacteria with diverse properties for use as starting material for development through the remainder of the project.

- In addition to isolates already in our microbial collection, initiate sourcing of specific strains highlighted in the literature.

Output 1B. Establishment of laboratory methods and initiation of screening of the microbial collection.

- Methods will be researched and tested to examine traits such as production of SO_2, acidification, and activities of polysaccharides, proteases, lipases and pectinases.

Output 2A. Data on the definition of starting conditions with appropriate stresses to be applied to LAB in directed evolution (DE) experiments.

- New targets for DE will be defined based on background work or emerging industry priorities. Initial targets will include increased tolerance to low pH and high SO_2. In each case minimum inhibitory concentrations (MIC) will be established and the diversity of the starters increased through EMS mutagenesis pre DE.

Output 2B. Yeast and bacterial strains identified with the potential to eliminate addition of additives or processing and data demonstrating relative efficacy of strains exhibiting such traits.

- Microbial collection will be tested to examine traits such as production of SO_2, polysaccharides, acidification, proteases, lipases and pectinases.

- Proof-of-concept GM yeast engineered for improved desirable fermentation capabilities utilising modern molecular biology such as CRISPR/Cas9.

Output 3E. Information on antagonistic properties of new yeast strains

- Develop methods to detect potential inhibitory agents*
- Develop inhibition assays between potential antagonistic yeast and *Brettanomyces*

Results and Discussion

Output 1A. A collection of candidate yeast and bacteria was assembled from environmental (collected as part of this project) and industrial samples and other culture collections. Environmental samples were collected from sugar rich niches of 19 tree and insect species located in the Waite Arboretum. Industrial samples were obtained from active wine fermentations and winery surfaces, other beverages (kombucha, sour beer, sourdough and sauerkraut) and cultures of particular interest, as highlighted in the literature. From discussions with collaborators, we also included isolates from other wine yeast culture collections (UC Davis and the NRRL culture collection). Clonal cultures were purified by streak plating onto a variety of media to

allow those with specific growth requirements to be maintained. Pure cultures were then identified by amplification and sequencing of the 16S (bacteria) and internal transcribed spacer (ITS, yeast) regions of ribosomal RNA. Sequences were then compared to the NCBI and QIIME databases for classification to the species level where possible. In all, 275 isolates of yeast and 241 bacteria were collected and identified. These spanned 41 yeast species, with an additional 10 of which could not be classified with these methods and are potentially new species. So far, four bacterial species have been classified with 204 isolates still remaining to be typed. Importantly many species common to wine were isolated, for example, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Aureobasidium pullulans*, *Kazachstania sp.*, *Lachancea sp.*, *Oenococcus oeni and Lactobacillus plantarum*. This collection was also evaluated for the presence of species harmful to humans, and those found were excluded. Throughout this project, another in-house collection of environmental isolates was also investigated for useful isolates. This extra collection of 77 yeasts, previously identified by ITS phylotyping, was obtained from uninoculated and fermenting Shiraz must (Hardy's vineyard, 2007, McLaren Vale, South Australia). Together this collection of 352 yeast and 241 bacteria isolates is diverse in species and highly likely to contain microbes useful for winemaking. Further characterisation will define the nature of the diversity in oenologically relevant phenotypes.

Output 1B. Methods were established to conveniently analyse properties of interest of the collection and each of these was designed with high throughput screening capabilities. This included screening yeast for lipases, proteases, β-glucosidases, antimicrobial capabilities (inhibition of *Brettanomyces bruxellensis*) and reduction of the undesirable compound acetic acid from wine. For bacteria, a screen for malolactic fermentation efficiency in the presence of sulfur dioxide was also designed, as well as analysis of other enzymatic properties. In particular, extensive troubleshooting was required to design an appropriate screen for malolactic efficiency of LAB in the presence of SO_2. Here we found that preconditioning and concentration of the inoculant as well as vessel type (microtiter plate *vs* tube), were important parameters to ensure the success of the experiment. For instance, robust LAB precultures were achieved by growing in 75% wine and 25% MRS with a small addition of potassium metabisulfite (15 mg/L). The quantum of inoculum was, however, difficult to measure accurately in this medium as the mixture of MRS and wine resulted in a precipitate that confounded optical density measurements. Instead, preliminary experiments were performed where growth over time was quantified with colony forming units to gain an understanding of typical cell numbers from each specific medium. Generally, an inoculation rate of 1-2% (v/v) from a 3 to 4-day old culture initially inoculated to 0.04 OD_{600} nm units of a 7-day old MRS liquid culture was found to be appropriate. We also found that when performing MLF in tubes, strains grew and metabolised malic acid more efficiently in glass tubes rather than plastic. Importantly we have also developed a protocol for spectrophotometric analysis of free and total SO_2 at micro-scale by modification of a commercial kit (Vintessentials).

Several other methods for enzymatic properties were also investigated for use here

and protocols designed (proteases, urease, pectinase), through which isolates performing well are reported below. Due to time constraints, only subsets of the microbial collection were evaluated for some properties, for instance, a protocol was designed for measurement of haze-forming proteins in juice pre- and post-fermentation by high-throughput reverse HPLC but was not applied to the entire library due to lack of time and funding. Within this Aim we have also reported on the capabilities of our high throughput fermentation system (Van Holst *et al*., 2021), a key piece of infrastructure used throughout this project and available for use by other researchers.

Collectively, these results and facilities provide invaluable resources to springboard future research in this area, especially where methodological design is often critical to outcome. As such, we have disseminated this information through comprehensive descriptions of these methods in peer reviewed publications and theses arising from this project.

Output 2A. Starting conditions were defined with appropriate stresses to LAB in directed evolution experiments. To advance our previous work utilising directed evolution (DE) to further improve strains of *Oenococcus oeni* suitable for malolactic fermentation, two bacterial strains were chosen as starter cultures. These were the previously evolved strain JJ 3-83 (GWR Ph1308), chosen for its rapid consumption of L-malic acid and tolerance when exposed to combined low pH, high ethanol, SO_2 or decanoic acid stressors. The second, B9, was selected through discussions with industry. This strain, currently being developed by Lallemand, has many desirable fermentation properties, in particular it enhances 'fruity' characteristics and mouthfeel of wines.

To further increase the genetic diversity prior to DE, UV mutagenesis of JJ 3-83 and B9 was undertaken, and clones isolated. Starting conditions for DE were determined by undertaking Minimum Inhibitory Assays focussing on tolerance to high sulfur dioxide and low pH. Following this, a screen for SO_2 tolerance of these strains helped define starting conditions for DE and to identify any further improved isolates. Once these were determined, we attempted DE of these in continuous culture. Unfortunately, continuous culture of JJ 3-83 and B9 in Merlot or a red blend (Cabernet Sauvignon/Shiraz/Grenache) wine with even low concentrations of SO_2 (20 mg/L) was extremely problematic. We found that extended culturing resulted in poor cell viability and thus limited, if any, improvement. Both batch and continuous cultures were fully explored with many modifications to the medium and other culture conditions, but due to limited success and time constraints this part of the project was ultimately abandoned.

Instead, we undertook screening of both the bacterial isolates in our Wine Australia microbial library (192 isolates) and clonal isolates of the UV mutagenised collection of B9 for malolactic efficiency in the presence of high concentrations of SO_2. As described above, this required quite extensive design of an appropriate high throughput screen, importantly including trials to determine the appropriate starting concentration of SO_2 for each wine batch. For instance, trials of 60-120 mg/L SO_2 revealed that 60 mg/L was most appropriate (for this given red wine) as there was a clear difference in malolactic fermentation duration, while most strains were still able to utilise all available malic acid (Figure 1).

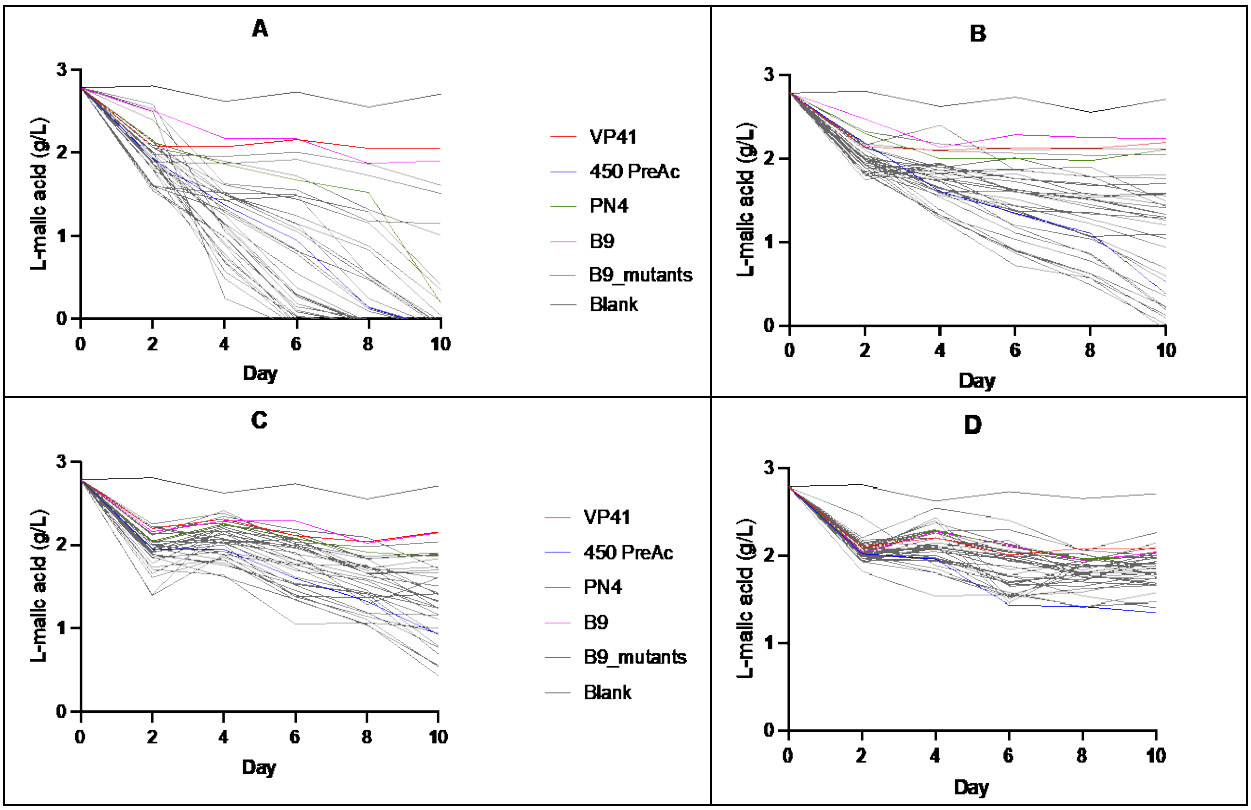


Figure 1. Malic acid utilisation during malolactic fermentation in red wine spiked with 60 (A), 80 (B), 100 (C) or 120 (D) mg/L SO_2 by B9 EMS mutants in comparison to the parent strain B9 and commercial cultures VP41, 450 PreAc and PN4.

Screening of 207 isolates (including strains from the environmental and B9 EMS mutant collections) revealed that 16 were capable of utilising all malic acid within 19 days, whereas remaining strains, including the widely used commercial culture Lalvin VP41, only used an average 32% of malic acid in this time frame. These robust strains included commercial strains, PN4, B9 and 450 PreAc, eight B9 EMS mutants and five environmental isolates. Upon re-testing of these in larger scale and replicated (10 mL) tube assays, two isolates (LOR_CHA_LAB5 and B9_M1) were found to have robust MLF in 60 mg/L SO_2 (Figure 2). These are being further explored by Miss Yingxin Liu as part of her ongoing PhD project (UoA funded). She is aiming to determine the exact mechanism allowing efficient malolactic fermentation in the presence of SO_2. For instance she is exploring the impact on known contributors to SO_2 tolerance, such as ATPase activity and the maintenance of intracellular pH.

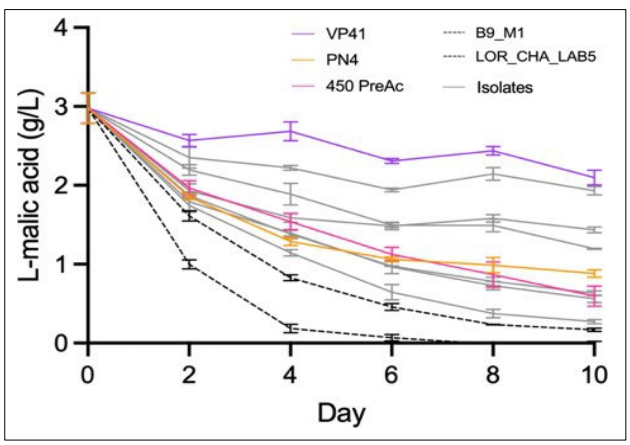


Figure 2. Malic acid utilisation during malolactic fermentation in small scale (10 mL) wine fermentations spiked with 60 mg/L SO_2 by B9 EMS mutant B9_M1 and several environmental isolates (including LOR_CHA_LAB5) in comparison to commercial cultures VP41, 450 PreAc and PN4.

Since some sulfur is important for LAB growth, our work has also investigated which sources are most important or detrimental for successful malolactic fermentation. Recent work by PhD student Miss Emily Hocking has shown that nine amino acids were essential for LAB growth across a range of commercial LAB. Focussing on those containing sulfur, only methionine was essential for all strains, whereas cysteine was also required by some. Further to this she also found that when methionine is supplied above a certain threshold during alcoholic fermentation, it suppresses the production of SO_2 by yeast, thereby allowing the subsequent malolactic fermentation to be more efficient. Here, alcoholic fermentations were conducted in Chemically Defined Grape Juice supplemented with a full complement of amino acids except with varying concentrations of methionine (0, 25, 50, 100 or 150 mg/L), Figure 3. Fermentation with three different yeasts showed that supplementation with at least 50 mg/L of methionine was required to maintain total SO_2 below 20 mg/L in each case, even in the presence of ample serine (a precursor of sulfur containing amino acids). This concentration of SO_2 is generally considered ideal in commercial winemaking, but higher amounts may inhibit the action of LAB in malolactic fermentation.

These findings suggest that winemakers and suppliers of commercial products supplying nutrients for robust malolactic fermentations should carefully consider the impact of supply of sufficient amino acids, in particular methionine, to enable robust and complete malolactic fermentations.

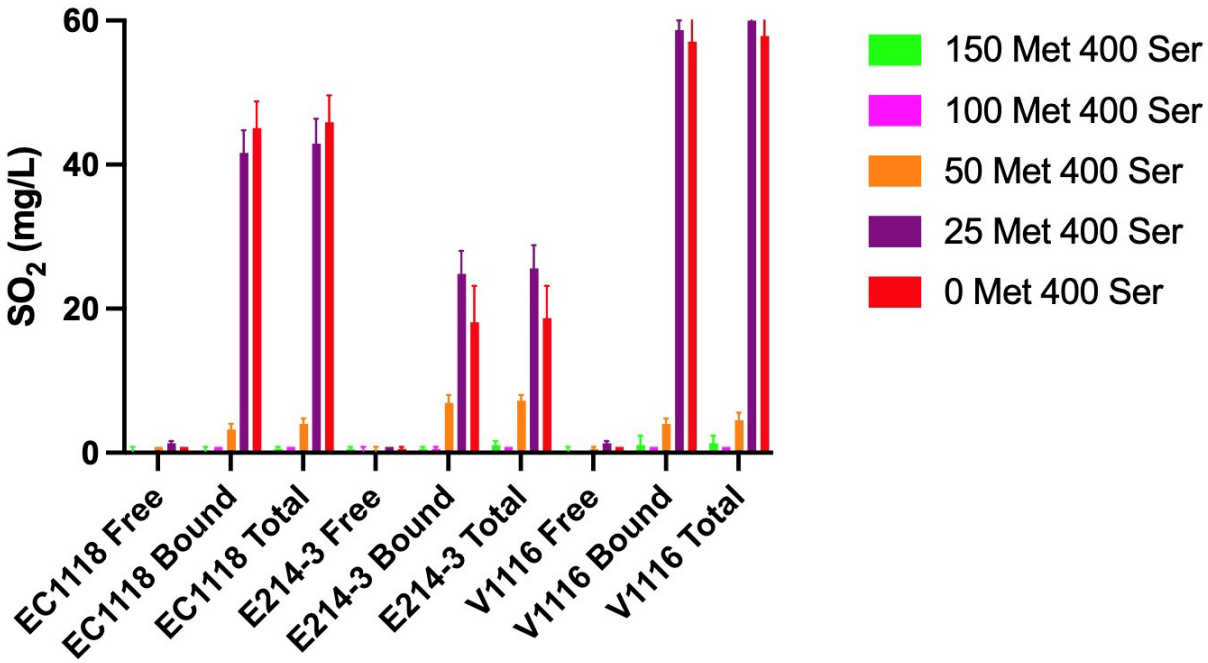


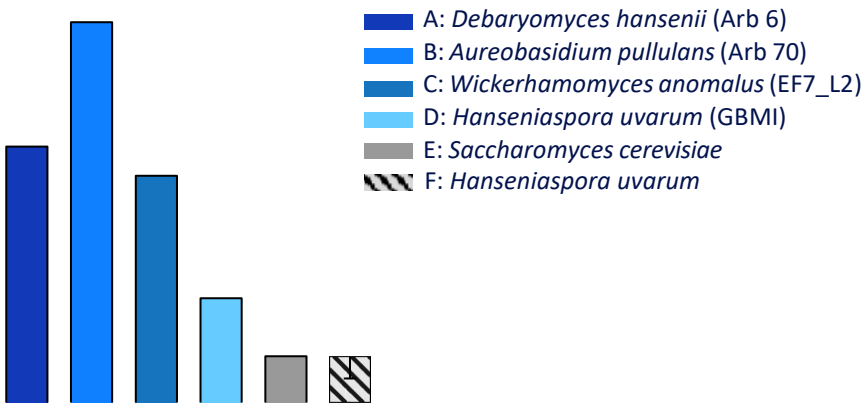
Figure 3. Free, bound and total sulfur dioxide measured in wines made with yeasts EC1118, E214-3 or V1116 when supplied with a full complement of amino acids (including serine at 400 mg/L), but with different amounts of methionine (0-150 mg/L).

Output 2B. Yeast and bacterial strains have been identified with the potential to eliminate addition of additives and improvement of processing through screening of our microbial collection for properties of interest and construction of proof-of-

concept strains using CRISPR Cas9 technologies (Output 2B Part 2). Here we report on the most promising candidates.

The β-glucosidase production of 253 yeast strains was evaluated using a multi-staged approach. Initially β-glucosidase activity of 1.2 mL cultures was analysed, with EC1118 (one of the most widely used *S. cerevisiae* in the Australian wine industry) and *Hanseniaspora uvarum*, as controls. The 50 best performing isolates (19.76% of those screened) were selected and rescreened as 3 mL cultures (in triplicate), revealing that 36 isolates had 30% higher activity than the control. Species with high activity were *Aureobasidium pullulans* (12 isolates), *Saccharomycodes ludwigii* (1), *Wickerhamomyces anomalus* (5), *Debaromyces sp.* (7), *Kazachstania aerobia* (2), *Hanseniaspora sp*. (4), *Saccharomycete sp.* (2) and *Yarrowia lipolytica* (1). In particular, Arb 6 (*Debaryomyces hansenii*), Arb 70 (*Aureobasidium pullulans*) and EF7 L2 (*Wickerhamomyces anomalus*) had five to eight-fold higher activity than the *S. cerevisiae* control (EC1118, Figure 4). The best 12 were then further evaluated in sequential fermentations with EC1118. Fermentation performance was evaluated in Gewürztraminer juice (100 mL) and finished wines examined for any impact on terpenes and other volatiles, as measured by GC-MS. Two isolates produced five to eight-fold more, these being *Debaryomyces hansenii* (Arb 6) and *Aureobasidium pullulans* (Arb 70), and were chosen for 20 L Gewürztraminer fermentations (AIM 2).

Figure 4. Beta-glucosidase activity of key environmental yeast isolates. **** statistical significance at $p < 0.0001$.



A separate collection of 77 vineyard isolates from a South Australian vineyard was also screened for β-glucosidase, as well as protease and lipase activity, and sulfide production. Results are fully reported in Lin *et al*., (2020). Importantly this collection contains a number of *K. aerobia*, *K. servazzii*, *A. pullulans* and *M. guilliermondii*, which were found to have increased protease activity under wine relevant conditions. This

is the first report of such findings. Three of these strains were therefore selected for further analysis (AIM 2).

274 yeast isolates were evaluated for their potential to metabolise acetic acid as a means of reducing volatile acidity, a common problem during winemaking, with some juices from contaminated fruit already containing marked concentrations. Small-scale (30 mL) fermentations were co-inoculated with EC1118 in Chardonnay juice spiked with 1 g/L acetic acid (initially containing 0.01 g/L). Among the isolates, 70% were able complete co-inoculated fermentation (<2.5 g/L sugar) thereby showing that many have the potential to contribute to fermentation or not be antagonistic to EC1118. Acetic acid and other major metabolites (i.e., other organic acids, sugars and ethanol) in the finished wines were measured by HPLC. 83 isolates were able to reduce acetic acid to below 1 g/L with five isolates reducing acetic acid by up to 0.4 g/L: these were Arb7, Arb8 (both *Debaryomyces hansenii*), AWRI1032 (*Hanseniaspora occidentalis*), 50-15 and 61-510 (both *Lachancea thermotolerans*). In 1L must trials where monocultures were allowed to ferment for 72 h before inoculation with EC1118, Arb7 and Arb8 fermentations reduced acetic acid by 0.3 g/L, more than EC1118 monocultures (Figure 5). Interestingly, most acetic acid was utilised between 72 and 96 h. To maximise acetic acid reduction, we repeated the 1L trial with an extra inoculation of test stains when more than two thirds of total sugar had been consumed. However, in this trial, acetic acid reduction was equal to the control (EC1118 alone), suggesting that the inoculation regime is important. Unfortunately, we were unable to continue this strategy due to time constraints. Future studies should focus on determining the most useful inoculation strategy. A successful outcome would provide a means by which winemakers could reduce acetic acid in unsound fruit, ideally to below rejection levels.

