Isolation and Characterisation of Yeasts with Biotechnological Properties

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Abstract

The primary aim of this research was to identify and characterise novel non-*Saccharomyces* species from a vineyard and assess their potential use as starter cultures, either as an alternative or complement to *Saccharomyces cerevisiae*. The first step towards this aim was to access the vast potential of non-*Saccharomyces* yeasts to produce desired oenological characteristics which may be absent in *S. cerevisiae* with a view towards their application in winemaking. In particular, their ability to produce high levels of aroma compounds (esters, higher alcohols) and secrete enzymes (β-glucosidases, esterases, lipases, proteases) releases aroma compounds from odourless precursors to positively enhance the sensorial profile of wines. The 77 yeast isolates previously isolated from a South Australian vineyard were identified by sequencing the internal transcribed space regions of the 5.8S rRNA gene (ITS1-5.8S-ITS2) region. The isolates consisted of 7 species belonging to 5 genera (*Aureobasidium*, *Kazachstania*, *Meyerozyma*, *Wickerhamomyces* and *Torulaspora*). The indigenous isolates were evaluated for oenological properties, specifically ethanol tolerance, enzymatic activities, and hydrogen sulfide production.

To improve the overall wine aroma complexity, research has devised various techniques from cultivation and harvesting (of grapes) to vinification. One such method is the use of mixed starter cultures (non-*Saccharomyces* and *S. cerevisiae*) to impart distinctive aroma/flavour profiles. Whilst commercial non-*Saccharomyces* belonging to *Metschnikowia pulcherrima*, *Lachancea thermotolerans* and *Torulaspora delbrueckii* are available as commercial wine starter cultures there is a diverse range of indigenous non-*Saccharomyces* unexplored. The next step was to conduct fermentation trials with 17 representative strains from each species in chemically defined grape juice media (CDGJM). None of the isolates could complete fermentation (as monocultures) in CDGJM (200 g/L sugar), with *T. delbrueckii* isolates

utilising sugar the fastest, following by *Kazachstania aerobia*, *Kazachstania servazzii* and *Wickerhamomyces anomalus*. Based on their fermentation profile in CDGJM, 7 isolates were selected for use in sequential fermentation with a commercial *S. cerevisiae* strain in sterile Viognier juice to evaluate their contribution to fermentation kinetics and production of key metabolites, including volatile compounds. These laboratory-scale fermentations showed that species belonging to *Kazachstania* (*K. aerobia* and *K. servazzii*) produced wines with elevated levels of phenylethyl alcohol and isoamyl alcohol, as well as their corresponding acetate esters. The *Kazachstania* spp. sequential wines also reduced alcohol (ethanol), by ~1% (v/v) compared to the *S. cerevisiae* control.

Because of this interesting result the fermentative efficiency of *Kazachstania* spp. in non-sterile red musts (Merlot and Shiraz) was evaluated. Three isolates of *Kazachstania* spp. (2x *K. aerobia* and 1x *K. servazzii*) were inoculated in sequential fermentations with *S. cerevisiae*. In contrast to *S. cerevisiae*, *Kazachstania* spp. wines had significantly increased phenylethyl acetate and isoamyl acetate in both Merlot and Shiraz, as well as increased glycerol concentrations and decreased ethanol concentration (Merlot only, not in Shiraz due to the high initial °Brix of Shiraz must). With respect to Shiraz wines, *Kazachstania* spp. treatments enhanced the wine sensory appeal; the wines were perceived as 'jammy', 'red fruit' and higher aroma intensity compared to *S. cerevisiae* control ('cooked vegetable', 'earthy', 'forest floor' and 'savoury').

In addition to the collection of 77 isolates, 5 isolates belonging to *Hanseniaspora uvarum* isolated from a Victorian vineyard were included for characterisation. The effects of *H. uvarum* on the terpene content of white wines were initially evaluated in Viognier, which was overall lower in the sequential wines compared to the *S. cerevisiae* control. To further validate this

phenomenon, tests were conducted in chemically defined grape juice medium spiked with linalool, and two more aromatic varietals (Muscat and Riesling), which included uninoculated treatments as negative controls. The results show that *H. uvarum* neither increased or reduced the linalool concentrations, except in Riesling which had higher linalool concentration, but still lower compared to the *S. cerevisiae* control. These findings suggest that the terpene content in white wines could be matrix- and/or temperature-dependent.

As *Kazachstania* spp. consistently produced higher concentrations of acetate esters compared to *S. cerevisiae* in both white and red wines, their genomic and metabolic features (particularly acetate ester biosynthesis) were investigated. Complete genome sequences of *K. aerobia* and *K. servazzii* isolates were obtained at contig level (*de novo* assembly) based on PacBio sequencing reads. The genome size and GC content was 12.5 Mb and 35.8% for *K. aerobia* and 12.3 Mb and 34.4% for *K. servazzii*. Furthermore, comparative analyses were performed with putative orthologous genes involved in acetate ester and higher alcohol formation. Unlike *S. cerevisiae*, where both alcohol acetyltransferase (AATase) encoding genes *ATF1* and *ATF2* are present, both *K. aerobia* and *K. servazzii* have only one orthologue in their genome.

This study will expand the knowledge on the application of non-*Saccharomyces* yeasts in winemaking, particularly *Kazachstania* spp. which have demonstrated their potential to be employed as pure or mixed-starter cultures.

Declaration

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

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Mandy Man-Hsi Lin — Date

List of Outputs

Published manuscripts included in this thesis:

Chapter 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, 1-12.

Chapter 3: Lin, M. M-H., 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, 1-11.

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

Chapter 4: Lin, M. M-H., Boss, P.K., Sumby, K.M., Walker, M.E., Jiranek, V. Modification of terpenes in white wines by *Hanseniaspora uvarum*. Prepared for submission to Food Chemistry.

Chapter 5: Lin, M. M-H., Walker, M.E., Jiranek, V., Sumby, K.M. Whole-genome analysis of two *Kazachstania* spp. isolates from grape must fermentations: identification of genes related to acetate ester production. Prepared for submission to Microbial Genomics.

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CHAPTER 1

Literature Review and Research Aims

Overview and context

This literature review was originally written during the first 6 months of MPhil candidature (Feb 2018), as a part of the Core Component Structure Program (CCSP) and revised again (for extended research) prior to PhD upgrade (Nov 2019). It has now been updated to include publications to the end of 2021, and has been written in context of the research undertaken during this PhD candidature as outlined below.

The overall aim of this project was to identify and characterise novel non-*Saccharomyces* yeasts, and evaluating their oenological potential that can contribute to wines with unique aroma profiles. This project started by identifying isolates from the lab culture collection which had previously been isolated from un-inoculated fermenting Shiraz musts. From the 77 isolates 5 genera were identified and characterised: *Aureobasidium*, *Kazachstania*, *Meyerozyma*, *Torulaspora*, *Wickerhamomyces* (Lin et al., 2020; Chapter 2). Following this initial publication, an upgrade to the PhD program was secured and research continued to investigate non-*Saccharomyces* yeast from other un-inoculated ferments and how they might be used in winemaking. To that end, 9 yeast isolates were isolated (2 genera; *Hanseniaspora uvarum* and *Saccharomyces cerevisiae*) originating from grape musts from the Heathcote (Victoria) wine region. These were characterised as with previous isolates but the *S. cerevisiae* isolates were ultimately omitted from this project, as the project focused on non-*Saccharomyces* (mainly on *K. aerobia*, *K. servazzii* and *H. uvarum*) and their metabolic characteristics and contribution to wine.

Despite the fact that none of the non-*Saccharomyces* isolates could complete fermentation as monocultures in synthetic media and sterile juice, the *Kazachstania* spp. isolates (mono- and sequential culture ferments) produced high levels of isoamyl acetate (banana, fruity, pear aroma) and phenylethyl acetate (rose, honey aroma) in Viognier. Specific

focus was then given to *K. aerobia* and *K. servazzii*, as very little was known about how they perform and their dominance during fermentation in non-sterile environments and their impact on wine aroma (Lin et al., 2022; Chapter 3).

H. uvarum, which is typically isolated from early/initial stages of un-inoculated fermentation, was the sole non-*Saccharomyces* yeast (total of 5 isolates) in samples obtained from fermenting Malvesia and Pecorino musts. The ability of *H. uvarum* to release wine terpene glycosides via enzymatic hydrolysis (secretion of β-glucosidase) has been reported to increase terpenes in aromatic white wines. Strains belonging to *Hansenisapora/Kloeckera* (*H. guilliermondii*, *H. osmophila*, *H. uvarum* and *H. vineae*) exhibited β-glucosidase and β-xylosidase activities, and have previously been shown to increase the levels of terpene (hotrienol, β-phenylethanol and 2,6-dimethyl-3,7-octadien-2,6-diol) in treated Muscat wines (López et al., 2014). Hence it was of interest to evaluate the effects of these *H. uvarum* isolates on the terpene content of wine produced from aromatic grape varieties (Chapter 4).

The genetic basis of ethyl and acetate ester formation in *Kazachstania* spp. is the topic of Chapter 5, where the genomes of two isolates (*K. aerobia* and *K. servazzii*) were sequenced and analysed with regards to identifying orthologs of the corresponding *S. cerevisiae* genes involved.

1.1. Introduction

Wine is the product of complex biochemical interactions between grapes (typically *Vitis vinifera*) and microorganisms (yeast, bacteria and fungi) present in grape must. In the process of alcoholic fermentation, yeasts transform sugars in the grape must under anaerobic conditions into ethanol and carbon dioxide (Jolly et al., 2014; Lleixà et al., 2016; Maicas 2020). The yeast *Saccharomyces cerevisiae* is well-known for being responsible for the completion of alcoholic fermentation. In modern, large-scale winemaking, selected starter cultures of *S. cerevisiae* are widely used to guarantee the desirable features of the individual strain and ensure complete fermentation (Harsch et al., 2009; Pretorius 2000; Walker and Stewart, 2016), and thus assuring consistency in wine quality. To date, inoculated (pure culture) fermentations are favoured over un-inoculated ones, which rely on the indigenous yeast flora naturally present on grape skins (Philipp et al., 2021). Un-inoculated fermentations are often deemed unpredictable, not only in the production of off-flavours imparted into the wine (Ciani et al., 2010), but also the relative low resistance to alcohol by the indigenous yeasts can result in sluggish or even stuck fermentations with high residual sugar levels (Medina et al., 2012; Padilla et al., 2016).

Un-inoculated fermentations may be associated with increased risk of spoilage, characterised by undesirable high levels of volatile acidity (VA) related to ethyl acetate produced by indigenous yeasts (mainly non-*Saccharomyces*) (Morata et al., 2021). However, the same yeast may be considered beneficial as specific metabolites can contribute to the complexity of wines (Benito et al., 2019; Ciani et al. 2016a; Ciani et al., 2010; Fleet 2006). The role of these yeasts in winemaking has been revisited as winemakers from Old World regions considered a wine's unique traits to be influenced by 'terroir-driven' characteristics (Jolly et al., 2006). The term 'terroir' is defined as a cultivated ecosystem at a given place, in which grapevines interact with soil and climate (Eder et al., 2017). In vineyards, variables such

as soil composition, climate conditions and agricultural practises have an effective role in shaping the microbial composition (Capozzi et al., 2015; Bokulich et al., 2013). Studies have demonstrated that the grape microbial composition, or the so-called 'microbial terroir' significantly correlates with regional wine characteristics (Capozzi et al., 2015; Garofalo et al., 2016; Jara et al., 2016). As most of the grape microbiome has an impact on vine health and wine quality, there has been mounting interest in non-conventional yeasts as a source of new and improved strains which can produce wine flavours which are reflective of the 'terroir' (Barata et al., 2012; Pretorius 2020).

The inclusion of non-*Saccharomyces* strains in either controlled mixed or multi-starter culture is regarded as a realistic approach to add complexity to the wine, whilst the presence of *S. cerevisiae* prevents the premature termination of fermentation (Ciani et al., 2006; Comitini et al., 2011; Zohre and Erten 2002). Specific requirements must be met for the selection of autochthonous starter cultures, namely reasonable ethanol tolerance for yeast viability to enable sugar utilisation, together with resistance to sulfur dioxide (SO_2), which is routinely used as an antimicrobial/antioxidant to prevent wine faults. The desired oenological attributes related to wine production, are reduced alcohol and acidity (total and volatile), enhanced wine aroma (Contreras et al., 2014; Padilla et al., 2016) and enhanced colour stability (in the case of red wines) (García-Estévez et al., 2017). One of the most common wine flaws/faults is excessive amounts of acetic acid that contribute to volatile acidity. Wine acidity (perceived as sourness) is most often reduced through the metabolism of lactic acid bacteria (LAB) during malolactic fermentation (decarboxylation of L-malic to L-lactic acid; MLF) (Olguin et al., 2021). However, the success of MLF is dependent upon several factors, such as alcohol content, pH, temperature, SO_2 concentration etc (Krieger-Weber et al., 2020). A study by Benito et al. (2015) suggested an alternative to MLF: *Schizosaccharomyces pombe*, which degrades malic acid to ethanol and CO_2. Colour is often associated with quality, together with sensory attributes in red

wines. However, MLF undertaken as part of red wine production can reduce anthocyanin content and colour stability by 10 to 23% (Benito et al., 2015), due to the cell adsorption and bacterial enzymatic activities (Benito et al., 2015). The use of *S. pombe* has the potential to remedy this, as the corresponding wines exhibit higher anthocyanin concentrations, resulting in higher colour intensity. Furthermore, *S. pombe* is reported to produce up to 5-times more pyruvic acid compared to *S. cerevisiae* (Benito et al., 2012), which can react with malvidin 3-glucoside to form the highly stable pyranoanthocyanin pigment, vitisin A (Marquez et al., 2013).

The use of indigenous microbiota contributes to increased aroma complexity in wines from different grape varieties through the production of particular aroma and flavour compounds not observed in typical *Saccharomyces* fermentations (Liu et al., 2016). The phenotypic biodiversity associated with such yeasts has its importance in the selection and characterisation of novel strains with distinct attributes, which can be valuable to the wine industry (Benito et al., 2019; Perrusquía-Luévano et al., 2019). The development of molecular DNA typing techniques allows for the discrimination of yeasts to the species (and/or sub-species) and strain level (Ivey and Phister, 2011; Tofalo et al., 2011). Methods range from simple PCR to real-time PCR (or quantitative PCR) to the more sophisticated matrix-assisted laser desorption time-of-flight mass spectrophotometry (MALDI-TOF-MS) (García et al., 2017; Gutiérrez et al., 2017). The rapid and precise identification and typing of yeast isolates is critical to understanding microbial biodiversity whether in a vineyard or a wine growing region, or monitoring the population dynamics during fermentation.

1.2. Microbial diversity of grapes and during fermentation (wine microbiome)

Understanding the diversity and evolution of yeast species during alcoholic fermentation is important for better control of wine production. Yeasts naturally present in grape musts originate from two main sources: the grapes and the vineyard, and the surface of winery/cellar equipment (Jolly et al., 2014; Varela and Borneman, 2016). The latter is only a minor source of non-*Saccharomyces* yeasts, as most yeast microbiota are derived from the surface of grape skins (Fleet 1990; Kántor and Kačániová, 2015).

There is a temporal succession of fungi during the development of the grape berry. Initially, grape berries are susceptible to fungal pathogens until véraison when sugars accumulate in the tissue and diffuse into the skin, prompting yeast growth (Renouf et al., 2005). At harvest, apiculate yeasts of *Hanseniaspora/Kloeckera*, *Metschnikowia* and *Candida* species dominate the grape surface flora (Fleet 2003; Renouf et al., 2005). Basidiomycete yeasts (e.g., *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp.) and the ascomycetous yeast-like *Aureobasidium pullulans* are also highly abundant on grape berry surfaces; the population and numbers dependent upon grape variety (Bozoudi and Tsaltas, 2018; Castrillo et al., 2019).

Environmental factors, such as temperature and humidity changes, ultraviolet radiation, nutrient use and agrochemical applications are known to affect the microbial ecosystems on grape surfaces (Renouf et al., 2005). It is noteworthy that the use of fungicides and pesticides in conventional vineyards can also alter the microbial population, resulting in the decline in species diversity in grape micro-ecosystems during maturation (Escribano-Viana et al., 2018; Rantsiou et al., 2020). In contrast, some fungal species (including fungal pathogens and non-*Saccharomyces*) can also develop resistance to fungicides, which has led to the investigation of the underlying mechanisms of fungicide resistance (Comitini and Ciano, 2008). For example, *A. pullulans* exhibits not only low sensitivity to some of the most commonly used chemical fungicides (e.g., cypronidil) (Magoye et al., 2020), but can outcompete other resident fungal

species on grapes treated with fungicide (Agarbati et al., 2019). Further debate exists as to whether the reduction in microbial diversity on fungicide-treated grapevines, and associated spontaneous (un-inoculated) fermentations, can only be prevented through organic/biodynamic practises (Liu et al., 2019).

After crushing, the freshly processed must is characterised by a diverse array of indigenous non-*Saccharomyces* species. The apiculate *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) is reported to be predominant during the initial stages of un-inoculated fermentation, together with *Candida* species (Albertin et al., 2016; Di Maro et al., 2007; Martin et al., 2018). Species such as *Kluyveromyces* spp., *Metschnikowia* spp., *Pichia* spp., *Schizosaccharomyces* spp. and *Torulaspora* spp. are typically found in the middle stages of fermentation, when ethanol concentration rises to 3-4% (Coulon et al., 2019; Pretorius 2000). These species persist until factors such as nutrient limitation, alcohol, and competition cause a decline in population. The low initial low numbers of *Saccharomyces cerevisiae* by mid-fermentation, are able to outcompete and establish as the dominant species responsible for completing fermentation (Ciani et al., 2016b; Esteve-Zarzoso et al., 1998). To date, the competitive 'fitness' of *S. cerevisiae*, due to its ability to produce ethanol and survive increasing ethanol conditions, has made it the preferred yeast for use as mono-cultures in commercial winemaking.

Sulfite (SO_2) as potassium metabisulfite is primarily used as an antimicrobial agent to inhibit the growth of indigenous/non-*Saccharomyces* yeasts on harvested grapes and processed must, but also as an antioxidant, to prevent browning from polyphenol oxidase activity (Santos et al., 2011) of the juice and wine. The addition of SO_2 can be problematic as it can adversely affect microbial diversity, with the standard addition (50 ppm SO_2) reported to prevent the growth of non-*Saccharomyces* populations (Albertin et al., 2014). As such, SO_2 reduction strategies are the focus of research and industry attention. One example is the use of selected

strains, such as *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (ZYMAFLORE® ÉGIDE, Laffort) together with a reduced addition of SO_2 (20 ppm), which not only prevent indigenous yeasts establishing on grapes and in juice, but also have a positive organoleptic effect on the final wine, because of the high rate of implantation (Coulon et al., 2019). To date, several non-*Saccharomyces* species are commercially available as active dry yeast, fresh liquid yeast, cream yeast, active frozen yeast or encapsulated yeast (e.g., *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Pichia kluyveri, Wickerhamomyces anomalus, Zygosaccharomyces bailii*) either as pure or mixed-cultures; each displaying different and various aroma/flavour characteristics to help winemakers obtain the desired wine profile.

1.3. Molecular identification and typing of yeast isolates

Plating methods have been traditionally used to identify yeast by colony morphology, for example the discrimination of *Saccharomyces* versus non-*Saccharomyces* yeasts on lysine agar (Heard and Fleet, 1986; Di Maio et al., 2011). Lysine as the sole nitrogen source inhibits the growth of *S. cerevisiae*, therefore only non-*Saccharomyces* will grow on this selective medium. Another established medium is Wallerstein Laboratory Nutrient (WLN) agar, routinely used for the detection of wild yeasts for use in industrial fermentation processes, and following microbial population dynamics (Pallmann et al., 2001). Yeast species typically found in wine fermentations can be distinguished by colony morphology or the colour on WLN medium (Cavazza et al., 1992). The bromocresol green (pH indicator) in the medium acts as a dye that yeasts can take up but not necessarily metabolise. *Brettanomyces* spp., a spoilage yeast species found in wineries, on the other hand can metabolise the dye – the colony colour ranging from light to dark green, and cyan. A green ring (resembling a halo) may be present on top of the colony (depending on species). Whilst plating techniques allows for the morphological-

physiological identification of yeasts (especially in mixed-cultures), it cannot be relied to accurately identify unknown yeast species.

The advent of molecular typing techniques now enables the rapid identification of yeast isolates at species or strain level. Strategies and methods used to identify microbes throughout the fermentation process can be categorised as either indirect or direct techniques (Table 1a). When a sample has been evaluated using conventional methods such as plating, which require the growth of the microbe, the subsequent analysis using molecular methods can be used to identify the organisms present at a genus, species or even strain level (Ivey and Phister, 2011). Alternatively, molecular methods allow microbes to be identified directly from the sample itself (Franco-Duarte et al., 2019). An example of direct analysis would be studying the microbial biodiversity and dynamics during fermentation, where DNA is extracted directly from the sample and the target region is amplified by PCR then sequenced. The two advantages that direct methods have over indirect are firstly, organisms can be identified that are viable but non-culturable, and secondly, speed. Contaminants can be detected in less time compared to plating, for example quantitative real-time PCR (qPCR) can be used to identify *Brettanomyces* spp. within a day instead of several (Tofalo et al., 2012). A disadvantage of direct methods versus the indirect traditional approach is that it cannot differentiate between living and dead (yeast) cells. DNA can also enter and persist in the environment, resulting in extracellular DNA and the passive release of DNA from dead cells which remains stable, and is indistinguishable from DNA in living cells (Emerson et al., 2017).

Whilst there are advantages and drawbacks to individual methods, it is important to choose that which is most suitable to provide an accurate identification, which can assist in problem diagnosis when monitoring fermentations and in the screening of isolates suitable for industrial use (Table 1b). The most relevant molecular method for the identification of yeasts is the use of ITS-PCR involving the amplification of the ribosomal DNA (rDNA) internal

transcribed spacer (ITS) region consisting of the highly conserved 5.8S rRNA gene and flanked by two ITS regions (ITS1 and ITS2) (Sun et al., 2009). Amplification of the ITS region provides a DNA barcode marker of the fungi kingdom. Because of the high degree of interspecific variability and intraspecific homogeneity of the spacer sequences, they allow the unambiguous identification of closely related yeast species (Sun et al., 2009; Li et al., 2012; White et al., 1990). Since the molecular size of PCR-amplified products may not be enough to distinguish between species within the same genus without DNA sequence analysis, the digestion of PCR products using restrictive enzymes (e.g., *Hae*III) is required for species differentiation (Renouf et al., 2005). However, the most diagnostic method is by sequence comparison of the PCR amplicon to a DNA sequence database such as GenBank® (https://www.ncbi.nlm.nih.gov/genbank/) using a DNA/protein alignment search tool such as BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Wang et al., 2015).

The 600 nucleotide D1/D2 domain at the 5' end of a large subunit of (26S) rDNA, is also commonly used for species identification, because of the low intraspecific polymorphism and high interspecific variability due to the concerted evolution of these ribosomal regions (Hesham et al. 2014). The combined use of universal (NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′)) and species-specific primers derived from the D1/D2 region of the 26S rDNA followed by the sequencing of this domain enables fast and accurate species identification of *Saccharomyces* and other wine yeasts (Hutzler et al., 2018).

In *Saccharomyces*, the delta (δ) sequences are elements that flank the Ty1 retrotransposons. Other than retrotransposons, there are solo δ elements of which 300 are described in the genome of the *S. cerevisiae* S288C strain which are ideal candidate targets for identifying polymorphisms (Legras and Karst, 2003). Such polymorphism in *S. cerevisiae* is exploited for genotyping by interdelta PCR, which is often used for the routine analysis of yeast

strains (Legras and Karst, 2003; Ness et al., 1993). The δ_{12} and δ_2 primers are commonly used as they detect a larger degree of polymorphism in terms of band patterning visible by gel electrophoresis. The use of different combinations (e.g., δ_{12}/δ_{21} (Legras and Karst, 2003), $\delta 1_2/\delta_2$ (Legras and Karst, 2003), δ_1/δ_2 (Ness et al., 1993)) can allow identification of a large number of individuals.

Besides molecular-based identification techniques, novel biochemical methods to detect microorganisms have emerged in recent years. Methods based on mass spectrometry (MS) have gained popularity for microbial typing due to their speed, cost efficiency and simplicity (Huschek and Witzel, 2019). When MS is coupled with other separation and ionisation techniques, such as matrix-assisted laser desorption ionisation time-of-flight mode (MALDI-TOF), the result is a powerful and reliable tool for the rapid and high-resolution characterisation of microorganisms (Franco-Duarte et al., 2019). The MALDI-TOF-MS method is based on the measurement of the molecular mass of ions generated from abundant proteins (which are typically conserved within a species), of which the molecular fingerprint for a particular organism is obtained in the form of a spectral profile (Huschek and Witzel, 2019). The spectrum is then matched with an existing database to determine the identity of the microorganism. However, one limitation of this method is the number of spectral libraries available (as a reference), which would require further work to be expanded, as strain differentiation (for *S. cerevisiae*) could not be achieved, and difficulties encountered when differentiating among *Metschnikowia* species (Kačániová et al., 2020).