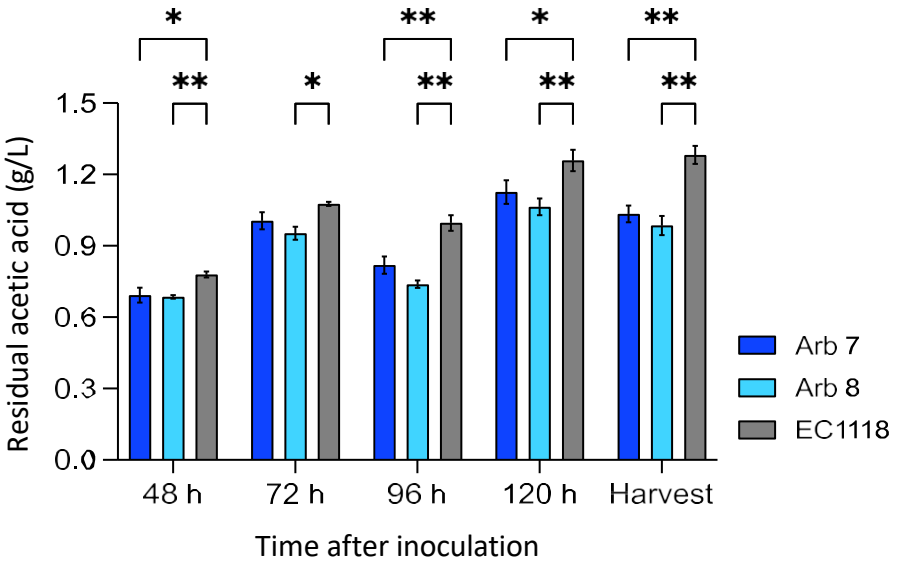


Figure 5. Residual acetic acid in 1 L fermenting red must over time and in finished (Harvest) wines. Fermentations were first inoculated with monocultures of novel environmental isolates (Arb7, Arb 8, 50-15, 61-510), AWRI1032 or the control Lalvin EC1118. After 72 hours a co-inoculum of Lalvin EC1118 was added.

Output 2B Part 2. Proof-of-concept genetically modified yeast strains were produced as an example of the potential for modification of certain metabolic pathways. This work sought to build up a capability to manipulate industrial wine yeast (*Saccharomyces cerevisiae*) using CRISPR/Cas9 technologies and was successfully implemented. Extending on this in the latter stages of the project we also successfully designed and constructed a mutant (EC1118 *can1*) that would be considered non-GMO under the current Australian legislation. This mutant falls within the non-GMO category since the deletion can be achieved without the introduction of foreign DNA, utilising non-homologous end joining (NHEJ) in conjunction with CRISPR/Cas9. Essentially this modification is undertaken without a repair template. Development of this method is important, as it is a critical step towards construction of 'non-GMO' yeast strains suitable for wine production under current Australian regulations.

For the proof-of-concept strains we chose genetic modifications that were previously shown to result in 1) efficient yeast fermentation, 2) enhanced proline metabolism, 3) reduction of ethyl carbamate, or 4) reduction of hydrogen sulfide production. Strains harbouring mutations in genes that affect fermentation efficiency in *S. cerevisiae* (*ECM33* and *SER1*), urea production (*CAN1*), proline (*PRO1* and *PUT2*) and sulfide accumulation (*SUL1* and *SUL2*) were constructed. Modified strains were made with the CRISPR/Cas9 system as this allowed for homozygous gene disruption directly in diploid prototrophic industrial strains instead of needing to rely on haploid derivatives whose phenotypes are often not representative of the industrial strain.

Fermentation efficiency, enhanced nutrient utilisation and reduced production of undesirable compounds were the targets of the use of these strains, ideally resulting in cheaper wine production and enhanced quality. Such benefits could arise through shorter occupancy of fermentation tanks, a reduced need for nutrient additives along with a reduced likelihood of the formation of off-flavours, aromas and other undesirable compounds (such as ethyl carbamate).

We previously showed that disruption of *ECM33*, encoding a GPI-anchored protein with relatively unknown function, resulted in reduced fermentation duration, and thus better fermentation efficiency. Here we disrupted *ECM33* in the commonly used commercial wine yeast Lalvin EC1118 (Lallemand). This mutant displayed a 13-20% reduction in fermentation duration, which was influenced by available nitrogen, and was dependent upon the strain (now tending to aggregate) remained suspended (Lang *et al*., 2021).

There is also a market for yeasts that are *less* fermentation efficient since they allow fermentation to be modulated for a chosen situation. For instance, non-*Saccharomyces* yeast are increasing in popularity, however, many strains show slower growth or fermentation and/or have lower alcohol tolerance. When co-inoculated with *Saccharomyces* to ensure fermentation completion, *Saccharomyces* will often dominate, preventing desirable characteristics of the non-*Saccharomyces* strains being evident. Winemakers currently tackle this problem by sequential inoculation, however, this is ultimately inconvenient. Instead, the availability of a *Saccharomyces* that is slower growing, but still maintains the ability to strongly complete fermentation would be very useful in this context and allow a single co-inoculation. As such, we engineered a loss-of-function mutant of *SER1*, known to

result in slow growth, with CRISPR/Cas9 editing, and examined the profiles of Sauvignon Blanc fermentations when co-inoculated with commercial *Metschnikowia pulcherrima* or *Lachancea thermotolerans* starters. In both cases, use of the *SER1* mutant resulted in timely completion of fermentation while permitting the growth and influence of the non-*Saccharomyces* strain (Lang *et al*., 2022).

Strains with enhanced nutrient uptake and utilisation are of benefit as they would make the best possible use of available, often limiting nutrients and achieve timely fermentation completion. We explored this concept further by constructing strains theoretically capable of making better use of under-utilised proline, abundant in grape juice but inaccessible during anaerobic fermentation. We created mutations (I150T, P247S or E415K) in *PRO1*, a gamma-glutamyl kinase that catalyses the first step in proline biosynthesis. Such mutants are relieved of the repression preventing proline uptake. We examined the impact of these mutations alone as well as in combination with deletion of the mitochondrial targeting sequence of *PUT2* the delta-1-pyrroline-5-carboxylate dehydrogenase (P5C) resulting in rerouting of P5C to the cytosol to produce glutamate from proline and thus feed the Central Nitrogen Pool. Each of these single mutants and combinations did accumulated proline in defined media, in some cases up to five-fold more (Lang, PhD thesis, 2021). Enhanced proline uptake was further confirmed by the fact that mutants survived in the presence of the toxic proline analogue, azetidine-2-carboxylate, a characteristic of cells that exhibit de-repressed proline accumulation. Fermentation dynamics and major metabolite production of mutants in both defined media and Muscat grape juice with sufficient nitrogen (350 or 256 mg YAN/L) were similar to the parent (EC1118). In some cases, fermentations were 8% shorter. Wines are currently being analysed for amino acid and ethyl carbamate content to determine which biochemical pathways were improved. The LCMS analysis required to quantify all alpha amino acids was made available at no cost by our collaborator, Hiroshi Takagi (Nara Institute of Science and Technology, Japan). Local analysis costs were beyond the reduced budget of the project. A publication on the findings will be prepared should resources become available.

The common production of H_2S often occurs in response to nitrogen and sulfur availability. Sulfate is abundant in grape juice and is taken up through both high (Sul1p and Sul2p) and low affinity (Soa1p) transporters. Sulfate is converted to SO_2 and H_2S by the sulfur assimilation pathway, before incorporation into sulfur-containing amino acids, cysteine and methionine. Under nitrogen limitation, the lack of nitrogenous amino acid precursors results in a build-up and release of H_2S. It has long been known that low H_2S producing yeasts have defective sulfite reductase. Recently developed strains based on these observations will, however, often produce high concentrations of SO_2, at times inhibiting malolactic fermentation. We revisited previous work in our laboratory where we targeted sulfate uptake and generated six wine yeast mutants in *SUL1* and *SUL2* that produced both less H_2S and SO_2. Two mutations (*SUL1* [1138 G>R], *SUL2* [1408 G>R]) were selected for introduction into EC1118 using CRISPR/Cas9 gene editing, as they were predicted to impact protein function (Walker *et al*., 2021). Neither mutant had compromised fermentation performance, and each did indeed reduce H_2S production (by 88-92%). Importantly, this occurred with no concomitant increase in SO_2 compared to the parent. This process proves both the causal relationship of these mutations and provides another example of the possibilities of genetic engineering using CRISPR/Cas9 technology. Reconstruction of these strains by non-homologous end joining (NHEJ, see above) would yield

candidates for immediate commercialisation, but is dependent on funding.

Finally, the high affinity arginine permease (Can1p) was targeted for disruption since arginine increases the intracellular pool of urea and, in turn, ethyl carbamate, unwanted in wine as it is a carcinogen. As described above, *CAN1* disruption was undertaken using NHEJ mediated CRISPR/Cas9 editing as an example of how yeast genomic DNA can be edited without introducing extraneous DNA, thereby designating such strains as non-GM (see AIM 4, Output 4B for further details). The two *can1* mutants (frame shift mutations at amino acids 67 and 69) were found to significantly reduce urea production during fermentation of a defined medium containing arginine, thus validating the technique (Lang, PhD thesis, 2021). The fermentation duration of these mutants in genuine grape juice (Chardonnay and Muscat, each with added arginine) was not altered compared to the parent. A modest amount of funding would permit work to determine residual urea and ethyl carbamate in terminal fermentation samples and prepare the findings for publication.

Few studies have focused on the possibilities of engineering wine yeast strains using the CRISPR/Cas9 protocol and thus this early attempt to adapt the technology to wine yeast was very important in highlighting the possibilities offered by these techniques for the sector. Not only have we shown that these techniques can be used for a wide range of desirable attributes, but also we demonstrate methodology that is currently directly applicable to the wine industry since the resultant strains are not considered GMO under the current Australian legislation (AIM 4, Output 4B).

Output 3E. Development of methods to detect potential inhibitory agents of yeast against the spoilage yeast *Brettanomyces* was achieved. Here a simple plate-based screen was designed to detect growth inhibition of *Brettanomyces* by individual non-*Saccharomyces*. This method utilised agar overlays inoculated with *Brettanomyces bruxellensis* AWRI1613 to assess the inhibition by isolates from our collection. Zones of clearing/growth inhibition indicated antimicrobial activity (Figure 6). In brief, the indicator and test strains were grown to confluency in rich liquid medium (YPD), the indicator strain was then mixed with cooled, molten malt agar and poured into rectangular plates. Test strains were then spotted onto the indicator agar and incubated at 22 °C for 2-4 days before checking for a halo of growth inhibition.

274 yeast isolates were examined for their capacity to inhibit *Brettanomyces bruxellensis* on malt agar. Twenty-three yeast and an isolate subsequently identified as a bacterium from our collection inhibited *Brettanomyces* growth, with ten doing so in both aerobic and, importantly, anaerobic conditions. The latter were: Arb 60 (*Gibbsiella sp.*), Arb 67 (*S. cerevisiae*), Arb 72 *(Rhodotorula sp*), YD4 (*Wickerhamomyces anomalus*), M2/1 (*Torulaspora delbrueckii*), 61-245 (*Lachancea thermotolerans*), T40, (*Hanseniaspora sp*. or *Taiwanica*), T52 *(Sirobasidiaceae* sp.), T76 (*Naganishia sp.* or *Cryptococcus sp*.), and T78 (*Bulleromyces sp.*).

Arb 67, being an *S. cerevisiae*, was the most directly applicable to industry, and thus was chosen for further study. It was also of interest due to its unique source - the *Brachychiton australis* (Broad-leaved bottle tree). We tested the ability of Arb 67 to reduce *Brettanomyces* spoilage (AWRI1613) in 100 mL Cabernet Sauvignon juice fermentations into which these yeast had been co-inoculated at various ratios (0.1, 1 and 10%). Recognising that the reduction of volatile phenols (VPs) is most important in managing *Brettanomyces*, these compounds were measured by GC-MS in terminal

fermentation samples. Unfortunately, this analysis revealed no reduction in 4 ethyl-phenol or 4 ethyl-guaiacol in this particular juice in the presence of Arb 67 (Figure 7). Since the experimental conditions, in particular, competition amongst the other yeast present, may be minimising the impact of Arb 67 or changing fermentation dynamics, we sought to separate the yeasts and use spent media. The latter should still be inhibitory by virtue of containing the inhibitor or lacking a key metabolite. Accordingly, *Brettanomyces* cultures were inoculated into spent media from the test isolates. A preliminary experiment in 0.2 mL cultures where spent media was used as part of the culture medium from each of the 34 test strains (initially cultured in Chemically Defined Grape Juice Medium, CDGJM) highlighted that five strains warranted further analysis given they significantly inhibited growth (OD_{600}) of *Brettanomyces* after an 11 day incubation. The bacterial strain (*Gibbsiella sp*.) completely inhibited growth.

The impact of the five yeast strains on *Brettanomyces* was further analysed in an experiment where they were again cultured for 48 h in CDGJM, the spent medium collected, diluted (1:1) with fresh CDGJM and inoculated with *Brettanomyces*. Optical density was tracked over the course of 20 days revealing that spent medium from 72-29 (*L. thermotolerans*) was able to delay the onset of *Brettanomyces* growth by ~24 h, in comparison to a *Brettanomyces* monoculture control (spiked with 3 or 6% ethanol). Interestingly, spent medium from other strains, in particular *Schizosaccharomyces pombe,* enhanced the growth of *Brettanomyces*. Time and funding constraints necessitated that this work be terminated at this point. Future work is, however, warranted and should include detailed determination of the basis of *Brettanomyces* growth and VP inhibition by these (and other) isolates. Ideally this work would lead to identification of isolates that are compatible with co-inoculation/fermentation or could be used as the source of an extract, in each case as a means of suppressing the adverse impacts of *Brettanomyces*.

As an additional line of investigation in this theme, the group also preliminarily examined propolis. This resinous mixture produced by honey bees is proposed to possess many and varied anti-microbial properties. Propolis was therefore included in plate-based inhibition assays, similar to the above. Clear zones of inhibition were observed with both small pieces of propolis and propolis tincture. As we were unable to recruit a student to this topic, this line of investigation was also abandoned at this point. Again, further investigation of this very promising result is warranted.

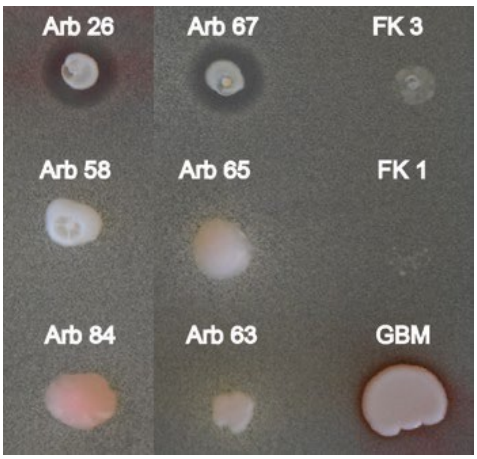


Figure 6. Two yeast strains, Arb 26 (*Penicillium glabrum*) and Arb 67 (*Saccharomyces cerevisiae*) inhibit growth of AWRI1613 (*Brettanomyces bruxellensis*) on malt agar.

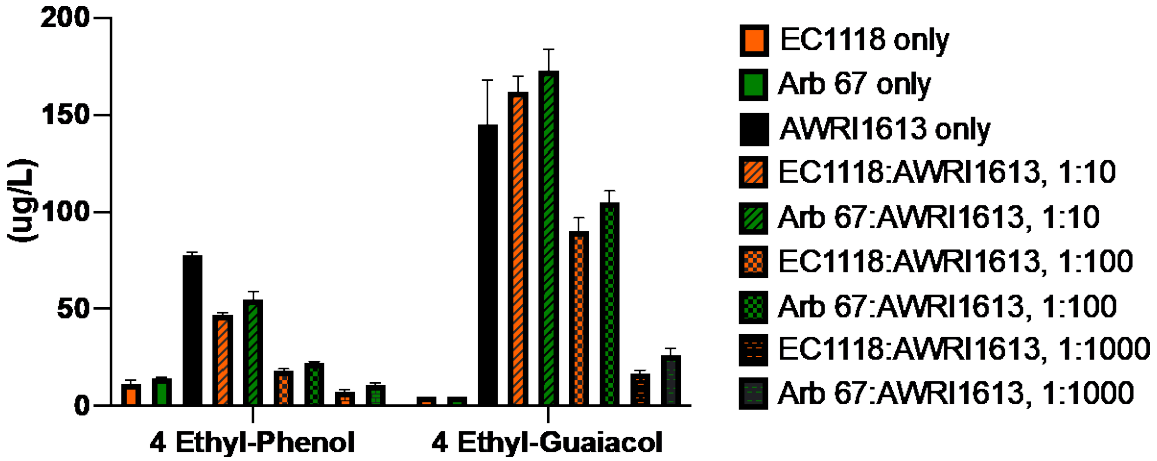


Figure 7. Residual volatile phenols in finished wines made with increasing ratios of test (Arb 67) or control (EC1118) to *Brettanomyces bruxellensis* (AWRI1613).

Publications arising from AIM1:

1. Gardner, J.M., Walker, M.E., Zhang, J., Van Holst, N., Jiranek, V. (2020) Searching new frontiers for winemaking microbes with unusual and useful activities. *Australian Grapegrower Winemaker*, 680, 87-88.

2. Lin, M. M-H., Boss, P. K., Walker, M. E., Sumby, K. M., Grbin, P. R., Jiranek, V. (2020) Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures. *International Journal of Food Microbiology,* 312. doi.org/10.1016/j.ijfoodmicro.2019.108373.

3. Van Holst N., Walker M.E., Watson T.L. Jiranek V. 'TeeBot' (2021) A high throughput robotic fermentation and sampling system. *Fermentation*, 7, 205. doi.org/10.3390/fermentation7040205.

4. González-Alonso, I., Walker, M.E., Vallejo-Pascual, ME. Naharro-Carrasco G., Jiranek V. (2021) Capturing yeast associated with grapes and spontaneous fermentations of the Negro Saurí minority variety from an experimental vineyard near León. *Scientific Reports* 11, 3748.

5. Lang, T., Walker, M., Jiranek, V. (2021) Disruption of *ECM33* in diploid wine yeast EC1118: Cell morphology and aggregation and their influence on fermentation performance. *FEMS Yeast Research*. 21, Issue 5, August 2021, foab044.

6. Walker, M.E., Zhang, J., Sumby, K., Lee, A., Houlès, A., Li, S-J., Jiranek, V. (2021) Sulfate transport mutants affect hydrogen sulfide and sulfite production during alcoholic fermentation. *Yeast* 38(6):367-381. doi: 10.1002/yea.3553.

7. Tom Lang, PhD thesis, the University of Adelaide (2021). Yeast strain construction using CRISPR/Cas9: A tool for research and strain optimisation.

8. Lang, T.A., Walker M.E., Boss, P.K., Jiranek V. (2022) Effect of 'loss of function' mutation in *SER1* encoding 3-phosphoserine aminotransferase in wine yeast EC1118: Fermentation characteristics when co-inoculated with non-*Saccharomyces*. *OenoOne* 56(2), 47-6.

9. *Yingxin Liu, Masters of Viticulture and Oenology (2021) Identification of high sulfur dioxide tolerant lactic acid bacteria for malolactic fermentation.

AIM 2: Information on the impacts of the new strains on wine composition, processing and stability. Recommendations on the use of new strains (must composition and microbial integrity and wine style)

Contributors

Research staff: Drs Michelle Walker, Jin Zhang, Jennie Gardner, Krista Sumby, Joanna Sundstrom, Mr Nick van Holst and Prof Vladimir Jiranek. These students also contributed: Ms Mandy Lin, Ms Emily Hocking, Mr Yiming Huo, Ms Chitra Nair.
Collaborator: Dr Paul Boss, CSIRO Agriculture and Food, Waite Campus, Urrbrae, SA

Background

A fundamental requirement of winemaking is reliable and predictable fermentations. Reliable in terms of trouble-free completion and predictable in terms of fermentation duration, yeast/bacterial nutritional needs and appropriate production of desirable and undesirable metabolites. To this day, winemakers still report fermentations that are too slow or fail to complete, often requiring amelioration with nutrients or chemicals, heating, modification of acidity or reinoculation, which is both time consuming and costly. Winemakers continue to seek to reduce additives (incl. nutrients) or are pursuing low temperature fermentation for stylistic reasons, and are having to handle more juices that are problematic (e.g. higher in sugar, lower in nutrients, more disease affected/carry higher loads of spoilage microbes/requiring more SO_2). While there is a large number of commercial yeast and bacteria available, not all have the ability to conduct fermentation reliably under these conditions.

In this study, we have built upon the research achieved in Aim1 and Wine Australia-funded project UA 1302 by further testing eight new microbial strains in larger volumes, allowing for a more in-depth evaluation of their performance and the impact they would have in addressing a number of winemaking needs. Strains selected for further analysis were three strains improved through directed evolution, as reported in UA 1302, and five with enhanced enzymatic activities as identified in this study: two with enhanced β-glucosidase activity and three with protease and lipase activities. Importantly confirmation of the ability of these strains to perform reliably in industrial conditions and when in the presence of common stressors, has been undertaken. Evaluation of strains at these larger scales allows for a more comprehensive analysis, improving the predictability of performance in a commercial setting. The fermentation performance of cultures in a range of juices as well as various metabolite compositions and effects on sensory profiles of finished wines have been analysed.

Further to this we have also examined the impact of juice dilution upon fermentation performance, since Australian legislation recently changed to allow additions of water to must under specific conditions. The aim of this change was to help winemakers mitigate the impacts of juices with high sugar concentrations, in an attempt to facilitate processing. Dilution with water is allowable pre-fermentation to juices with apparent Baumé of 13.5° or more, as long as the final °Bé is not less than 13.5 °Bé. In this study we examined what impact juice dilution had on both primary and secondary fermentation, both in terms of fermentation durations and also impact on metabolites in finished wines. Further to this, we also examined the impacts of nutrient

supplementation to diluted juices in an attempt to replenish diluted nutrients and alleviate any undesirable outcomes. This part of the project sought to provide further understanding of the impacts (both positive and negative) of this process rather than simply facilitating fermentation progress. This information will aid winemakers in the decision about when, how and what to expect if undertaking this practice.

Summary of Outcomes

Evaluation of fermentation performance at small industrial scale (20-40 L) by eight key yeasts highlighted by screening undertaken in this project (AIM 1) and previously in this lab revealed that all strains are worthy of further investigation and contribute individual desirable processing advantages or attributes to wines made from them.

The fermentation durations of evolved strains FM16_C7H, C7H_B4 and RM5-15, each *S. cerevisiae*) in these experiments, were very similar to control strains. It is promising that they perform as well as EC1118, one of the most widely used commercial strains, and we expect them to excel in juices where nutritional needs might not be met with supplementation. Further to this, sensory profiling by the expert panel was very positive about wines made with evolved isolates, particularly C7H_B4, whose aroma was described as more perfumed and complex with darker blackberry fruits, better body and length and increased tannin perception. Statistical analysis from individual rankings also supported wine made with C7H_B4 being of higher aromatic quality to wine made with 71B, but not to EC1118 or parent strains. RATA analysis undertaken with the public, however, found no significant differences. The colour of wines made with C7H_B4 was also increased in blue to yellow and GC-MS analysis revealed many compounds were significantly different, those detected above their aroma threshold being isobutyl acetate, ethyl hexanoate and ethyl octanoate.

Similarly, non-*Saccharomyces* strains Arb 6 (*Debaryomyces hansenii),* Arb 70 (*Aureobasidium pullulans*)*,* PF_9_W20 (*Kazachstania servazzii*) and two *Kazachstania aerobia strains* (PF_8_W29 and PF_9_W18) were also capable of robust fermentation in comparison with the commercial strains and were found to contribute positively to aromas. The increased β-glucosidase activity of Arb 6 and Arb 70, resulted in higher amounts of specific terpenes (nerol and β-citronellol) and, in turn, enhanced floral aromas, specifically lychee and rose and perfumed aromas. Each *Kazachstania* isolate also contributed attributes such as, rose, honey and fruity aromas and upon chemical profiling these were associated with elevated phenylethyl acetate and isoamyl acetate. Genome sequences of two of these *Kazachstania* isolates were then assembled and annotated and an *in silico* analysis revealed duplications of genes that are likely to contribute to this elevated ester production.

We have also shared three evolved lactic acid bacteria strains of SB3 (A90, 2-49 and 3-83) with the commercial starter culture company Laffort for further investigation. Their studies have so far reported few if any improvements in comparison to the parent strain, however, these are yet to be evaluated in examples of highly stressful environments to which they were evolved.

In the second part of this Aim, juice dilution was found not to impede microbial fermentation, but as expected, instead resulted in reduction of both alcoholic and

malolactic fermentation duration and changes to the chemical composition of wines. Nutrient addition was also effective in shortening fermentation duration in some conditions. Shortened alcoholic fermentation duration reported here would be of great interest to the Australian winemaking community as a reduction of up to 42% (or 28% for dilutions to 13.5 °Bé or above in accordance with Australian standards) would be considered an extremely useful outcome. Thus, use of juice dilution or addition of nutrient could allow for more efficient use of often limited tank space. Winemakers do, however, need to carefully consider the impact on wine aroma and flavour. As shown here, juice dilution resulted in changes (mostly reductions) to the concentrations of 22 volatile compounds in three or more treatments, with a single treatment resulting in modifications to as many as 15 different volatile compounds.

Outputs and Performance Targets: AIM 2A

Note, the following Results from this Aim are divided into two parts, A and B to report separately on the work addressing the impact of juice dilution on fermentation.

Output 1C. Data on the fermentation performance of the most promising one or two DE yeast strains from UA 1302 to complete strain evaluation.

- Evolved yeast strains selected previously for rapid and reliable fermentation will be evaluated in juice trials at the UA winery (50 kg red must fermentations in triplicate). Such volumes will allow provision of sensory profiles via triangle tests, preference tests and RATA (to be conducted in July-August 2018).

Output 2C. Data on the sensory impact of the most promising one or two DE yeast strains from UA 1302 to complete strain evaluation and publication.

- Sensory profiles of evolved yeast strains selected previously for rapid and reliable fermentation will be undertaken via triangle tests, preference tests and RATA. If funding allows, this will also include analysis of 20L white juice fermentations.

Output 3A. Two new yeast or LAB strains with desirable attributes and data on their evaluation available for the wine industry. Information on the impacts of the new strains on wine composition, processing and stability

- Strain performance will be evaluated in comparison to current industry best practice for each target trait under lab and pilot-scale (>20 L) conditions.

Output 4A. Data on the fermentation performance of the best yeast/LAB strains (20L) in juice.

- Liaise with starter culture suppliers for in-house trials
- Conduct further vintage (2021) trials (if necessary) with promising strains

Output 4F. Up to four new yeast or LAB strains with desirable attributes and data on their evaluation available for the wine industry

- Provide data on strain evaluations under lab scale (<20L) conditions
- Offer strains to yeast suppliers for evaluation prior to commercialisation

Results and Discussion

Output 1C. Data on the fermentation performance and sensory impact (Output 2C) of the most promising DE yeast strains from UA 1302 and this project (Output 4A and 4F) were generated. Small scale, industrial fermentations were performed with three *Saccharomyces* strains improved through directed evolution (ex project UA 1302) and five novel non-*Saccharomyces* yeast collected from the environment in this project and shown in small scale trials to have potential for desirable fermentation attributes (Table 1). The non-*Saccharomyces* yeast were from diverse species not previously reported to be beneficial to wine fermentations i.e. Arb 6 (*Debaryomyces hansenii)*, Arb 70 (*Aureobasidium pullulans)*, PF_8_W29 and PF_9_W18 (*Kazachstania aerobia)* and PF_9_W20 (*Kazachstania servazzii)*.

Table 1: Yeast isolates chosen from small-scale studies for 20L trials in juice.

Isolate	**Species**	**Source**	**Attribute in small scale studies**
FM16 C7H	*Saccharomyces cerevisiae*	Evolved strain of L2056, (McBryde *et al*., 2006, Jiranek laboratory)	Efficient sugar catabolism
C7H_B4	*Saccharomyces cerevisiae*	Colony isolate from FM16 C7H evolved for 158.3 generations. (UA 1302)	Efficient sugar catabolism
RM5-15	*Saccharomyces cerevisiae*	Colony isolate from sporulated and random mated population (C7H x 71B) evolved for 37 generations. (UA 1302)	Efficient sugar catabolism
Arb 6	*Debaryomyces hansenii*	Sap of the White cedar tree, *Melia azedarach* in the Waite Arboretum, Urrbrae, SA	High glucosidase activity
Arb 70	*Aureobasidium pullulans*	Flowers of the Broad Leaved Bottle Tree, *Brachychiton australis,* in the Waite Arboretum, Urrbrae, SA	High beta-glucosidase activity
PF_8_W29	*Kazachstania aerobia*	Uninoculated fermenting Shiraz grape must, Hardy's vineyard, 2007, McLaren Vale, SA	High enzymatic activities, ethanol tolerance, low H_2S
PF_9_W18	*Kazachstania aerobia*	Uninoculated fermenting Shiraz grape must, Hardy's vineyard, 2007, McLaren Vale, SA	High enzymatic activities, ethanol tolerance, low H_2S
PF_9_W20	*Kazachstania servazzii*	Uninoculated fermenting Shiraz grape must, Hardy's vineyard, 2007, McLaren Vale, SA	High enzymatic activities, ethanol tolerance, low H_2S

The three *S. cerevisiae* strains were evolved using different protocols, as described in the Final Report for project UA 1302: Fit for Purpose, Yeast and Bacteria via Directed Evolution. In brief, FM16 C7 was evolved in batch culture in a bioreactor with oxidative stress, C7H_B4 was further evolved from FM16 C7 in bioreactor batch culture with ethanol content increasing from 8 to 12.5%, and RM5-15 is a colony isolate first selected from random mating of C7H and 71B spores and then further evolved in batch culture in 100 mL flasks with increasing contents of sugar (100–320 g/L), ethanol (0–6%) and medium chain fatty acids (octanoic and decanoic acids, 3–6 mg/mL) and decreasing pH (3.5–3.1) and yeast assimilable nitrogen (450–350 mg/L).

Fermentations with these DE strains were undertaken by WIC Winemaking Services in the Hickinbotham Winery at the Waite Campus, as outlined by the standard small batch red winemaking protocol (Appendix 5). In brief, four replicated 40 kg lots of Adelaide Hills Merlot from the 2018 vintage were fermented with each yeast (evolved strains FM16 C7, C7H_B4 and RM5-15). Commercial strains Lalvin EC1118 and Lalvin 71B (both from Lallemand), were included for reference. In all, 20 separate wines were made. Each fermentation was co-inoculated with the commercial lactic acid bacteria Lalvin VP41 (Lallemand) after five days. Alcoholic fermentation was complete after 13 days, when wine was separated from grape pomace (pressed) and stored in separate kegs for MLF to completed (16 days). Wines were racked seven days after completion of MLF, cold settled and then bottled after four months. The fermentation profiles of each strain under these conditions were similar (Appendix 5, Figure 19). Pre-bottling analysis was undertaken and basic wine parameters varied as follows: alcohol, 14.3-14.6%; glucose and fructose, <0.3 g/L; pH, 3.51-3.55; TA, 5.6-6.4 g/L; malic acid, <0.05 g/L; VA, 0.25-0.56; free and total SO_2 of 45-49 and 76-93 mg/L, respectively.

Basic wine metabolite analysis was undertaken by HPLC with no significant differences found (i.e., < 5% variance). GC-MS analysis of volatiles was undertaken in collaboration with Dr Paul Boss, with the methodology and results described in Lin *et al*., (2020).

Detailed sensory profiling of wines (Merlot) made with the three evolved (C7H, C7H_B4 and RM5-15; UA 1302) and two commercial (EC1118 and 71B) yeast strains was undertaken by Assoc. Prof. Sue Bastian's (UA) sensory laboratory five months after bottling. Ten industry experts examined the wines both individually, and as a group where extensive notes were taken in a round table discussion. Excitingly, many preferred wines had been made with the evolved strains, in particular C7H_B4, which exhibited desirable aromatic qualities. The wine experts completed two tasks. In the first task, wine experts were asked to rate the aroma and flavour of the 20 Merlot wines and score them using a 20 point wine quality scoring system. For the second task, the experts were asked to rate the quality against a reference wine using a seven point scale ranging from 1 (much lower quality) to 7 (much higher quality), with 4 representing the same quality. The reference wine was a blend of 500 mL each of the four EC1118 replicates. Wine samples (30 mL) were provided in black, covered ISO glasses at ambient temperature (21–23 °C) labelled with random four digit codes. The experts were asked to make comments and notes for each task explaining their reasoning for their answers.

The results of the ANOVA from the expert panel sensory feedback showed that significant differences between yeast treatments were only observed for the aroma attribute (Tables 1 and 2). *Post hoc* tests revealed that C7H_B4 scored higher compared to 71B, but with no difference between the other wines.

Quality scores from the seven point scoring system demonstrated that the quality of 71B was significantly lower compared to C7H-B4 (Table 3). Importantly there was no significant difference between the reference wine, when compared to itself.

Table 1: one-way ANOVA results. Significant differences are marked in bold.

Attribute	F (4,195)	p-value
Aroma	**2.945**	**0.022**
Body / Texture	0.183	0.947
Total Acidity	0.763	0.551
Flavour / Taste	0.328	0.859
Length	0.238	0.917
Astringency	0.712	0.585
Overall Quality	0.458	0.767
SCORE	0.281	0.890

Table 2: Mean attribute potential scores. Lower case letters indicate significant differences at p<0.05 based on Tukey post-hoc comparison.

Attribute	**71B**	**C7H**	**C7H_B4**	**EC1118**	**RM5-15**
Aroma	2.23[a]	2.73[ab]	3[b]	2.82[ab]	2.55[ab]
Texture	3.05	2.93	2.95	3.03	2.93
Acidity	0.74	0.66	0.64	0.66	0.74
Flavour/Taste	1.85	1.95	1.85	1.80	1.72
Length	2.05	2.05	1.95	2.10	2.03
Astringency	0.66	0.61	0.58	0.65	0.56
Overall Quality	2.07	2.35	2.30	2.32	2.15
Score	12.65	13.28	13.26	13.39	12.68

Table 3: Quality means relative to reference. Lower case letters indicate significant differences at p<0.05 based on Tukey post-hoc comparison.

Attribute	**71B**	**C7H**	**C7H_B4**	**EC1118**	**RM5-15**
Quality	3.35[a]	3.45[ab]	4.27[b]	4.03[ab]	4.08[ab]

The Rate-All-That-Apply (RATA) technique was utilised for sensory profiling of wines by 53 participants from the general public. RATA analysis of the 20 Merlot wines occurred across two 2 hour sessions, a week apart in May 2019. Red wine consumers were recruited from the School of Agriculture, Food and Wine staff and post-graduate students who had previous red wine experience (36 female, average age 41 yr). Participants gave written informed consent prior to the tasting.

The first 10 wine samples (30 mL were presented in the first session, monadically, blind and in randomised order across panellists, served in International Standards Organisation (ISO) covered black glasses at room temperature labelled with random 4-digit codes at each tasting. Tasters were asked to rate the intensity only of the aroma, flavour and mouthfeel attributes they perceived, using their own Merlot, and or, red wine experience as their reference/comparison point. Panellists rated 25 aroma intensity, three taste, 25 flavour intensity, six mouthfeel intensity and two length of aftertaste attributes. In the second session, a week later, the participants were presented with the second set of 10 red wine samples (30 mL) and asked to rate the intensity of the same attributes on aroma, flavour, mouthfeel and length of aftertaste, following the same protocol as the first session. Both tasting sessions were conducted in individual booths in the Sensory lab at the University of Adelaide, Waite Campus.

Data were analysed using one-way ANOVA to identify significant differences between wine samples and wine yeast strains for colour measurements and expert quality scoring. For RATA, two-way ANOVA with participants as random factor and sample/yeast as fixed factor was used to test for significant differences for all attributes. PCA was used to visualise significant attributes. Figure 8 indicates that there was more variability between reps within treatment than between treatments, with the exception of C7H_B4, reps 1, 2 and 3, which have all grouped together. To a lesser extent also C7H rep 1,3 and 4.

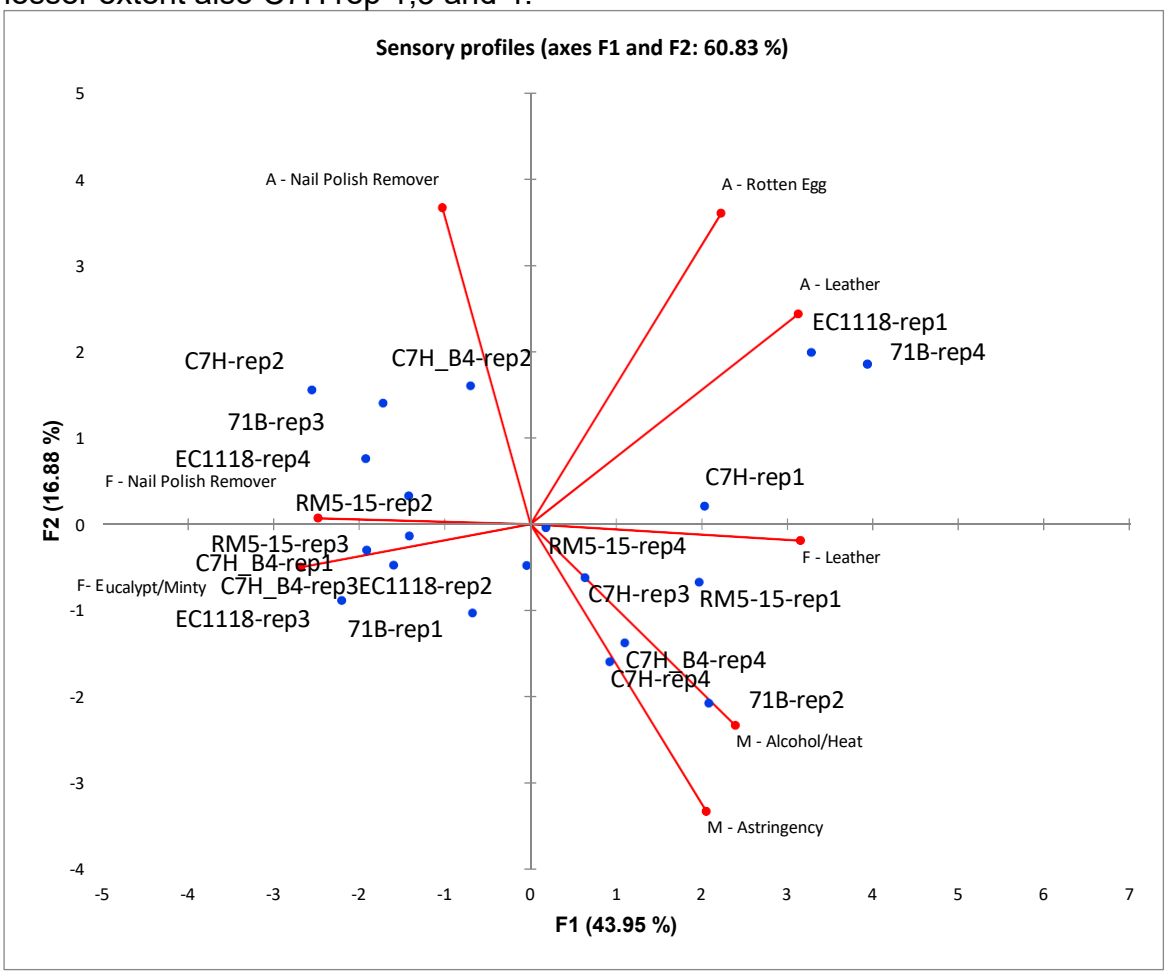


Figure 1. Principal Component Analysis (PCA) of statistically significant means of sample configuration wine reps.

Figures 8 shows the results of PCA analysis of the RATA sensory attributes overlaid with the expert quality score as supplementary data. Factor 1 and 2 explain 60.83% of the variability of the samples in the sensory space. The wines on the lefthand side had more eucalypt while wines on the righthand side were associated with leather. Wines in the upper half of the plot were more intense in VA (nail polish) and rotten egg (H_2S) aroma. Wines in the bottom half were more associated with astringency and alcohol. Investigating differences in more detail it was found that, despite being significant, the extent of the differences and the intensity of the faults were small.

Table 4: Statistically significant mean values between treatments for aroma (A) and flavour (F) based on two-way ANOVA ($p < 0.05$), different lowercase letters indicate significant differences between samples based on Tukey *post hoc* comparison ($p<0.05$).

	71B	**C7H**	**RM5-15**	**EC1118**	**C7H_B4**
A - Nail Polish Remover	0.928^{ab}	1.000^{a}	1.017^{a}	0.867^{ab}	0.706^{b}
F - Red Fruit	2.850^{ns}	3.056^{ns}	3.011^{ns}	2.706^{ns}	2.733^{ns}
M - Astringency	3.489^{ab}	3.533^{a}	3.428^{ab}	3.378^{ab}	3.178^{b}

Chemical profiling of these wines was also undertaken by HPLC. Phenolics and colour were analysed in-house as well as volatiles by GC-MS (Paul Boss). Colour analysis was performed with a Cintra 4040 (GBC Scientific Equipment) double monochromator spectrometer. Results were expressed as CIE L*a*b values:

- L* = Lightness from 0 (Black) to 100 (White)
- a* = Green (-) to Red (+)
- b* = Blue (-) to Yellow (+)

ANOVA results indicate significant differences between yeast treatments for all three colour parameters (Table 5). Further examination of the *post hoc* comparisons show that CH7 resulted in darker (lower L* value) and slightly more red (higher a* value) compared to all other treatments. CH7_B4 and EC1118 had slightly higher b* values compared to CH7, however, these differences were less than 0.5 points and therefore most likely not relevant.

Table 5: One-way ANOVA colour analysis by yeast treatment.

Parameter	F (4/35)	p-value
L*	18.14	<0.001
a*	20.82	<0.001
b*	4.79	0.004

Table 6: Mean values and post-hoc comparisons of colour parameters by yeast treatment.

Parameter	CH7	RM515	71B	CH7-B4	EC1118
L*	79.48^{a}	81.23^{b}	81.25^{b}	81.86^{b}	81.98^{b}
a*	23.33^{a}	21.17^{b}	21.33^{b}	20.48^{b}	20.38^{b}
b*	0.7^{ab}	0.77^{ab}	0.64^{a}	0.98^{b}	0.95^{b}

In summary, the expert panel was very positive about wines made with evolved isolates, in particular C7H_B4, for which they found the aroma to be more perfumed and complex with darker blackberry fruits, better body and length and increased tannin perception. Statistical analysis also supported this strain yielding higher quality wine when considering aroma. RATA analysis using the public, however, found no significant differences between the wines. The colour of wines made with C7H_B4 was also slightly increased in blue to yellow and GC-MS analysis revealed many compounds were significantly altered in wines made by C7H_B4 (Appendix 5, Figures 20-23). Those above their aroma threshold being isobutyl acetate, ethyl hexanoate and ethyl octanoate, while below threshold were, methyl octanoate, isoamyl hexanoate, hexanoic acid and octanoic acid. Some compounds were also found to be

decreased in the C7H_B4 mutant, these being hexyl acetate, isobutyl acetate, ethyl 2-methylbutryate, 4-methyl-1-pentanol, 3-methyl-1-pentanol, acetic acid, 2-methylbutanoic acid and 2-phenylethyl acetate. Future work should test these strains in different grape varieties/wine styles and under limitations to which they were selected to be resistant. Further testing at the industrial scale would also prove useful in securing licencing arrangements to see them commercialised and made available to the Australian industry. Candidate yeast starter culture companies would necessarily participate in the design of such trials and we would benefit from our existing and extensive links with these companies.

The fermentation performance and sensory attributes of wines made with two yeast isolated from the environment during this project (Arb 6, *Debaryomyces hansenii* and Arb 70, *Aureobasidium pullulans*) were also evaluated in 20 L juice fermentations (2020 Gewürztraminer, Barossa Valley, Treasury Wine Estates). Again, as these were non-*Saccharomyces*, fermentations were sequentially inoculated with EC1118 after 72 h. Fermentations progressed efficiently with all sugars utilized within 10 days (Figure 9). Sensory and GC-MS analyses were undertaken on the finished wines, with statistical and PCA of results (Figure 10). We found that the non-*Saccharomyces* contributed significantly to the aroma and chemical profile of the wines. Here the increased β-glucosidase activity resulted in higher production of specific terpenes (nerol and beta-citronellol) and, in turn, enhanced floral aromas, specifically lychee and rose and perfumed aromas, while wines made with just EC1118, were more tropical, ginger and had bruised apple aromas. These strains would be useful in winemaking and brewing where more pronounced fruity and floral aromas are desired. Future work should trial these strains with a focus on evaluation of the production of terpineols in different grape varieties and with different co-inoculation strategies.

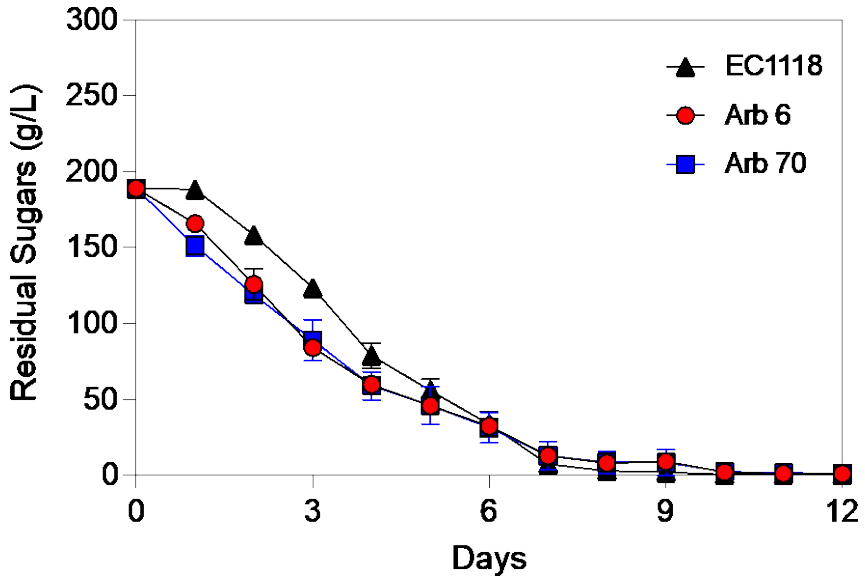


Figure 9. Residual sugars in fermentations of Gewürztraminer (20 L) with high β-glucosidase yeasts, Arb 6 (*D. hansenii*) or Arb 70 (*A. pullulans*), compared to EC1118 (*S. cerevisiae*). Values are the average of triplicates (error bars = stdev).