Table 1a. Direct versus indirect methods used in the area of microorganism identification (adapted from Franco-Duarte et al., 2019)

Type	Basis
Indirect	Isolation and culture of microorganisms on selective medium and the determination of their various phenotypic characteristics. DNA is then isolated from sample which is used for identification (e.g., sequencing, RAPD, mt-RFLP)
Direct	Identification of specific microbes in a mixed population as well as identify non-culturable microbes. DNA or RNA is isolated directly from the sample and used for further analyses (e.g., PCR, real-time PCR (qPCR)). Less specific compared to indirect methods.

Table 1b. Molecular methods used in detection and identification of wine-related microbes (fungi) (updated from Ivey and Phister, 2011).

Type of method	Identification method	Level of discrimination	References
Hybridisation methods			
Flow cytometry	Direct	Genus and species	Guzzon and Larcher, 2015; Page and Kurtzman, 2020
Complete genome hybridisation	Indirect	Groups to specific strains	Dequin and Casaregola, 2011
Sequencing methods			
Ribosomal, *actin-1* or *rpoB* DNA sequencing	Indirect	Species	Daniel and Meyer, 2003
Multilocus sequencing typing	Indirect	Species and strain (better for bacteria than *Saccharomyces*)	Muñoz et al., 2009
Whole genome sequencing	Indirect	Strain	Gopalakrishnan and Winston, 2019
Fingerprinting methods			
ITS-RFLP	Indirect	Species	Guillamon et al., 1998
26S rDNA-RFLP	Indirect	Species	Hesham et al., 2014; Yang et al., 2006
Karyotyping	Indirect	Strain	Dunn et al., 2005
mt-RFLP	Indirect	Strain-yeast	Rodríguez et al., 2011
AFLP	Indirect	Strain	Baselga et al., 2017; de Barros Lopes et al., 1999
RAPD-PCR	Indirect	Strain	Cordero-Bueso et al., 2011; Tompkins et al., 2018
δ-sequence amplification	Indirect	Strain-yeast	Legras and Karst, 2003
Microsatellite	Indirect	Strain-yeast	Bruke et al., 2012
DGGE/TGGE	Direct	Usually species by may identify strains depending on targets for PCR	Escribano-Viana et al., 2018; Hernán-Gómez et al., 2001
PCR detection			
Yeast targets	Direct	Species to strain	Hierro et al., 2004
qPCR			
Yeast targets	Direct	Species	Navarro et al., 2020

1.4. Interaction of *Saccharomyces cerevisiae* and non-*Saccharomyces* during alcoholic fermentation

The use of selected non-*Saccharomyces* with *S. cerevisiae* in a controlled mixed-starter culture has received increasing interest for improving the complexity and quality of wines. As mentioned previously, the inability of non-*Saccharomyces* to ferment grape juice to dryness has the potential to produce undesirable flavour compounds, which are considered as a wine fault. However, these behave differently in a mixed-culture environment, as these yeasts behave differently when *S. cerevisiae* is included to complete fermentation.

In order to develop autochthonous starter culture(s) to produce the desired sensory properties, suitable candidates with novel or improved oenological properties must be identified from a population of microorganisms, and then rigorously tested in the presence of *S. cerevisiae* in 'controlled' mixed or sequential fermentations prior to commercialisation (Comitini et al., 2011). Several co-inoculation studies involving *S. cerevisiae* and other wine yeasts have shown increase viability of the non-*Saccharomyces* cells in a mixed population (Table 2), alluding to sequential inoculation as being an attractive tool for enhancing the competitive behaviour of non-*Saccharomyces* yeasts, whereby inoculation of non-*Saccharomyces* is subsequently followed by that of *S. cerevisiae* (Ciani et al., 2014). This allows the metabolism of the first inoculated yeast to be exploited with minimal influence from the second. The duration between the first and second inoculation is important for non-*Saccharomyces* to express their metabolic activity (e.g., low ethanol yield) and to improve their competitiveness towards indigenous yeasts in the must.

Studies on co- (mixed) cultures related to the timing of *S. cerevisiae* inoculation on the presence of non-*Saccharomyces* and the contribution towards the wine aromatic profile. Gobbi et al. (2013) demonstrated *Lachancea thermotolerans* viability increased when inoculation of *S. cerevisiae* was delayed from 24 to 48 hours. When *Saccharomyces* was delayed to 48 h, *L.*

thermotolerans was the dominant species in the end of the 24-day ferment, whilst earlier inoculation of *Saccharomyces* after 24 hours, resulted in *Lachancea* only persisting for half of the ferment (11 days). In another study, *M. pulcherrima* was used in mixed (sequential) cultures with *S. cerevisiae* – the addition of *M. pulcherrima* at time 0 and *S. cerevisiae* 24 hours later influenced the characteristics of both white and red wines (Duarte et al., 2019). Wines produced generally had higher glycerol, reducing sugars, total dry matter and reduction in alcohol content; all are valorised features of the consumer wine market (Duarte et al., 2019).

The dynamic interaction between yeasts can be influenced by several abiotic factors. Competition for nutrients such as sugars, vitamins and nitrogen compounds have a significant impact on fermentation kinetics (Ciani et al. 2016b). Oxygen availability affects lipid biosynthesis and therefore growth of wine yeasts (Ciani et al. 2016a; Ciani et al. 2016b). Low oxygen conditions as in fermentation, decrease survival of non-*Saccharomyces* belonging to *Hanseniaspora* and *Torulaspora* genera, while *S. cerevisiae* is well adapted to anaerobic conditions. The nitrogen status in grape must can influence the fermentative behaviour of yeast strains in mixed (co-inoculated) and sequential fermentations (Bordet et al., 2020; Gobert et al., 2017; Medina et al. 2012). Non-*Saccharomyces* initially present in an un-inoculated fermentation utilise vitamins and amino acids and limit the subsequent growth of *S. cerevisiae* (Medina et al., 2012). The lack of yeast assimilable nitrogen (YAN), can result in stuck/sluggish fermentation, as reported in a study by Taillandier et al. (2014) where YAN was almost exhausted by *T. delbrueckii* in a 48-hour interval sequential fermentation.

Fermentation temperature also plays an important role in yeast interactions, as it affects the sensitivity of yeasts to factors such as ethanol, influencing growth rate and viability (Şener et al. 2007). A study conducted on mixed fermentation showed that *K. apiculata* grew and survived longer at lower temperatures (Gao and Fleet 1988). The influence of temperature on growth is an important consideration, with white wines generally fermented at lower

temperatures to preserve the aromatic compounds, whilst higher temperatures are favoured in red wine production, for enhanced pigment and tannin extraction. Gao and Fleet (1988) showed that *K. apiculata* grew and survived at lower temperatures in mixed fermentations. Similarly, Bilbao et al. (1997) showed that *K. apiculata* persisted at lower temperatures in pure and mixed cultures (with *S. cerevisiae*) in apple juice fermentations, producing high amounts of ethyl acetate and glycerol at 10 °C. On the other hand, *S. cerevisiae* suppressed the production of ethyl acetate by *K. apiculata* in mixed cultures. This could be further explored in wine ferments, considering the optimum temperature for vinification can vary, as yeasts differ in response to temperature (Liszkowska and Berlowska, 2021; Molina et al., 2007).

By understanding the interactions of different yeasts in a fermentation setup, the potential contribution of non-*Saccharomyces* to winemaking can be fully explored and exploited through the eventual commercialisation of new strains and species. To date, whilst there are several available on the market, either as pure or mixed cultures (Table 3), the number is considerably less than that of *S. cerevisiae*, where the large phenotypic diversity is exploited in terms of regionality (cool and warm climate winemaking), wine style and typicity.

Table 2. Influence of non-*Saccharomyces* yeasts in mixed-fermentations. (Adapted from Ciani et al. 2010, with updated references).

Aim	Non-*Saccharomyces* co-fermented with *S. cerevisiae*	Method	References
Reduction of acetic acid production	*Torulaspora delbreuckii*	Sequential cultures	Ciani et al., 2006; Puertas et al., 2017
Malic acid degradation	*Schizosaccharomyces pombe*	Sequential cultures or immobilised cells (batch and continuous)	Ciani and Ferraro, 1996; Canonico et al., 2016; Ciani and Ferraro, 1998; Loira et al., 2018
Enhancement of glycerol content	*Candida stellata*	Immobilised cells (pre-treatment or sequential cultures)	Ferraro et al., 2000
Enhancement of glycerol content	*Candida cantarelli*	Mixed or sequential cultures	Toro and Vasquez 2002
Improvement of wine aroma complexity	*Hanseniaspora uvarum* (also known as *Kloeckera apiculata*)	Mixed or sequential cultures	Ciani et al., 2006; Martin et al., 2018; Pietrafesa et al., 2020
Reduction of acetic acid production Increase lactic acid and reduces pH	*Kluyveromyces thermotolerans* (now known as *Lachancea thermotolerans*)	Sequential cultures	Hranilovic et al., 2021; Morales et al., 2019
Reduction of malic acid content	*Issatchenkia orientalis* (also known as *Pichia kudriavzevii*)	Mixed fermentation	Kim et al., 2008; Del Mónaco et al., 2014
Increase in aroma and complexity	*Pichia fermentans*	Sequential cultures	Clemente-Jimenez et al., 2005
Increased varietal thiol	*Pichia kluyveri*	Mixed fermentation	Anfang et al., 2008; Jolly et al., 2003; Zohre and Erten, 2002
Improvement of wine aroma profile	*Candida pulcherrima* (now known as *Metchnikowia pulcherrima*)	Mixed fermentation	Hranilovic et al., 2020; Jolly et al., 2003; Zohre and Erten, 2002
Increase in geraniol concentration	*Debaryomyces vanrijii*	Mixed fermentation	Garcia et al., 2002
Influence of sensorial and physico-chemical properties of wine	*Schizosaccharomyces* spp. *Saccharomycodes* spp. *Pichia* spp.	Ageing over the less during wine maturation	Palomero et al., 2009

Table 3. Summary of commercial non-*Saccharomyces* currently available on the market. (Adapted from Vejarano et al., 2021, with information obtained from suppliers' website that commercialise them in winemaking).

Yeast species	Brand format* (Company)	Impact on aroma profile	Other contributions to wine	Commercial website
Torulaspora delbrueckii	**Level 2 Biodiva™ TD291** ADY (Lallemand)	Higher content of esters, terpenes and thiols, Low production of volatile acidity, acetaldehyde	High production of glycerol. Osmophilic yeast (late harvest and ice wines). High production of polysaccharides: mouth-feel.	https://www.lallemandwine.com/en/australia/products/catalogue/wine-yeasts/54/level2-biodiva/
Torulaspora delbrueckii	**Prelude™** ADY (CHR Hansen)	Higher intensity and aromatic complexity High production of medium-chain fatty-acid esters (more stable esters) Improvement of fruit flavours (thiols, esters). Low production of volatile phenols, volatile acidity, acetaldehyde, H_2S.	High production of polysaccharides: mouth-feel.	https://www.chr-hansen.com/en/food-cultures-and-enzymes/fermented-beverages/cards/product-cards/prelude
Torulaspora delbrueckii	**Zymaflore Alpha** ADY (Laffort)	Co-fermentation with *S. cerevisiae*: higher expression of thiols 3SH and A3SH. Higher production of 2-phenylethanol and esters. Low production of volatile phenols, volatile acidity, acetaldehyde, acetoin, diacetyl, H_2S.		https://laffort.com/en/products/zymaflore-alpha/
Torulaspora delbrueckii	**Viniferm NSTD** ADY (Agrovin)	Higher expression of thiols: grapefruit, boxwood. Higher flowery aroma: 2-phenylethanol. Low production of volatile acidity, acetaldehyde, acetoin, H_2S.	High production of polysaccharides: mouth-feel. Co-fermentation with *S. cerevisiae*: lower alcohol content.	https://www.agrovin.com/en/producto/viniferm-nstd/

Torulaspora delbrueckii	**EnartisFerm Qτ** ADY (Enartis)	Contribution of fruity aromas (esters). Low production of volatile acidity, H_2S.	Osmophilic yeast (high sugar grape must).	https://www.enartis.com/datasheets/TECHNICAL-DATA-SHEET/EN/TDS-EN-FermQTau.pdf
Torulaspora delbrueckii	**EnartisFerm Qτ Liquido** CRY (Enartis)	Contribution of fruity aromas (esters). Low production of volatile acidity, H_2S.	Increasing of mouth-feel: high production of glycerol and polysaccharides. Osmophilic yeast (high sugar grape must). Capacity to ferment in monoculture. Improvement of foam persistence in base wine (sparkling wine production).	https://www.enartis.com/datasheets/TECHNICAL-DATA-SHEET/IT/TDS-IT-FermQtauLiquido.pdf
Torulaspora delbrueckii	**Oenovin Torulaspora Bio** ADY (Oeno)	Contribution of fruity aromas. Red fruit. Low production of volatile acidity.		https://www.oeno.it/wp-content/uploads/ST-Oenovin-Torulaspora-Oeno-BIO-2018-rev1.pdf
Torulaspora delbrueckii	**Torulaspora delbrueckii** FLY (Probiotec)	Higher floral aroma: 2-phenylethanol. Low production of volatile acidity, acetaldehyde. No production of H_2S.	High production of glycerol: mouth-feel.	http://www.probiotec.it/schede/Torulaspora-delbrueckii.pdf
Torulaspora delbrueckii	**Torulaspora delbrueckii 12.2** FLY (Probiotec)	Higher floral aroma: 2-phenylethanol. Low production of volatile acidity, acetaldehyde. No production of H_2S.	High production of glycerol: mouth-feel. Capacity to ferment in monoculture. Ability to second fermentation in sparkling wines.	http://www.probiotec.it/schede/Torulaspora-delbrueckii-12.2.pdf
Lachancea thermotolerans	**Level 2 Laktia™** ADY (Lallemand)	Higher aromatic complexity. Low production of volatile acidity.	High production of glycerol. High lactic acid production: acidity + freshness. Biocompatible for co-inoculation with malolactic bacteria (malolactic fermentation, MLF).	https://www.lallemandwine.com/en/australia/products/catalogue/wine-yeasts/109/level-2-solutions-laktia/

Lachancea thermotolerans	**Concerto™** ADY (CHR Hansen)	Higher intensity and aromatic complexity. Integration of red fruit (strawberry), black fruit, spices. Low production of volatile acidity, acetaldehyde, H_2S.	High lactic acid production: acidity + freshness. Biocompatible for co-inoculation with malolactic bacteria (MLF). Production of polysaccharides: mouth-feel. More rounded and smoother mouthfeel.	https://www.chr-hansen.com/en/food-cultures-and-enzymes/fermented-beverages/cards/product-cards/concerto
Lachancea thermotolerans	**Viniflora® Octave** ADY (CHR Hansen)	Esters: enhance fruit flavours. Stone fruits (peach, apricots) and pear notes. Low production of acetic acid, phenols, H_2S.	Capacity to increase the lactic acid content: increased acidity and pH reduction. Reduction of alcohol content. Bioprotection in the pre-fermentative stage. Reduction of added SO_2 doses used. Disadvantage: Inhibits MLF.	https://www.chr-hansen.com/en/food-cultures-and-enzymes/fermented-beverages/cards/product-cards/octave
Lachancea thermotolerans	**EnartisFerm Qfk** CRY (Enartis)	Higher production of 2-phenylethanol (rose, flower). Low production of volatile acidity.	Capacity to increase the lactic acid content: increased acidity and pH reduction. Reduction of alcohol content. High production of glycerol.	https://www.enartis.com/it/prodotti/vino/lieviti/lieviti-liquidi/enartisferm-q%C6%99/
Lachancea thermotolerans	**Excellence X-Fresh** ADY (Lamothe Abiet)	More “fresh and fruity” aromatic profile.	Capacity to increase the lactic acid content: increased acidity and pH reduction. Reduction of alcohol content.	https://lamothe-abiet.com/wp-content/uploads/FT/EN/FT_EN_X_FRESH.pdf
Lachancea thermotolerans	**LEVULIA Alcomeno** ADY (AEB)	Low production of volatile acidity.	Capacity to increase the lactic acid content: increased acidity and pH reduction. Reduction of alcohol content. More freshness and balance on the palate.	https://www.aeb-group.com/media/catalogo-unico/levulia_alcomeno-2886/docs/us/LEVULIA_ALCOMENO_TDS_EN_0040516_OENO_USA.pdf
Lachancea thermotolerans	**Kluyveromyces thermotolerans** FLY (Probiotec)	Higher production of 2-phenylethanol (rose, flower). Low production of acetaldehyde.	Capacity to increase the lactic acid content: increased acidity and pH reduction. High production of glycerol.	http://www.probiotec.it/schede/Kluyveromyces-thermotholerans.pdf

Metschnikowia pulcherrima	**Flavia® MP346** ADY (Lallemand)	Higher expression of terpenes and thiols (α-arabinofuranosidase activity). Low production of volatile acidity, acetaldehyde	More perception of acidity and freshness. Polysaccharides releasing and early autolysis: mouth-feel	https://catalogapp.lallemandwine.com/uploads/yeasts/docs/36209185ea4f5b1375e1d3b85562dd99f9cd775b.pdf
Metschnikowia pulcherrima	**Oenoferm® MProtect** ADY (Erbslöeh)	Low production of acetic acid and ethyl acetate. Prevention of off-flavours.	Biocontrol of spontaneous grape microbiota. Reduction of added SO_2 doses used.	https://erbsloeh.com/fileadmin/user_upload/pdf/Wine/technical_data_sheet/GB/oenoferm_mprotect-technical_data_sheet-english-erbsloeh.pdf
Metschnikowia pulcherrima	**AWRI Obsession** ADY (AB Biotek)	Improvement of dark fruit flavour. Capability to mask green characters. Low production of volatile acidity.	Increasing of colour and complexity in red wines.	https://www.abbiotek.com/perch/resources/next-generation-awri-obsession-product-information-may-2019-web.pdf
Metschnikowia pulcherrima	**LEVULIA® PULCHERRIMA** ADY (AEB)	Monoculture: Increase of higher alcohols and terpenes. Co-culture: Increase of higher alcohols, ethyl esters, phenyl-acetate, isoamyl acetate and terpenes. Low production of volatile acidity.	Capacity to ferment in monoculture. Capacity to finish the fermentative process.	https://www.aeb-group.com/us/levulia-pulcherrima-8749
Metschnikowia pulcherrima	**PRIMAFLORA® VB BIO** ADY (AEB)	Preservation of enzymes. Contributes to the flavour and aromatic complexity. Prevention of off-flavours: H_2S, butyric odours, volatile phenols, acetic acid, etc. Less extraction of unpleasant flavours.	Lower production of biogenic amines. Bioprotection in the pre-fermentative stage. Reduction of added SO_2 doses used.	https://www.aeb-group.com/au/primaflora-vb-bio-10480.
Metschnikowia pulcherrima	**Excellence® B-Nature®** ADY (Lamothe Abiet)	Improvement of the aromatic complexity.	Bioprotection in the pre-fermentative stage. Reduction of added SO_2 doses used.	https://lamothe-abiet.com/wp-content/uploads/FT/EN/FT_EN_B_NATURE.pdf

Metschnikowia fructicola	**Levia Nature** ADY (Oeno)	Low production of volatile acidity.	Bioprotection in the pre-fermentative stage. Reduction of added SO_2 doses used. Facilitates the implantation of *S. cerevisiae*.	https://www.oeno.it/wp-content/uploads/ST_Levia_NATURE_rev3.pdf
Metschnikowia fructicola	**IOC Gaïa™** ADY (Lallemand)	Improvement of the sensory expression and preserving varietal character. Low production of volatile acidity.	Competitive factor: active K2 (bioprotection in the pre-fermentative stage). Reduction of added SO_2 doses used. Facilitates the implantation of *S. cerevisiae*.	https://catalogapp.lallemandwine.com/uploads/yeasts/docs/fb8e1d0b8c8bb51c62288183495020f49c99e0f4.pdf
Wickerhamomyces anomalus	**Anti Brett 1** FLY (Probiotec)	No production of acetic acid. Low production of H_2S.	Active mycocin against *Brettanomyces*. Synergy with *Kluyveromyces wickerhamii*.	http://www.probiotec.it/schede/anti-brett-1.pdf
Kluyveromyces wickerhamii	**Anti Brett 2** FLY (Probiotec)	No production of acetic acid. Low production of H_2S.	Active mycocin against *Brettanomyces*. Synergy with *Wickerhamomyces anomalus*.	http://www.probiotec.it/schede/anti-brett-2.pdf
Schizosaccharomyces pombe	**Atecrem 12H** CRY (BioEnologia)	Low production of acetic acid, volatile acidity.	Malic acid degradation. Gluconic acid degradation. High production of glycerol (until 15 g/L). High production of polysaccharides. Production of vitisin A. Reduction of ochratoxin A (OTA).	https://www.bioenologia.com/vino/atecrem-12h
Schizosaccharomyces pombe	**Promalic** ENCY (Proenol)	Improvement of the freshness and aromatic profile	Malic acid degradation. No contact between yeast and must/wine. Easy removal of encapsulated yeast after deacidification process.	https://www.proenol.com/web/produtos/leveduras-encapsuladas/promalic-detail
Stamerella bacillarus	**Atecrem 11H** CRY (BioEnologia)	Medium production of volatile acidity	High production of glycerol (until 14 g/L).	https://www.bioenologia.com/vino/atecrem-11h

Zygosaccharomyces bailii	**Fructoferm W3** ADY (Lallemand)		Fructophilic yeast for the treatment of stuck fermentations	https://www.yumpu.com/de/document/view/36907518/fructoferm-w3
Zygosaccharomyces parabailii	**Hardened Spaniard** FLY (Mainiacal Yeast)	It lends notes of fresh cut apples and earthy/hazelnut like flavours.	"Flower-film yeast" for Sherry wines.	https://mainiacalyeast.com/pro-brewers
Pichia kluyveri	**Frootzen®** AFY (CHR Hansen)	Expression of thiols 3SH and A3SH. Low production of volatile phenols, volatile acidity, H_2S.		https://www.chr-hansen.com/en/food-cultures-and-enzymes/fermented-beverages/cards/product-cards/frootzen-first-ever-pichia-kluyveri-yeast
Pichia kluyveri	**Pichia kluyveri MIP-001** FLY (Propagate Lab)		According to the technical datasheet, is a yeast strain commonly found in wine.	https://www.propagatelab.com/pichia-kluyveri
Pichia kluyveri, *Kazachstania servazzii*	**Trillyeast** CRY (BioEnologia)	High production of esters: rose, peach, pear and apple. Strong notes of olea fragrans and liquorice.	High production of glycerol.	https://www.bioenologia.com/vino/trillyeast
Torulaspora delbrueckii + *Saccharomyces cerevisiae*	**Oenoferm® Wild & Pure** ADY (Erbslöeh)	Production of fruity esters. Higher expression of terpenes. Support the ripe and exotic fruit aroma.	Creamier, long-lasting, pleasant flavour and mouth-feel.	https://erbsloeh.com/fileadmin/user_upload/pdf/Wine/technical_data_sheet/GB/oenoferm_wild_and_pure_f3-technical_data_sheet-english-erbsloeh.pdf
Torulaspora delbrueckii + *Saccharomyces cerevisiae*	**New Nordic Ale Yeast** FLY (White Labs)		The technical datasheet only mentions its use in white and red winemaking.	https://www.whitelabs.com/yeast-single?id=177&type=YEAST&style_type=0#:~:text=Isolated%20from%20spontaneously%20fermented%20apples,cerevisiae%20and%20one%20Torulaspora%20delbrueckii).
Torulaspora delbrueckii + *Metschnikowia pulcherrima*	**Zymaflore® Égide** ADY (Laffort)		Bioprotection in the pre-fermentative stage). Reduction of added SO_2 doses used. Facilitates the implantation of *S. cerevisiae*.	https://laffort.com/en/products/zymaflore-egide/

Metschnikowia pulcherrima + *Saccharomyces cerevisiae*	**Primaflora® VR Bio** ADY (AEB)	Preservation of enzymes. Contributes to the flavour and aromatic complexity. Prevention of off-flavours: butyric odours. Less extraction of unpleasant flavours.	Lower production of biogenic amines and acetamides. Bioprotection in the pre-fermentative stage). Reduction of added SO_2 doses used.	https://www.aeb-group.com/au/primaflora-vr-bio-10496
Lachancea thermotolerans + *Saccharomyces cerevisiae*	**Viniflora® Symphony** ADY (CHR Hansen)	Enhancement of fruity flavours: thiols and esters. White wines: floral aroma, tropical fruity notes. Red wines: complex and round flavours. Low production of acetic acid, volatile acidity, volatile phenols, H_2S.	Limited lactic acid production from sugars. Biocompatible with malolactic bacteria.	https://irp-cdn.multiscreensite.com/747494ab/files/uploaded/16-CHR-yeast%20Viniflora%20Symphony%20TDS.pdf
Lachancea thermotolerans (40%) + *Saccharomyces cerevisiae* (60%)	**Rhythm™** ADY (CHR Hansen)	Improvement of fruity flavours: thiols and esters. Low levels of acetic acid, volatile acidity, volatile phenols, H_2S.	Lactic acid production from sugars. Enhancement of complexity and mouth-feel. Improve palate weight. Biocompatible with malolactic bacteria.	https://catalogs.gusmerenterprises.com/view/397297212/10/
Lachancea thermotolerans (10%) + *Torulaspora delbrueckii* (10%) + *Saccharomyces cerevisiae* (80%)	**Harmony™** ADY (CHR Hansen)	Improvement of fruity flavours: thiols and esters. Low levels of acetic acid, volatile acidity, volatile phenols, H_2S.	Medium production of polysaccharides. Biocompatible with malolactic bacteria.	https://catalogs.gusmerenterprises.com/view/397297212/10/
Lachancea thermotolerans + *Torulaspora delbrueckii* + *Saccharomyces cerevisiae*	**Melody™** ADY (CHR Hansen)	White wine: improvement of aromatic intensity and increase of tropical fruit flavour: thiols and esters. Red wine: more pronounced fruity and spicy notes. Low production of volatile phenols, volatile acidity, H_2S.	Facilitates the malolactic fermentation. Medium production of polysaccharides: mouth-feel.	https://www.chr-hansen.com/en/food-cultures-and-enzymes/fermented-beverages/cards/product-cards/melody

***** ADY = active dry yeast, FLY = fresh liquid yeast, CRY = cream yeast, AFY = active frozen yeast, ENCY = encapsulated yeast.