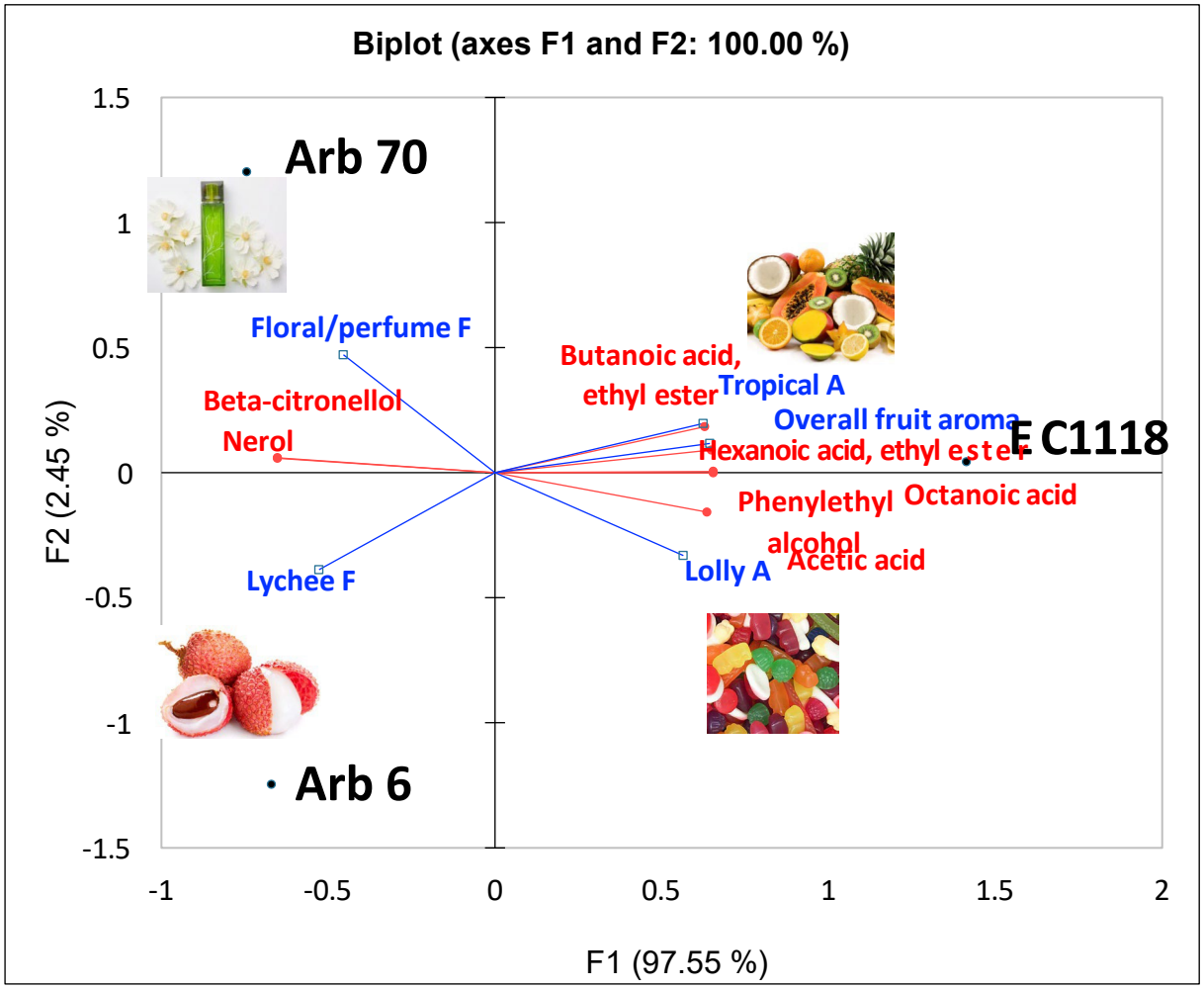


Figure 10. Principal component analysis of the sensory attributes of wines made with high β-glucosidase yeasts, Arb 6 (*D. hansenii*) or Arb 70 (*A. pullulans*), in comparison to EC1118 (*S. cerevisiae*).

Additional fermentation trials were conducted to define the fermentation attributes and sensory impact on wines made with the *Kazachstania species* found here with proteolytic and lipase activities. These were originally isolated from a South Australian vineyard and resulted in wines with increased esters, phenylethyl acetate and glycerol. Two *Kazachstania aerobia* isolates (KAW18 and KAW29) and *Kazachstania servazzii* isolate KSW20 were previously evaluated as potential starter cultures for sequential fermentation using 100 mL filter-sterile Viognier juice (Lin *et al*., 2020). The fermentation attributes of these three *Kazachstania* isolates in Shiraz, Merlot and Muscat were further evaluated in small-scale industrial fermentations (Lin *et al*., 2022). Each *Kazachstania sp*. was sequentially inoculated with the commercial wine yeast EC1118 (*S. cerevisiae*). Sensory and chemical data with statistical and Principal Component Analyses revealed that each *Kazachstania* contributed distinct desirable aroma attributes such as, rose, honey and fruity aromas and these were associated with elevated phenylethyl acetate and isoamyl acetate. To characterise these promising isolates further, the genome sequences of two isolates have been assembled and annotated. An *in silico* analysis of genes that may contribute to ester biosynthesis has been completed and published (Lin *et al*., 2023). Results from this Output have also been presented as a poster at the 18th AWITC (poster #85), with the wines being available for tasting at the University of Adelaide booth in the AWITC Trade

Display in 2022, to inform industry and attract starter culture company interest.

During screening of the environmental collection of yeasts for the capability to utilise acetic acid, we also conveniently collected data on their capability to utilise sugars in wine-like environments, a good indicator of their fermentative capacity and potential as commercial starter cultures. In this way we selected three yeasts that completed fermentation during small-scale screening. These turned out to be *S. cerevisiae*, despite having been recovered from a novel niche, a native bee 'hotel'. We analysed their fermentation attributes in replicated, small-scale fermentations (100 mL) in CDGJM and found that both their sugar utilisation profiles and impact on major metabolite concentrations of finished wines were similar to the common commercial strain, Lalvin EC1118 (Figure 11). Preliminary genetic comparisons suggest that the isolates are distinct from EC1118. As such, they warrant further investigation given their EC1118-like fermentation robustness, but with potentially novel sensory contributions.

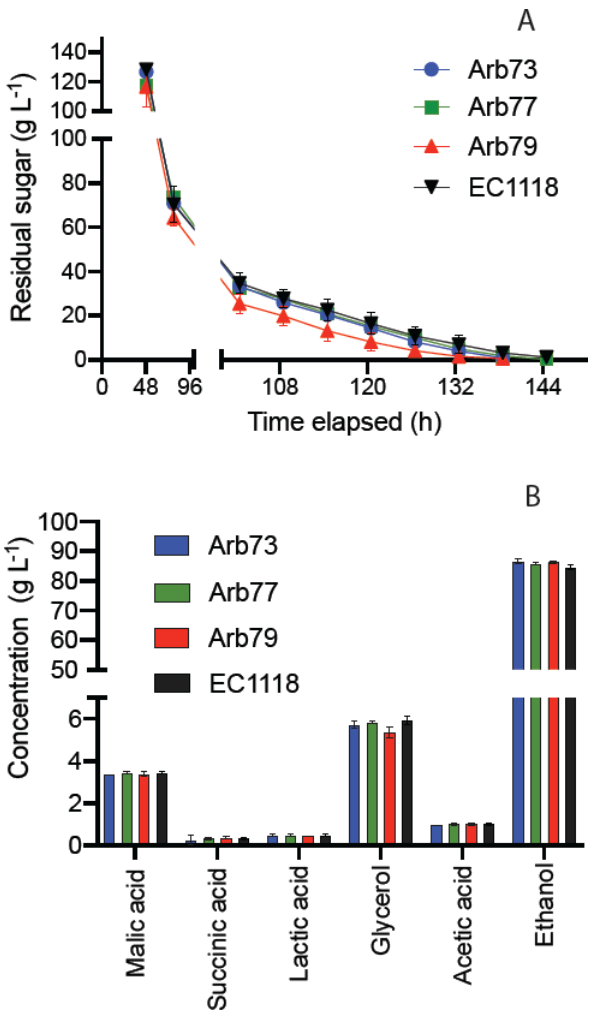


Figure 11. Novel yeast strains Arb 73, Arb 77 and Arb 79 (all *S. cerevisiae*) isolated from a native bee 'hotel' are able to ferment sugars similarly to a commercial yeast Lalvin EC1118 in chemically defined grape juice (A) resulting in no adverse changes to the major metabolites esp. acetic acid (B).

Output 3A. New LAB strains with desirable attributes and data on their evaluation has been made available for the wine industry. In a previous project (UA 1302) an *Oenococcus oeni* isolate (KS21#6), evolved from a pre-commercial Lallemand strain, and others derived from Laffort's SB3 (in cryo-storage) displayed robust malolactic fermentation in harsh multi-stressor conditions in laboratory scale trials thereby making these candidates for further commercial development. In light of this, three evolved strains of SB3 (A90, 2-49 and 3-83) were provided to Laffort in Nov 2018, for genotyping and evaluation in synthetic wine (13% alc, pH 3.5, 3 g/L malic acid). In our analyses A90 and 2-49 outperformed SB3 (and 3-83). Laffort prepared lyophilisates to assess performance of these strains in a commercialised format and tested performance in Merlot and Cabernet Sauvignon wines by direct inoculation or after acclimatisation. They found that 2-49 behaved similarly to SB3 or worse than current strains on the market when tested in typical conditions. Future work should involve testing these under the more challenging conditions (>14.5% alc.), akin to those under which they were evolved.

Outputs and Performance Targets: AIM 2B

Output 1D. Data on the adverse impacts of dilution of high sugar content juices on nutrient balance and therefore yeast and LAB fermentation reliability.

- Fermentation trials of juice from grapes picked well beyond desirable maturity (high sugar content) after dilution and with varying nutrient supplementation will be undertaken.

Output 2D. Fact sheets (advice to winemakers) based on detailed characterisation of risks arising from dilution of high sugar juices.

- Analysis of fermentation performance of yeast and bacteria matched with analysis of volatile and/or non-volatile composition of wines from diluted juices. Specific risks and impacts to be evaluated include nutrient and vitamin dilution/imbalances and use of mains (chlorinated) water. Fact sheets generated.

Output 3C. Information on the impacts of juice dilution of high sugar juices

- Results from high throughput evaluation of diluted juices (with addition of nutrient also examined) to be compiled and conclusions reported either as a fact sheet, poster, journal article or news article for the Wine Australia website.

Results and Discussion

Outputs 1D, 2D and 3C. Data on the adverse impact of juice dilution on microbial fermentation was generated and communicated. The fermentation performance of yeast EC1118 and Lalvin R2, and bacteria Lalvin VP41 (Lallemand, Australia) in a range of diluted juices (16, 14.5, 13.5 and 12.5 °Bé) with and without the addition of nutrient (complex organic nutrient, FERMAID® O, Lallemand) was also tested. Permission to use these strains and nutrient supplement in this context was granted

by Lallemand after helpful in-depth discussions to ensure an experimental design that was appropriate to the industry context.

Due to the number of conditions tested, this was performed in high throughput utilising our robotic fermentation platform (Tee-bot 2.0) where 48 conditions were tested in quadruplicate (192 x 30 mL fermentations). Please refer to Gardner *et al*. (2022) for detailed methodology and outcomes. Chemical compositions of wines were evaluated by HPLC and GC-MS. As expected, juices with reduced °Bé were not impeded and instead completed fermentation sooner (Figure 12). This included both alcoholic and malolactic fermentations. Nutrient addition reduced fermentation durations in some cases. In general, GC-MS revealed that wines made with diluted juices had a reduced concentration of volatile compounds, presumably since dilution reduced the concentration of juice derived precursors. Interestingly, some volatiles were also increased with dilution. Of the 38 volatile compounds tested, 22 were found to be significantly different in three or more treatments. Of these, 19 were influenced by dilution and 17 by nutrient addition. Reduction of certain volatiles could be viewed as desirable, for instance isoamyl acetate was reduced to below aroma threshold by dilution of juices fermented by Lalvin R2, commonly thought to overpower white wines made with this particular yeast. The production of acetic acid, considered a spoilage compound at higher concentrations, was also reduced to below aroma threshold (AT) when juice was diluted to 13.5 °Bé and fermented with Lalvin R2 (333 *vs* 107 mg/L, AT: 200 mg/L). Intriguingly, the production of methionol (sweet potato aroma) increased in concentration with juice dilution to 14.5 °Bé, however, only with the use of Lalvin R2 (1.9 to 4.2 mg/L). Some major yeast metabolites were also impacted, as measured by HPLC, and decreased in proportion to juice dilution, these being malic and succinic acid as well as glycerol and ethanol (Gardner *et al*., 2022).

We were particularly interested to determine whether the addition of nutrient could compensate for diluted precursors and restore yields of compounds seen in undiluted juices. We found that indeed many (44 of 56 data points) increased, however, rarely to the same concentration found in undiluted juices. Compounds found to increase with the addition of nutrient in more than one condition included 3- and 4-methyl-1-pentanol (up to 129 %), butanoic acid (up to 166 %), ethyl butanoate (up to 120 %), hexanoic acid (up to 141 %) and octanoic acid (up to 346 %), ethyl lactate (up to 131 %) and isobutyric acid (up to 168 %; Figure 13).

In summary, we found that dilution of juices prior to fermentation did have wide consequences beyond merely moderating final ethanol content and winemakers should carefully consider these. Future work would be beneficial to test a selection of key conditions using chlorinated water as the diluent since this is often the only source of water available (or indeed used by choice) in industry.

Two manuscripts, one peer reviewed (Gardner *et al*., 2022) and one technical (Gardner *et al*., 2020), in place of a fact sheet have been published on this Output. Also, a poster on this work was presented at the 17th AWITC conference, held in Adelaide (21-24 July 2019). Session: Fermentation, clarification and maturation. Poster 49: JM Gardner, ME Walker, P Boss, V Jiranek. “Don’t like banana in your Riesling? Juice dilution could be your solution”.

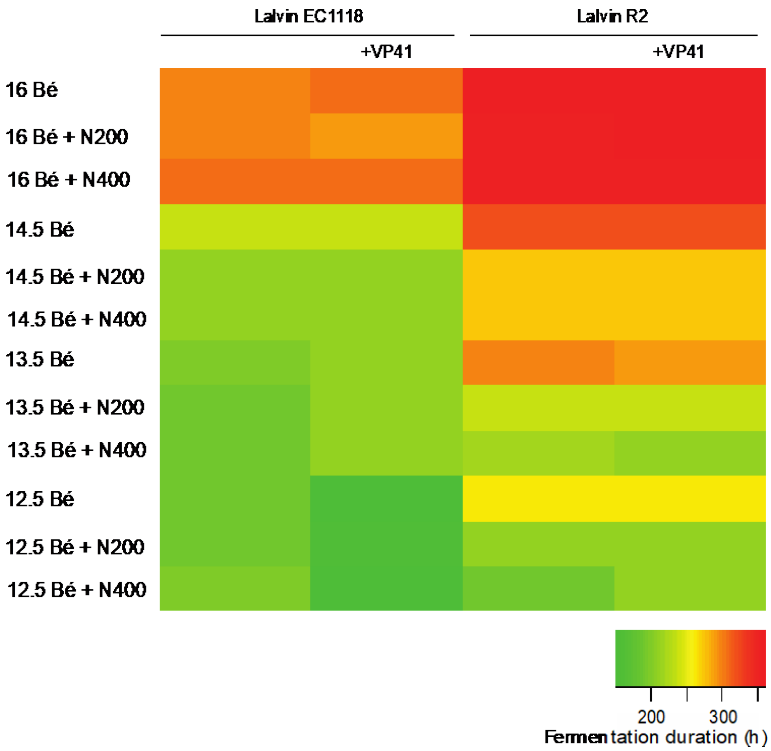


Figure 12. Alcoholic fermentation duration of Lalvin EC1118 and Lalvin R2, with (+VP41) and without (-VP41) Lalvin VP41 malolactic bacteria and with the addition of complex yeast nutrient at 0, 200 mg L^{-1} (N200) or 400 mg L^{-1} (N400), presented as a heat map. 16 °Bé juice was either fermented neat, or diluted with water to 14.5, 13.5 or 12.5 °Bé. Data presented are the average of triplicate fermentations (Gardner *et al.,* 2020)

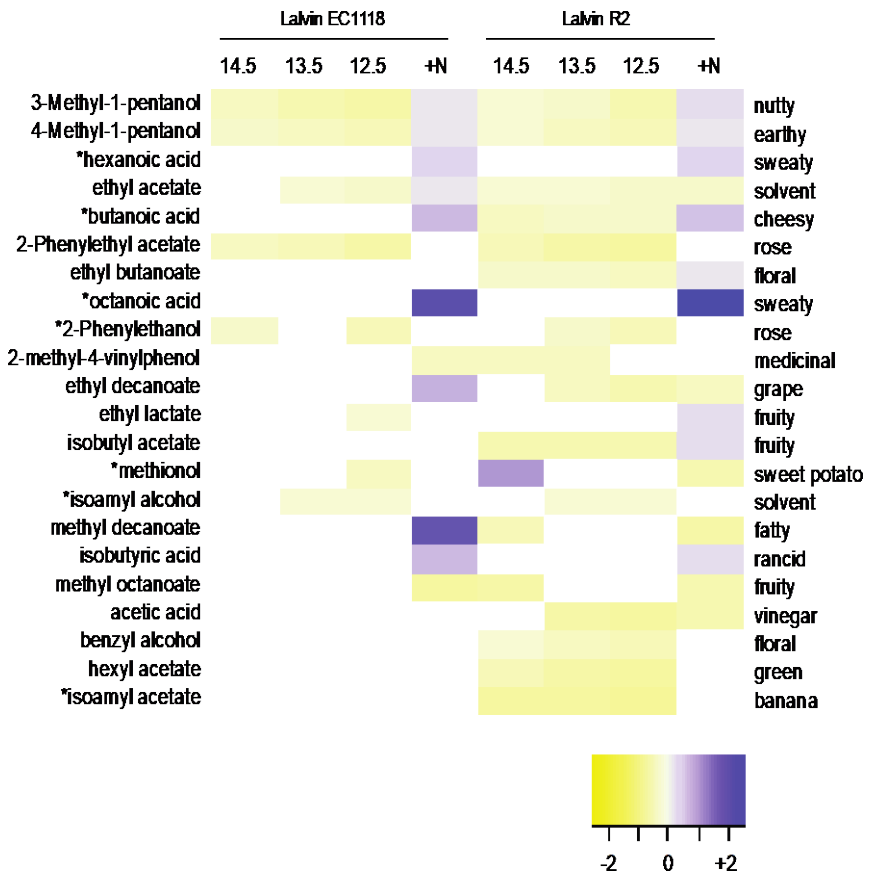


Figure 13. Change in volatiles detected in wines that were significantly different to the matched control (16 °Bé juice fermented by either Lalvin EC1118 or Lalvin R2 with no exogenous nutrient added, or where nutrients were added (+N), comparison was to the same °Bé juice with no nutrients added. +N represents combined results from diluted juices with 200 or 400 mg L^{-1} of added complex nutrient. Where a significant difference was detected (one-way ANOVA, $p < 0.05$), values are the ratio of treatment:control -1. Volatiles that increased with treatment (blue) and decrease (yellow). *Volatiles detected above the aroma threshold in at least one treatment (Gardner *et al*., 2020).

Publications arising from AIM 2:

1. Gardner, J.M., Walker, M.E., Boss, P., Jiranek, V (2020) Is juice dilution your solution? How do microbes feel about it? *Wine & Viticulture Journal*. 34(5).

2. Lin, M., Boss, P.K., Walker, M.E., Sumby, K.M., Jiranek, V. (2022) Influence of *Kazachstania* spp. on the chemical and sensory profile of red wines. *International Journal of Food Microbiology*, 362, 2 February 2022, 109496.

3. Gardner, J.M., Walker, M.E., Boss, P., Jiranek, V. (2022) The effect of grape juice dilution and complex nutrient addition on oenological fermentation and wine chemical composition. *Journal of Food Composition and Analysis*, 105, 104241.

4. Lin, M. PhD Thesis (2022): Isolation and characterisation of yeasts with biotechnological properties. The University of Adelaide.

AIM 3: Basis for microbial enhancement determined by genome, RNA sequencing and/or QTL analysis, as appropriate.

- ***Data compiled from 'omics experiments and/or QTL analyses. Latest gene technologies coupled with bioinformatic processing tools will be used to decipher how desirable strain properties are conferred***

Contributors

Research staff: Drs Michelle Walker, Jin Zhang, Jennie Gardner, Krista Sumby and Professor Vladimir Jiranek.
Postgraduate students: Tom Lang, Mandy Lin, Yan Berkovich

Background

Understanding of the basis for enhancement of key microbial traits is especially important in the context of wine production and more broadly food and beverage production, since there is still a lack of understanding of how particular biochemical pathways interact, how they may impact other important process during wine production and how further improvement can be targeted. Historically, literature has focused on analyses of single microbes, but this approach is limited to that particular strain or isolate and often only reviewed in the context of one particular environment or variable. Due to the recent availability of many publicly accessible datasets and the transparency in methodology of published literature, an excellent opportunity lies in data mining relevant published research in what is already a rich resource. Obviously to best interrogate published data sets, collection, interpretation and cross comparisons are necessary, as well as a sound understanding of this area. Our group specifically has this expertise, proven by our track record in publications of screening for improved microbes and the use of Directed Evolution with selected wine microbes (pioneered in 2002 via UA 01/04 and GWR Ph0901) to generate superior (non-recombinant) yeast and bacteria in batch or continuous culture with key fermentation stressors. Further, this is an excellent opportunity to incorporate in-house data allowing further expansion of knowledge around specific strains of interest relevant to winemaking. This is of particular interest for strains improved with Directed Evolution as the impact of the often many genomic changes can be difficult to interpret. The same can be applied during analyses of novel environmental isolates, in particular non-*Saccharomyces* strains, of which only a few report on the genetic base for phenotypic traits. Comparison between many strains with a similar trait is a powerful way to reveal common genes modifications, or modifications of a specific biochemical pathway, only achievable when using a 'top down' approach such as this.

Previously we collated data from many studies to build a database, the **F**ermentation **R**elevant **Y**east **G**enes (FRYG) database, an in-house database of 'omics experiments collected from both our own and other data. As above, this allows for cross comparison of specific traits across different experimental set ups and in different strain backgrounds. Importantly, it also includes our in-house data from a screen of the laboratory yeast deletion library (BY4743 ORF::KanMX (Winzeler *et al*., 1999) where 93 genes essential for the timely completion of high sugar fermentation referred to as Fermentation Essential Genes (Walker *et al*., 2014) were discovered. Essentially

these genes are required for yeast to sense and respond to the multiple stresses encountered during growth in grape juice to allow utilisation of all sugars. These stresses change over the course of fermentation, occurring consecutively from early through to late stage fermentation. 93 genes were identified, which when deleted, either gave rise to protracted fermentation (84 mutants) or fermentation arrest (9 mutants) under conditions of high sugar (200 g/L) and optimal nitrogen (450 mg/L FAN) but did not affect cell growth. The set also includes genes identified from screens of two prototrophic libraries - a prototrophic version of the laboratory yeast library - BY4741P (Mulleder *et al*., 2012) and a wine yeast deletion library in AWRI 1631 (Varela *et al*., 2012) under both high (450 mg/L FAN) and low nitrogen (75 mg/L FAN) conditions. Genes whose deletion results in shortened fermentation duration were of particular interest as these may be ideal targets for later strain development. Within this current project we sought to build upon and interrogate this database specifically with single nucleotide polymorphisms (SNPs) discovered in genomic sequences of both natural and evolved isolates, key strains with specific desirable traits, as generated during this project (Aims 1 and 2).

Summary outcomes

The **F**ermentation **R**elevant **Y**east **G**enes (FRYG) database was extended upon here and encompasses genes reported by us and others to influence fermentation. This includes genes whose deletion or over-expression confers shortened or lengthy fermentation and was most recently updated to include in-house gene deletion data from former Wine Australia-funded PhD students (Jasmine Peters [GWR Ph1313] and Max Huang [GWR Ph1314]). We have also included SNPs from six in-house evolved wine yeast strains [UA 1302] selected for their fermentation efficiencies, made possible by sequencing of these strains as part of this project.

Together with the six evolved strains, we also sequenced the whole genomes of two *Kazachstania* species, of interest due to their influence on the rose wine aroma produced through increased production of acetate esters (Aim 1 and 2). Here we found duplication of acetate ester genes, which are a very likely cause. Comparisons of all eight new genomes were made to their parent and/or related strains to reveal gene lists that are also likely related to the desirable phenotypes of interest. In regard to the evolved strains, between three and 1535 single nucleotide polymorphisms (SNPs) were identified in each of these and comparison to the FRYG database revealed 177 SNPs common to other reported evolved strains, with 36 of these common between our evolved strains. Gene Ontology analysis further revealed enrichment in regulation of the cellular component of the plasma membrane and cell periphery. This highlights how this type of analysis can rapidly reveal which genes to focus on for future studies.

Several of these genes would be an excellent focus of further study to better understand how the yeast can undergo successful fermentation in ‘difficult’ juices or how to improve wine aroma. Use of gene editing technologies, as we have also shown with this project to be adaptable and useful in this context, would be the next logical step to engineer beneficial genetic changes to yeast, in particular the NHEJ CRISPR technique we have shown to be applicable without the addition of bacterial DNA sequences to the genome (Aim 1). Otherwise, these genes could also be potential targets for strain improvement in breeding programs whereby natural genetic variants

(mutations) giving similar phenotypes could be selected and crossed into current commercial strains to further improve them. Such breeding programs would be facilitated by PCR tracking of the 'targeted' genes from individual strains, similar to the 'marker assisted' breeding approach in plants.

Outputs and Performance Targets: AIM 3

Output 3B. Information on which yeast genes play a role in successful wine fermentation

- Data compiled from 'omics experiments. In-house databases related to fermentation attributes will be updated

Output 4C. Yeast or bacterial databases of genetic changes that putatively confer improved phenotypes. Experimental design proposed for future strain improvement

- Databases of genetic variations of strains leading to putative improved phenotypes will be built. This will encompass those identified from sequencing and/or QTL analysis of novel isolates and the fermentome (expanded in here), as well as from literature and other available yeast databases.
- Interrogation of databases will be undertaken to build on key microbial metabolic pathway models directly related to chosen microbial enhancement(s).
- Bioinformatic analysis and experimental design. Specific gene targets identified as highlighted'.

Results and Discussion

In Output 3B and 4C, we have expanded the information known about which genes play a role in successful wine fermentation and analysed how this could be best taken advantage of through compilation and comparison of genetic databases. Critical analysis of genomic data collected during this study was enabled through the use of our in-house database of **F**ermentation **R**elevant **Y**east **G**enes (FRYG), as was initially reported in Walker *et al*., (2014), GWRDC Projects UA 05/01 and UA 05/06. Key genes and alleles discovered during this analysis were incorporated into the FRYG database, which allowed cross-experimental comparison to both our and other researchers' data relating to fermentation profiling. Thus far, the database encompasses genes reported by others to influence fermentation, genes whose deletion or over-expression confers shortened or lengthy fermentation (identified from genome-wide screens in this laboratory) and more recently, SNPs identified from genomic sequencing of six in-house evolved wine yeast strains. This project was also updated to include the gene-deletion data from former Wine Australia-funded PhD students (Jasmine Peters [GWR Ph1313] and Max Huang [GWR Ph1314]). This database is very useful in uncovering key genes (and lists thereof) that impact yeast fermentation capabilities under multiple experimental conditions. For instance, compiled gene lists were generated and then compared using Gene Ontology

to reveal the most relevant pathways contributing to specific strain capabilities. Importantly this approach allowed the discovery of novel gene targets for future wine yeast improvement.

Whole genome sequencing of six previously evolved yeast strains [UA 1302] was undertaken and comparisons made to their parent and related strains. Phenotypic data of these were used to help identify and predict impacts of key genetic changes. This was done in collaboration with Chris Large from University of Washington. Between three and 1535 single nucleotide polymorphisms (SNPs) were identified in gene coding regions (Table 8) with 0-182 of these predicted to affect protein function (SNPas). Regions of loss of heterozygosity were also identified in some strains. Comparison of SNPas to our updated Fermentome database has highlighted many new genes putatively impacting fermentation enhancement. Particular focus was upon comparison of SNPas to genes involved in fermentation enhancement rather than required for fermentation, specifically to identify those gene modifications likely to result in improved fermentation. Comparison of SNPas between our evolved strains and to other (published) evolved strains identified 36 genes (Table 9) and 177 genes, respectively (Table 8). 158 common genes were identified between the SNPas and gene modifications (deletions or over-expression) previously found by us to enhance fermentation (Table 8). Gene Ontology analysis of these sets revealed enrichment in regulation of cellular and biological processes, including specifically the cellular component of the plasma membrane and cell periphery. In general, little enrichment was found, which may reflect the likelihood of multiple pathways that can be manipulated to enhance fermentation. Whilst not unexpected, as many mechanisms are likely to be responsible for fermentation efficiency, four genes involved in the DNA integrity checkpoint process, are of note. These are *TEL1* and *ADE6* (SNPs of evolved strains Q7 and Tee9), *TOF1* (SNPs of Tee9 and FM16-C7) and *COQ4* (C7HB4 and FM16-C7). We have also made broader comparisons to gain more insight into the genetics of fermentation efficiency. For example, comparison of all evolved SNPs predicted to affect protein function to those genes known to impact fermentation efficiency (within in-house screens), revealed that over half (90 of 158) are involved in biological regulation and often linked with the cell membrane or periphery (42 of 158).

Table 8: Summary of yeast genes playing a role in successful fermentation completion identified through fermentome database comparison. In this analysis a particular focus was on genes identified from evolved yeast (FM16 C7, Tee9, RM5_15, RM3_25, Q7 and C7HB4) with single nucleotide polymorphisms that are predicted to affect protein function (SNPa). Coloured genes highlight matches between sets.

Table 8: (continued)

	FM16 C7	Tee9	RM5_15	RM3_25	Q7	C7HB4	Number of unique genes found
Number of genes with SNPs in ORFs	1335	1280	4	5	72	270	ND
Number of genes with predicted affected protein function (SNPa)	182	70	1	0	29	70	ND
Number of genes with mutation shared with other evolved strains							
Tee9	6	X	O	O	5	8	
RM5_15	0	0	X	O	0	0	36
RM3_25	0	0	O	X	0	0	
Q7	0	5	O	O	X	5	
C7HB4	12	8	O	O	5	X	
Number of genes common to Jiranek SNPas and other reported evolved strains	52	61	0	0	11	62	177
Number of genes (SNPa) common to LIT_FRYG	64	21	0	1	12	25	ND
Number of genes (SNPa) common to SGD_FRYG	84	42	0	1	10	36	ND
Number of genes (SNPa) common to fermentation effifcient genes ID in library screening	38	50	0	0	18	61	158
Genes identified in ≥2 conditions of in house evolved (SNPa) or fermentation efficiency library screens.	53	62	0	0	25	81	

GIP4	NTG1			TEL1	URA7	
VPS15	TEL1			PBP2	YBR209W	
KTR4	VPS15			TSC11	PCS60	
DER1	ISW1			ARN1	SYP1	
PCL5	RAD23			YDL144C	RRT12	
FAA3	UTR4			TPS2	TSC11	
RNR3	DNF1			UGA1	KAP123	
MND2	LAG1			ADE6	SWI4	
UFD4	RIM101			SYS1	PEA2	
PRY2	WSC4			MAD3	SPT2	
GLG1	SSZ1			YLR125W	ECM29	
CCP1	YHR112C			GIS4	MSC7	
ESL2	OYE2			RAD33	SSM4	
COQ4	SRX1			TCB3	HXT12	
MSC2	HSL1			BUL2	HCS1	
CHL4	OAC1			YMR090W	PGM1	
PIB2	NTR2			YMR206W	NTR2	
YGL082W	GLG1			ALG6	YFL051C	
RCK1	SIR1			DNL4	HXT10	
SKI8	MET10			HIR2	YDL109C	
PBP1	PMT5			TEA1	RGT2	
TDH3	YDL144C			MGR2	ARG82	
PMT6	RGA2			BEM3	COQ4	
CWC22	SEH1			HOS3	MSC2	
MNN14	YGL114W			NTO1	HIM1	
NTA1	RSM23				SWA2	
TOR1	YGL260W				PHO92	
ENT3	MSB2				EFT2	
YJR154W	ADE6				SXM1	
LMO1	YGR153W				PEX29	
AVL9	CHO2				COQ8	
HRD3	XKS1				RCK1	
FRE1	MAD3				GSC2	
COA4	BBC1				ELP2	
DUS3	FAR1				YGR237C	
UBX2	TPK1				CWC22	
NUP188	LAA1				TPK1	
MYO5	HSP104				LAA1	
ESC1	IZH3				CPR7	
UBP15	ADE16				GEF1	
NIS1	EMP70				NTA1	
MKT1	AVL9				TOR1	
YNL193W	VPS36				POM34	
TOF1	GLO1				FRE1	
SIN3	GAL80				EST1	

	RTS1
	CIN5
	WHI2
	IRC23
	RAS1
	RGL1
	KAP120
	YPR003C
SNPa genes common to ≥3 fermentation effifcient genes (from Jiranek library screening experiments)	AAD15
	LDS1
	PAM1
	SKY1
	XRN1
	COA4
	HOG1
	YJR38
	YLR407W
	IDC3
	YTA12
	WHI2
SNPa genes common to evolved strains reported in the literature (Payen et al 2016 "benefical AD" and "E" gene sets)	FAB1
	MSW1
	SSD1
	PEP7
	VPS45
	HOG1
	SIN3
	WHI2
	LTE1
	RRG1
	Vps45
Most common genes (SNPa) to LIT_FRYG	HOG1
	UBP15
	RTS1
Most common genes (SNPa) to SGD_FRYG	

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Systematic name	Common Name	Description
YBL088C	TEL1	TELomere maintenance
YBR097W	VPS15	Vacuolar Protein Sorting
YDL144C	TSC11	Temperature-sensitive Suppressors of Csg2 mutants
YDR204W	NTR2	NineTeen complex Related protein
YDR205W	GLG1	Glycogenin-Like Gene
YER093C		
YGL158W	COQ4	COenzyme Q
YGR061C	MSC2	Meiotic Sister-Chromatid recombination
YGR278W	RCK1	Radiation sensitivity Complementing Kinase
YJL013C	ADE6	ADEnine requiring
YJL164C	CWC22	Complexed With Cef1p
YJL207C	MAD3	Mitotic Arrest-Deficient
YJR062C	TPK1	Takashi's Protein Kinase
YJR066W	LAA1	Large AP-1 Accessory
YKR022C	NTA1	N-Terminal Amidase
YKR058W	TOR1	Target Of Rapamycin
YLR114C	AVL9	Apl2 Vps1 Lethal
YLR214W	FRE1	Ferric REductase
YML059C	NTE1	Neuropathy Target Esterase
YML072C	TCB3	Three Calcium and lipid Binding domains (TriCalBins)
YML103C	NUP188	NUclear Pore
YML111W	BUL2	Binds Ubiquitin Ligase
YMR003W	AIM34	Altered Inheritance rate of Mitochondria
YMR096W	SNZ1	SNooZe
YMR109W	MYO5	MYOsin
YMR207C	HFA1	
YMR219W	ESC1	Establishes Silent Chromatin
YNL273W	TOF1	TOpoisomerase I-interacting Factor
YNR067C	DSE4	Daughter Specific Expression
YOL004W	SIN3	Switch INdependent
YOR028C	CIN5	Chromosome INstability
YOR038C	HIR2	HIstone Regulation
YPL066W	RGL1	Rho1 GEF Localizing
YPL115C	BEM3	Bud EMergence
YPR003C		
YPR031W	NTO1	NuA Three Orf

Table 9: Yeast genes with single nucleotide polymorphisms that are predicted to affect protein function (SNPa) common in at least two Jiranek group evolved yeast strains (FM16 C7, Tee9, RM5_15, RM3_25, Q7 and C7HB4). Coloured genes highlight matches in sets of Table 1.

Within this Output we were also were able to complete the final data analysis and publication regarding the wine yeast Tee9 produced in our laboratory by directed evolution (GWT 1305) and capable of enhanced fructose utilisation during wine fermentations. Details can be found in the publication by Walker and co-workers (2022). Here the genomic analysis and comparison to our in-house databases revealed 371 mutations, 95 in non-coding and 276 in coding regions where 297 genes are predicted to be impacted. Some of these were further analysed by introduction of single mutations in the parent background, but none so far solely confer the fructophilic profile (Berkovich 2020).

Comparison of SNPas to our Fermentome database subsets covering all genes known to be involved in specific phenotypes required for successful fermentation (LIT_FRYG

and SGD_FRYG) enabled classification of how SNPas may confer fermentation efficiency. Importantly, these analyses have also been vital to make informed decisions while designing yeast modification protocols such as the use of CRISPR Cas9 technology to develop proof-of-concept genetically modified strains addressed in AIM 1, Output 2B. As reported above, this included construction and evaluation of strains harbouring mutations in genes that affect fermentation efficiency in *Saccharomyces cerevisiae* (*ECM33* and *SER1*), urea production (*CAN1*), proline (*PRO1* and *PUT2*) and sulfide accumulation (*SUL1* and *SUL2*).

Due to the interesting sensory impact of the *Kazachstania* sp. as reported in AIM 2, Output 1C and 2C, we also sequenced two of these strains to better understand how they conferred their high acetate ester production and subsequent intense rose aromatic profile in wines. This was particularly useful since only sparse information regarding these attributes and the genetic architecture that may support them is available and only two other *Kazachstania* sp. have been fully sequenced (*K. africana* and *K. naganishii*). This analysis revealed that both *Kazachstania* sp. carried duplications in genes related to acetate ester production and results are published (Lin *et al*., 2023).

Publications arising from AIM 3:

1. Yan Berkovich, Masters of Viticulture and Oenology (2020) Yeast Glucose Induced Degradation protein 7 (Gid7) and Fructose-Bisphosphate Aldolase (Fba1): Effect of Gid7 (E726K) and Fba1 (G135S) variants on fructose metabolism during alcoholic fermentation.

1. Walker, M.E., Watson, T.L., Large, C.L., Berkovich, Y., Lang T.A., Dunham M.J., Jiranek V (2022). Directed evolution as an approach to increase fructose utilisation in synthetic grape juice by wine yeast AWRI 796. *FEMS Yeast Research* 22:1-17.

2. Lin M., Walker, M., Jiranek V., Sumby, K. (2023) Genomic analysis of *Kazachstania aerobia* and *Kazachstania servazzii* reveals duplication of genes related to acetate ester production. *Microbial Genomics*. MGEN-D-23-00003R.

AIM 4: Knowledge on the efficacy of various strain improvement methods.

- ***As we expect new technologies to be utilised for strain improvement, we will identify shortcomings (if any) and benefits of these as applied in the wine microbiology space.***

Contributors

Research Staff: Drs Michelle Walker, Jin Zhang, Jennie Gardner, Mr Lucien Alperstein and Professor Vladimir Jiranek.

Background

Modification of wine aroma and flavour by careful selection and use of microorganisms is a well proven method for winemakers when faced with challenges, such as juices outside of specification and matching constantly evolving consumer preferences. Certainly, the option for simply choosing a certain culture for fermentation is flexible, rapid and cost efficient, especially in comparison to other options such as replacing a vineyard or developing a completely different wine style. As we have described, there are many new technologies coming online and as such, Australian industry leaders should take advantage of opportunities to embrace and further develop microbes in this space. Clearly, research in this area is vital for Australia to be at the forefront of such technologies.

Summary of Outcomes

Here we reviewed and reported (Alperstein *et al*., 2020, Gardner *et al*., 2023) on the most up-to-date literature regarding currently used and soon to be implemented strain improvement technologies. Within these articles we also highlighted where strategies had been successful. This also included surveying what strains are commercially available, how they were isolated and improved upon and their use in industry, by way of a wine industry survey. We found that only 5% of strains currently available were developed with the use of genetic modification or evolution technologies, with these strains being only recently introduced. A wide range of organisms is in commercial use, predominantly *Saccharomyces cerevisiae* and *Oenococcus oeni*, but also non-*Saccharomyces* strains and includes those relatively new to market reflecting interest and uptake of new technologies. It was clear that winemakers value the availability of commercial cultures, in particular yeast, with 68% of winemakers choosing to inoculate 75% or more of fermentations. Winemakers still, however, report processing difficulties that are microbiologically related such as spoilage and fermentation control, which highlight areas where new technologies may offer solutions. This was also reflected when asking which were the most desirable attributes for commercial cultures, with stress tolerance of both yeast and bacteria being the most sought-after. The need, uptake and small market share of novel improved microbes, as reported here, highlights an opportunity within the market to provide new strains.

Outputs and Performance Targets: AIM 4

Output 4B. Survey of current and upcoming strain improvement technologies. Provide wine industry relevant examples (Wine Australia-funded projects). Dissemination of findings as peer or industry magazine article, web blog or conference seminar.

- Review literature on current and upcoming methods for strain improvement, including legislation.
- Survey (online) winemakers and yeast supply companies as to number of strains per improvement methodology and uptake by industry, compare this to 2019 AWRI industry practices survey.
- Analyse Wine Australia-funded projects (UA and AWRI) as to success of strategies implemented.

Results and Discussion

Output 4B. We compiled the latest publications in relation to strain improvement technologies and used this information to design and undertake a survey of the Australian wine industry to determine uptake and future requirements. Current and upcoming strain improvement technologies (Figure 14) involving gene technology, with a focus on yeasts were initially investigated by gaining a full understanding of the current Australian regulations in this space. At present, techniques involving bacteria within food production are still in their infancy. The Gene Technology Regulations 2001 (Compilation No. 9) legislation www.legislation.gov.au/Details/F2020C00957 outlines the regulations regarding techniques not considered gene technology (Schedule 1A, pg 25). Yeasts modified by repair of single or double stranded breaks in genomic DNA using CRISPR Cas9 and a nucleic acid template to guide Homology Directed (HD) repair are considered GMOs (Schedule 1B, pg 26). Yeasts made with permitted technologies are non-GMO (Schedule 1, pg 27). This includes yeasts made with CRISPR technology without a repair template, which relies on Non-Homologous End Joining (NHEJ) repair (Schedule 1, Item 4). 'Self-cloned' yeast may also be considered non-GMOs when the modified traits in the original yeast are a result of gene technology not considered GMO and are crossbred (Schedule 1 Item 6). The same applies to yeasts modified as a result of DNA exchange between donor species and the host species, if it is a naturally occurring process (Schedule 1 Item 7), as in Quantitative Trait Loci (QTL) Assisted Breeding, which is being adopted by several yeast companies. Examples of all include Lalvin ICV OKAY (Lallemand) and Zymaflore series (RX60, CX9, X5, X16, Xpure; Laffort).

We fully investigated current and upcoming methodologies for yeast strain improvement by way of literature reviews throughout the life of the project, summaries of which were published (Alperstein *et al*., 2020, Gardner *et al*., 2023). Initially we began with the topical debate of how to decide where wine microbes should be best sourced, i.e. bioprospecting *vs* synthetic biology or whether to rely on the indigenous population (Alperstein *et al*., 2020). This review explored the pros and cons of each

technique and included commentary around current consumer sentiment.

Gardner *et al*., (2023) expanded upon the above concepts where we reviewed what yeast cultures are currently available for the alcoholic beverage industry from commercial suppliers worldwide, importantly also including a comprehensive list of strains with descriptions of their species, supplier, technology used in their development and attributes in specific beverages. This important work provides a fully transparent and unbiased summary of the variety of available isolates and highlights the currently quite narrow variety of techniques utilised. For instance, the vast majority of isolates (76%) were simply selected from nature, whereas 18% were developed with breeding technologies and only 5% were developed with the use of genetic modification or evolution technologies. At the time, 248 strains were available on the market from 23 suppliers, of which there were nine different species available with the most (71%) being *Saccharomyces cerevisiae*.

We also reviewed which technologies that may be used in the near future, by exploring recent advances in fermentation relevant strain improvement techniques. For example, genetic modification through mutagenesis, QTL-aided breeding, directed evolution, CRISPR Cas9 and use of the synthetic yeast genome, including some of the latest research undertaken by the AWRI and Macquarie University involving construction of a neo-chromosome of pangenomic elements unique to industrial/environmental rather than laboratory isolates (Gardner *et al*., 2023).