1.5. Potential of non-*Saccharomyces* to reduce alcohol/ethanol content in wine

The role of non-*Saccharomyces* in wine production has been re-visited as a potential to positively contribute to wine complexity and novelty, rather than as a source of microbial spoilage in wine. The expansion of the wine market and changes in consumer preferences has led to the development of strategies to produce varied styles of wine (Fleet 2008). Furthermore, there has been progressive trend towards increased alcohol content in wines, resulting in part from increased sugar content and early ripening (shortened, hotter vintages due to global climate change), as well as altered winemaking practises towards fuller bodied wines (Ciani et al., 2016a; García et al., 2020; van Leeuwen and Darriet, 2016). The need to reduce the presence of ethanol in wine, is evident not only in terms of wine production, but also public concern. For the industry, the high sugar musts result in ethanol levels toxic to the microbiota (yeast and bacteria), whilst high alcohol content in wines can mask the bouquet of the wine, altering its sensory attributes to the consumer. But more importantly, health concerns linked to alcohol consumption has led to an increase in recommendations to reduce alcohol content (Contreras et al., 2014).

Approaches to limit excessive alcohol production prior to fermentation have been made using various techniques aimed at reducing fermentable sugars. These include altered viticulture practises (e.g., irrigation), and pre- (e.g., water addition, enzyme additions) (Gardner et al., 2022; Ozturk and Anli, 2014) and post-vinification practises (e.g., reverse osmosis, evaporative perstraction) (Schmitt and Christmann, 2019), and microbial strategies (Varela et al., 2015). The use of non-*Saccharomyces* is a pivotal strategy to a holistic approach to producing reduced-alcohol wines of high quality and aromatic complexity. A study done by Hranilovic et al. (2020) compared the timing of the secondary inoculation (24 h, 48 h and 72 h) of *M. pulcherrima* and *S. cerevisiae* (as sequential cultures) and their effects on ethanol production. The longer the delay of the inoculation of *S. cerevisiae* the greater the ability of

the *M. pulcherrima* to modulate the aroma/flavour profile in wines with 0.6 to 1.2 (ABV)% less ethanol than the control (*S. cerevisiae*) (Hranilovic et al., 2020). The presence of *M. pulcherrima* had also altered the volatile profiles (i.e., reduction of ethyl esters of medium-chain fatty acids, and lower acetic acid content), with no off-flavours detected (Hranilovic et al., 2020), which demonstrated their ability (like several other non-*Saccharomyces* species) to influence wine aroma.

1.6 Contribution of non-*Saccharomyces* to wine aroma/flavour profile

Aroma is one of the most important indicators to the quality of wine. Wine aroma can be subdivided into three groups: i) primary (from the grape variety), ii) secondary (from fermentation) and iii) tertiary (from aging of the wines) (Padilla et al., 2016). Non-*Saccharomyces* yeasts can influence the primary and secondary aroma through the production of enzymes and metabolites (Tufariello et al., 2021). Formation of primary aroma during the ripening of grapes can be attributed to a small number of chemical families, such as methoxypyrazines, C_{13}-norisoprenoids, volatile sulfur compounds and terpenes (Padilla et al. 2016). Methoxypyrazines are products of amino acid metabolism and are associated with green characters (e.g., vegetal, capsicum) in wine (Lei et al., 2018). C_{13}-norisoprenoids, derived from the degradation of carotenoids, largely contribute to varietal characters in aromatic varieties (e.g., Riesling), for example, β-damascenone which has a fruity aroma, is said to enhance the intensity of other fruit-smelling compounds. Of the many terpenoids (e.g., monoterpenes) found in all grape cultivars, they occur in highest concentrations in aromatic varieties such as Muscat and Gewürtztraminer (Park and Noble, 1993). Most primary aroma compounds occur as bound non-volatile glycosides, which are released during fermentation through the action of yeast (and bacterial) enzymes (i.e., glycosidases) (Michlmayr et al., 2012; Padilla et al., 2016). The formation of volatile aroma compounds can occur as part of yeast metabolism (glycolysis,

amino acid and fatty acid metabolism). Alternatively, they are the product of enzymatic released from non-volatile precursors via breakage of glycosidic or carbon-sulfhydryl linkages (Table 4). Because of the highly diverse non-*Saccharomyces* yeast species present in un-inoculated (spontaneous) fermentations, it is expected that a wider range of extracellular enzymes would be produced compared to a monoculture fermentation with *S. cerevisiae*. Consequently, a wider variety of volatile compounds will be formed in uninoculated/spontaneous fermentations (Borren and Tian, 2021).

Table 4. Major volatile compounds produced during fermentation, along with the genes (in *S. cerevisiae*) and enzymes responsible, initial substrates and their effect on the aroma (updated from Borren and Tian, 2021).

Volatile compounds	Gene(s) responsible	Enzyme responsible	Substrates	Aroma descriptors
Esters	*ATF1/ATF2, IAH1, EEB1/EHT1, EAT1*	Alcohol acetyltransferase, esterase	Alcohol + acid	Floral, fruity
Terpenes	*BTS1, COQ1, ERG12, ERG20, EXG1, HMG1, MVD1*	Glycosidase	Terpenoid glycosides	Floral, varietal
Higher alcohols	*ADH1-5, ARO3, ARO4, ARO7, ARO10, BAT1, BAT2*	Alcohol dehydrogenase	Amino acids	Low: fruity High: ethereal
Volatile phenols	*PAD1, FDC1*	Phenol reductase, decarboxylase	Carboxylic acids	Low: smoky, bacon High: barnyard
Sulfur containing compounds	*IRC7*	Sulfur lyase, alcohol dehydrogenase	Amino acids, thiols, natural sulfur	Sulfite: rotten eggs Thiols: tropical
Volatile fatty acids	*FAS1, FAS2*	Decarboxylase, fatty acid synthase	Acetyl-coA, malonyl-coA	Vinegar, rancid, pungent

1.6.1 Glycosidases

Glycosidases play a pivotal role in the winemaking process, through the release of aroma component (aglycone) from the sugar moiety (glycone) in the odourless aroma glysoside precursor (Padilla et al., 2016). These enzymes (e.g., β-D-glucosidase, α-L-arabinofuranosidase) are involved in hydrolysis of terpene glycosides by cleaving to the intersugar bonds (depending on conjugate), which releases monoterpenes (Esteve-Zarzoso et al., 2002). Screening studies have shown species belonging to *Candida*, *Debaryomyces*, *Hanseniaspora*/*Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces,* and *Zygosaccharomyces* can produce β-D-glucosidases (Maicas and Mateo, 2016; Rosi et al., 1994). Additionally, the enzymes of *Hanseniaspora* spp. displayed higher efficiency compared to other yeast species in releasing desirable aromas during the early stages of fermentation, whilst *Pichia anomala* (now known as *Wickerhamomyces anomalus*) exhibited higher β-D-glucosidase towards the end of fermentation (Swangkeaw et al., 2009).

1.6.2 β-lyases

Volatile sulfur compounds (e.g., mercaptans and thiols) are also detected in grapes as odourless precursors after the release of primary aroma compounds due to yeast enzymatic activity. Volatile thiols, which give their characteristic tropical aroma in Sauvignon Blanc wines (Padilla et al., 2016; Roncoroni et al., 2011) are generated through the odourless cysteinylated precursors cysteine-3-mercaptohexan-1-ol (Cys-3MH; conjugated thiol) and cysteine-4-mercapto-4-methylpentan-2-one (Cys-4MMP; conjugated thiol) by the action of carbon-sulfur-lyases to form 4-mercapto-4-methylpentan-2-one (4MMP) (box-tree, broom), 3MH (grapefruit) and 3MHA (3-mercaptohexyl acetate; enzymatically converted from 3MH) (box-tree, passionfruit) (Swiegers et al., 2007). The yeast (*S. cerevisiae* and *S. paradoxus*) *IRC7* gene encodes for β-lyases, which elevated the production of 4MMP after fermentation. The

deletion of *IRC7* gene reduced 4MMP production, but did not affect the yield of 3MH (Roncoroni et al., 2011).

1.6.3 Ethyl and acetate ester formation

Regarding the production of esters, the vast majority are produced during fermentation and are divided into two main groups: ethyl esters and acetate esters which contribute to the fruity aroma, boosting the fruit and floral characteristics of the grape varietal. The biosynthesis of esters is formed in either of the following two ways: 1) the direct equilibrium reaction between alcohol and acid (enzyme free), and 2) enzyme catalysed reactions (Tufariello et al., 2021). Formation of ethyl esters involves the activation of the acid with coenzyme A (coA) before reacting with the alcohol to form an ester. The intermediates can be either acetyl-coA (through pyruvate (glycolysis)) or acyl-coA (formed by acyl-coA synthetase).

Fatty acid ethyl esters (e.g., ethyl butanoate, ethyl hexanoate) are formed by the esterification of ethanol with fatty acid or fatty acyl-coA (Saerens et al., 2006). Acetate esters (e.g., isoamyl acetate, phenylethyl acetate) are formed from acetyl-coA and alcohol (degraded from amino acids, carbohydrates, lipids) and catalysed by alcohol acetyltransferase (AAT) (Yoshioka and Hashimoto, 1984). Non-*Saccharomyces* are known to be high ester producers, and has been linked with negative effects in wine associated with high ethyl acetate production (Padilla et al., 2016). Species belonging to *Hanseniaspora* (*H. guillermondii* and *H. osmophila*) have been reported to produce remarkable levels of phenylethyl acetate (3- to 9-fold compared to *S. cerevisiae* pure cultures) (Viana et al., 2009). A fairly novel ascomycete yeast, *Kazachstania gamospora* produced more esters (200-times more, specifically ethyl proprionate (a desirable floral aroma)) compared to the *S. cerevisiae* control (Beckner Whitener et al., 2015).

1.6.4 Proteases and lipases

Aside from these aroma-related enzymes, other beneficial enzymes in wine-related yeasts (e.g., protease, lipases) are also important in improving various technological and sensory features of the wine (Claus and Mojsov, 2018). Wine haze derived from proteins (e.g., chitinase and thaumatin-like proteins) is an aesthetic problem especially in white wines (van Sluyter et al., 2015). Currently, protein and haze removal in wines is achieved by bentonite addition, which can be detrimental to the wine quality (Millarini et al., 2020). Microbial proteases could be a potential alternative, or supplement to bentonite for removal of unwanted wine proteins. To date, fungal acid proteases (e.g., aspartic acid protease from *Botrytis cinerea* (van Sluyter et al., 2013) are considered as potential alternatives. As most *S. cerevisiae* strains do not possess extracellular protease activity (Schlander et al., 2017), non-*Saccharomyces* yeasts may provide alternative sources either as purified preparations or through co-culture. Preliminary work by Schlander et al. (2017) demonstrated strains of *M. pulcherrima* and *W. anomalus* to degrade bovine serum albumin (a model protein) through secreted proteases during growth in grape juice.

Extracellular pectinolytic enzymes of non-*Saccharomyces* yeasts, could be utilised to avoid the costly preparations of commercial enzymes (i.e., polygalacturonase, pectinlyase, pectinesterase) to remove unwanted pectin haze and filter-clogging polysaccharides in white wines (Claus and Mosjov, 2018). The use of selected wine yeasts (or prepared enzymes derived from these yeasts) with such characteristics would minimise the disadvantages that the harsh fining and clarification brings to the loss of important wine aroma/flavour compounds.

1.7. Potential use of non-*Saccharomyces* as bio-control agents during pre- and post-fermentation

Among the biotic factors affecting the interaction of yeasts in a controlled multi-starter fermentation, killer toxins are the most studied. Killer yeasts can kill sensitive yeasts by secreting proteinaceous toxins to which they, themselves, are immune (Marquina et al., 2002). Killer toxins are secreted polypeptides encoded by extrachromosomal elements which are found in *Saccharomyces,* as well as some non-*Saccharomyces* species (*Candida*, *Cryptococcus*, *Debaromyces*, *Hanseniaspora, Pichia, Torulaspora,* and *Zygosaccharomyces*) (Vélazquez et al., 2015). The mode of action differs; with toxins produced by *S. cerevisiae* triggering early cell death in *Hanseniaspora guillermondii* (Albergaria et al., 2010), whilst non-*Saccharomyces* killer toxins exhibited a wider range of activities, inhibiting strains belonging to both *Saccharomyces* and non-*Saccharomyces* genera (Andorra et al., 2012). Non-*Saccharomyces* killer yeasts have potential as biocontrol agents, e.g. the commercialised *Kluyveromyces wickerhamii* (Anti Brett 2, Probiotec) produces mycocin to control *Brettanomyces*/*Dekkera* spoilage yeasts, and *Pichia membranifaciens* secretes the killer toxins PMKT and PMKT2 that is inhibitory to *Brettanomyces bruxellensis* (Belda et al., 2017). The production of phenolic off-flavours (POF) (e.g., 4-ethylphenol, 4-ethylguiacol and 4-ethylcatechol) by these yeasts lends to undesirable sensory characteristics (barnyard, horse stable) in the wine. Mixed cultures of *S. cerevisiae* and *W. anomalus* displayed killer activity on *B. bruxellensis*, showing the potential of using non-*Saccharomyces* killer yeasts to produce wines with controlled quality. Additionally, the use of non-*Saccharomyces* killer toxins to control apiculate yeasts could possibly displace the use of SO_2 as a pre-fermentative treatment to control microbial contamination in grape musts (Mehlomakulu et al., 2015).

Whilst current research is in its infancy, further studies beyond the scope of the project, are warranted to maximise the use of non-*Saccharomyces* to winemaking in wine production;

both in process efficiency and wine quality and composition.

1.8 Genetic basis of yeast-derived wine aroma

1.8.1 Genome sequencing as a tool to characterise genetic variation between wine yeasts

In recent years, whole-genome sequencing (WGS) has become an affordable and accessible tool for yeast genotyping. Analysis of the entire yeast genome not only provides insights into the genetic diversity/architecture but also the genetic variation and changes underlying adaptation at a genome-wide scale. Advances in WGS technologies (e.g., Illumina, Nanopore, PacBio) and analysis tools/pipelines (publicly accessible and propriety) have boosted the output speed as well as the lowered costs of WGS (Amarasinghe et al., 2020). The yeast genome has been widely studied since the *S. cerevisiae* S288C genome was fully sequenced and made available (Goffeau et al., 1996). This allowed the rapid progress in understanding of fermentation and the wine microbiome. The approximate 6000 genes in *S. cerevisiae* provides a model system for studying fundamental genetics and complex biological pathways.

1.8.2 Genome architecture of wine related non-*Saccharomyces*

Among the non-*Saccharomyces*, *Metschnikowia* is one of the most investigated genera (with over 80 species in this genera) since it exerts moderate fermentative power and have the ability to modulate production of volatile compounds and improve the sensory profile of wines. The most common *Metschnikowia* associated in grape and wine-related environments are *M. fructicola*, *M. pulcherrima*, and *M. viticola*. At present, the complete genome sequences of different strains for *M. fructicola* and *M. pulcherrima* are available publicly (Hershkovitz et al., 2013; Hirao et al., 2019, Piombo et al., 2018; Venkatesh et al., 2018).

In fermented beverages, flavour trait/phenotype is deemed an important characteristic when developing yeast screening methods within the beverage (or food) industry (Carrau et al., 2017). Although non-*Saccharomyces* yeasts have been reported as beneficial for winemaking,

since they contribute to aroma/flavour complexity to wines, their genomic features are still poorly explored (not detailed compared to *S. cerevisiae*). Like several non-*Saccharomyces* yeasts, *Kazachstania* spp. have been reported to produce high levels of acetate esters (Beckner Whitener et al., 2015; Lin et al., 2020). As previously mentioned (in Section 1.6.3), acetate esters (e.g., phenylethyl acetate) are synthesised by alcohol acetyltransferase (AATase) from acetyl coenzyme A (coA) and their corresponding alcohols. The *ATF1* encodes one type of AATase, which catalyses the synthesis of acetate esters from acetyl coA and several kinds of alcohols (Fujii et al., 1994; Fujiwara et al., 1999). In *S. cerevisiae*, various genes have been identified as contributors to acetate esters, ethyl esters and higher alcohol biosynthesis, which have remained uncharacterised in most non-*Saccharomyces* yeast species. Wolfe et al. (2015) reported briefly on whole-genome approaches of *K. africana* and *K. naganishii*, but with limited information regarding their genome annotation (Wolfe et al., 2015). Ester genes and their biosynthesis pathways are explained in more detail in Chapter 5.

1.9 Project summary and thesis structure

In this literature review, the conventional to molecular approaches in yeast identification, and the interaction mechanisms between yeasts and their contribution to the wine aroma/flavour profile have been discussed. The negative perception about non-*Saccharomyces* yeasts is changing due to the increased research interest and publications demonstrating their importance in contributing to sensory attributes in wines. Selection of non-*Saccharomyces* yeasts for use as wine starter cultures with the potential to positively influence the organoleptic properties of wine, requires the critical evaluation of physical and nutrient requirements of the yeast, as well as their phenotypic behaviour and the stability of such phenotypic traits.

This project aimed to characterise indigenous non-*Saccharomyces* yeast isolates on their behaviour during alcoholic fermentation and their metabolic activities involved in wine aroma production. Furthermore, this project aimed to study the genetic basis of the newly discovered *Kazachstania* spp. (*K. aerobia* and *K. servazzii*), specifically the functional genes involved in flavour-active ester biosynthesis/production.

In order to achieve the project aims, the following objectives were pursued and outlined in the following chapters of this thesis:

1. Identify, characterise non-*Saccharomyces* yeast isolates derived from a South Australian vineyard environment for enzymatic activities/oenological properties and evaluating their fermentation efficiency in CDGJM and sterile juice (Chapter 2)

2. Investigate the impact of *Kazachstania* spp. isolates in a non-sterile wine environment and sensory effects (Chapter 3)

3. Explore the potential effects of *Hanseniaspora uvarum* isolates on the terpene compounds in white wines (Chapter 4)

4. Study the genome of *Kazachstania* spp. (*K. aerobia* and *K. servazzii*) and the genes involved in ester biosynthesis (Chapter 5)

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CHAPTER 2

"Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures"

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Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures

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ABSTRACT

The use of non-*Saccharomyces* yeast in conjunction with *Saccharomyces cerevisiae* in wine fermentation is a growing trend in the wine industry. Non-*Saccharomyces*, through their distinctive production of secondary metabolites, have the potential to positively contribute to wine sensory profile. To discover new candidate strains for development as starter cultures, indigenous non-*Saccharomyces* were isolated from un-inoculated fermenting Shiraz musts from a South Australian vineyard (McLaren Vale wine region) and characterised. Among the 77 isolates, 7 species belonging to 5 genera (*Kazachstania*, *Aureobasidium*, *Meyerozyma*, *Wickerhamomyces* and *Torulaspora*) were identified by sequencing the internal transcribed spacer regions of the 5.8S rRNA gene (ITS1-5.8S-ITS2 region). The indigenous isolates were evaluated for oenological properties, namely, ethanol tolerance, enzyme activity, and H_2S production. To determine their potential industrial use as starter cultures, representative isolates of each species were assessed in a sterile chemically defined grape juice and Viognier grape juice to evaluate their contribution to fermentation kinetics and production of key metabolites, including volatile compounds.

1. Introduction

Wine is the product of alcoholic fermentation and involves the interactions of microorganisms especially yeasts, transforming sugars in grape must into ethanol and carbon dioxide. Whilst non-*Saccharomyces* species present in grape must were previously considered potential spoilage yeasts (Jolly et al., 2014), being associated with stuck or sluggish fermentations, and at times, undesirable sensory properties; several non-*Saccharomyces* strains, such as *Kluyveromyces marxianus*, *Metschnikowia pulcherrima*, and *Pichia kluyveri* do positively contribute to wine quality (Anfang et al., 2008; Hranilovic et al., 2018; Rollero et al., 2018; Ruiz et al., 2018). The role of non-*Saccharomyces* yeasts present in un-inoculated must fermentations is receiving increasing attention, as their ability to secrete enzymes and produce desirable secondary metabolites could potentially improve the aromatic profile of wine, as well as minimise processing during wine production.

The abundance and variability of indigenous yeast species on grapes is thought to be highly dependent on a region's climate, soil, terrain and harvesting procedures (Barata et al., 2012; Capozzi et al., 2015). The resulting biodiversity of grapevine-associated microbiota could potentially identify a vineyard, linking the wine characteristics specifically to the '*terroir*' or environment (Eder et al., 2017; Knight et al., 2015). Detailed studies on grapevine-associated microbiota may lead to the identification of indigenous strains of oenological value, for enhancing regional characteristics in wine.

Traditionally, yeasts have been classified by morphological and physiological methods based on taxonomic keys. However, as new species and known species are renamed due to variations in molecular characteristics and genomic differences, these methods cannot be relied upon for routine identification of yeast cultures. Advances in molecular techniques have enabled the differentiation of yeast isolates to species and strain level. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) are reliable methods for molecular typing of yeasts. PCR amplification and sequencing of the two gene regions, namely D1/D2 domain of 26S rDNA region, and the internal transcribed spacer (ITS) region between the 18S and 28S ribosomal RNA (rRNA) genes using the specific primers ITS1 and ITS4 are frequently used for rapid identification of yeasts (Kurtzman and Robnett, 1998; Sun et al., 2009). The ITS region contains the highly conserved 5.8S rRNA gene with higher interspecific differences compared to 18S

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and 28S rRNA genes. This has proven to be useful in measuring genealogical relationships between close fungal species. Combined with RFLP, greater polymorphism can be achieved by applying restriction enzymes to amplified genes to differentiate species or strains from one another (Granchi et al., 1999).

In the early stages of fermentation, the majority of the microbiota are represented by aerobic and apiculate yeast, and yeast-like fungi, which reside on the surfaces of grape berries or winery equipment (Grangeteau et al., 2016; Pretorius, 2000). Despite the wide variability of yeast species, apiculate yeasts belonging to *Hanseniaspora/Kloeckera* and *Candida* species represent the dominant microbial population early in fermentation, followed by species belonging to *Metschnikowia* and *Pichia* (Padilla et al., 2016). Other yeast species such as *Schizosaccharomyces, Lachancea*, *Torulaspora*, *Rhodotorula* and *Zygosaccharomyces* are also identified (Taillandier et al., 2014). As fermentation progresses, the population of non-*Saccharomyces* yeasts decline and *S. cerevisiae* dominates and completes the fermentation (Fleet, 2003).