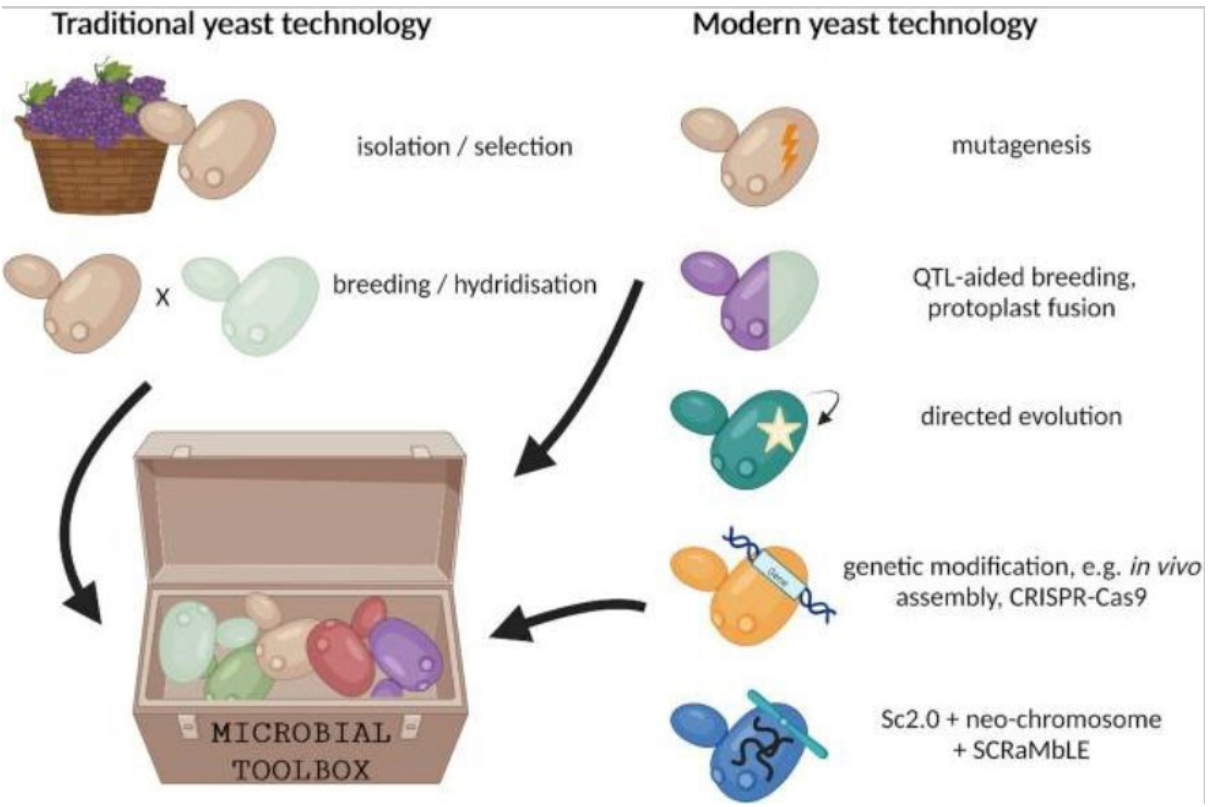


Figure 14: Traditional and modern yeast technologies to produce yeasts for the wine industry (from Gardner *et al*., 2023)

Finally, we also reported (Gardner *et al*., 2023) on the current use of microbes in the Australian wine industry, as declared by winemakers, as well as regulations around,

and sentiment about the potential use of GMOs in the future. To achieve this, we conducted an online survey of winemakers with more than 1200 survey invitations distributed. Complete results of the survey were published separately (Alperstein *et al*., 2022). 41 responses were recorded from a roughly even distribution of winery sizes. Both the types of microbes and methods used in industry were captured along with opinions about where future research should focus and the value and use of genetically modified organisms. Answers to the comprehensive questionnaire highlight the opinion that research in wine microbiology is very important, along with many other industrial trends. For example, most primary and secondary fermentations are purposefully inoculated (Figure 15), a wide range of strains are used, including non-*Saccharomyces* with the aim of increasing wine complexity. Stress tolerance was the attribute with the highest priority with the most desirable outcomes being control of fermentation dynamics and spoilage organisms. Desirable microbial attributes were also identified and notably 53% of winemakers expressed interest and acceptance of the use of microbes modified by genetic engineering (including techniques that leave no foreign DNA, such as CRISPR Cas9).

An interview-style survey of microbial suppliers (the major five in Australia) was also undertaken and mirrored the comments from winemakers. Importantly suppliers highlighted that in their opinion, face-to-face exchange of information (via the suppliers) was the most effective method of information dissemination, highlighting the importance of close ties between research providers and industry. Responses from both winemakers and suppliers were also compared to those collected during the 2019 AWRI Industry Practices survey and commentary on the similarities and differences can be found in Gardner *et al*. (2023). A poster was presented at the 18th AWITC (poster #50), and a complete summary of the survey was made available as a brochure at the University of Adelaide booth at the meeting.

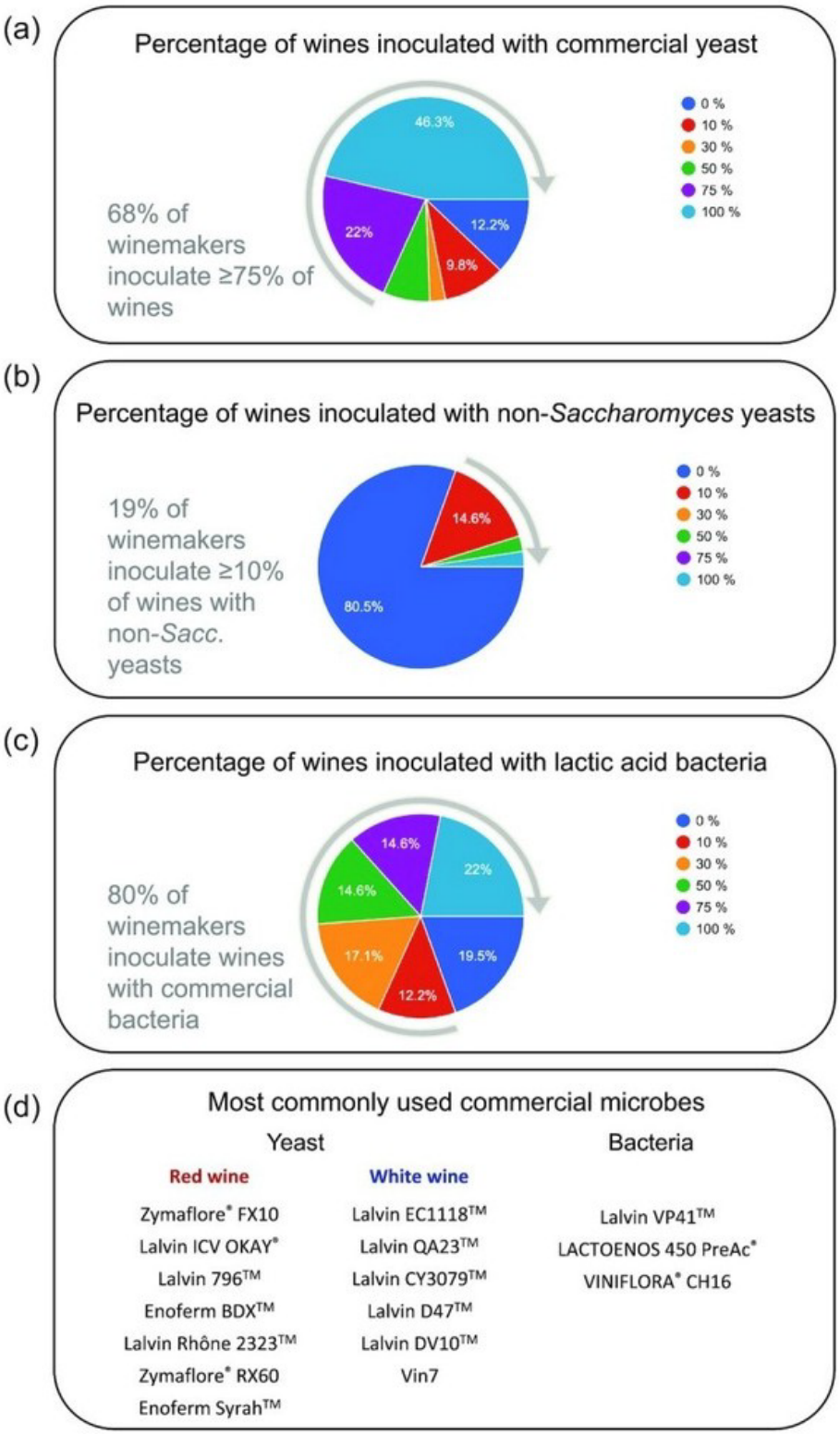


Figure 15: Key results from a Wine Industry Survey conducted in 2021 (full results in Alperstein *et al*., (2022)). Forty-one winemakers responded and represented an equal distribution of winery sizes and microbial expenditure budgets. The percentage of winemakers that reported inoculation with commercial yeast (**a**), non-*Saccharomyces* yeast (**b**) and lactic acid bacteria (**c**). The most commonly used commercially available yeast and lactic acid bacteria (**d**), from Gardner *et al*., (2023).

Publications arising from AIM 4:

1. Alperstein, L., Gardner, J. M., Sundstrom, J. F., Sumby, K. M., Jiranek, V. (2020) Yeast bioprospecting versus synthetic biology–which is better for innovative beverage fermentation? *Applied Microbiology and Biotechnology*, 104(5), 1939-1953. https://doi.org/10.1007/s00253-020-10364-

2. Alperstein, L., Gardner, J.M., Walker, M.E.W. *et al*. Microbes in Oenology, Industry Practices Survey. In: Adelaide TUo (ed.). Figshare, 2022. 10.6084/m9.figshare.20526816

3. Gardner, J.M., Alperstein, L, Walker, M.E., Zhang, J, Jiranek V. (2023) Modern yeast development: Finding the balance between tradition and innovation in contemporary winemaking. *FEMS Yeast Research* 23, foac049, https://doi.org/10.1093/femsyr/foac049.

AIM 5: Role of bacteriophage agents in stuck or sluggish MLF defined and quantified from a number of fermentations and wineries.

- ***Data on the presence of novel inhibitors or bacteriophage in wineries with failed MLF***

Contributors

Research Staff: Drs Jin Zhang, Jennie Gardner, Michelle Walker and Professor Vladimir Jiranek and PhD candidate Miss Zhecun Xu.
Collaborators: Dr Jiao Jang, College of Enology, Northwest A & F University, Yangling 712100, China and Professors Rob Edwards and Elizabeth Dynsdale, FAME Laboratory, College of Science and Engineering, Flinders University, Bedford Park, SA, Australia.

Background

As is well understood, sluggish or stuck malolactic fermentation delays wine production and can lead to an increased risk of wine spoilage. Most commonly, problematic MLF is thought to relate to wine composition that reduces the efficiency of lactic acid bacteria. Recently, however, some groups have renewed claims that bacteriophage (viruses that infect bacteria) may also impact LAB efficiency during winemaking.

Bacteriophage are naturally present in almost all environmental niches, including wine and as such, understanding their occurrence and impact on winemaking is important. These viruses survive by infecting and replicating in bacteria, which can result in lysis and subsequent death of the host. For instance, in the dairy industry, it is well known that infection with some phage results in incomplete fermentation, leading to reduced product quality. However, the impact of bacteriophage, or more specifically oenophage, (those specific to *O. oeni*) on MLF and wine composition is relatively unknown, with phage predation only proposed as a potential cause of unsuccessful MLF, and thus far being based only on anecdotal evidence.

Since each bacteriophage specifically infects a certain bacterial species whilst being harmless to human cells, the application of certain phage could be a strategy for targeting spoilage bacteria. Indeed, this method of ‘phage therapy’ is also being explored for human health as an alternative to antibiotics. In a winemaking context, perhaps bacteriophage could be useful antibacterial agents, replacing chemicals such as SO_2.

Since so little is known about the prevalence, risk and potential use of bacteriophage in the context of wine, this part of the project aimed to expand our understanding. We expect that knowledge in this area will support future research fundamental to risk management and if necessary, intervention strategies to prevent phage infections as well as the potential for useful application of phage as potential antimicrobials in the context of winemaking.

Summary of Outcomes

Prophages were detected within 130 *O. oeni* isolates from Australian and Chinese wineries, and their integrase/prophage-like sequences were found to be highly homologous to published sequences. The number and type of prophage harboured in these bacteria in Australian samples was diverse, often appearing in multiple prophage types. Eight Australian *O. oeni* isolates containing prophage were selected and artificially induced to undergo lysis and free oenophage were collected and purified. A preliminary analysis of the impact of these oenophage on L-malic acid degradation in a defined medium revealed delayed fermentation in the presence of some phage on some hosts, though only in select conditions. Further exploration of these interactions is necessary, particularly regarding the influence of oenophage on MLF in red and white wines under various stress conditions. These findings highlight that oenophage still have the potential to influence MLF outcome and future research will provide valuable insights for fermentation management practices.

Outputs and Performance Targets: AIM 5

Output 3D Determination of the importance of bacteriophage in inhibiting MLF

- Develop PCR method to detect bacteriophage (oenophage) in *Oenococcus oeni**.
- Identify phage types by DNA extraction from isolated phage and restriction digestion*.

Mimic sluggish/stuck MLF by transfection of *O. oeni* with purified oenophage*.

Output 4D. Validation as to whether bacteriophage can cause failed MLF

- Survey commercial preparations and samples from representative, problematic and successful MLFs for the presence of phage

Results and Discussion

Output 3D. We have determined that bacteriophage can influence malolactic fermentation and continue with work on whether they can cause failed MLF (Output 4D). This Aim is ongoing as of 2023 as this part of the project was significantly delayed. The PhD student leading this activity (Ms Zhecun Xu) was delayed in China due to COVID-19 border restrictions at the beginning of the project, and thus this work was delayed for >12 months. Ms Xu was remotely enrolled in early April 2021, but took extended leave of absence to preserve her candidature whilst being unable to access laboratories. In the interim, Dr Jin Zhang undertook some experiments where time permitted. Ms Xu arrived back in Australia in June 2022 to continue. While absent, she wrote a comprehensive literature review on bacteriophage specific to wine lactic acid bacteria, which has been published (Xu *et al*., 2021).

In December 2021, Ms Xu visited and worked with collaborator Dr Jiao Jiang at Northwest Agricultural and Forestry University, China. Unfortunately, this was only for two weeks, being limited due to COVID restrictions. However, during this time, she had access to a collection of lactic acid bacteria isolated from Chinese wines, which she

analysed for their bacteriophage type and content (as described below).

Wine samples with stuck or retarded MLF, as well as efficient MLF (as a control) were collected from industry and these were screened for the presence of phage. This was achieved first by development of a PCR method to detect bacteriophage infected and phage-free *O. oeni* isolates. Following this, at least 5-10 bacterial isolates from each sample were collected for DNA extraction. Amplification of phage-specific regions (Integrase and Endolysin genes) was then performed on each sample. Other studies have so far reported six integrase types (Int A-F). This survey of 96 Australian and Chinese wine *O. oeni* isolates revealed that temperate phage infection was highly prevalent in these wines with 73% being phage positive (Figure 16). Classification of phage type, in terms of number and integration type (Int A-F), was undertaken and many diverse profiles were uncovered. The major phage types were also found to be different from those in publications from other groups, highlighting the potential diversity in populations of environmental phage that is yet to be uncovered. Many strains also harboured multiple phage (i.e. were poly-lysogenic). This treasure trove of diverse profiles included isolates that appear to be phage free, single and poly-lysogens with all except for integration types B and E discovered.

Methodology was also developed to efficiently induce and purify phage from these wine derived bacteria, as well as subsequent infection of wine derived bacterial isolates with phage from different hosts (Figure 17). This includes quantification via plaque forming assays and identification via PCR of purified phage. This is especially significant since this is a rare example of phage that have been purified from wine bacteria and purposefully used to infect a non-host wine bacterial isolate. Importantly this further supports the hypothesis that phage naturally occurring in wine are capable of infecting local wine bacteria, potentially causing cell death and thus impacting malolactic fermentation.

As Ms Xu is continuing with her PhD (submission due in 2025), experiments are still underway to examine the impact of phage infection on malolactic performance of phage-positive and negative strains. Preliminary data have shown that the growth medium is important for phage impact on bacterial growth and more so on malolactic fermentation efficiency with some phage and strain combinations resulting in MLF being delaying by 4-5 days (Figure 18). We suspect a range of environmental factors may also render wine bacteria more susceptible to phage infection, and thus a matrix of environmental stressors will be introduced (i.e. increasing acidity and ethanol and decreasing temperature). Future experiments are continuing and will also include imaging of wine derived bacteriophage with Transmission Electron Microscopy to help determine phage family grouping and tailing type. We have also developed a collaboration with Professor Rob Edwards and Liz Dinsdale (FAME laboratory, Flinders University) whose group specialises in discovery of phage from the natural environment. This collaboration continues with the Edwards lab in the process of sequencing the genome of five bacteriophage isolated from Australian wine *O. oeni* isolates. Professor Rob Edwards has also agreed to act as Ms Xu's external advisor. The work that will be achieved will depend on available resources and our ability to cross-subsidise the project.

Results from this Output have so far been presented as a Master's Thesis (Xu 2020) and a poster at the 18th AWITC (Xu *et al*., 2022). We have also published a review of this area highlighting the key concepts of this Output (Xu *et al*., 2021).

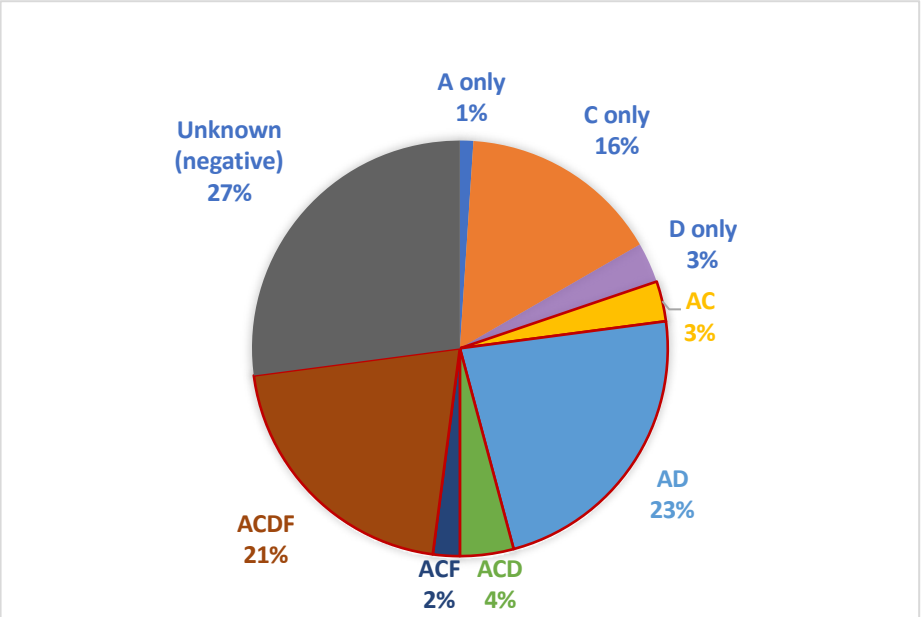
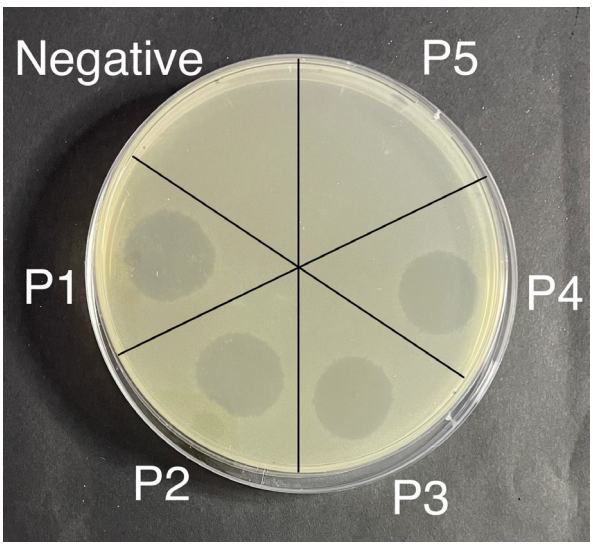


Figure 16: Distribution of Integrase type (A-F) of phage found in bacterial isolates from Australian and Chinese winery samples.



Lysates	LAB strains		
	G1	SB3	450PreAc
P1	√	√	×
P2	√	×	√
P3	√	×	√
P4	√	×	×
P5	×	×	×
P6	√	×	×
P7	√	√	×
P8	√	×	×

Figure 17. Ability of purified phage lysates to lyse a laboratory (G1) and commercial (wine) *Oenococcus oeni* lactic acid bacterial strains (SB3 and 450PreAc).

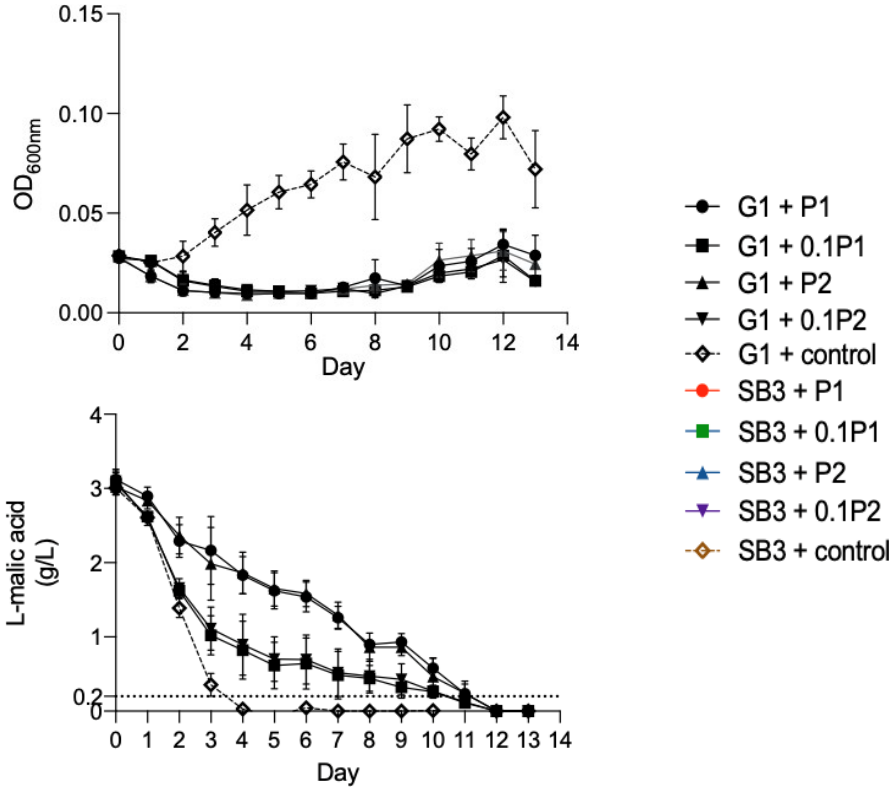


Figure 18. Growth of lactic acid bacteria as estimated by optical density (OD) at 600 nm as well as L-malic acid utilisation over time when host bacteria (G1 or SB3) were exposed to phage (P1 or P2) at varying infection rates (e.g., P1 or 0.1 P1, being representative of 1 x 10^6 or 1 x 10^5 phage/mL, respectively)

Publications arising from AIM 5:

1. Zhecun Xu (Jessie), Masters of Viticulture and Oenology (2020) Investigation and characterisation of bacteriophage in stuck and sluggish malolactic fermentation.