Current commercial production of wine favours the use of selected *S. cerevisiae* strains as pure starter cultures, due to the advantage of achieving successful alcoholic fermentation. Despite this, wines produced with mono-cultures can lack aromatic complexity and distinct characteristics, which are introduced by indigenous yeasts (Fleet, 2008; Padilla et al., 2016). In this context, the inclusion of non-*Saccharomyces* along with *S. cerevisiae* has been proposed to mimic un-inoculated fermentations (Ciani et al., 2010; Hranilovic et al., 2017) whilst avoiding the risk of stuck fermentation. To ensure the viability of these yeasts and their positive impact during the winemaking process, the selection of yeasts *via* appropriate screenings is necessary, to confirm whether they can convert substrates in grapes into desirable aromatics in wine, in order to be considered as novel starter cultures (Padilla et al., 2016).

Selection of a wine yeast strain is based on specific attributes that guarantee the desirable features of the specific wine style. Flavour and aroma are undoubtedly the most important distinguishing features of wine. It is well established that several secondary metabolites found in grapes and musts are either in free or bound form (Rodriguez et al., 2004); the latter is flavourless and non-volatile, which must be hydrolysed through the action of wine yeasts and bacteria to have a flavour impact (Padilla et al., 2016). The yeast enzymes involved in the hydrolysis of glycosidic precursors are glycosidases, such as β-glucosidase, which release monoterpenes from their glycosylated form (Maicas and Mateo, 2015). Glucosidase activity has been reported in strains of *Candida*, *Hanseniaspora* and *Pichia* (Charoenchai et al., 2008). Besides glucosidases, proteolytic and lipolytic activity are deemed important due to their potential to degrade haze proteins and lipids (Strauss et al., 2001). Other beneficial attributes include tolerance to ethanol and low hydrogen sulfide production (Šuranská et al., 2016). No one strain will necessarily have the ideal combination of all traits, thus a palate of strains with mixed properties is a useful resource for the winemaker.

This study focuses on the identification of autochthonous non-*Saccharomyces* isolates derived from a South Australian vineyard environment. As a preliminary step to selecting isolates for use as starter cultures or as winemaking additives, isolates were first characterised based on potentially useful enzymatic activities (β-glucosidase, protease and lipase) and unwanted activities (H_2S production) and ethanol tolerance. In order to determine whether these new isolates were suitable for winemaking and to investigate whether there were any isolate specific differences between fermentations, their ability to complete alcoholic fermentation and subsequent secondary metabolite production was studied in both chemically defined grape juice media and in juice. Fermentation kinetics under laboratory conditions were compared along with secondary metabolite differences analysed using HPLC and GC–MS.

2. Material and methods

2.1. Yeast strains

The indigenous yeast strains used in this study were isolated from un-inoculated fermenting Shiraz grape must fermentations at different stages (pre-, mid- and end) using grapes sourced from McLaren Vale, South Australia (Hardy's vineyard; 2007). The yeast isolates were originally selected on Wallerstein (WL) nutrient medium (Pallmann et al., 2001) and Lysine medium (Lin, 1975), to allow for detection of morphological differences between species and growth of indigenous non-*Saccharomyces* yeasts, respectively. The cryogenically preserved isolates (−80 °C in 40% glycerol) were revived on YPD agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plates for this study, and are listed in Supplementary Table 1.

2.2. Molecular identification of yeast

Genomic DNA was isolated from yeast according to Adams et al. (1998). Species identification was by ITS PCR, with the fungal specific primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) to amplify the internal transcribed spacer (ITS) and 5.8S rDNA gene regions (White et al., 1990). PCR amplification reactions were performed in a 25 µL reaction using Mango Taq DNA polymerase (Bioline, USA) according to manufacturer's instructions, and 50 pmol of each primer and ~200 ng genomic DNA as template. PCR was initiated at 95 °C for 5 min, followed by 35 amplification cycles (95 °C, 2 min; 53 °C, 2 min; 72 °C, 2 min) and terminated with a final 10 min extension at 72 °C.

PCR products were resolved by 1.5% (v/v) agarose gel electrophoresis and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) prior to DNA sequencing (AGRF, Adelaide). Yeast species were identified by sequence comparison to the Genbank® NIH genetic sequence database (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST sequence analysis tool (Raja et al., 2017).

2.3. Screening of oenological properties

Plate-based assays were used to screen for H_2S production and specific enzymatic activities (β-glucosidase, protease and lipase) (Strauss et al., 2001; Šuranská et al., 2016). YPD cultures were grown from single colonies (1 mL; 24 h growth) and used as inocula (4 µL). Yeast isolates were screened for H_2S production on BiGGY (bismuth glucose glycine yeast) (Sigma, USA) agar. The media were spot-inoculated and incubated at 28 °C for 3 days prior to assessment for colour. *Saccharomyces cerevisiae* strains F15 *ICR7* and BY4741 *Δmet17* were included as positive controls for H_2S production. AWRI1631 *Δmet5* was used as a negative control (Supplementary Fig. 1).

Extracellular β-glucosidase activity was determined using arbutin (glucosylated hydroquinone), added to YNB agar (0.67% yeast nitrogen base (Difco), 0.5% arbutin (Sigma), 2% agar). The medium was pH adjusted to 5 before autoclaving and 2 mL of 1% ferric ammonium citrate solution (sterile-filtered) were added per 100 mL of medium prior to plate pouring (Rosi et al., 1994). Plates were spot-inoculated (4 µL) and incubating at 28 °C for 3 days. The ß-glucosidase activity was detected as a brown halo around the colonies.

Extracellular protease activity was determined by spot-plating yeast cultures (4 µL) on skim milk agar plates (10% skim milk powder, 2% agar) and incubating for 5 days at 28 °C. Proteolytic yeasts hydrolyse casein to form nitrogenous compounds indicated as clear zone surrounding the colonies.

Lipase activity was determined using YPD supplemented with 0.3% (v/v) tributyrin (Sigma-Aldrich) as agar plates. The lipolytic yeast *Yarrowia lipolytica* (Mycology Culture Collection, SA Pathology) was included as a positive control. Plates were incubated for 7 days at 28 °C. A clear halo around the colony indicated the presence of lipase activity.

Ethanol tolerance were screened in 96-well microtiter plates with Chemically Defined Grape Juice Medium (CDGJM, 200 g/L sugar, 350 mg/L FAN (Henschke and Jiranek, 1993)) supplemented with 2, 4, 6, 8, 10 and 12% (v/v) ethanol. Each well contained 200 µL of CDGJM, which was inoculated with 4 µL of cell suspension (previously grown overnight in YPD at 28 °C). The initial cell concentration was approximately 2×10^6 cells/mL ($OD_{600} \approx 0.1$). Plates were sealed with a sterile gas-permeable film (BreathEasy®; Molecular Solutions). The culture density was measured every 12 h using a microplate spectrophotometer (TECAN, Switzerland) at 600 nm over 3 days.

2.4. Fermentation of selected isolates in CDGJM

From the screening survey, representative strains from each species were selected for initial fermentation trials (Supplementary Table 2). Fermentations were conducted in triplicate in 100 mL of CDGJM (100 g/L glucose, 100 g/L fructose) and optimal nitrogen (350 mg/L FAN) at 20 °C, with shaking (130 rpm). Yeast cultures were grown in two steps: overnight in YPD at 28 °C before transferring to CDGJM starter (CDGJM with 50 g/ L glucose, 50 g/ L fructose (Henschke and Jiranek, 1993) at a rate of 1×10^6 cells /mL^{-1} and incubated overnight at 28 °C prior to inoculation at a rate of 5×10^6 cells/mL^{-1} in 250 mL conical flasks fitted with airlocks and sampling ports.

2.5. Sequential fermentation in grape juice

Filter-sterilised Viognier juice (2017; Waite vineyard, South Australia) was used for the sequential fermentation experiment. The sugar content (22.8°Brix; 250 g/L) was measured with a hand-held refractometer. Yeast assimilable nitrogen (initial YAN; 236 mg N/L) was determined on a Chemwell 2910 automated analyser (Awareness Technology Inc.) using the Primary Amino Nitrogen (K-PANOPA) and Ammonia (K-AMIAR) enzymatic kits (Megazyme, USA). Additional nitrogen was added in the form of diammonium phosphate (150 mg/L), and YAN was adjusted to 386 mg/L in the juice.

Sequential fermentations were performed in triplicate in 250 mL fermentation flasks equipped with air-locks and containing 100 mL of grape juice. Non-*Saccharomyces* starter cultures, grown overnight in YPD at 28 °C, were transferred to CDGJM starter at 1×10^6 cells/mL and incubated overnight before inoculation at 5×10^6 cells/mL into grape juice. The commercial wine yeast strain EC1118 (Lallemand, Australia), in active dried yeast form, was rehydrated according to the manufacturer's instructions prior to inoculating at 5×10^6 cells/mL after 72 h. Fermentations were sampled every 12 h, and sugar concentration was measured by enzymatic analysis. At the end of fermentation (residual sugar < 2 g/L), wine samples were centrifuged and stored for subsequent analyses for wine metabolites and volatiles.

2.6. Profiling of wine composition

Organic acids (malic, succinic, acetic), glucose, fructose, glycerol and ethanol were determined in terminal samples by HPLC (Li et al., 2017). Undiluted samples were injected onto an Aminex H7C-8H column (300 × 7.8 mm, Bio-Rad) on an Agilent 1100 series HPLC system (Agilent Technologies). An aqueous solution of H_2SO_4 (2.5 mM) was used as mobile phase, at a flow rate of 0.5 mL/min and a column temperature of 60 °C. Signals were detected at 210 nm with an Agilent G1315B diode array and an Agilent G1362A refractive index detector. Compounds were detected by their retention time and quantified by comparison to known standard solutions using Agilent ChemStation software (Li et al., 2017). Quantification was achieved using calibration curves ($R^2 > 0.99$) relating to the concentration of compounds (analytes) from prepared standard solutions. HPLC data were subjected to one-way ANOVA using GraphPad Prism 7.02 (GraphPad, USA). Mean comparisons were performed by Tukey's multiple comparison test at $p < .01$.

2.7. Profiling of volatile compounds produced in wine

Wine samples from the monoculture and sequential fermentations in Viognier juice were analysed for volatile compounds using headspace–solid phase micro extraction – gas chromatography with mass spectrometry (HS-SPME-GC–MS) (Schelezki et al., 2018). Prior to SPME-GC–MS analysis, the wines were diluted 1 in 100 or 1 in 2 with ultra-pure water to a final volume of 10 mL and NaCl (3 g) was added to each SPME vial before being vortexed thoroughly. Samples diluted 1 in 2 were then spiked with 10 µL of an internal standard mix comprised of d13-hexanol (920 mg/L); d11-hexanoic acid (930 mg/L); d13-octanal (82.1 mg/L); d3-linalool (1.73 mg/L) or 10 µL of a 1 in 10 dilution of this mix in the 1 in 100 diluted samples. Volatile compounds were quantified using calibration curves as outlined in Schelezki et al. (2018).

Standards in model wine (12% aqueous ethanol, pH adjusted to 3.2 with tartaric acid) were prepared in triplicate at five evenly spaced concentrations across the range for quantifying the analytes. The highest standard concentration was approximately 150% of the highest concentration observed in the wines for each analyte. Calibrations were linear throughout the range with $R^2 = 0.94–0.99$. All calibration samples were prepared and analysed according to the protocols outlined above.

Statistical evaluation of volatile compounds produced by yeast isolates was carried out by one-way ANOVA using GraphPad Prism 7.02 (GraphPad, USA). Volatile compounds with means calculated not to be statistically different between treatments ($p > .01$), were eliminated from further analyses. Volatile compounds with means that are significantly different ($p < .01$) across the treatments were analysed by principal component analysis (PCA), using the statistical package XLSTAT (version 2018.06, Addinsoft, SARL, Paris, France).

3. Results

3.1. Selection and identification of yeast species

A total of 77 yeast isolates were revived from a collection of 200 yeast, bacterial and fungal isolates sampled from a 2007 Shiraz must (Supplementary Table 1). The yeasts were originally isolated from different stages (pre-, mid- and end) of three un-inoculated fermentations, which were undertaken outside of a winery environment. Colonies were initially isolated on WL nutrient medium to allow the differentiation non-*Saccharomyces* yeasts (and bacteria) based on colony morphology and colour, and Lysine medium which enriches non-*Saccharomyces* (Lin, 1975; Pallmann et al., 2001). Species identification was undertaken using PCR amplification of the ITS regions of the ribosomal DNA (Díaz et al., 2013) and DNA sequence analysis comparison to known fungal ITS regions (https://www.ncbi.nlm.nih.gov/genbank/).

Six ascomycete species belonging to five different genera were identified: *Aureobasidium pullulans*, *Meyerozyma guilliermondii*, *Kazachstania aerobia*, *Kazachstania servazzii*, *Wickerhamomyces anomalus* and *Torulaspora delbrueckii* (Table 1). *A. pullulans* was the most abundant species, representing 48% of the non-*Saccharomyces* present in the fermenting must. The second most abundant species was *K. aerobia* (20%), followed by *W. anomalus* and *T. delbrueckii* at equal percentages (13%), with the latter being present during later stages of fermentation. Both *K. servazzii* and *M. guilliermondii* represented 1% of the total yeast isolates, while a total of 3 isolates remain unidentified; having no similarity to the fungal sequences in the Genbank database.

3.2. Screening of selected oenological properties of indigenous yeasts

Hydrogen sulfide has a detrimental effect on a wine's organoleptic properties, due to its characteristic unpleasant aroma. H_2S production by the isolates was assessed on BiGGY agar by comparing the colour of

Table 1
Distribution of indigenous yeast species in un-inoculated Shiraz grape musts (2007 vintage).

Species	No. of isolates	Proportion (%)
Aureobasidium pullulans	37	48
Kazachstania aerobia	15	20
Kazachstania servazzii	1	1
Meyerozyma guilliermondii	1	1
Wickerhamomyces anomalus	10	13
Torulaspora delbrueckii	10	13
Unidentified[a]	3	4

[a] Uncultured fungi and unknown isolates are grouped together (Refer Supplementary Table 1).

the colonies. All except one isolate (MF_8_L1 (a)), produced H_2S, with greater production being indicated by a darker colony colour (Supplementary Table 1; Supplementary Fig. 1). The colony colours ranged from light brown through brown, dark brown and black. The positive controls, *IRC7* and *Δmet17*, produced darker coloured colonies whilst *Δmet5* (negative control) produced a white colony (Supplementary Fig. 1).

To characterise β-glucosidase activity in the indigenous isolates, assays were performed on agar plates that contained a β-glucosidic substrate. In this study, arbutin (β-D-glucoside) was used as the sole carbon source. Inclusion of ferric ammonium citrate in the YNB-arbutin plates, produced a browning of the medium around β-glucosidase positive colonies (Rosi et al., 1994). Results from screening for presence of β-glucosidase activity are presented in Supplementary Table 1. Based on the results obtained (Supplementary Table 1), most isolates lacked β-glucosidase activity as indicated by the absence of a brown precipitate around the colonies. Nevertheless, β-glucosidase activity was observed in some isolates belonging to *A. pullulans* (PF_7_L1, PF_7_L35, MF_8_L8 (2) and MF_9_L8) and unidentified isolates (PF_8_W30) (Supplementary Table 1; Supplementary Fig. 2).

Proteolytic activity was assessed in skim milk agar plates, which contained glucose and casein as carbon sources for growth promotion. In total, 31% of the yeast isolates were capable of synthesising proteases and formed clear zones around the colonies. Most species other than *W. anomalus* and *T. delbrueckii* included some isolates that were able to produce proteases (Supplementary Table 1; Supplementary Fig. 3).

Lipase activity was measured using the triglyceride tributyrin, which is an ester of glycerol and butyric acid (Budavari, 1996). All isolates exhibited lipase activity, where lipase production was depicted as a clear halo around the colony (Supplementary Table 1; Supplementary Fig. 4). The yeasts differed in their lipolytic activity, with halos ranging in size from 0.2 to 10 mm. Accordingly, four *A. pullulans* isolates (MF_8_L2 (a), MF_8_L9 (a), MF_8_L13 (a) and MF_9_L6) and one unidentified isolate (PF_8_W30) were observed to have the highest extracellular lipase activity.

The effect of ethanol concentration on the survival of non-*Saccharomyces* was evaluated (Supplementary Fig. 5). Ethanol tolerance varied among isolates of the same genus, with increasing ethanol concentration leading to reduced growth. An isolate of *W. anomalus* (PF_7_L32) was the most ethanol-tolerant whereas all of *K. aerobia* isolates were the least tolerant of the collection (Supplementary Fig. 5). Most *A. pullulans* isolates could tolerate up to 10% ethanol concentration before a marked decrease in viability. The viability of *T. delbrueckii* had decreased significantly after 6% ethanol, as compared to the control (in the absence of ethanol) where the growth density was the highest (Supplementary Fig. 5). At all ethanol concentrations, very little growth was observed in several *K. aerobia* and *A. pullulans* isolates (Supplementary Fig. 5).

3.3. Fermentation in CDGJM using single cultures of non-Saccharomyces yeast

From the screening of selected oenological properties, 17 yeast isolates were chosen as pure non-*Saccharomyces* starter cultures for evaluation of fermentation efficiency in CDGJM (Supplementary Fig. 6). Isolates representative of each species (including the unidentified isolates) were selected based on the following criteria: low H_2S production, high enzyme activity and moderate ethanol tolerance (to 10%). None of the isolates could complete fermentation in CDGJM (200 g/L sugar), with residual sugar ranging from 197 g/L to 74 g/L (Supplementary Fig. 6). When comparing sugar consumption during fermentation, *T. delbrueckii* strains utilised sugar the fastest, followed by *K. aerobia, K. servazzii* and *W. anomalus*. Overall the *A. pullulans* isolates consumed minimal amounts of sugar (Supplementary Fig. 6).

3.4. Sequential fermentation of Viognier juice using non-Saccharomyces yeasts and Saccharomyces cerevisiae

To evaluate the potential influence of non-*Saccharomyces* isolates in winemaking, 7 isolates were selected from the initial trial in CDGJM for use in sequential fermentation with a *Saccharomyces cerevisiae* strain. Isolates belonging to *K. aerobia* (PF_8_W29, PF_9_W18), *K. servazzii* (PF_9_W20), *W. anomalus* (PF_7_L32, EF_7_L3) and *T. delbrueckii* (EF_8_L3, EF_8_L7) were used to ferment Viognier juice. The sequential fermentations were compared directly to monoculture fermentations of the 7 non-*Saccharomyces* yeasts and the commonly used *S. cerevisiae* strain, EC1118 (Lallemand).

For the EC1118 fermentation, sugar utilization was rapid, finishing in under 96 h (Fig. 1), whilst the non-*Saccharomyces* monoculture fermentations did not complete as per CDGJM (Fig. 2; Supplementary Fig. 6). All sequentially inoculated fermentations finished by 234 h (Fig. 1). Following the sequential inoculation with EC1118, *K. servazzii* completed fermentation the fastest (180 h), followed by *K. aerobia* (210*h*), *W. anomalus* and *T. delbrueckii* (both at 234 h).

3.5. Wine composition analysis by HPLC

Organic acid (malic, succinic, acetic), sugar (glucose and fructose), glycerol and ethanol analyses were performed at the end of the Viognier wine fermentations. Concentrations of or changes to these compounds were evaluated. In general, the amounts of malic and succinic acid were largely unchanged when comparing the sequential wines and

the monoculture wines (Fig. 2 (a) and (b)). However, the acetic acid concentrations had decreased in sequential wines except for those of *W. anomalus* isolates (WA), which were nonetheless below the legal limit of 1.2 g/L. Residual sugar concentrations were high in monoculture wines, while glucose was depleted in sequential wines leaving some fructose (< 1 g/L). The overall glycerol concentration had increased by approximately 2 g/L in the sequential wines compared to the monoculture wines, where the sequential inoculation of *Kazachstania* isolates (*K. aerobia* and *K. servazzii*) followed by *S. cerevisiae* produced the highest glycerol concentrations compared to others (Fig. 2b). Ethanol concentrations in the wines produced by sequential fermentation were about 1% ($^{v}/_{v}$) less than the single-inoculum control (EC1118) which was ~ 12% ($^{v}/_{v}$).

3.6. Volatile compound analysis by GC–MS

Wine samples were collected at the end of the Viognier wine fermentations for volatile analysis. A total of 76 volatile compounds were identified and quantified by SPME-GC–MS in the monoculture wines, and a total of 66 compounds in the sequential wines. Because of the large number of compounds present in the fermentations conducted with each isolate as mono- and sequential cultures, it was necessary to focus on the compounds that were significantly different between the

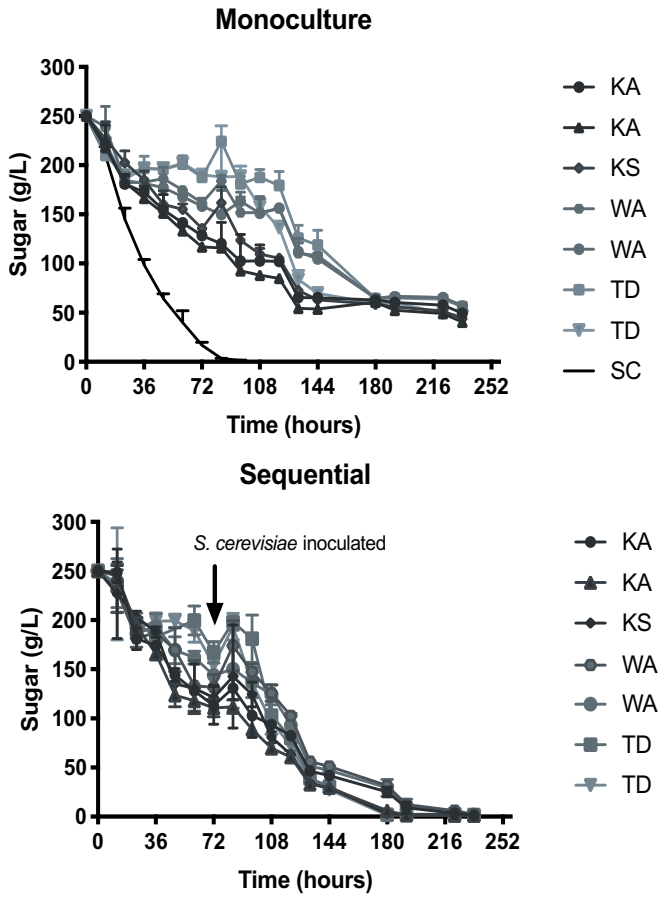

Fig. 1. Fermentation profile of selected yeast isolates in pure culture (monoculture) and sequential fermentation with *S. cerevisiae* (EC1118) in Viognier juice. KA – *K. aerobia*, KS – *K. servazzi*, WA – *W. anomalus*, TD – *T. delbrueckii*, SC – *S. cerevisiae*.

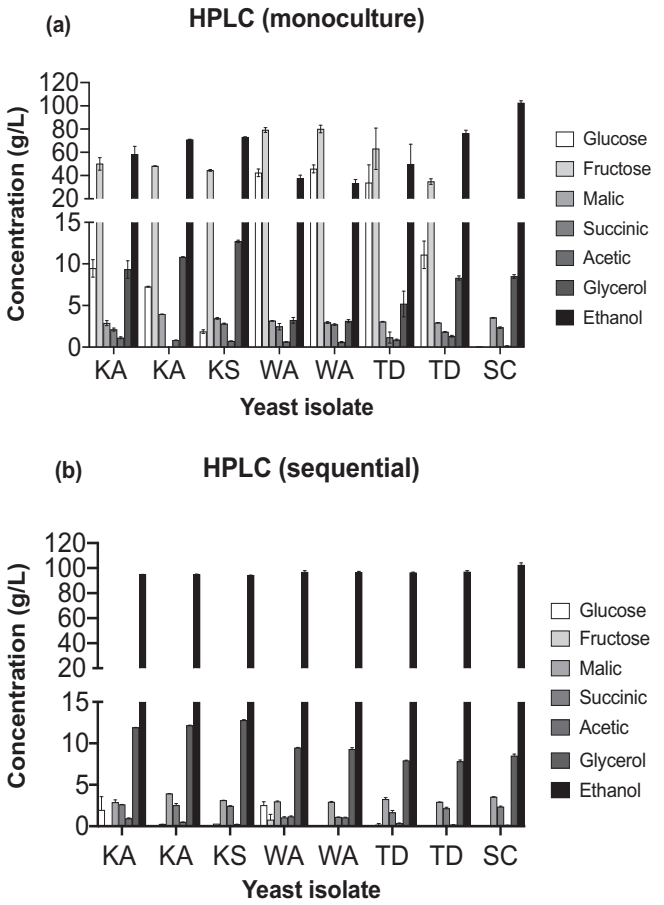

Fig. 2. Secondary metabolite profiling of Viognier wines produced by selected isolates in monoculture (a) and sequential fermentation (b) with *S. cerevisiae* (EC1118). KA – *K. aerobia*, KS – *K. servazzi*, WA – *W. anomalus*, TD – *T. delbrueckii*, SC – *S. cerevisiae*.

wines fermented with each isolate. To achieve this, the SPME-GC–MS data were analysed using ANOVA, and those compounds that did not differ significantly ($p > .01$) in monoculture *vs* the sequential wines were eliminated from further analyses. There were 58 compounds from the monoculture fermentations and 48 compounds from the sequential fermentations that showed significant differences ($p < .01$) when comparing the means for volatile compounds among the wines fermented with each isolate (Tables 2 and 3).