2. Xu, Z., Walker, M.E., Zhang, J., Gardner, J.M., Sumby K., Jiranek V. (2021) Exploring the diversity of bacteriophage specific to *Oenococcus oeni* and *Lactobacillus* spp and their role in wine production. *Applied Microbiology and Biotechnology* 05(23):8575-8592. DOI: 10.1007/s00253-021-11509-2. PMID:34694447.

3. Xu, Z., Zhang, J., Gardner, J., Walker, M., Jiranek, V. (2022) Do bacteriophage influence malolactic fermentation? Poster (#48) presented at the 18th Australian Wine Industry Technical Conference, June 2022, Adelaide.

4. Zhecun Xu (Jessie), PhD (2021-): Investigation and characterisation of bacteriophage in stuck and sluggish malolactic fermentation.

AIM 6: Dissemination of new knowledge to Industry

- **At least five peer-reviewed or industry publications on improved strains and knowledge gained on genetic basis of strain improvement**

Output E&F.

Contributors

Research Staff: Drs Michelle Walker, Jin Zhang, Jennie Gardner, Krista Sumby, Joanna Sundstrom, Mr Nick van Holst and Professor Vladimir Jiranek.

Summary of Outcomes

The Jiranek group has so far disseminated findings from this project through 41 peer reviewed and technical articles, invited seminar presentations and poster and oral presentations at a number of national and international conferences. Vladimir Jiranek (Project Supervisor) has publicised the project to visitors to the Department of Wine Science and during discussions with yeast suppliers (Laffort, Lallemand, Christian Hansen, AB Biotek, Fermentis) regarding newly generated bacterial strains and new knowledge gained about their best use.

Outputs and Performance Targets: AIM 6

Output 2E. One peer reviewed publication and one technical publication reporting promising one or two DE yeasts from UA 1302

Output 3F. One peer reviewed publication and one technical publication reporting novel desirable fermentation attributes discovered in microbial culture collection screening

Output 4E. Three peer reviewed publications and two technical publications reporting on generation of improved strains and their enological attributes

Output 4G. Submission of MSc/PhD thesis on parallel work that coincides with one or two of the project aims

- Preparation of publications.
- Supervise MSc/PhD student(s) towards degree completion

Results and Discussion

Output 2E, 3F, 4F and 4G. A total of 41 publications have been achieved for this project. This includes:

14 scientific journal articles (two more in draft form)

Two technical articles

One technical brochure
13 conference presentations
Seven accepted theses (with two more PhD students yet to complete)

Published Papers:

1. Lin, M. M-H., Boss, P. K., Walker, M. E., Sumby, K. M., Grbin, P. R., Jiranek, V. (2020) Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures. *International Journal of Food Microbiology,* 312. doi.org/10.1016/j.ijfoodmicro.2019.108373

2. Alperstein, L., Gardner, J. M., Sundstrom, J. F., Sumby, K. M., Jiranek, V. (2020) Yeast bioprospecting versus synthetic biology–which is better for innovative beverage fermentation? *Applied Microbiology and Biotechnology*, 104(5), 1939-1953. https://doi.org/10.1007/s00253-020-10364-x

3. Gardner, J.M., Walker, M.E., Zhang, J., Van Holst, N., Jiranek, V. (2020) Searching new frontiers for winemaking microbes with unusual and useful activities. *Australian Grapegrower Winemaker*, 680, 87-88.

4. Gardner, J.M., Walker, M.E., Boss, P., Jiranek, V (2020) Is juice dilution your solution? How do microbes feel about it? *Wine & Viticulture Journal.* 34(5).

5. Walker, M.E., Zhang, J., Sumby, K., Lee, A., Houlès, A., Li, S-J., Jiranek, V. (2021) Sulfate transport mutants affect hydrogen sulfide and sulfite production during alcoholic fermentation. *Yeast* 38(6):367-381.doi: 10.1002/yea.3553. Epub 2021 Mar

6. Lang, T., Walker, M., Jiranek, V. (2021) Disruption of *ECM33* in diploid wine yeast EC1118: cell morphology and aggregation and their influence on fermentation performance. *FEMS Yeast Research*. 21, Issue 5, August 2021, foab044, https://doi.org/10.1093/femsyr/foab044

7. González-Alonso, I., Walker, M.E., Vallejo-Pascual, ME. Naharro-Carrasco G., Jiranek V. (2021) Capturing yeast associated with grapes and spontaneous fermentations of the Negro Saurí minority variety from an experimental vineyard near León. *Scientific Reports* 11, 3748. https://doi.org/10.1038/s41598-021-83123-1

8. Xu, Z., Walker, M.E., Zhang, J., Gardner, J.M., Sumby K., Jiranek V. (2021) Exploring the diversity of bacteriophage specific to *Oenococcus oeni* and *Lactobacillus* spp and their role in wine production. *Applied Microbiology and Biotechnology* 05(23):8575-8592. DOI: 10.1007/s00253-021-11509-2. PMID: 34694447.

9. Van Holst N., Walker M.E., Watson T.L. Jiranek V. 'TeeBot' (2021) A High throughput robotic fermentation and sampling system. *Fermentation*, 7, 205. https://doi.org/10.3390/fermentation7040205.

10. Gardner, J.M., Walker, M.E., Boss, P., Jiranek, V. (2022) The effect of grape juice dilution and complex nutrient addition on oenological fermentation and wine chemical composition. *Journal of Food Composition and Analysis*, 105, 104241.

11. Walker, M.E., Watson, T.L., Large, C.L., Berkovich, Y., Lang T.A., Dunham M.J., Jiranek V (2022). Directed evolution as an approach to increase fructose utilisation in synthetic grape juice by wine yeast AWRI 796. *FEMS Yeast Research* 22:1-17.

12. Lang, T.A., Walker M.E., Boss, P.K., Jiranek V. (2022) Effect of 'loss of function' mutation in *SER1* encoding 3-phosphoserine aminotransferase in wine yeast EC1118: fermentation characteristics when co-inoculated with non-*Saccharomyces*. *OenoOne* 56(2), 47-61 https://doi.org/10.20870/oeno-one.2022.56.2.4908.

13. Huang C-W., Deed, R. C., Parish-Virtue, K., Pilkington, L. I., Walker, M.E., Jiranek, V., Fedrizzi, B. (2022) Characterization of polysulfides in *Saccharomyces cerevisiae* cells and finished wine from a cysteine-supplemented model grape medium. *Food Microbiology*, FM-D-22-00407R1 (accepted for publication 25/08/2022).

14. Lin, M., Boss, P.K., Walker, M.E., Sumby, K.M., Jiranek, V. (2022) Influence of *Kazachstania* spp. on the chemical and sensory profile of red wines. *International Journal of Food Microbiology*, 362, 2 February 2022, 109496 https://doi.org/10.1016/j.ijfoodmicro.2021.109496

15. Gardner, J.M., Alperstein, L, Walker, M.E., Zhang, J, Jiranek V. (2023) Modern yeast development: Finding the balance between tradition and innovation in contemporary winemaking. *FEMS Yeast Research* 23, foac049, https://doi.org/10.1093/femsyr/foac049.

16. Lin M., Walker, M., Jiranek V., Sumby, K. (2023) Genomic analysis of *Kazachstania aerobia* and *Kazachstania servazzii* reveals duplication of genes related to acetate ester production. *Microbial Genomics*. MGEN-D-23-00003R (accepted for publication 7/4/23).

Drafted papers:

17. Walker, M.E., Gardner, J.M., Large, C., Watson, T.L, Zhang, J., Van Holst, N., Boss, P.K, Dunham, M., Jiranek, V. (2023) Genomics and performance of yeast strains produced by directed evolution for winemaking.

18. Zhang, J., Gardner, J.M., Walker, M.E., Van Holst, N., Boss, P.K, Ristic, R., Huo, Y., Nicholson, E., Jiranek, V. (2023) Evaluation of environmental yeast isolates for high beta-glucosidase activity and their oenological traits in vinification.

Conference Presentations:

19. Session 8: Oral Presentation 198: Sulfate transport mutants effect hydrogen sulfide production during alcoholic fermentation. Walker, M. E., Zhang, J., Sumby, K., Lee, A., Houlès, A., Li., S-J., Watson, T., Jiranek, V. 34th International

Specialized Symposium on Yeasts, Bariloche, Argentina.2019

20. Session 4: e-Poster 161: Investigation of yeast genes required for the modulation of colour in model red wine. Walker, M., Watson, T., Roncoroni, M., Huang, C-W., Gardner, R., Jiranek, V. 34th International Specialized Symposium on Yeasts, Bariloche, Argentina. 2019

21. Session: Fermentation, clarification and maturation. Poster 49: J.M. Gardner, M.E. Walker, P. Boss and V. Jiranek "Don't like banana in your Riesling? Juice dilution could be your solution" 17th Australian Wine Industry Technical Conference, Adelaide, Australia. July 2019

22. Oral Presentation 45: Lin M. Effect of *Kazachstania* spp. on the aroma/volatile profile of white and red wines. Crush 2021, the Grape and Wine Symposium, June 2021, Adelaide.

23. Oral Presentation 53: Gardner J.M. The effect of grape juice dilution on alcoholic and malolactic fermentation duration. Crush 2021, the Grape and Wine Symposium, June 2021, Adelaide.

24. Oral Presentation 55: Zhang J, Evaluation of environmental yeast isolates for high beta-glucosidase activity and their oenological traits in vinification. Crush 2021, the Grape and Wine Symposium, June 2021, Adelaide.

25. Oral Presentation 57: Walker M.E. Disruption of arginine permease (CAN1) in EC1118 by CRISPR gene editing without a DNA mutational template. Crush 2021, the Grape

26. Oral Presentation: Jiranek V. Kazachstania spp; exotic yeasts with winemaking potential. ICY15, International Conference on Yeast Genetics and Molecular Biology, Session 5.2, August 2021, Vienna.

27. Poster Presentation (#48): Xu Z., Zhang J., Gardner J., Walker M., Jiranek V. Do bacteriophage influence malolactic fermentation? 18th Australian Wine Industry Technical Conference, June 2022, Adelaide.

28. Poster Presentation (#50): Liu Y., Zhang J., Gardner J., Jiranek V. A quest for malolactic bacteria efficient in the presence of sulfur dioxide. 18th Australian Wine Industry Technical Conference, June 2022, Adelaide.

29. Poster Presentation (#55): Zhang J., Gardner J., Walker M., van-Host N., Ristic R., Boss P., Huo Y., Nicholson E., Jiranek V. Novel winemaking microbes for your tool kit. 18th Australian Wine Industry Technical Conference, June 2022, Adelaide.

30. Poster Presentation (#85): Lin, M., Boss, P.K., Walker, M.E., Sumby, K.M., Jiranek, V. *Kazachstania* spp. : A non-*Saccharomyces* yeast to enhance fruity and floral aromas in wine. 18th Australian Wine Industry Technical Conference, June 2022, Adelaide.

31. Oral Presentation: Zhang, J. Non-*Saccharomyces*: Exotic yeasts with winemaking potential? The 13th International Symposium on viticulture and Enology and High Quality Development Forum of Yellow River Ancestral Wine Region, April 2023, Xuzhou, Jiangsu Province, China.

Post graduate students (*completed)

32. *Zhecun Xu (Jessie), Masters of Viticulture and Oenology (2020): Investigation and characterisation of bacteriophage in stuck and sluggish malolactic fermentation. Supervised by Vladimir Jiranek, Jennifer Gardner and Jin Zhang.

33. Zhecun Xu (Jessie), PhD (2021-): Investigation and characterisation of bacteriophage in stuck and sluggish malolactic fermentation. Supervised by V Jiranek, J Gardner, J Zhang.

34. Yingxin Liu, PhD (2022-) Identification of high SO_2 tolerant lactic acid bacteria for malolactic fermentation. Supervised by C Varela, V Jiranek. J Gardner.

35. *Yan Berkovich, Masters of Viticulture and Oenology (2020): Yeast Glucose-Induced Degradation protein 7 (Gid7) and Fructose-Bisphosphate Aldolase (Fba1): Effect of Gid7 (E726K) and Fba1 (G135S) variants on fructose metabolism during alcoholic fermentation. Supervised by V Jiranek, M Walker

36. *Mandy Lin, Honours, Viticulture and Oenology (2017): Characterisation of yeasts isolated from a vineyard for winemaking use. Supervised by V Jiranek, K Sumby, M Walker

37. *Mandy Lin, PhD Thesis (2022): Isolation and characterisation of yeasts with biotechnological properties. Supervised by V Jiranek, K Sumby, M Walker

38. *Yin Liu, Masters of Viticulture and Oenology (2017) Developing new starter cultures of lactic acid bacteria for co-inoculation and sequential malolactic fermentation in high alcohol wine. Supervised by V Jiranek, K Sumby.

35. *Yingxin Liu, Masters of Viticulture and Oenology (2021) Identification of high sulfur dioxide tolerant lactic acid bacteria for malolactic fermentation. Supervised by V Jiranek, J Gardner, J Zhang.

39. *Tom Lang, PhD (2018-2021) Yeast strain construction using CRISPR/Cas9: A tool for research and strain optimisation. Supervised by V Jiranek, M Walker

Technical fact sheets / brochures:

41. Alperstein, Lucien; Gardner, Jennifer; Walker, Michelle; Zhang, Jin; Jiranek, Vladimir (2022): Microbes in Oenology, Industry Practices Survey. figshare. Book. https://doi.org/10.6084/m9.figshare.20526816.v1

11.0 Outcomes and Conclusion

This project was undertaken to both generate and add to the knowledge base of how best to generate 'new and improved' starter cultures for the wine industry. Predictable and reliable fermentations that are also adaptable to winemaker specifications are vital for process efficiency and to enable production of the highest quality wine from a given juice. We surveyed the wine industry to understand what the needs for and current usage of wine microbes are and then used this knowledge, together with a review of the most up-to-date technological advances in the field, to shape the direction of our research.

We developed, refined and undertook a number of technical approaches to both isolate novel strains from nature, screen them for desirable traits, and where possible improve them. We then trialled the most promising strains in small industrial-scale fermentations where performance was measured, in an effort to predict industrial performance, as well as to yield enough wine for sensory trials. Where time permitted, we then sequenced the genomes of particular yeast of interest to identify the likely genetic drivers of the novel characteristic of that isolate, as well as to support IP protection. These predictions were underpinned by analyses using our in-house *Fermentation Relevant Yeast Gene* Database where the most likely causal genes/mutations are highlighted by comparison across hundreds of data sets. Finally, we engineered strains using CRISPR Cas9 DNA editing with mutations of interest as both a proof-of-cause of phenotype as well as to generate examples of engineered wine strains, since there are few currently reported. This multi-pronged approach resulted in isolation, identification and development of strains with the following capacities: yeast with enhanced β-glucosidase, protease and esterase activities leading to desirable flavour profiles, robust fermentation reliability in yeast and bacteria, in the presence of high sulfur dioxide.

In this project, we have also examined two relatively under-reported influencers on fermentation: juice dilution and bacteriophage. These were examined in an effort to evaluate and address their potential risks. The impact of juice dilution on alcoholic and malolactic fermentation was examined, since legislation recently changed in Australia allowing its use under prescribed conditions. Here, as expected, we found that dilution shortened fermentation durations (by up to 28% when considering additions within legal limitations) and resulted in changes to the volatile chemical profile of wines. The work with bacteriophage is ongoing, but so far, we have found that they are widespread, diverse in profile and have the capacity to delay malic acid degradation.

The ambitious nature of this project and the extensive time needed to obtain improved strains meant that there were often delays throughout the project. Additionally, a significant change to the level of funding awarded to the project in the final year(s) proved extremely challenging to accommodate, particularly while ensuring supervisory support for associated students was not compromised. However, on the whole,

performance targets were met. We also took a ‘guided’ approach to the design of experiments, incorporating findings from relevant academic literature, industry, as well as our own unpublished experiences, thus the timing of experiments and the capacity to perform them were at times limited.

The outcomes of this project have the potential to generate many benefits for winemakers, primarily by decreasing the risk of problem fermentations. By decreasing this risk, profit margins will improve, not only via preservation of the annual loss in potential value or product, but also in significantly reduced production costs associated with management of problematic fermentations. More reliable (shorter) fermentations will require less inputs (energy, additives, labour, etc.) and result in less wastage with the salvage of poorer quality fruit, thereby reducing overall environmental impact. Greater profitability and sustainability will help the wine industry maintain its critical employment role, particularly in regional areas. This will be achieved by enhancing profitability and expanding markets domestically (through new products), as well as helping provide sought after, ideally high value wine to emergent overseas markets.

12.0 Recommendations

We recommend that research into the development and use of improved microbes for the Australian wine industry be continued to ensure that the Australian Wine Industry maintains the best possible market presence due to the high-quality products it produces sustainably. From the industry survey data reported here, we found the needs and desire for novel stains such as these was high. Thus, work reported in this project should be extended, particularly where strains of yeast and bacteria were identified with useful capabilities. Specifically, non-*Saccharomyces* and evolved *Saccharomyces* strains with enhanced enzymatic capabilities and efficient fermentation performance that also translate to desirable aromatic profiles (strains Arb6, Arb 70, PF_8_W29, PF_9_W18 and PF_9_W20 and C7H-B4) should be trialled by commercial starter culture companies. Strains discovered here with the following capabilities should also be further examined in a laboratory context, in preparation for future commercialisation: those with antimicrobial capabilities, the ability to utilise acetic acid or displaying efficient MLF in the presence of SO_2. Work involving the gene edited (but non-GMO) yeast EC1118 *can1* should also be completed by analysis of its ethyl carbamate production during fermentation and the findings published to ensure maximum benefit for the research community and industry.

We advise winemakers to continue to explore the use of new microbes as they become commercially available and work closely with manufacturers and researchers to ensure best practice. This also extends to other processing that may impact the performance of yeast and bacteria. In this space, this project specifically addressed the impact of juice dilution, and reports that although it is an effective method to allow fermentation to progress efficiently, winemakers must be aware that it will impact the volatile profile of wines, which cannot fully be recovered to pre-dilution concentrations with the addition of nutrients. We also recommend that further research should be undertaken to better understand the minimum requirement of juices for methionine to ensure suppression of yeast-derived production of SO_2 in wines where MLF is desirable. We also recommend continuing the work in understanding the risk of bacteriophage in wine by further isolation of more native examples and testing their specific impacts in industrial wine processing.

Appendix 1: Communication

Communication and dissemination of findings from this research have been achieved through publications, conference, workshop and seminar presentations, annual meetings (with AWRI and Wine Australia) and discussions with various yeast and bacteria manufacturers (including Lallemand and Laffort). Outlines of the work have also been provided to wine industry groups and international academics visiting the Waite Campus. In addition, the work has been detailed as cutting-edge technology in wine microbiology through lectures to undergraduate and postgraduate oenology/viticulture students as well as BSc (Biotechnology) students at the University of Adelaide.

Appendix 2: Intellectual Property

IP of commercial significance is linked to strains generated through the project. Also, where commercial strains from industry were improved upon a material transfer agreement was put in place. Specifically for the use of B9, an MTA was put in place with Lallemand (15 May 2019), with the strain being received on the 18 July 2019. We aim to seek the commercialisation of such IP via our established links with key suppliers of yeast and bacterial cultures. IP generated by the project will be co-owned by the University of Adelaide and Wine Australia in proportions determined. Wine Australia will share in intellectual property and royalties, according to the contribution of each party to the creation of the project intellectual property, including financial contributions and un-reimbursed in-kind contributions of background intellectual property, expertise, materials, equipment, infrastructure and/or labour to the project. Management of commercialisation and IP licensing will be undertaken by the University of Adelaide division 'University of Adelaide Enterprise' (replacing the former Adelaide Research and Innovation Pty Ltd as from July 2016).

Appendix 3: References

All relevant references are included at the end of the related section above.