Significant differences were observed in the concentrations of esters in the wines produced with either mono- or sequential cultures (Tables 2 and 3). Among the ethyl esters, one of the more noticeable effects was a > 10-fold increase in ethyl propanoate concentration in the wines produced with *K. servazzii* (KSW20) as monocultures compared to the control wines (C) (Table 2). Contrastingly, most of the other ethyl esters quantified in the non-*Saccharomyces* monoculture wines had significantly lower concentrations than wines made from *S. cerevisiae*. Ethyl acetate and propyl acetate were similar to ethyl propanoate in that they were significantly higher in KSW20 wines compared to other monoculture wines, including the control. The other acetate esters were either most abundant in *S. cerevisiae* wines or those of *Kazachstania* spp. (KAW18, KAW29 and KSW20) monoculture wines, and were generally present in lowest concentrations in the *W. anomalus* (WAL3 and WAL32) and *T. delbrueckii* (TDL3 and TDL7) monoculture wines. One striking difference was observed in the concentration of phenylethyl acetate, which was 140 times higher in *K. aerobia* (KAW18) monoculture wines compared to the control but was also greatly increased in other *Kazachstania* spp. monoculture wines. The *W. anomalus* monoculture wines had higher of 3-methyl-butanal and isovaleric acid compared to the other wines but had the lowest concentrations of 4-methyl-1-pentanol, 3-methyl-1-pentanol and isoamyl alcohol (Table 2).

Of the other alcohols measured in the monoculture wines, 2-methyl-1-propanol and isoamyl alcohol concentrations were approximately 5- and 3-fold higher in KAW29 and KAW18 wines respectively, whereas methionol was 4-times higher in the control wines than TDL7 and WAL3 wines (Table 2). Acetic acid (15–32 mg/L) and acetoin (0.042–0.068 mg/L) concentrations were significantly higher in the *Kazachstania* spp. wines compared to the controls. The concentration of 2-nonanone and 2-undecanone were significantly lower in all the non-*Saccharomyces* monoculture wines compared to the control, as was true for the β-citronellol and 4-vinyl-guaiacol (Table 2). Dihydro-2-methyl-3(2H)-thiophenone concentrations were approximately 6-fold higher in the KAW29 wines and 3-fold higher in the TDL3 wines compared to the control. The *T. delbrueckii* and *W. anomalus* monoculture wines were also notable because of the low concentrations of α-bergamotene in these wines (Table 2).

Again, there was a > 8-fold increase in ethyl propanoate concentration in *K. servazzii* (KSW20) wines sequentially fermented with *S. cerevisiae* compared to the control (Table 3). As most of other ethyl esters quantified had significantly lower concentrations than the control, wines made sequentially from TDL3 had higher amounts of ethyl decanoate, ethyl 9-decenoate, and ethyl dodecanoate compared to the other wines including the control (Table 3). All sequential wines, except for TDL7 had significantly higher concentrations of ethyl acetate and isoamyl acetate than the control. It was again observed that the phenylethyl acetate concentrations were the highest in *Kazachstania* spp. wines, which were 110 times higher than the control in *K. servazzii* (KSW20) sequential wines. Other acetate esters were also generally lower in *W. anomalus* and *T. delbrueckii* sequential wines compared to *Kazachstania* spp. and the control wines. Similar to their respective

Table 2
Mean concentrations (μg/L) of volatile compounds in pure fermentation by yeast isolates and *S. cerevisiae* (EC1118). Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated.

Compound	Yeast isolate[a]							
	C (EC1118)	KAW18	KAW29	KSW20	TDL3	TDL7	WAL3	WAL32
Esters								
Ethyl propanoate	31.97^{ab}	18.31^{c}	23.72^{bc}	400.16^{a}	28.43^{bc}	48.00^{b}	13.07^{c}	13.10^{c}
Ethyl isobutyrate	1.29^{bc}	1.31^{bc}	3.41^{a}	0.67^{c}	3.29^{a}	2.73^{ab}	1.10^{bc}	1.04^{bc}
Ethyl butanoate	20.35^{a}	0.75^{c}	0.95^{c}	1.42^{bc}	2.10^{bc}	4.04^{b}	0.86^{c}	1.14^{bc}
Ethyl 2-methylbutyrate	1.70^{a}	0.24^{d}	0.27^{d}	0.40^{d}	0.73^{cd}	0.60^{cd}	1.12^{b}	1.10^{bc}
Ethyl isovalerate	0.12^{a}	0.020^{cd}	0.018^{d}	0.008^{d}	0.032^{bcd}	0.043^{bc}	0.047^{b}	0.045^{b}
Ethyl decanoate	522.35^{a}	61.57^{b}	56.09^{b}	24.36^{b}	10.00^{b}	5.95^{b}	2.49^{b}	0.92^{b}
3-Methylbutyl octanoate	0.53^{a}	0.10^{b}	0.07^{b}	0.04^{b}	0.008^{b}	0.008^{b}	0.003^{b}	0.002^{b}
Ethyl 9-decenoate[1]	100.00^{a}	4.19^{b}	6.50^{b}	1.88^{b}	0.33^{b}	1.65^{b}	0.07^{b}	0.09^{b}
Ethyl 4-hydroxybutanoate[2]	0.74^{a}	0.53^{b}	0.99^{b}	1.11^{b}	0.04^{b}	0.10^{b}	0.03^{b}	0.04^{b}
Ethyl dodecanoate	3.48^{a}	3.01^{a}	2.20^{ab}	1.80^{ab}	0.16^{b}	0.09^{b}	0.04^{b}	0.06^{b}
Phenylethyl propanoate[3]	77.11^{ab}	98.46^{a}	76.69^{ab}	90.69^{a}	40.60^{bc}	40.21^{bc}	26.99^{c}	30.82^{c}
Ethyl hexanoate	27.27^{a}	0.97^{b}	1.28^{bc}	1.37^{b}	2.00^{b}	2.93^{b}	1.14^{b}	1.22^{b}
Ethyl octanoate	33.77^{a}	1.06^{b}	1.22^{b}	0.84^{b}	0.33^{b}	0.78^{b}	0.11^{b}	0.11^{b}
Ethyl phenylacetate	0.56^{a}	0.37^{b}	0.22^{c}	0.45^{ab}	0.043^{d}	0.029^{d}	0.054^{d}	0.051^{d}
Acetates								
Ethyl acetate	2852.26^{c}	2805.50^{c}	5772.80^{bc}	18,576.48^{a}	9032.93^{bc}	3370.77^{bc}	9609.26^{b}	9699.64^{b}
Propyl acetate	33.53^{d}	13.80^{d}	41.61^{cd}	188.04^{a}	66.14^{bcd}	14.20^{d}	95.42^{bc}	99.95^{b}
Isobutyl acetate	5.99^{b}	3.17^{c}	9.93^{a}	5.87^{b}	3.77^{bc}	1.45^{c}	2.25^{c}	2.58^{c}
Hexyl acetate	4.78^{b}	0.61^{bc}	0.90^{bc}	3.03^{ab}	0.095^{c}	0.089^{c}	0.057^{c}	0.078^{c}
(*E*)-3-Hexen-1-yl acetate	73.62^{b}	181.01^{b}	199.57^{b}	448.95^{a}	2.96^{b}	4.23^{b}	1.66^{b}	2.12^{b}
(Z)-3-Hexen-1-yl acetate	19.01^{bc}	92.23^{b}	86.11^{b}	171.46^{a}	3.07^{c}	5.61^{c}	0.95^{c}	1.32^{c}
(E)-2-Hexen-1-yl acetate	1.19^{c}	186.75^{a}	59.52^{bc}	103.11^{ab}	0.87^{c}	0.69^{c}	0.92^{c}	0.90^{c}
Benzyl acetate	3.82^{c}	7.44^{c}	16.45^{b}	112.19^{a}	0.78^{c}	0.62^{c}	0.78^{c}	0.79^{c}
Geranyl acetate[3]	9.03^{ab}	6.92^{ab}	6.07^{ab}	15.67^{a}	0.79^{b}	1.21^{ab}	0.55^{b}	0.40^{b}
Citronellol acetate[3]	39.70^{a}	0.13^{b}	0.45^{b}	0.27^{b}	0.13^{b}	0.12^{b}	0.17^{b}	0.13^{b}
Isoamyl acetate[4]	210.46^{a}	28.55^{cd}	57.10^{bc}	75.11^{b}	21.62^{c}	2.35^{c}	24.76^{cd}	30.10^{cd}
Phenylethyl acetate[4]	11.82^{d}	988.52^{a}	585.01^{c}	771.44^{b}	2.70^{d}	2.76^{d}	2.18^{d}	5.00^{d}
Alcohols								
Benzyl alcohol	243.13^{abc}	292.30^{a}	249.70^{abc}	259.51^{ab}	180.42^{bc}	179.96^{bc}	165.21^{bc}	190.52^{bc}
2-Methyl-1-propanol	37,440.21^{c}	105,960.88^{b}	186,207.40^{a}	59,570.03^{c}	46,570.15^{c}	25,970.52^{c}	24,629.93^{c}	29,796.98^{c}
4-Methyl-1-pentanol	492.02^{b}	1015.39^{a}	531.72^{b}	325.53^{c}	186.45^{cd}	200.24^{cd}	164.33^{d}	185.84^{d}
2-Heptanol	1.07^{b}	2.31^{a}	2.34^{a}	2.15^{a}	0.86^{b}	1.02^{b}	0.82^{b}	0.87^{b}
3-Methyl-1-pentanol	270.12^{b}	135.14^{c}	103.76^{c}	527.09^{a}	39.83^{d}	28.70^{d}	29.50^{d}	30.07^{d}
1-Hexanol	1143.52^{b}	1452.82^{a}	1458.96^{a}	1230.77^{ab}	1358.38^{ab}	1215.85^{ab}	1348.82^{ab}	1431.99^{a}
(E)-3-Hexen-1-ol	72.48^{ab}	60.67^{bc}	56.63^{c}	37.27^{d}	72.09^{ab}	72.14^{ab}	77.86^{a}	81.11^{a}
3-Ethoxy-1-propanol	2691.97^{b}	4634.20^{ab}	9645.31^{ab}	7906.83^{ab}	7709.35^{ab}	11,986.21^{a}	3423.02^{b}	2455.56^{b}
(Z)-3-Hexen-1-ol	34.60^{a}	22.54^{b}	19.94^{b}	13.33^{b}	35.30^{a}	34.86^{a}	34.86^{a}	35.57^{a}
2-Nonanol	4.06^{a}	2.79^{ab}	1.32^{bc}	1.99^{bc}	0.30^{c}	0.34^{c}	0.24^{c}	0.34^{c}
1-Nonanol	3.52^{a}	1.97^{ab}	2.64^{ab}	2.64^{ab}	1.26^{b}	1.03^{b}	0.92^{b}	1.11^{bc}
Methionol	6210.62^{b}	12,673.93^{a}	6683.01^{b}	7827.54^{b}	2928.59^{c}	2351.38^{cd}	748.79^{d}	890.87^{d}
Isoamyl alcohol	172,254.53^{b}	255,572.10^{a}	238,619.34^{a}	165,817.12^{b}	95,901.21^{cd}	99,241.34^{c}	60,945.27^{d}	66,193.92^{cd}
Phenylethyl alcohol	213,589.23^{bc}	702,231.35^{a}	224,208.81^{bc}	280,697.33^{b}	63,310.98^{c}	85,415.99^{c}	37,216.29^{c}	48,965.65^{c}
Acids								
Acetic acid[5]	473.16^{c}	16,784.47^{ab}	31,206.68^{a}	15,230.12^{b}	8153.19^{bc}	13,044.05^{bc}	5711.90^{bc}	7289.86^{bc}
Isovaleric acid[5]	3615.65^{b}	808.05^{c}	1049.63^{c}	760.56^{c}	3654.42^{b}	1806.18^{bc}	8184.75^{a}	6855.65^{a}
Aldehydes								
3-Methyl-butanal[6]	2.04^{b}	2.06^{b}	1.21^{b}	1.96^{b}	4.39^{b}	0.44^{b}	26.12^{a}	17.96^{a}
Benzaldehyde	2.38^{abc}	3.88^{ab}	3.14^{abc}	2.17^{abc}	1.66^{bc}	1.35^{c}	3.80^{ab}	4.17^{a}
Benzeneacetaldehyde[6]	3.61^{ab}	7.57^{ab}	3.84^{ab}	5.11^{ab}	3.24^{b}	2.16^{b}	10.01^{a}	7.32^{ab}
Ketones								
2-Propanone[6]	43.40^{b}	51.64^{ab}	45.72^{b}	43.88^{b}	54.70^{ab}	51.24^{ab}	77.97^{ab}	84.13^{a}
3-Hydroxy-2-butanone (Acetoin)[6]	1.52^{b}	67.86^{a}	67.65^{a}	42.16^{a}	1.08^{b}	0.28^{b}	1.06^{b}	1.11^{b}
2-Nonanone[6]	51.41^{a}	0.96^{b}	0.77^{b}	0.59^{b}	0.36^{b}	0.22^{b}	0.32^{b}	0.19^{b}
Dihydro-2-methyl-3(2H)-thiophenone[6]	40.75^{de}	92.29^{bcd}	231.64^{a}	66.19^{cde}	131.18^{b}	117.82^{bc}	10.97^{e}	22.08^{e}
2-Undecanone[6]	10.53^{a}	0.91^{b}	0.62^{b}	0.37^{b}	0.14^{b}	0.18^{b}	0.20^{b}	0.19^{b}
Terpenes								
α-Bergamotene[7]	0.11^{a}	0.04^{abc}	0.05^{abc}	0.11^{ab}	0.01^{bc}	0.01^{bc}	0.005^{c}	0.005^{c}
β-Farnesene	0.04^{b}	0.05^{b}	0.06^{ab}	0.13^{a}	0.02^{b}	0.04^{b}	0.003^{b}	0.004^{b}
β-Citronellol	3.65^{a}	1.37^{bc}	0.81^{c}	0.83^{c}	1.50^{bc}	2.23^{b}	0.74^{c}	0.76^{c}
Nerolidol[7]	0.05^{ab}	0.09^{ab}	0.08^{ab}	0.16^{a}	0.03^{b}	0.05^{ab}	0.001^{b}	0.001^{b}
Lactones								
γ-Butryolactone[6]	12.30^{abc}	14.57^{ab}	17.58^{a}	18.83^{a}	6.82^{c}	7.17^{c}	8.67^{bc}	9.98^{bc}
Volatile phenols								
4-Vinyl guaiacol	392.54^{a}	18.48^{b}	31.11^{b}	7.32^{b}	114.86^{b}	7.85^{b}	121.44^{b}	145.40^{b}
4-Vinylphenol[8]	160.97^{a}	13.66^{cd}	11.77^{cd}	8.87^{d}	67.80^{bcd}	6.04^{d}	92.30^{abc}	116.05^{ab}

(*continued on next page*)

Table 2 (*continued*)

Compound	Yeast isolate[a]							
	C (EC1118)	KAW18	KAW29	KSW20	TDL3	TDL7	WAL3	WAL32
2,4-Bis(1,1-dimethylethyl)-phenol[8]	3321.78^{c}	16,284.87^{ab}	21,381.36^{a}	21,084.89^{a}	8101.27^{bc}	5802.47^{c}	6532.59^{c}	8255.27^{bc}

Concentration values of compounds are expressed in equivalents of:
[1] Ethyl decanoate.
[2] Ethyl butanoate.
[3] Benzyl acetate.
[4] Ethyl octanoate.
[5] Hexanoic acid.
[6] Benzaldehyde.
[7] (E)-Nerolidol.
[8] Benzyl alcohol.
[a] Means within rows with different letters are significantly different at $p < .01$. Tukey's multiple comparison test.

monoculture wines, the *W. anomalus* sequential wines had higher isovaleric acid compared to other wines, as well as having the lowest concentrations of 4-methyl-1-pentanol, 3-methyl-1-pentanol and isoamyl alcohol (Table 3). While isoamyl alcohol concentrations remained similar in KAW29 and KAW18 sequential wines compared to their corresponding monoculture wines, 2-methyl-1-propanol were approximately 5- and 8-fold higher than the control (Table 3). Acetic acid was significantly higher in *Kazachstania* spp. and *W. anomalus* sequential wines compared to the control. Of volatile phenols, *W. anomalus* and *T. delbrueckii* wines had higher concentrations of 4-vinyl-guaiacol and 4-vinylphenol compared to the other sequential wines (Table 3). Dihydro-2-methyl-3(2H)-thiophenone concentrations were approximately 3-fold higher in KAW29 wines and the same in TDL3 wines compared to the control (Table 3).

To visualise the relationship between yeast isolates and their combination with a commercial strain (treatments) with the chemical composition of Viognier wines, principal component analysis (PCA) was performed. Only the compounds that were found to be significantly different ($p < .01$) among monoculture wines (58 compounds) and sequential wines (48 compounds) were used for the analysis. For the relationship between monoculture isolates and chemical compounds produced in Viognier, the first two Principal Components (PCs) accounted for 73.2% of the variation in the volatile compounds, with PC1 explaining 41.4% of the variance and PC2 31.9% (Fig. 3). The first PC separated the wine samples based on the volatile composition of different isolates, with *Kazachstania* spp. (KAW18C, KAW29C and KSW20C) on the right-hand side of the plot and *T. delbrueckii* (TDL3C, TDL7C) and *W. anomalus* (WAL3C, WAL32C) located on the left-hand side of the plot (Fig. 3). The compounds that appear to be driving the separation along PC1 are 3-methyl-butanol, isovaleric acid, 2-propanone and both (E) and (Z)-3-hexanol on the left of the plot (Fig. 3). The *Kazachstania* spp. wines on the right of PC1 were associated with high concentrations of several acetate esters (benzyl acetate, hexen-1-yl acetate, phenylethyl acetate) and alcohols (2-methyl-1-propanol, phenylethyl alcohol). Separation on PC2 was driven by a number of ethyl esters towards the top of the plot, co-localising with the control wines (Fig. 3). Higher concentrations of 2-nonanone, 2-undecanone, 4-vinylphenol and guaiacol, β-citronellol and isoamyl acetate were also associated with the control wines. The compounds driving separation of PC2 towards the bottom of the plot are 1-hexanol, acetic acid, 3-ethoxy-1-propanol and 2,4-bis(1,1-dimethylethyl)-phenol.

The PCA of the composition of sequential wines displayed a clear separation between the isolates (Fig. 4). There was a clear separation according to different isolates on PC1, which accounted for 46.6% of the overall variation among wine samples (Fig. 4). PC2, which constitutes 21.6% of the variation separated wines made from *T. delbrueckii* (TDL3S, TDL7S) and *W. anomalus* (WAL3S and WAL32S) sequentially fermented with *S. cerevisiae* to those of *Kazachstania* spp. (KAW18S, KAW29S, KSW20S) (Fig. 4). The compounds driving the separation along PC1 are 4-vinyl-phenol and guaiacol and both (E) and (Z)-3-hexanol on the left of the plot (Fig. 4). Again, the *Kazachstania* spp. sequential wines on the right of PC1 are associated with high concentrations of acetate esters and alcohols (Fig. 4). Separation on PC2 is driven by ethyl isobutyrate, isobutyl acetate, propyl acetate and acetic acid towards the top of the plot, which are closely associated with *W. anomalus* sequential wines. Higher concentrations of a number of ethyl esters and terpenes (β-citronellol) are associated with the control and *T. delbrueckii* wines. The compounds that appear to be driving the separation of PC2 towards the bottom of the plot are 1-nonanol, 1-heptanol, 2-undecanone, ethylphenyl acetate, 3-methyl-1-petanol and methionol (Fig. 4).

4. Discussion

In this study, a total of 77 non-*Saccharomyces* yeast isolates belonging to 5 genera (*Aureobasidium*, *Kazachstania*, *Meyzerozyma*, *Wickerhamomyces*, *Toluraspora*) were identified by sequencing the 5.8S-ITS rRNA region. The use of WL nutrient and Lysine media enabled the selection of indigenous non-*Saccharomyces* yeasts present in the vineyard environment and in wine musts undergoing un-inoculated fermentation. Fermentations were conducted with the un-inoculated Shiraz grape musts (2007) in sterile bags, prior to isolation of yeast, thus omitting the influence of the resident winery microflora. To our knowledge, some species in the overall yeast microbiota of Shiraz grape must reported here, namely *Kazachstania aerobia* and *Kazachstania servazzii*, have not been reported in wine-related environments in Australia. Some predominant yeast species that are usually present during the early stages of fermentation (*e.g. Hanseniaspora/Kloeckera* spp.) (Capozzi et al., 2015) were not found in this study. It is possible that vineyard treatments, such as fungicides may have altered the microbial profile. *A. pullulans* is able to detoxify copper sulphate, whilst *H. uvarum* is sensitive (Agarbati et al., 2019). The influence of vineyard treatments should be considered in future studies, as changes in microbial population could have some impact on fermentation process and wine composition.

Because not all non-*Saccharomyces* isolates belonging to the same species will have all the desired characteristics relevant to wine production, certain oenological criteria need to be met as a fundamental step in selecting yeast isolates for potential use as starter cultures. The specific requirements evaluated were the production of beneficial enzymatic activities, tolerance to ethanol and low H_2S production. The 77 non-*Saccharomyces* isolates were firstly identified by molecular methods, and then screened for selected technological properties. The list of results, which are summarised by oenological properties important to winemaking is provided in Supplementary Table 1.

The production of extracellular β-glucosidase, protease and lipase enzymatic activities by the yeast isolates were evaluated, as they were considered as properties of potential oenological interest. Glucosidases,

Table 3
Mean concentrations (μg/L) of volatile compounds in sequential fermentation by yeast isolates and *S. cerevisiae* (EC1118). Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated.