Appendix 4: Staff

The following staff contributed to this project:

The University of Adelaide
Professor Vladimir Jiranek (Project Supervisor)

Postdoctoral fellows
Dr Michelle Walker
Dr Jennie Gardner
Dr Jin Zhang
Dr Krista Sumby
Dr Joanna Sundstrom

Technical staff
Mr Nick Van Holst

PhD students
Mr Tom Lang
Ms Mandy Lin
Ms Zhecun Xu
Ms Yingxin Liu
Miss Emily Hocking
Mr Yiming Huo
Ms Chitra Nair
Ms Carla Virdis

Honours and MVO students
Ms Zhecun Xu
Ms Yingxin Liu
Mr Yan Berkovich

Collaborators:
Dr Paul Boss, CSIRO

Prof Robert Edwards and Prof Elizabeth Dinsdale, Flinders University

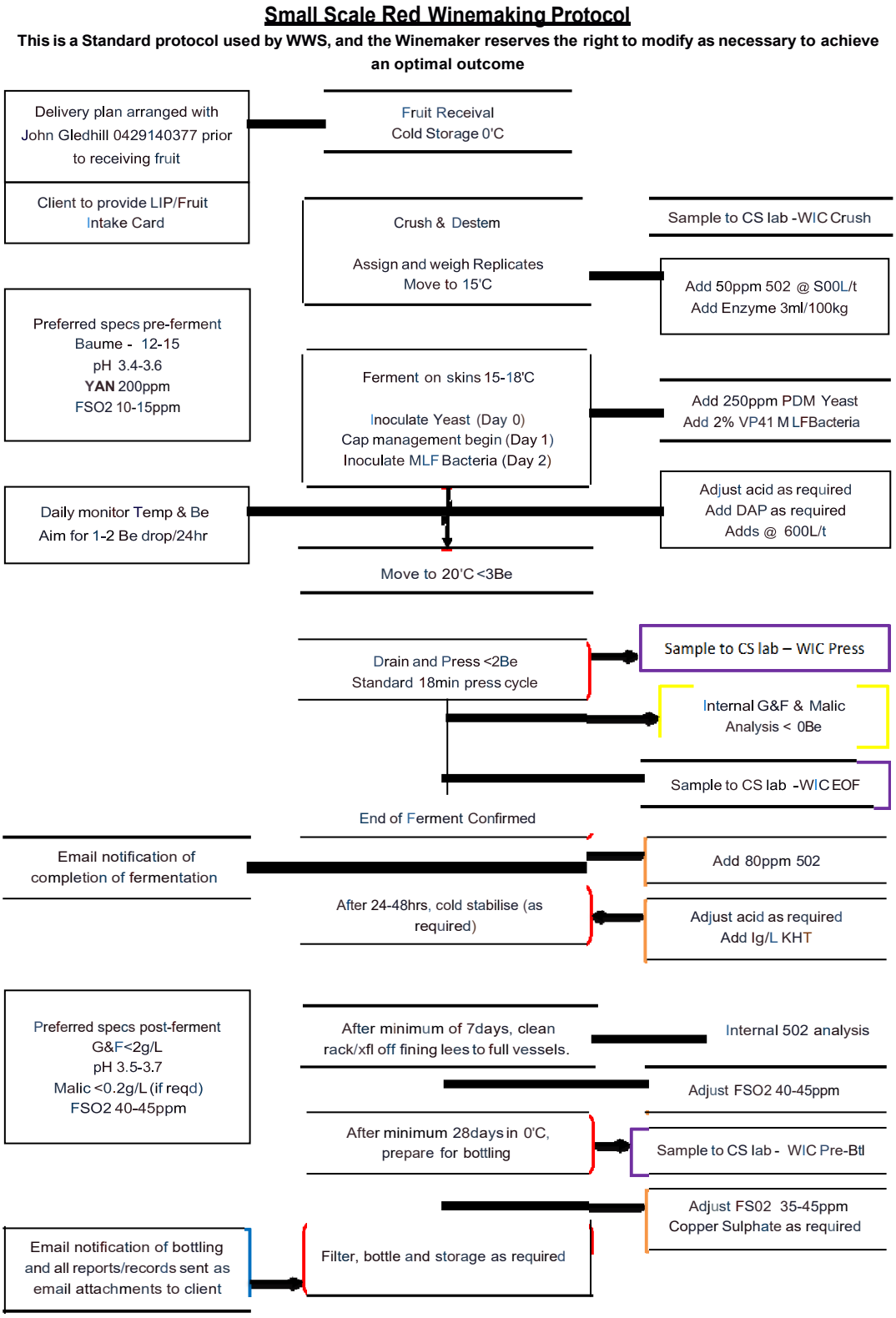
Chris Large and Prof Matrieya Dunham, University of Washington

Wolf Blass - Treasury Wine Estates (Nuriootpa, Barossa Valley)

The University of Adelaide's Hickinbotham Roseworthy Wine Science Laboratory (Waite Campus)

Lallemand Australia

Appendix 5: Supplementary Data



1 . Red Wine Making (WIC WM services)



40 kg Ferments, 15°C
Day 1: 5 Yeast (4 reps)
Day 5: LAB VP41 (MLF),
DAP & TA adjusted
Day 9: Transferred to 20°C



Day 13: Pressed ferments
& transfer to 30L steel kegs

Day 18: AF and MLF finished

Day 23: Racked Wines (20L steel Kegs. Sulfured & cold settled (4°C) 31/03/2018

July 2018 bottled wines
2 cartons/rep (24 bottles)

Nov-Dec 2018
Sensory analysis

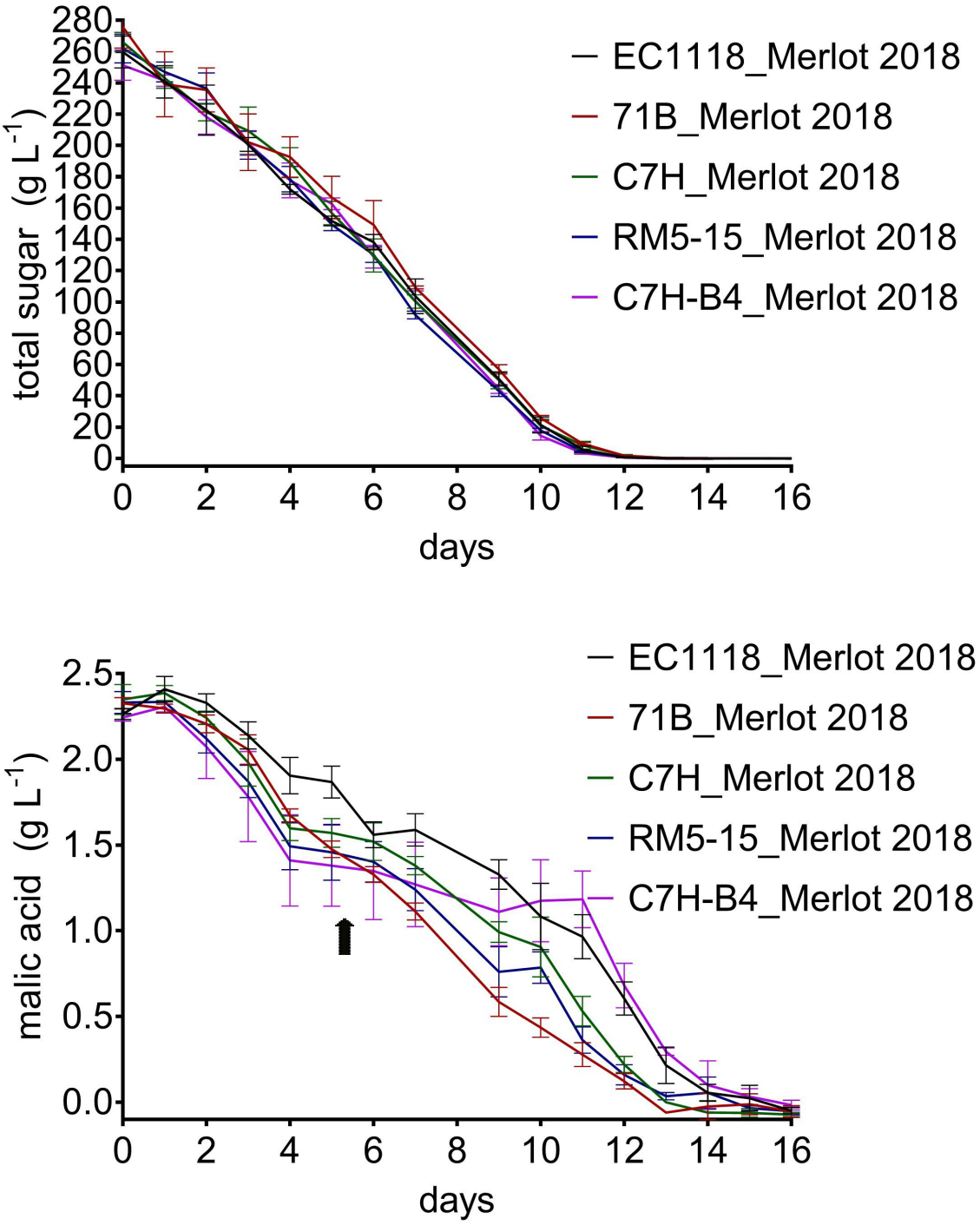


Figure 19. Primary and secondary fermentation progress of DE strains in 2018 Merlot wine, industrial scale fermentations as undertaken by WIC winemaking services

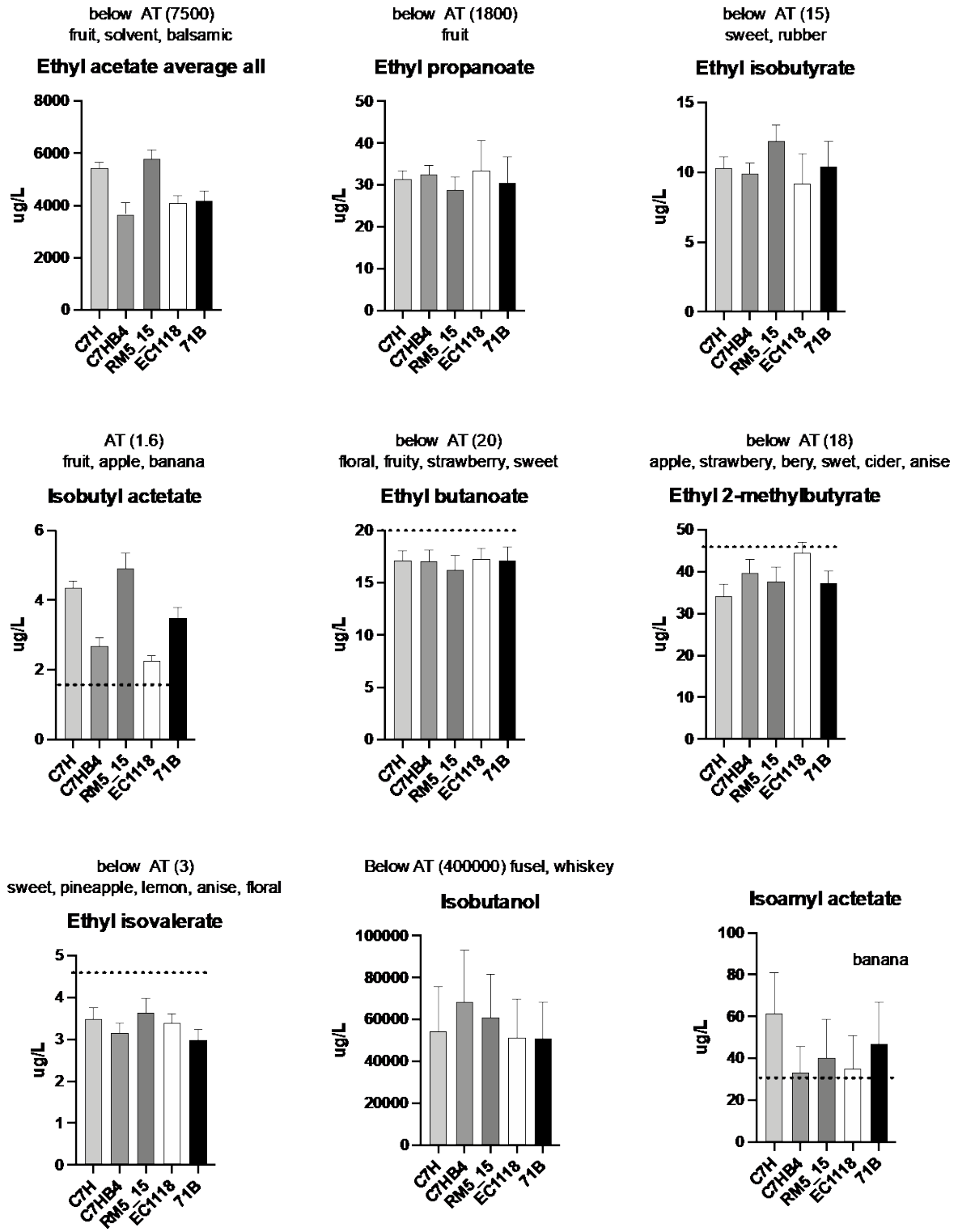


Figure 20. Compounds detected in final Merlot wines made with evolved yeasts, C7H, C7HB4 and RM5-15 in comparison to commercial strains EC1118 and 71B.

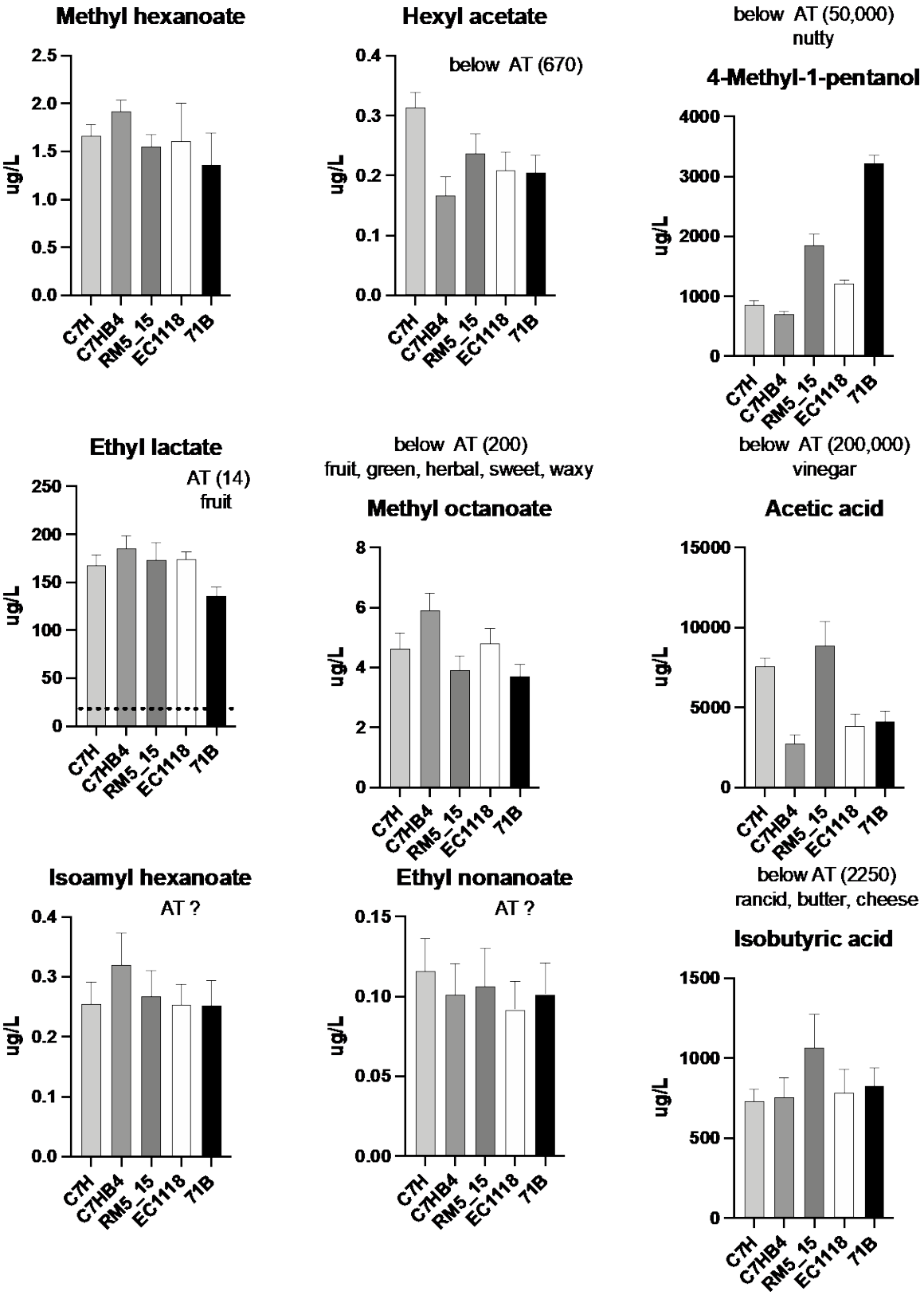


Figure 21. Compounds detected in final Merlot wines made with evolved yeasts, C7H, C7HB4 and RM5-15 in comparison to commercial strains EC1118 and 71B.

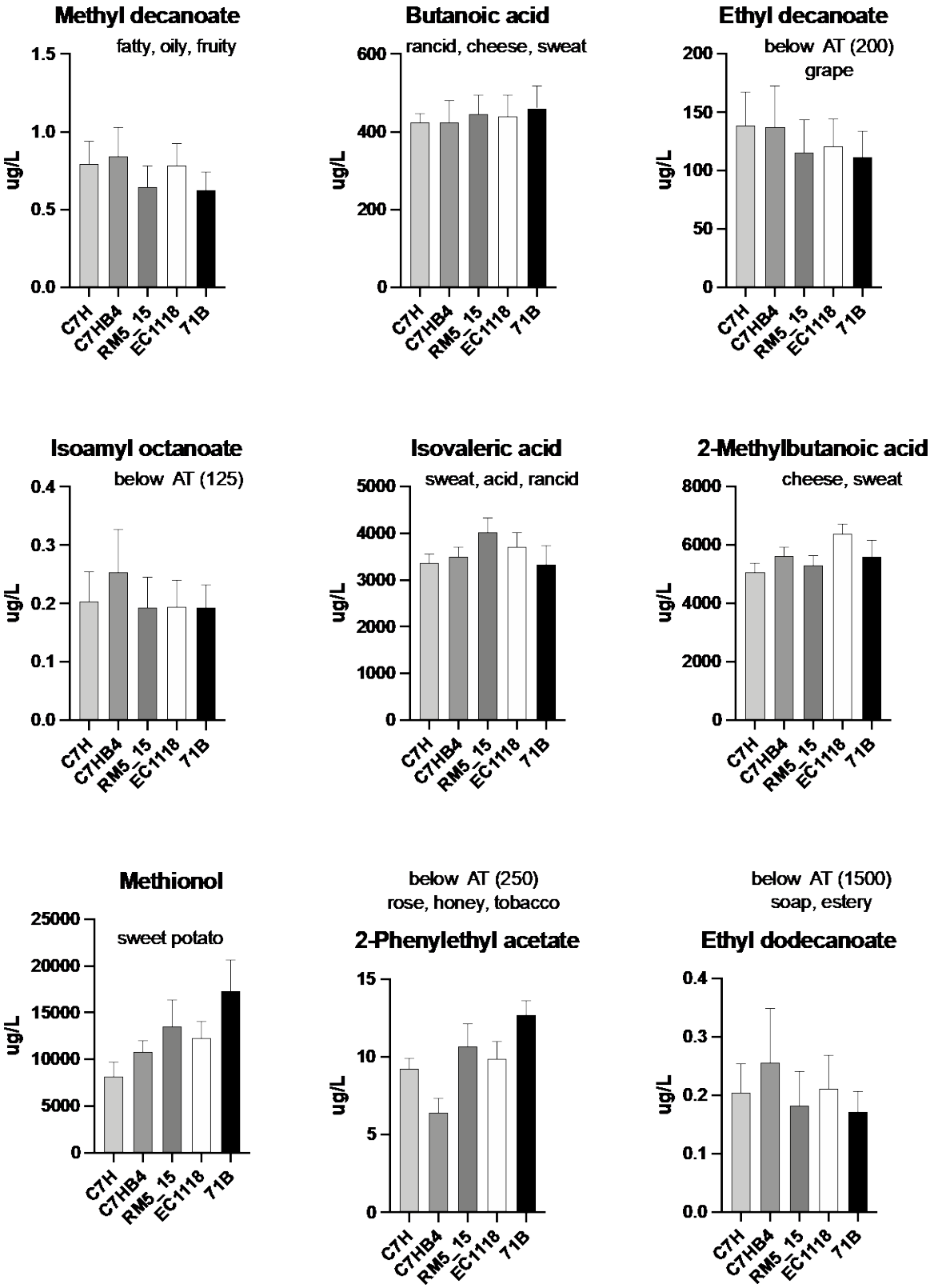


Figure 22. Compounds detected in final Merlot wines made with evolved yeasts, C7H, C7HB4 and RM5-15 in comparison to commercial strains EC1118 and 71B.

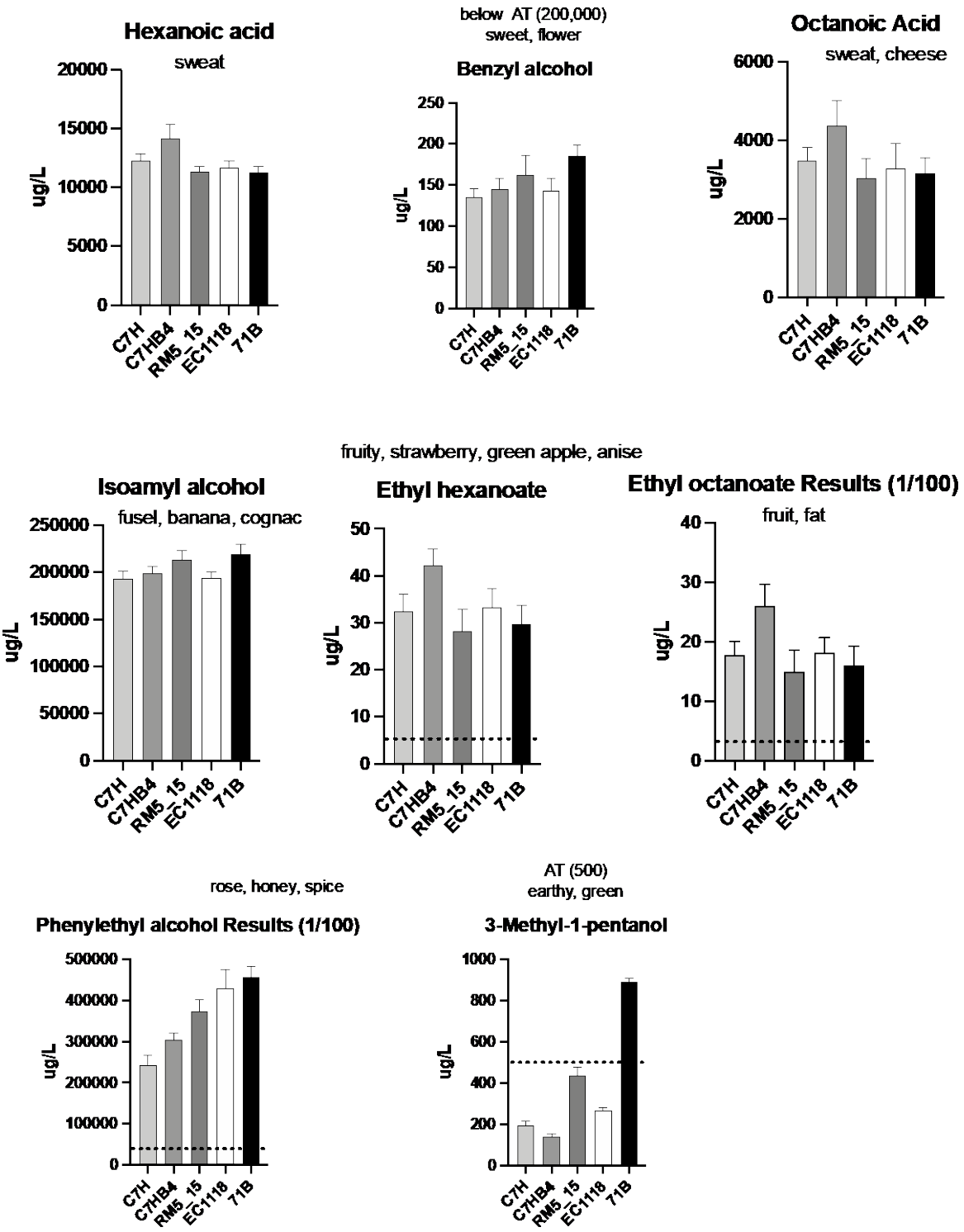


Figure 23. Compounds detected in final Merlot wines made with evolved yeasts, C7H, C7HB4 and RM5-15 in comparison to commercial strains EC1118 and 71B.