Compound	Yeast isolate[a]							
	C (EC1118)	KAW18	KAW29	KSW20	TDL3	TDL7	WAL3	WAL32
Esters								
Ethyl propanoate	31.97^{b}	18.39^{b}	24.40^{b}	303.00^{a}	22.72^{b}	30.50^{b}	16.54^{b}	16.45^{b}
Ethyl isobutyrate	1.29^{b}	2.02^{b}	4.10^{a}	0.75^{b}	1.49^{b}	1.69^{b}	4.34^{a}	4.11^{a}
Ethyl butanoate	20.35^{a}	4.71f	7.82^{ef}	9.87^{de}	18.40^{ab}	18.42^{ab}	14.65^{bc}	12.83^{cd}
Ethyl 2-methylbutyrate	1.70^{b}	0.50^{c}	0.49^{c}	0.84^{bc}	1.29^{bc}	1.99^{b}	4.20^{a}	4.42^{a}
Ethyl isovalerate	0.12^{bc}	0.05^{d}	0.04^{d}	0.03^{d}	0.09^{cd}	0.14^{b}	0.19^{a}	0.19^{a}
Ethyl decanoate	522.35^{ab}	80.78^{c}	99.49^{bc}	149.29^{bc}	849.85^{a}	277.82^{bc}	433.23^{abc}	354.80^{bc}
3-Methylbutyl octanoate	0.53^{a}	0.15^{b}	0.20^{b}	0.27^{b}	0.87^{a}	0.35^{a}	0.39^{a}	0.29^{a}
Ethyl 9-decenoate[1]	100.00^{a}	7.30^{b}	22.54^{a}	26.12^{b}	119.46^{a}	96.68^{a}	17.45^{b}	11.52^{b}
Ethyl dodecanoate	3.48^{ab}	1.19^{b}	1.20^{b}	1.14^{b}	6.35^{a}	1.93^{bc}	1.96^{b}	1.77^{b}
Ethyl hexanoate	27.27^{a}	2.72^{c}	4.76^{c}	8.00^{c}	22.19^{ab}	14.38^{abc}	14.23^{abc}	12.36^{bc}
Ethyl octanoate	33.77^{a}	1.98^{c}	3.92^{c}	7.12^{bc}	24.92^{ab}	14.36^{bc}	8.00^{bc}	6.12^{c}
Ethyl phenylacetate	0.56^{abc}	0.50^{abc}	0.29^{bc}	0.62^{ab}	0.44^{abc}	0.65^{a}	0.25^{c}	0.25^{c}
Acetates								
Ethyl acetate	2852.26^{c}	3677.57^{bc}	7395.93^{b}	17,840.64^{a}	16,984.63^{a}	2692.11^{c}	18,279.39^{a}	20,084.42^{a}
Propyl acetate	33.53^{d}	28.42^{d}	70.60^{cd}	179.22^{a}	97.35^{bc}	27.08^{d}	134.37^{ab}	132.60^{ab}
Isobutyl acetate	5.99^{e}	22.72^{cd}	65.00^{a}	14.19^{de}	15.29^{de}	5.42^{e}	37.36^{b}	30.04^{bc}
(*E*)-3-Hexen-1-yl acetate	73.62^{c}	251.73^{b}	275.22^{b}	562.10^{a}	147.04^{bc}	50.43^{c}	98.13^{c}	76.03^{c}
(Z)-3-Hexen-1-yl acetate	19.01^{c}	111.99^{b}	105.92^{b}	193.29^{a}	51.90^{c}	15.70^{c}	30.39^{c}	24.13^{c}
(E)-2-Hexen-1-yl acetate	1.19^{d}	226.81^{a}	78.19^{c}	128.78^{b}	2.17^{d}	2.03^{d}	2.44^{d}	0.79^{d}
Benzyl acetate	3.82^{c}	7.13^{bc}	11.04^{b}	50.34^{a}	9.58^{bc}	3.32^{c}	7.82^{bc}	6.16^{bc}
Geranyl acetate[2]	9.03^{bc}	20.94^{abc}	25.40^{ab}	33.30^{a}	12.39^{bc}	7.03^{c}	8.21^{c}	6.04^{c}
Isoamyl acetate[3]	210.46^{ab}	278.42^{ab}	521.89^{a}	326.68^{ab}	254.11^{ab}	113.37^{b}	317.14^{ab}	255.93^{ab}
Phenylethyl acetate[3]	11.82^{c}	786.43^{a}	437.60^{b}	393.91^{b}	15.80^{c}	9.48^{c}	18.51^{c}	16.61^{c}
Alcohols								
2-Methyl-1-propanol	37,440.21^{c}	177,325.46^{b}	290,374.18^{a}	69,767.86^{c}	49,902.58^{c}	53,445.80^{c}	106,437.24^{bc}	104,611.80^{bc}
4-Methyl-1-pentanol	492.02^{bcd}	1048.64^{a}	544.26^{b}	507.48^{bc}	438.29^{bcd}	515.38^{bc}	265.48^{cd}	224.44^{d}
1-Heptanol	22.78^{b}	25.64^{b}	25.73^{b}	30.32^{ab}	136.26^{a}	32.28^{ab}	8.23^{b}	9.63^{b}
2-Heptanol	1.07^{ab}	2.49^{a}	2.52^{a}	2.43^{a}	1.71^{ab}	1.25^{ab}	0.87^{b}	0.93^{b}
3-Methyl-1-pentanol	270.12^{b}	148.34^{cd}	110.90^{d}	403.78^{a}	138.11^{cd}	241.71^{bc}	54.26^{d}	46.59^{d}
1-Hexanol	1143.52^{b}	1429.64^{a}	1233.74^{b}	1164.27^{b}	1099.87^{b}	1198.43^{b}	1198.43^{b}	1268.83^{ab}
(E)-3-Hexen-1-ol	72.48^{a}	58.36^{bc}	50.35^{c}	39.06^{d}	69.26^{ab}	71.57^{a}	68.87^{ab}	73.31^{a}
3-Ethoxy-1-propanol	2691.97^{c}	10,713.84^{b}	17,064.77^{a}	14,962.85^{ab}	3059.03^{c}	3008.53^{c}	4791.21^{c}	4704.12^{c}
2-Nonanol	4.06^{bc}	4.74^{bc}	7.04^{a}	5.38^{ab}	4.54^{bc}	4.91^{bc}	2.87^{c}	3.03^{c}
1-Nonanol	3.52^{b}	4.10^{b}	2.50^{b}	2.87^{b}	12.35^{a}	4.32^{b}	1.88^{b}	1.82^{b}
Methionol	6210.62^{b}	9778.77^{a}	5103.59^{b}	5876.53^{b}	5299.70^{b}	6874.70^{b}	2036.54^{c}	2062.56^{c}
Isoamyl alcohol	172,254.53^{cd}	268,533.95^{a}	232,209.00^{b}	189,651.80^{c}	148,700.54^{de}	162,967.59^{cd}	129,159.73^{ef}	117,743.49^{f}
Phenylethyl alcohol	213,589.23^{bc}	698,705.09^{a}	243,838.44^{b}	287,223.34^{b}	114,726.45^{cd}	218,230.09^{bc}	80,372.12^{d}	82,187.05^{d}
Acids								
Acetic acid[4]	473.16^{b}	11,113.76^{ab}	20,295.89^{a}	2299.03^{b}	1969.89^{b}	662.19^{b}	5921.57^{b}	7024.98^{b}
Isovaleric acid[4]	3615.65^{b}	1123.39^{c}	1085.77^{c}	1185.33^{c}	4987.65^{b}	4183.95^{b}	13,386.30^{a}	11,512.16^{a}
Aldehydes								
3-Methyl-butanal[5]	2.04^{c}	17.27^{a}	6.56^{b}	3.17^{c}	2.16^{c}	2.21^{c}	1.82^{c}	2.57^{c}
Ketones								
3-Hydroxy-2-butanone (Acetoin)[5]	1.52^{bcd}	5.07^{a}	3.49^{ab}	3.23^{abc}	1.35^{bcd}	2.03^{bcd}	0.81^{d}	1.19^{cd}
2-Nonanone[5]	51.41^{a}	5.87^{c}	11.54^{bc}	11.59^{bc}	34.86^{ab}	54.60^{a}	9.95^{bc}	8.51^{c}
Dihydro-2-methyl-3(2H)-thiophenone[5]	40.75^{cd}	54.45^{bc}	121.31^{a}	68.17^{b}	27.86^{d}	32.01^{bc}	24.85^{d}	20.74^{d}
2-Undecanone[5]	10.53^{a}	5.06^{bc}	5.02^{bc}	4.43^{bc}	8.71^{ab}	9.25^{ab}	2.52^{c}	2.07^{d}
Terpenes								
β-Farnesene	0.04^{bc}	0.14^{a}	0.05^{bc}	0.07^{b}	0.04^{bc}	0.05^{bc}	0.03^{c}	0.03^{c}
β-Citronellol	3.65^{ab}	2.87^{ab}	1.44^{b}	3.00^{ab}	3.74^{ab}	5.03^{a}	3.15^{ab}	3.48^{ab}
Nerolidol[6]	0.05^{b}	0.23^{a}	0.05^{b}	0.12^{ab}	0.07^{b}	0.09^{b}	0.03^{b}	0.04^{b}
Volatile phenols								
4-Vinyl guaiacol	392.54^{ab}	124.60^{d}	163.99^{cd}	276.30^{bc}	514.82^{a}	430.56^{a}	508.72^{a}	454.75^{a}
4-Vinylphenol[7]	160.97^{bc}	88.64^{c}	109.20^{c}	171.96^{bc}	300.77^{a}	174.20^{bc}	237.95^{ab}	234.34^{ab}
2,4-Bis(1,1-dimethylethyl)-phenol[7]	3321.78^{d}	15,734.81^{ab}	10,202.74^{c}	18,203.45^{a}	11,600.85^{c}	9025.85^{c}	12,494.55^{bc}	12,743.37^{bc}

Concentration values of compounds are expressed in equivalents of:
[1] Ethyl decanoate.
[2] Benzyl acetate.
[3] Ethyl octanoate.
[4] Hexanoic acid.
[5] Benzaldehyde.
[6] (E)-Nerolidol.
[7] Benzyl alcohol.
[a] Means within rows with different letters are significantly different at $p < .01$. Tukey's multiple comparison test.

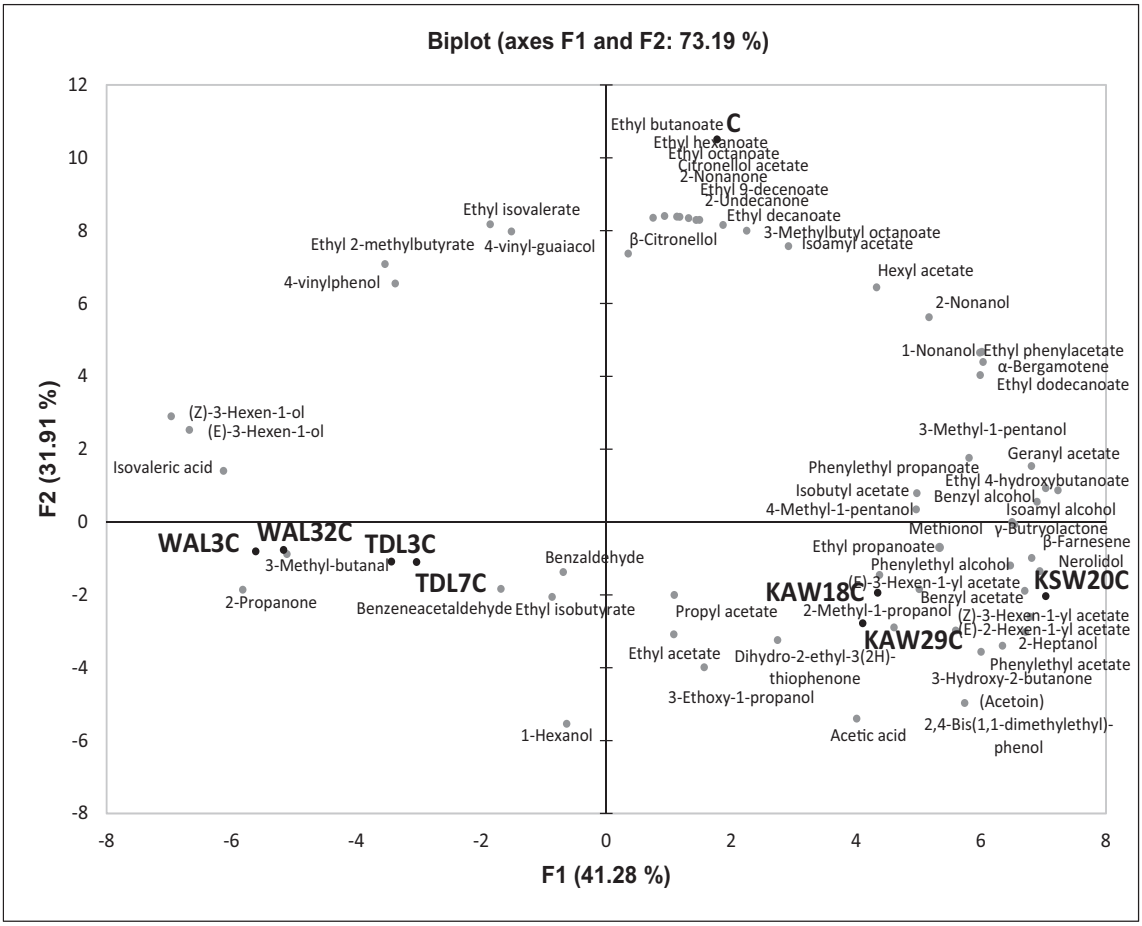

Fig. 3. Principal component analysis (F1 and F2) of the volatile profiles of mean sensory data for pure cultures in Viognier wines. Yeast isolates (treatments) represented black and aroma attributes represented in grey. C = *S. cerevisiae* (EC1118), KA – *K. aerobia*, KS – *K. servazzi*, WA – *W. anomalus*, TD – *T. delbrueckii*.

especially β-glucosidases are involved in the hydrolysis of glycol-conjugated aroma precursors in musts and wines (Rodriguez et al., 2004), and are widely associated with non-*Saccharomyces* wine yeasts. Whilst several studies report on the presence of higher β-glucosidase activity in indigenous non-*Saccharomyces* wine yeasts (eg. *Brettanomyces/Dekkera* spp., *Pichia* spp.), than those of *S. cerevisiae* (Fia et al., 2005; Rodriguez et al., 2004; Rosi et al., 1994), this was not fully reflected in the findings of this study. Only four *A. pullulans* isolates (PF_7_L1, PF_7_L35, MF_8_L8 (2) and MF_9_L8) exhibited extracellular β-glucosidase activity (identifiable by a halo around the colony), when grown in the presence of the substrate arbutin as a carbon source (Supplementary Table 1). The other *A. pullulans* isolates may produce β-glucosidase but intracellularly, as alluded to in a study of indigenous Brazilian *A. pullulans* isolates, with the authors suggesting that intracellular β-glucosidase could be used to liberate monoterpenes during winemaking (Baffi et al., 2013). The distribution of such activities among our isolates may be validated *in vitro* using permeabilised cells or cell extracts (for intracellular activity) and media or whole cells (for extracellular activity), using *p*-nitrophenyl-β-D-glucoside as a substrate.

Proteolytic activity of yeasts has been studied in relation to wine stabilization by prevention of protein haze, where proteins are hydrolysed into amino acids and peptides (Lagace and Bisson, 1990). Protein haze can be aesthetic problem in many white wines, but the use of bentonite has drawbacks, such as loss of wine volume and sensory properties (Maicas and Mateo, 2015). Although extracellular protease activity is not common in yeasts, it has been observed in a few species such as *Metschnikowia pulcherrima* and *Zygoascus meyerae* (Jolly et al., 2014). In this study, proteolytic activity was detected in several isolates belonging to *K. aerobia*, *K. servazzii*, *A. pullulans* and *M. guilliermondii* (Supplementary Table 1). These genera have not been previously reported as having proteases under wine-relevant conditions, and which should be checked for activity against the grape-specific thaumatin-like proteins that are responsible for instability in white wines (Schlander et al., 2017). The absence of extracellular proteases in *W. anomalus* corresponds to the study by Madrigal et al. (2013), in which the presence of proteolytic activity in *Wickerhamomyces* isolates was uncommon (11 out of 17 isolates did not express extracellular proteolytic activity).

Lipids originating from grapes and the autolysis of yeast lees can be degraded by lipase, which catalyse the hydrolysis of long chain triglycerides. Lipolytic activity is relevant to winemaking with regards to colour extraction (from red grape berries) and modulation of aroma (Charoenchai et al., 2008). Lipases are responsible for cleaving wine lipids to release volatile compounds such as ethyl esters and ethyl acetates, which have a strong influence on wine aroma. Interestingly, lipase activity was detected in all the yeast isolates in this study, which has not been reported elsewhere. Further characterization is required around substrate specificity with regards to ester hydrolysis given that the main esters responsible for wine aroma are short to medium (C2–C12).

Yeast cell membranes are considered the primary target of ethanol stress, as ethanol alters membrane organisation, thus affecting glucose uptake and fermentation rates under oenological conditions (Navarro-Tapia et al., 2018). Since non-*Saccharomyces* have a lower tolerance to ethanol compared to *S. cerevisiae*, it is expected that these indigenous isolates would not be able to grow in the presence of higher ethanol concentrations. The *A. pullulans* isolates were able to grow in CDGJM up to 10% ethanol concentration, but did not utilise glucose in CDGJM screening (Supplementary Fig. 6). Interestingly, one *W. anomalus* (formerly *Pichia anomala*) isolate (PF_7_L32) was tolerant at 12%, which

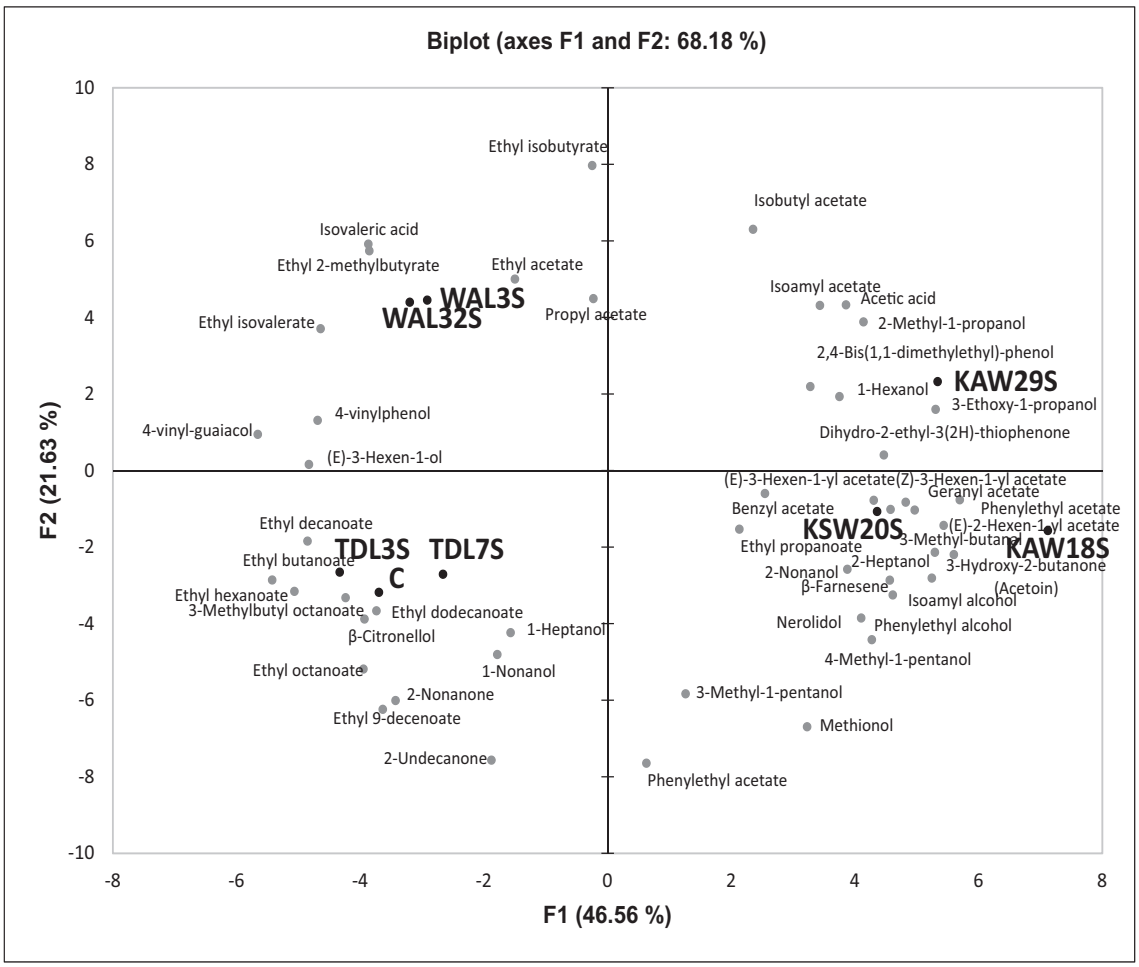

Fig. 4. Principal component analysis (F1 and F2) of the volatile profiles of mean sensory data for sequential cultures in Viognier wines. Yeast isolates (treatments) represented in black and aroma attributes represented in grey. C = *S. cerevisiae* (EC1118), KA – *K. aerobia*, KS – *K. servazzi*, WA – *W. anomalus*, TD – *T. delbrueckii*.

agrees with a study reporting on several *W. anomalus* strains being tolerant up to 12.5% (Padilla et al., 2018).

Hydrogen sulfide has a negative organoleptic effect in wines. Seventy-six isolates produced H_2S, ranging from low to high (Supplementary Table 1). Only one isolate (MF_8_L1, *A. pullulans*) did not produce H_2S, however, this isolate is not suitable for use as a starter culture due to the low ethanol tolerance and little to no production of protease and β-glucosidase. As the isolates were only tested in BiGGY agar, the H_2S potential of these indigenous non-*Saccharomyces* yeast isolates should be tested under oenological conditions, such as a grape juice indicator media containing bismuth citrate (Jiranek et al., 1995). The use of a chemically defined medium allows the standardization of nitrogen and sulfur concentrations, and eliminates the variation inherent in grape must.

From screening the oenological properties listed above, a total of 17 yeast isolates representing each species were selected to conduct small-scale fermentations in CDGJM. During the initial assessment of the selected isolates for their ability to ferment CDGJM, the *Kazachstania* spp. isolates were the fastest in sugar catabolism (Supplementary Fig. 6). Fermentations became sluggish around 4.5 days (108 h), leaving a higher than desired residual sugar (> 100 g/L) than the completed fermentation by EC1118 (< 2 g/L). All *A. pullulans*, *M. guilliermondii* and unidentified isolates were considered as poor fermenters as they consumed < 100 g/L of sugar and thus did not proceed to sequential fermentation trials in grape juice.

The use of mixed starter cultures of a *S. cerevisiae* wine strain with select non-*Saccharomyces* to exploit the beneficial attributes of the latter, is a promising means to improve wine complexity from otherwise *Saccharomyces* inoculated fermentations (Jolly et al., 2006; Padilla et al., 2016). In sequential fermentation, the non-*Saccharomyces* are fermented for a given time before *S. cerevisiae* is introduced, which then rapidly outcompetes the other yeast. When all 7 yeast isolates were sequentially fermented (1:1 ratio) with *S. cerevisiae* (inoculated at 72 h) in grape juice, all fermentations proceeded to dryness (< 2 g/L) (Fig. 1). These findings agree with a study which demonstrated the potential of *Kazachstania* spp. in mixed-culture fermentations in Sauvignon Blanc juice (Jood et al., 2017). All sequential fermentations of *Kazachstania* spp. completely consumed sugars, but were slower than the *S. cerevisiae* monoculture fermentations (Jood et al., 2017). In the same context, *W. anomalus* isolates fermented at a slightly slower rate in the presence of *S. cerevisiae*. The effects of the inoculum ratio in sequential fermentations on growth and fermentation rate of non-*Saccharomyces* were species and strain dependent (Comitini et al., 2011). Closer examination of the utilization of different culture ratios of non-*Saccharomyces* and *S. cerevisiae*, as well as the timing of each inoculation, in terms of effects of wine metabolome is warranted but beyond scope of this study.

In alcoholic fermentation, glycerol is the major metabolite produced after ethanol and is important in osmoregulation and redox balance in yeast cells (Klein et al., 2017). As a viscous and non-volatile liquid, glycerol can contribute to the sweetness and the mouthfeel of wines (Goold et al., 2017). Relating to ethanol formation in wines, all non-*Saccharomyces* yeast wines produced approximately 1% (v/v) less alcohol than wines conducted with *S. cerevisiae* only (~12.8% v/v; Fig. 2) in addition to higher glycerol levels, possibly due to redirection of carbon flux from ethanol towards glycerol (Goold et al., 2017). In most of the wines produced with non-*Saccharomyces*, glycerol concentrations were increased and were significantly higher than wines produced with

S. cerevisiae only (Fig. 2). Similar results were reported by Comitini et al. (2011) and Englezos et al. (2015) in wine fermentations conducted with L. *thermotolerans/S. cerevisiae* and *C. zemplinina/S. cerevisiae* mixed starters. However, du Plessis et al. (2017) reported on negative outcomes, namely the reduction of glycerol levels in Shiraz wines (without malolactic fermentation) produced using non-*Saccharomyces* yeasts (*Candida zemplinina*, *K. aerobia*, *K. gamospora*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *T. delbrueckii and Zygosacchaomyces kombuchanensis*). The differences in the amount of glycerol produced in wines could be due to the yeast strain and grape variety used (du Plessis et al., 2017).

The impact of non-*Saccharomyces* on aroma compound biosynthesis is evident from the volatile analysis of the pure culture and sequential wines. Yeast-derived aroma compounds produced during fermentation are strain and species-dependent (Cordente et al., 2012), which is useful in the selection of strains/isolates suited to a particular wine style. Nevertheless, the volatile compounds derived from yeast metabolism may have either a positive or negative effect on wine aroma and quality, dependent upon whether the odour threshold in wine is reached or their synergistic effect with other related aroma compounds.

The main component responsible for volatile acidity is acetic acid (Erasmus et al., 2004), which was found to be higher in monoculture and sequential wines produced by *Kazachstania* spp. compared to the control (Tables 2 and 3). However these amounts are not considered detrimental to the wine as they were below the detection threshold at 0.5 g/L (Ugliano et al., 2010). Higher alcohols are major volatile by-products of fermentation which contribute to the complexity of wine aroma at concentrations below 300 mg/L (Ivit and Kemp, 2018; Padilla et al., 2016). In this study, sequential wines of *K. aerobia* produced higher amounts of isoamyl alcohol (marzipan aroma) and phenylethyl alcohol (floral aroma) compared to the *S. cerevisiae* pure culture wines. The aroma importance of higher alcohols extends to other features of wine flavour, by serving as precursors of esters. Most volatile compound groups are comprised of esters (acetate and ethyl esters), as they are the most abundant compounds found in wine (Padilla et al., 2016). Among them are ethyl acetate, isoamyl acetate, isobutyl acetate and 2-phenylethyl acetate, which originate from acetic acid (Padilla et al., 2016). Non-*Saccharomyces* yeasts are well known to produce esters, in particular *W. anomalus* can yield high levels of ethyl acetate (fruity aroma) in wines, when compared to *S. cerevisiae*, with some isolates producing > 150 mg/L, close to the concentration at which this acetate ester can impart spoilage (nail-polish remover aroma) to wine (150–200 mg/L) (Padilla et al., 2016; Rojas et al., 2003). This was observed in *K. servazzii* and *W. anomalus* ferments, where the latter was described to be a high producer of ethyl acetate (Fan et al., 2019). The monoculture and sequential ferments with *W. anomalus* (WAL3 and WAL32) had approximately 2- and 6-fold increase respectively of ethyl acetate, when compared to the control wine (Tables 2 and 3). The wines fermented with *Kazachstania* isolates were mainly characterised by fruity and floral aromas, for example *K. aerobia* produced 110 times more phenylethyl acetate compared to *S. cerevisiae*, an ester associated with floral and honey scent (Cordente et al., 2012). The amount of isoamyl acetate (banana aroma) produced in *Kazachstania* sequential wines was twice that of pure culture wines of *S. cerevisiae*. *Kazachstania gamospora,* another newly discovered species of this genus also produces large quantities of esters compared to *S. cerevisiae,* in particular phenylethyl proprionate (another desirable floral aroma), which was not detected in this study (Whitener et al., 2015).

In conclusion, this study provides promising insights into potential new non-*Saccharomyces* starter cultures, with a number of species characterised in terms of potential effect on aroma profile following sequential fermentation with *S. cerevisiae* EC1118. Follow-up investigations to compare strain performance in different juices (red and white grapes varietals), as well as in non-sterile conditions are required to determine the usefulness of these individual isolates on a commercial scale. The enzymatic activities (β-glucosidase, lipase and protease) shown by these isolates may be suitable for specific vinification processes, such as juice clarification, whereby protease preparations may provide a substitute for the use of bentonite. Whilst such activities have potential positive benefits for wine production, it is important to determine whether they also have a negative impact, such as decolourisation (β-glucosidases). It is worth exploring the *Kazachstania* isolates, as they have demonstrated the potential to synthesise desirable metabolites and aroma compounds in wine. A more comprehensive study is warranted, on a larger scale which would include sensory evaluation to determine key sensory characteristics of the wine.

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Declaration of competing interest

The authors declare there are no conflicts of interest regarding the study.

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Supplementary Material

Title: Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures

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Supplementary Table 1. List of yeast isolates used and identified in this study and their enzymatic and oenological properties.

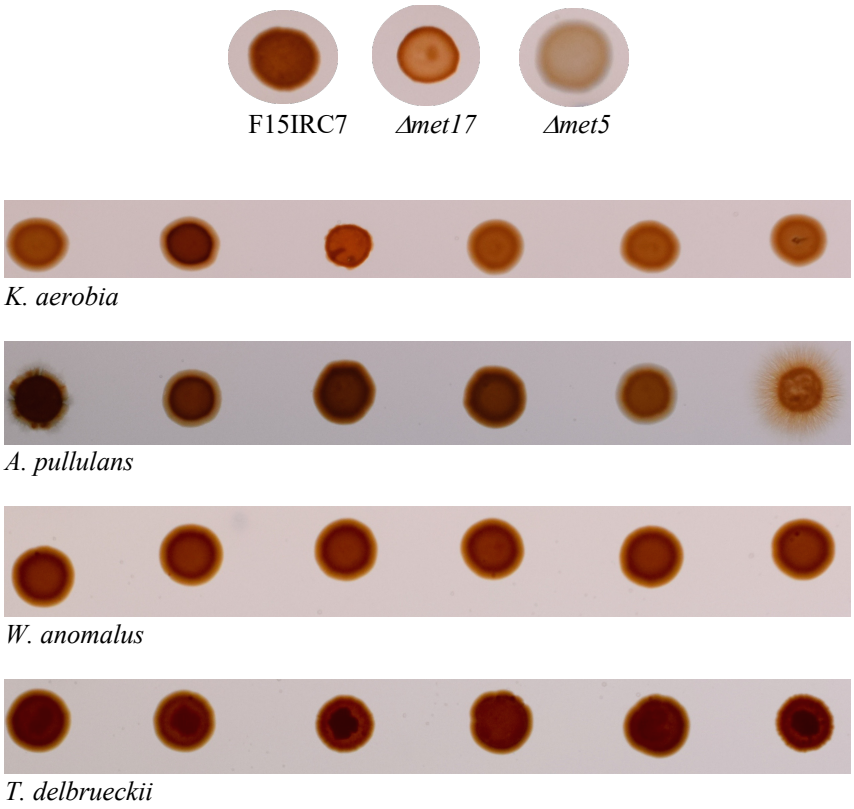
Species Isolate ID	**GenBank accession number**	**Protease** Skim milk	**Lipase** YPD + 0.3% tributyrin	**β-glucosidase** YNB + arbutin + ferric ammonium citrate	**H_2S** BiGGY
***K. aerobia* (15)**					
PF_8_W24	MN328363	-	+	w	+++
PF_8_W26	MN328364	++	+	w	+++
PF_8_W29 **(KAW29)**	MN328365	++	+	w	++
PF_8_W31(1)	MN328366	-	+	-	++
PF_8_W31(2)	MN328367	-	+	-	++
PF_9_W4	MN328368	++	+	w	++
PF_9_W5	MN328369	-	+	-	++
PF_9_W9	MN328370	++	+	-	++
PF_9_W11	MN328371	-	+	w	++
PF_9_W18 **(KAW18)**	MN328372	++	+	w	+++
PF_9_W24	MN328374	++	+	w	+++
PF_9_W27	MN328375	-	+	w	+++
PF_9_W28	MN328376	-	+	-	++
MF_9_W4	MN328377	-	+	-	++
EF_9_L1	MN328378	-	+	-	++
***A. pullulans* (37)**					
PF_8_W25	MN398476	++	++	w	+++
PF_9_W21	MN398477	++	+	w	++
PF_7_L1	MN398478	++	+	++++	+++
PF_7_L2	MN398479	-	++	w	++
PF_7_L3	MN398480	-	+	-	++++
PF_7_L4	MN398481	-	+	-	+++
PF_7_L5	MN398482	++	+	w	+++
PF_7_L6	MN398483	++	+	w	+++
PF_7_L7	MN398484	++	+	w	+++
PF_7_L8	MN398485	-	+	w	+++
PF_7_L9	MN398486	-	+	w	+++
PF_7_L10	MN398487	-	white	w	+++
PF_7_L11	MN398488	-	+	w	+++
PF_7_L12	MN398489	-	+	black	+
PF_7_L19	MN398490	-	+	w	+++
PF_7_L25	MN398491	-	+	w	+++
PF_7_L26	MN398492	-	+	w	+++
PF_7_L27	MN398493	+++	+	w	+++
PF_7_L30	MN398494	++	+	w	+++
PF_7_L35	MN398495	++	+	+++	++
PF_7_L40	MN398496	++	+	w	+++
MF_8_L1 (a)	MN398497	-	++	w	-
MF_8_L2 (a)	MN398498	-	+++	w	+++
MF_8_L7 (a)	MN398499	-	white	w	+
MF_8_L8 (a)	MN398500	-	+	w	+++

MF_8_L9 (a)	MN398501	-	+++	w	+++
MF_8_L10 (a)	MN398502	-	+	w	+++
MF_8_L11 (a)	MN398503	-	+	w	++
MF_8_L13 (a)	MN398504	-	+++	w	++
MF_8_L8 (2)	MN398505	+++	+	++++	++
MF_8_L10 (2)	MN398506	-	+	w	++
MF_8_L12 (2)	MN398507	-	+	w	++
MF_9_L6	MN398508	++	+++	-	++
MF_9_L8	MN398509	+++	+	+++	++
MF_9_L19	MN398510	-	+	w	++++
MF_8_L5(a)	MN400108	++	+	w	+++
MF_8_L12(a)	MN400109	++	+	w	+++
***M. guillermondii* (1)**					
PF_9_W13	MN336180	++	++	w	+++
***K. servazzii* (1)**					
PF_9_W20 **(KSW20)**	MN328373	-	++	w	+++
Unidentified (3)					
PF_8_W27	N/A	++	++	w	+++
PF_8_W30	N/A	+++	+++	+++	++
MF_7_W4	N/A	-	++	w	++
***W. anomalus* (10)**					
PF_7_L32 **(WAL32)**	MN398511	-	+	-	++
EF_7_L1	MN398512	-	+	-	++
EF_7_L2	MN398513	-	+	-	++
EF_7_L3 **(WAL3)**	MN398514	-	++	-	++
EF_7_L4	MN398515	-	++	-	++
EF_7_L5	MN398516	-	++	-	++
EF_7_L6	MN398517	-	++	-	++
EF_7_L7	MN398518	-	++	-	++
EF_7_L9	MN398519	-	++	-	++
EF_7_L10	MN398520	-	++	-	++
***T. delbrueckii* (10)**					
EF_8_L1	MN398521	-	++	-	++++
EF_8_L2	MN398522	-	++	-	+++
EF_8_L3 **(TDL3)**	MN398523	-	++	-	+++
EF_8_L4	MN398524	-	++	-	++++
EF_8_L5	MN398525	-	++	-	+++
EF_8_L6	MN398526	-	++	-	++++
EF_8_L7 **(TDL7)**	MN398527	-	++	-	++
EF_8_L8	MN398528	-	++	-	++++
EF_8_L9	MN398529	-	++	-	++
EF_8_L10	MN398530	-	++	-	++++

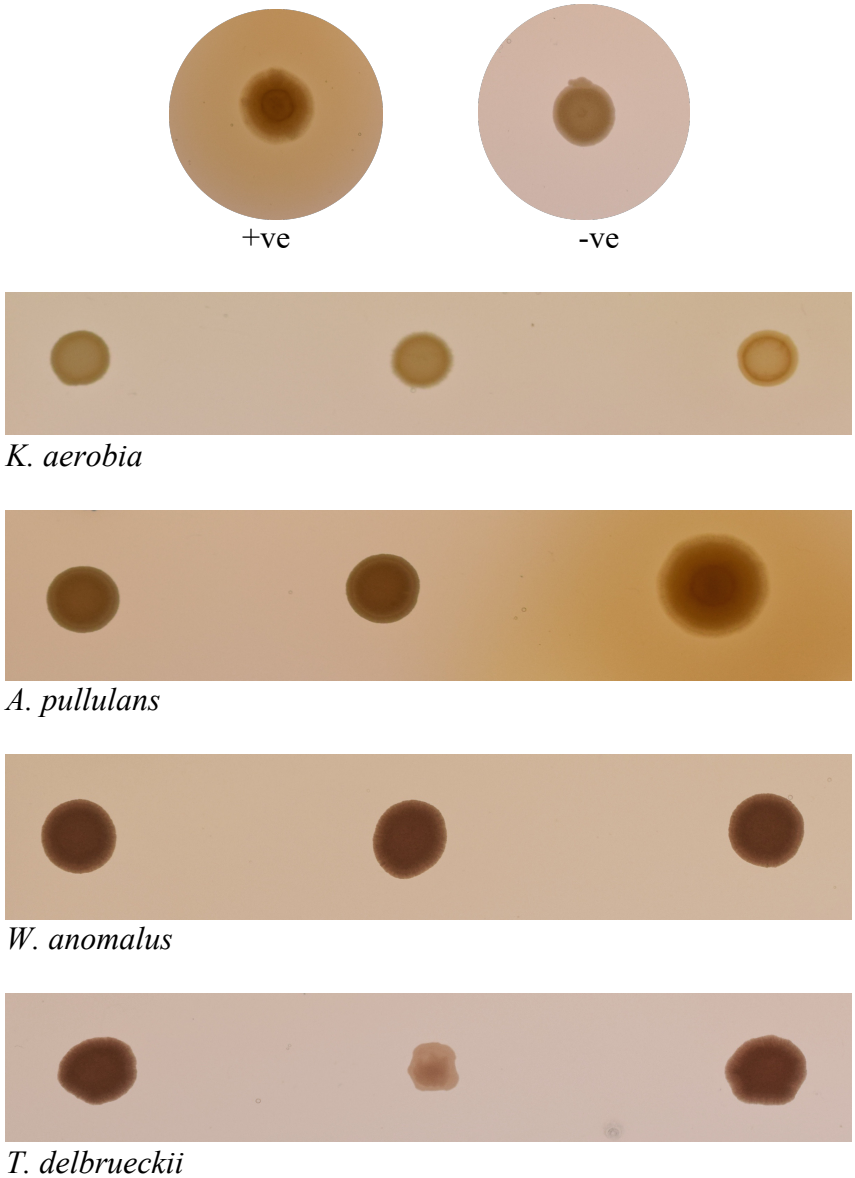
For isolate ID, isolates are named by: (stage of fermentation)_(fermentation number)_(selective medium). PF – pre-fermentation, MF – mid-fermentation, EF – end of fermentation, W – WLN media, L – lysine media. Isolates selected for sequential fermentation followed by chemical analyses are in bold eg. **(TDL7)**.

For enzymatic activity: + = Positive result (clear or fluorescent halo from the edge of colony more than 1 mm); w = weak production; - = negative result. For H_2S production: + = white colonies; ++ = light brown; +++ = brown; ++++ = dark brown; - = negative result

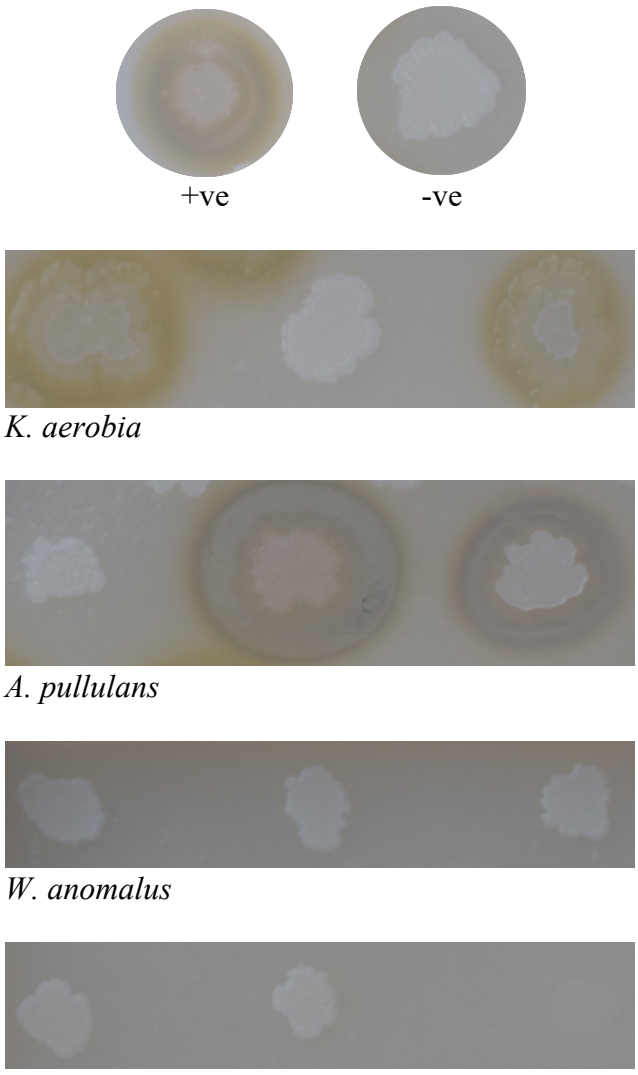
Supplementary Figure 1. Evaluation of H_2S production in yeast isolates. *S. cerevisiae* strains F15 and BY4741 *Δmet17* were used as positive controls. The AWRI1631 *Δmet5* was used as a negative control. Image shows representative samples of 77 yeast isolates.



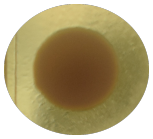
Supplementary Figure 2. Evaluation of ß-glucosidase activity in yeast isolates. β-glucosidase activity was evaluated using YNB agar plates containing arbutin and ferric ammonium citrate. β-glucosidase activity was detected as brown halo around colonies (+ve). Image shows representative samples of 77 yeast isolates.



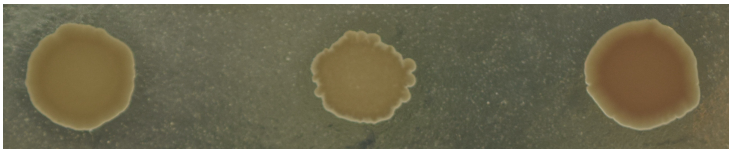
Supplementary Figure 3. Evaluation of protease activity in yeast isolates. Proteolytic activity was assessed using skim milk agar plates. Protease activity was identified as clear zones (+ve) around the colonies, whereby skim milk (protein source) was degraded. Image shows representative samples of 77 yeast isolates.



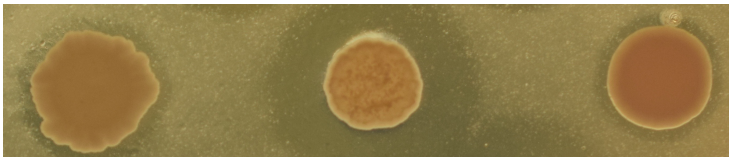
Supplementary Figure 4. Evaluation of lipase activity in yeast isolates. Lipase activity was assessed on YPD agar plates containing tributyrin. Lipase activity was identified as clear halos around the colonies. *Yarrowia lipolytica* was used as a positive control. Image shows representative samples of 77 yeast isolates.



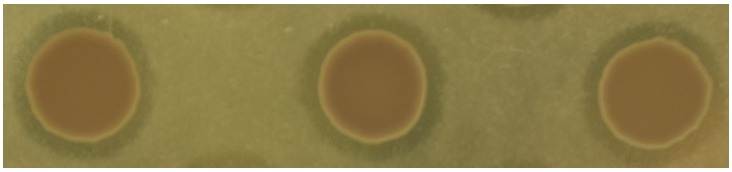
Y. lipolytica



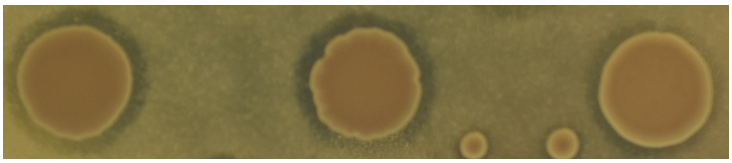
K. aerobia



A. pullulans

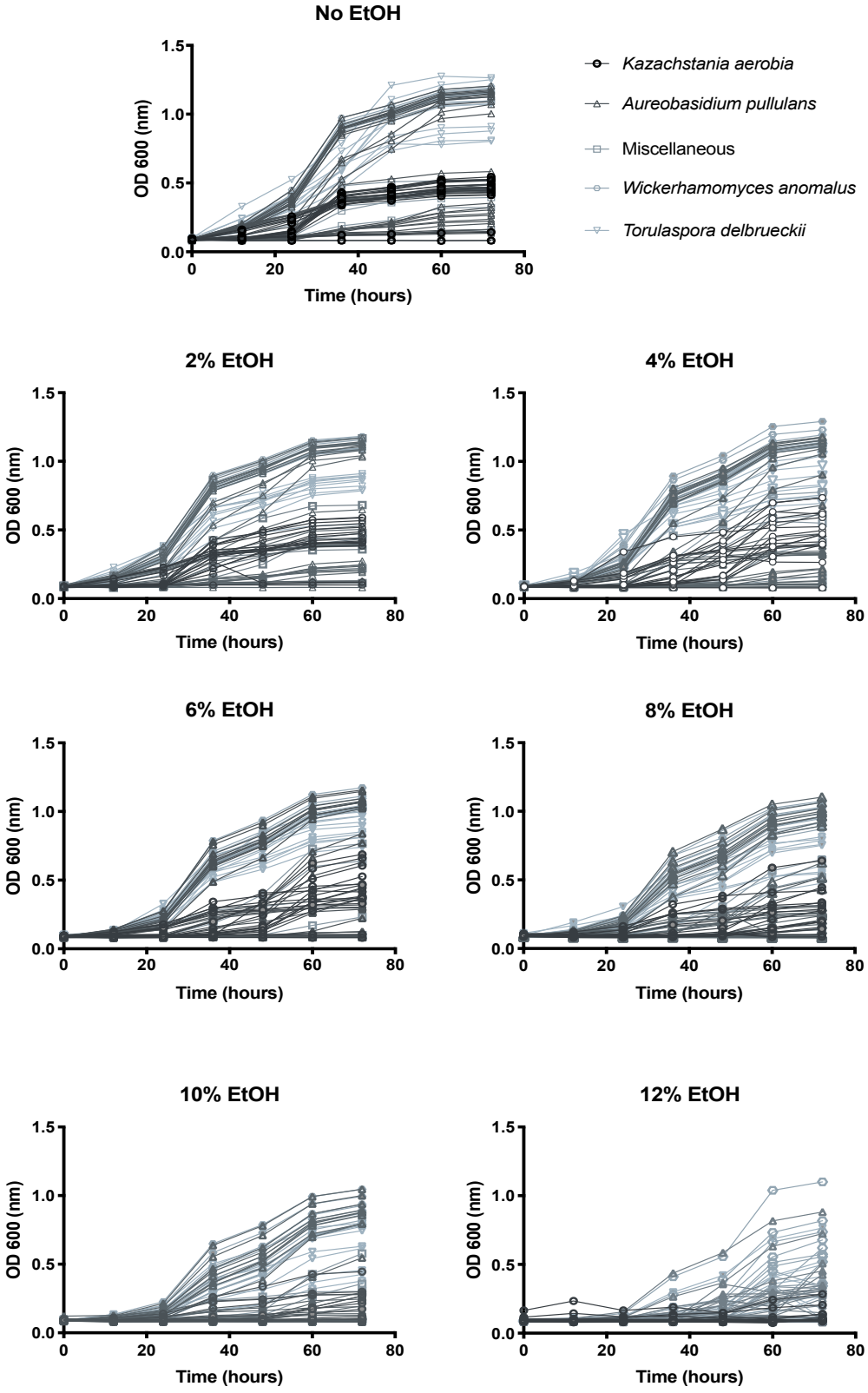


W. anomalus



T. delbrueckii

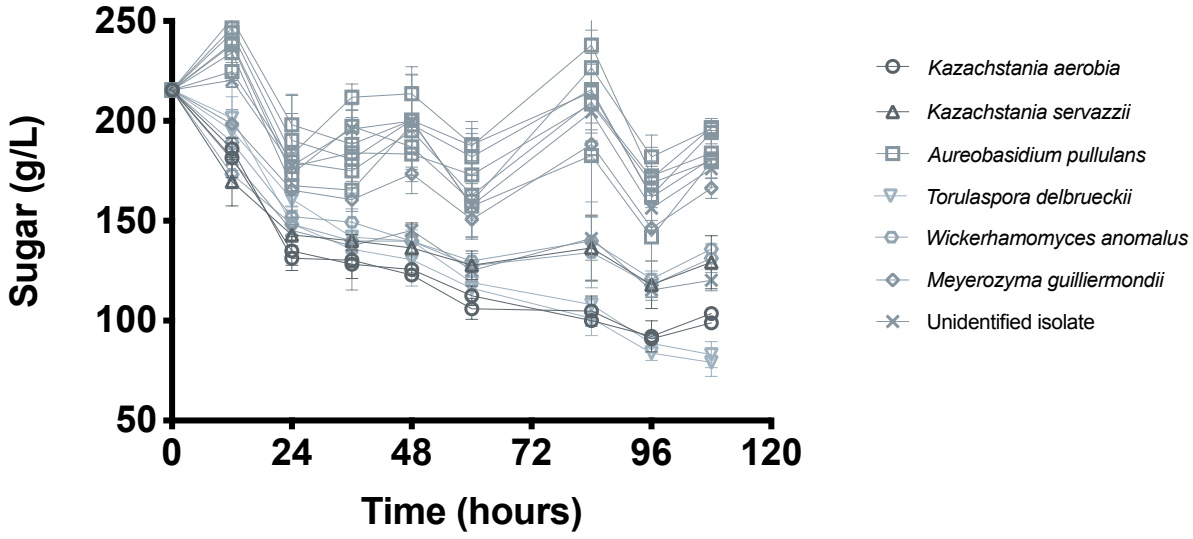
Supplementary Figure 5. Growth curves of indigenous yeast strains in CDGJM in the absence (0%) and presence of different ethanol concentrations (2, 4, 6, 8, 10 and 12%). *K. servazzii*, *M. guillermondii* and unidentified isolates are collated as 'Miscellaneous'.



Supplementary Table 2. Yeast isolates chosen for fermentation trial in CDGJM. Representative isolates of each species were chosen based from the evaluation of oenological properties.

Isolate ID	Species
PF_8_W29	*K. aerobia*
PF_9_W18	*K. aerobia*
PF_7_L1	*A. pullulans*
PF_7_L12	*A. pullulans*
PF_7_L27	*A. pullulans*
PF_7_L35	*A. pullulans*
MF_8_L1(a)	*A. pullulans*
MF_8_L8(2)	*A. pullulans*
PF_9_W13	*M. guilliermondii*
PF_9_W20	*K. servazzii*
MF_8_L5 (a)	*A. pullulans*
PF_8_W30	Unidentified
MF_7_W4	Unidentified
PF_7_L32	*W. anomalus*
EF_7_L3	*W. anomalus*
EF_8_L3	*T. delbrueckii*
EF_8_L7	*T. delbrueckii*

Supplementary Figure 6. Fermentation profile of selected indigenous yeast isolates from screening as monocultures in CDGJM. Results are the mean of triplicate fermentations.



Supplementary Table 3. Mean for peak areas for volatile compounds in pure fermentation by yeast isolates with *S. cerevisiae* (EC1118).

	Yeast isolate[a]							
Compound	**C (EC1118)**	**KAW18**	**KAW29**	**KSW20**	**TDL3**	**TDL7**	**WAL3**	**WAL32**
Esters								
Ethyl propanoate	0.45 ^{ab}	0.26 ^{c}	0.34 ^{bc}	5.68 ^{a}	0.40 ^{bc}	0.68 ^{b}	0.19 ^{c}	0.19 ^{c}
Ethyl isobutyrate	0.01 ^{bc}	0.01 ^{bc}	0.029 ^{a}	0.006 ^{c}	0.028 ^{a}	0.023 ^{ab}	0.009 ^{bc}	0.009 ^{bc}
Ethyl butanoate	1.00 ^{a}	0.04 ^{c}	0.05 ^{c}	0.07 ^{bc}	0.10 ^{bc}	0.20 ^{b}	0.04 ^{c}	0.06 ^{bc}
Ethyl 2-methylbutyrate	0.02 ^{a}	0.003 ^{d}	0.003 ^{d}	0.005 ^{d}	0.008 ^{cd}	0.007 ^{cd}	0.013 ^{b}	0.012 ^{bc}
Ethyl isovalerate	0.016 ^{a}	0.003 ^{cd}	0.002 ^{d}	0.001 ^{d}	0.004 ^{bcd}	0.0055 ^{bc}	0.006 ^{b}	0.006 ^{b}
Ethyl decanoate	11.86 ^{a}	2.19 ^{b}	1.00 ^{b}	0.87 ^{b}	0.36 ^{b}	0.21 ^{b}	0.09 ^{b}	0.03 ^{b}
3-Methylbutyl octanoate	0.13 ^{a}	0.026 ^{b}	0.016 ^{b}	0.009 ^{b}	0.002 ^{b}	0.002 ^{b}	0.001 ^{b}	0.001 ^{b}
Ethyl 9-decenoate	3.48 ^{a}	0.15 ^{b}	0.23 ^{b}	0.07 ^{b}	0.01 ^{b}	0.06 ^{b}	0.002 ^{b}	0.003 ^{b}
Ethyl 4-hydroxybutanoate	0.018 ^{bc}	0.013 ^{c}	0.024 ^{ab}	0.027 ^{a}	0.001 ^{d}	0.002 ^{d}	0.001 ^{d}	0.001 ^{d}
Ethyl dodecanoate	0.97 ^{a}	0.84 ^{a}	0.61 ^{ab}	0.5 ^{ab}	0.04 ^{b}	0.023 ^{b}	0.01 ^{b}	0.02 ^{b}
Phenylethyl propanoate	0.31 ^{ab}	0.402 ^{a}	0.31 ^{ab}	0.5 ^{a}	0.17 ^{bc}	0.16 ^{bc}	0.11 ^{c}	0.13 ^{c}
Ethyl hexanoate	0.37 ^{a}	0.01 ^{b}	0.02 ^{bc}	0.02 ^{b}	0.03 ^{b}	0.04 ^{b}	0.02 ^{b}	0.02 ^{b}
Ethyl octanoate	0.48 ^{a}	0.02 ^{b}	0.02 ^{b}	0.01 ^{b}	0.005 ^{b}	0.01 ^{b}	0.002 ^{b}	0.002 ^{b}
Ethyl phenylacetate	0.104 ^{a}	0.068 ^{b}	0.04 ^{c}	0.083 ^{ab}	0.008 ^{d}	0.005 ^{d}	0.01 ^{d}	0.01 ^{d}
Acetates								
Ethyl acetate	2.03 ^{c}	2.00 ^{c}	4.12 ^{bc}	11.32 ^{a}	6.44 ^{bc}	2.40 ^{bc}	6.85 ^{b}	6.92 ^{b}
Propyl acetate	0.04 ^{d}	0.02 ^{d}	0.05 ^{cd}	0.23 ^{a}	0.08 ^{bcd}	0.02 ^{d}	0.12 ^{bc}	0.12 ^{b}
Isobutyl acetate	0.11 ^{b}	0.06 ^{c}	0.18 ^{a}	0.11 ^{b}	0.07 ^{bc}	0.03 ^{c}	0.04 ^{c}	0.05 ^{c}
Hexyl acetate	0.63 ^{a}	0.08 ^{bc}	0.12 ^{bc}	0.34 ^{ab}	0.01 ^{c}	0.01 ^{c}	0.008 ^{c}	0.01 ^{c}
(E)-3-Hexen-1-yl acetate	0.05 ^{b}	0.12 ^{b}	0.13 ^{b}	0.3 ^{a}	0.002 ^{b}	0.003 ^{b}	0.001 ^{b}	0.001 ^{b}
(Z)-3-Hexen-1-yl acetate	0.012 ^{bc}	0.06 ^{b}	0.05 ^{b}	0.11 ^{a}	0.002 ^{c}	0.003 ^{c}	0.001 ^{c}	0.001 ^{c}
(E)-2-Hexen-1-yl acetate	0.0004 ^{c}	0.06 ^{a}	0.02 ^{bc}	0.03 ^{ab}	0.0003 ^{c}	0.0002 ^{c}	0.0003 ^{c}	0.0003 ^{c}
Benzyl acetate	0.02 ^{c}	0.03 ^{c}	0.067 ^{b}	0.46 ^{a}	0.003 ^{c}	0.003 ^{c}	0.003 ^{c}	0.003 ^{c}
Geranyl acetate	0.015 ^{ab}	0.012 ^{ab}	0.010 ^{ab}	0.026 ^{a}	0.001 ^{b}	0.002 ^{ab}	0.001 ^{b}	0.001 ^{b}
Citronellol acetate	0.14 ^{a}	0.0005 ^{b}	0.002 ^{b}	0.001 ^{b}	0.0004 ^{b}	0.0004 ^{b}	0.0006 ^{b}	0.0005 ^{b}
Isoamyl acetate	1.84 ^{a}	0.25 ^{cd}	0.5 ^{bc}	0.66 ^{b}	0.19 ^{c}	0.02 ^{c}	0.22 ^{cd}	0.26 ^{cd}
Phenylethyl acetate	0.169 ^{d}	14.1 ^{a}	8.36 ^{c}	11.10 ^{b}	0.04 ^{d}	0.04 ^{d}	0.03 ^{d}	0.07 ^{d}

Alcohols								
Benzyl alcohol	0.065 ^{abc}	0.078 ^{a}	0.067 ^{abc}	0.069 ^{ab}	0.048 ^{bc}	0.048 ^{bc}	0.044 ^{c}	0.051 ^{bc}
2-Methyl-1-propanol	0.17 ^{c}	0.49 ^{b}	0.85 ^{a}	0.27 ^{c}	0.21 ^{c}	0.12 ^{c}	0.11 ^{c}	0.14 ^{c}
4-Methyl-1-pentanol	0.044 ^{b}	0.09 ^{a}	0.047 ^{b}	0.029 ^{c}	0.016 ^{cd}	0.018 ^{cd}	0.015 ^{d}	0.016 ^{d}
2-Heptanol	0.021 ^{b}	0.045 ^{a}	0.045 ^{a}	0.042 ^{a}	0.02 ^{b}	0.02 ^{b}	0.02 ^{b}	0.02 ^{b}
3-Methyl-1-pentanol	0.24 ^{b}	0.12 ^{c}	0.09 ^{c}	0.47 ^{a}	0.04 ^{d}	0.03 ^{d}	0.03 ^{d}	0.04 ^{d}
1-Hexanol	0.77 ^{b}	0.98 ^{a}	0.98 ^{a}	0.83 ^{ab}	0.91 ^{ab}	0.82 ^{ab}	0.91 ^{ab}	0.96 ^{a}
(E)-3-Hexen-1-ol	0.034 ^{ab}	0.028 ^{bc}	0.026 ^{c}	0.017 ^{d}	0.034 ^{ab}	0.034 ^{ab}	0.036 ^{a}	0.038 ^{a}
3-Ethoxy-1-propanol	0.022 ^{b}	0.037 ^{ab}	0.077 ^{ab}	0.063 ^{ab}	0.062 ^{ab}	0.096 ^{a}	0.027 ^{b}	0.020 ^{b}
(Z)-3-Hexen-1-ol	0.011 ^{a}	0.007 ^{b}	0.006 ^{b}	0.004 ^{b}	0.011 ^{a}	0.011 ^{a}	0.012 ^{a}	0.012 ^{a}
2-Nonanol	0.019 ^{a}	0.013 ^{ab}	0.006 ^{bc}	0.009 ^{bc}	0.0014 ^{c}	0.0016 ^{c}	0.0011 ^{c}	0.0016 ^{c}
1-Nonanol	0.04 ^{a}	0.022 ^{ab}	0.029 ^{ab}	0.03 ^{ab}	0.014 ^{b}	0.01 ^{b}	0.01 ^{b}	0.012 ^{bc}
Methionol	0.17 ^{b}	0.35 ^{a}	0.19 ^{b}	0.22 ^{b}	0.08 ^{c}	0.07 ^{cd}	0.02 ^{d}	0.02 ^{d}
Isoamyl alcohol	7.01 ^{b}	11.04 ^{a}	9.71 ^{a}	6.74 ^{b}	3.9 ^{cd}	4.04 ^{c}	2.48 ^{d}	2.69 ^{cd}
Phenylethyl alcohol	1.08 ^{bc}	3.55 ^{a}	1.13 ^{bc}	1.42 ^{b}	0.32 ^{c}	0.43 c	0.19 ^{c}	0.25 ^{c}
Acids								
Acetic acid	0.07 ^{c}	2.63 ^{ab}	4.9 ^{a}	2.39 ^{b}	1.28 ^{bc}	2.05 ^{bc}	0.90 ^{bc}	1.14 ^{bc}
Isovaleric acid	0.13 ^{b}	0.03 ^{c}	0.04 ^{c}	0.03 ^{c}	0.13 ^{b}	0.06 ^{bc}	0.29 ^{a}	0.24 ^{a}
Aldehydes								
3-Methyl-butanal	0.02 ^{b}	0.02 ^{b}	0.01 ^{b}	0.02 ^{b}	0.04 ^{b}	0.004 ^{b}	0.25 ^{a}	0.18 ^{a}
Benzaldehyde	0.06 ^{abc}	0.099 ^{ab}	0.08 ^{abc}	0.055 ^{abc}	0.042 ^{bc}	0.034 ^{c}	0.096 ^{ab}	0.106 ^{a}
Benzeneacetaldehyde	0.028 ^{ab}	0.058 ^{ab}	0.029 ^{ab}	0.039 ^{ab}	0.02 ^{b}	0.02 ^{b}	0.077 ^{a}	0.056 ^{ab}
Ketones								
2-Propanone	0.29 ^{b}	0.35 ^{ab}	0.31 ^{b}	0.30 ^{b}	0.37 ^{ab}	0.34 ^{ab}	0.52 ^{ab}	0.57 ^{a}
3-Hydroxy-2-butanone (Acetoin)	0.04 ^{b}	1.84 ^{a}	1.84 ^{a}	1.14 ^{a}	0.03 ^{b}	0.007 ^{b}	0.03 ^{b}	0.03 ^{b}
2-Nonanone	1.27 ^{a}	0.024 ^{b}	0.019 ^{b}	0.015 ^{b}	0.009 ^{b}	0.005 ^{b}	0.008 ^{b}	0.010 ^{b}
Dihydro-2-methyl-3(2H)-thiophenone	0.58 ^{de}	1.32 ^{bcd}	3.31 ^{a}	0.95 ^{cde}	1.88 ^{b}	1.69 ^{bc}	0.16 ^{e}	0.32 ^{e}
2-Undecanone	0.29 ^{a}	0.025 ^{b}	0.017 ^{b}	0.01 ^{b}	0.004 ^{b}	0.005 ^{b}	0.005 ^{b}	0.005 ^{b}

Terpenes								
α-Bergamotene	0.45 [a]	0.18 [abc]	0.20 [abc]	0.44 [ab]	0.05 [bc]	0.06 [bc]	0.02 [c]	0.02 [c]
β-Farnesene	0.42 [b]	0.47 [b]	0.63 [ab]	1.37 [a]	0.26 [b]	0.38 [b]	0.04 [b]	0.04 [b]
β-Citronellol	3.65 [a]	1.37 [bc]	0.81 [c]	0.83 [c]	1.5 [bc]	2.23 [b]	0.74 [c]	0.76 [c]
Nerolidol	0.57 [ab]	0.85 [ab]	0.86 [ab]	1.79 [a]	0.3 [b]	0.5 [ab]	0.01 [b]	0.02 [b]
Lactones								
γ-Butryolactone	0.08 [abc]	0.1 [ab]	0.121 [a]	0.123 [a]	0.05 [c]	0.05 [c]	0.06 [bc]	0.07 [bc]
Volatile phenols								
4-Vinyl guaiacol	0.11 [a]	0.005 [b]	0.009 [b]	0.002 [b]	0.03 [b]	0.002 [b]	0.04 [b]	0.04 [b]
4-Vinylphenol	0.048 [a]	0.004 [cd]	0.004 [cd]	0.003 [d]	0.02 [bcd]	0.002 [d]	0.028 [abc]	0.035 [ab]
2,4-Bis(1,1-dimethylethyl)-phenol	1.0 [c]	4.88 [ab]	6.41 [a]	6.32 [a]	2.43 [bc]	1.74 [c]	1.96 [c]	2.47 [bc]

[a]Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.

Supplementary Table 4. Mean peak areas for volatile compounds in sequential fermentation among yeast isolates with *S. cerevisiae* (EC1118).

	Yeast isolate[a]							
Compound	**C (EC1118)**	**KAW18**	**KAW29**	**KSW20**	**TDL3**	**TDL7**	**WAL3**	**WAL32**
Esters								
Ethyl propanoate	0.45 ^{b}	0.26 ^{b}	0.35 ^{b}	4.3 ^{a}	0.32 ^{b}	0.43 ^{b}	0.23 ^{b}	0.23 ^{b}
Ethyl isobutyrate	0.011 ^{b}	0.017 ^{b}	0.035 ^{a}	0.006 ^{b}	0.013 ^{b}	0.014 ^{b}	0.037 ^{a}	0.035 ^{a}
Ethyl butanoate	1.0 ^{a}	0.23 ^{f}	0.38 ^{ef}	0.48 ^{de}	0.9 ^{ab}	0.9 ^{ab}	0.72 ^{bc}	0.63 ^{cd}
Ethyl 2-methylbutyrate	0.02 ^{b}	0.006 ^{c}	0.006 ^{c}	0.01 ^{bc}	0.01 ^{bc}	0.02 ^{b}	0.05 ^{a}	0.05 ^{a}
Ethyl isovalerate	0.018 ^{bc}	0.007 ^{d}	0.005 ^{d}	0.004 ^{d}	0.011 ^{cd}	0.018 ^{b}	0.024 ^{a}	0.024 ^{a}
Ethyl decanoate	11.86 ^{ab}	2.87 ^{c}	3.54 ^{bc}	5.31 ^{bc}	13.02 ^{a}	9.89 ^{bc}	11.54 ^{abc}	11.26 ^{bc}
3-Methylbutyl octanoate	0.131 ^{a}	0.037 ^{b}	0.05 ^{b}	0.067 ^{b}	0.215 ^{a}	0.086 ^{a}	0.096 ^{a}	0.072 ^{a}
Ethyl 9-decenoate	3.48 ^{a}	0.25 ^{b}	0.78 ^{a}	0.91 ^{b}	4.15 ^{a}	3.36 ^{a}	0.61 ^{b}	0.40 ^{b}
Ethyl dodecanoate	0.97 ^{ab}	0.33 ^{b}	0.33 ^{b}	0.32 ^{b}	1.76 ^{a}	0.54 ^{bc}	0.55 ^{b}	0.49 ^{b}
Ethyl hexanoate	0.37 ^{a}	0.037 ^{c}	0.065 ^{c}	0.11 ^{c}	0.30 ^{ab}	0.196 ^{abc}	0.194 ^{abc}	0.168 ^{bc}
Ethyl octanoate	0.48 ^{a}	0.03 ^{c}	0.06 ^{c}	0.10 ^{bc}	0.36 ^{ab}	0.21 ^{bc}	0.11 ^{bc}	0.09 ^{c}
Ethyl phenylacetate	0.104 ^{abc}	0.093 ^{abc}	0.053 ^{bc}	0.115 ^{ab}	0.082 ^{abc}	0.120 ^{a}	0.047 ^{c}	0.046 ^{c}
Acetates								
Ethyl acetate	2.03 ^{c}	2.62 ^{bc}	5.28 ^{b}	12.7 ^{a}	12.1 ^{a}	1.92 ^{c}	13 ^{a}	14.3 ^{a}
Propyl acetate	0.04 ^{d}	0.04 ^{d}	0.09 ^{cd}	0.22 ^{a}	0.12 ^{bc}	0.03 ^{d}	0.17 ^{ab}	0.17 ^{ab}
Isobutyl acetate	0.11 ^{e}	0.41 ^{cd}	1.16 ^{a}	0.25 ^{de}	0.27 ^{de}	0.1 ^{e}	0.67 ^{b}	0.54 ^{bc}
(E)-3-Hexen-1-yl acetate	0.049 ^{c}	0.168 ^{b}	0.184 ^{b}	0.376 ^{a}	0.098 ^{bc}	0.034 ^{c}	0.066 ^{c}	0.051 ^{c}
(Z)-3-Hexen-1-yl acetate	0.01 ^{c}	0.07 ^{b}	0.07 ^{b}	0.12 ^{a}	0.03 ^{c}	0.01 ^{c}	0.02 ^{c}	0.01 ^{c}
(E)-2-Hexen-1-yl acetate	0.0004 ^{d}	0.07 ^{a}	0.024 ^{c}	0.04 ^{b}	0.007 ^{d}	0.0006 ^{d}	0.0007 ^{d}	0.0002 ^{d}
Benzyl acetate	0.016 ^{c}	0.029 ^{bc}	0.045 ^{b}	0.21 ^{a}	0.039 ^{bc}	0.014 ^{c}	0.032 ^{bc}	0.025 ^{bc}
Geranyl acetate	0.015 ^{bc}	0.035 ^{abc}	0.042 ^{ab}	0.056 ^{a}	0.210 ^{bc}	0.012 ^{c}	0.014 ^{c}	0.01 ^{c}
Isoamyl acetate	1.84 ^{ab}	2.43 ^{ab}	4.56 ^{a}	2.86 ^{ab}	2.22 ^{ab}	0.99 ^{b}	2.77 ^{ab}	2.24 ^{ab}
Phenylethyl acetate	0.17 ^{c}	11.12 ^{a}	6.25 ^{b}	5.63 ^{b}	0.23 ^{c}	0.14 ^{c}	0.26 ^{c}	0.24 ^{c}
Alcohols								
2-Methyl-1-propanol	0.17 ^{c}	0.81 ^{b}	1.33 ^{a}	0.32 ^{c}	0.23 ^{c}	0.24 ^{c}	0.49 ^{bc}	0.48 ^{bc}
4-Methyl-1-pentanol	0.044 ^{bcd}	0.093 ^{a}	0.048 ^{b}	0.045 ^{bc}	0.039 ^{bcd}	0.046 ^{bc}	0.023 ^{cd}	0.02 ^{d}
1-Heptanol	0.13 ^{b}	0.14 ^{b}	0.14 ^{b}	0.17 ^{ab}	0.75 ^{a}	0.18 ^{ab}	0.05 ^{b}	0.05 ^{b}

2-Heptanol	0.021 ab	0.048 a	0.049 a	0.047 a	0.033 ab	0.024 ab	0.017 b	0.018 b
3-Methyl-1-pentanol	0.24 b	0.13 cd	0.1 d	0.36 a	0.12 cd	0.22 bc	0.05 d	0.04 d
1-Hexanol	0.77 b	0.96 a	0.83 b	0.78 b	0.74 b	0.8 b	0.8 b	0.85 ab
(E)-3-Hexen-1-ol	0.034 a	0.027 bc	0.024 c	0.018 d	0.032 ab	0.033 a	0.032 ab	0.034 a
3-Ethoxy-1-propanol	0.02 c	0.09 b	0.137 a	0.12 ab	0.02 c	0.02 c	0.04 c	0.04 c
2-Nonanol	0.019 bc	0.022 bc	0.033 a	0.025 ab	0.021 bc	0.023 bc	0.014 c	0.014 c
1-Nonanol	0.039 b	0.045 b	0.027 b	0.031 b	0.135 a	0.047 b	0.021 b	0.020 b
Methionol	0.17 b	0.27 a	0.14 b	0.16 b	0.15 b	0.19 b	0.06 c	0.06 c
Isoamyl alcohol	7.01 cd	11.09 a	9.44 b	7.71 c	5.05 de	6.63 cd	5.25 ef	4.79 f
Phenylethyl alcohol	1.08 bc	3.53 a	1.23 b	1.45 b	0.58 cd	1.10 bc	0.41 d	0.42 d
Acids								
Acetic acid	0.07 b	1.74 ab	3.18 a	0.36 b	0.31 b	0.1 b	0.93 b	1.10 b
Isovaleric acid	0.13 b	0.039 c	0.04 c	0.04 c	0.18 b	0.15 b	0.48 a	0.41 a
Aldehydes								
3-Methyl-butanal	0.02 c	0.16 a	0.06 b	0.03 c	0.02 c	0.02 c	0.02 c	0.03 c
Ketones								
3-Hydroxy-2-butanone (Acetoin)	0.041 bcd	0.138 a	0.095 ab	0.088 abc	0.036 bcd	0.055 bcd	0.022 d	0.032 cd
2-Nonanone	1.27 a	0.15 c	0.29 bc	0.29 bc	0.86 ab	1.35 a	0.25 bc	0.21 c
Dihydro-2-methyl-3(2H)-thiophenone	0.58 cd	0.78 bc	1.74 a	0.98 b	0.4 d	0.46 bc	0.36 d	0.3 d
2-Undecanone	0.29 a	0.14 bc	0.14 bc	0.12 bc	0.24 ab	0.25 ab	0.07 c	0.06 d
Terpenes								
β-Farnesene	0.42 bc	1.51 a	0.54 bc	0.78 b	0.46 bc	0.54 bc	0.32 c	0.38 c
β-Citronellol	3.65 ab	2.87 ab	1.44 b	3.00 ab	3.74 ab	5.03 a	3.15 ab	3.48 ab
Nerolidol	0.57 b	2.46 a	0.60 b	1.3 ab	0.80 b	0.99 b	0.35 b	0.41 b
Volatile phenols								
4-Vinyl guaiacol	0.114 ab	0.036 d	0.078 cd	0.081 bc	0.150 a	0.126 a	0.148 a	0.133 a
4-Vinylphenol	0.048 bc	0.027 c	0.032 c	0.052 bc	0.09 a	0.05 bc	0.07 ab	0.07 ab
2,4-Bis(1,1-dimethylethyl)-phenol	0.995 d	4.71 ab	3.06 c	5.45 a	3.48 c	2.7 c	3.74 bc	3.82 bc

[a]Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.

CHAPTER 3

"Influence of *Kazachstania* spp. on the chemical and sensory profile of red wines"

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

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

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

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

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Influence of *Kazachstania* spp. on the chemical and sensory profile of red wines

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ABSTRACT

We report the fermentative traits of two *Kazachstania* species (*K. aerobia* and *K. servazzii*) in non-sterile red wine and the resulting chemical and sensory properties. This builds on our previous work which revealed that *Kazachstania* spp. increased acetate esters in sterilised white wine. In this study *Kazachstania* spp. were initially evaluated in laboratory-scale fermentations (500 mL) in Merlot must to assess whether similar increases in chemical/volatile compounds would occur. The impact of malolactic fermentation (MLF) by *Oenococcus oeni* (VP41) on aroma composition was considered and found to reduce ester profiles in Merlot wines. The sensory implications of sequential inoculation with *Kazachstania* spp., followed by *Saccharomyces cerevisiae*, were then evaluated in small-lot fermentations (7 kg) of Shiraz must. Fungal diversity was monitored during early fermentation stages and was influenced by the early implantation of *Kazachstania* spp., followed by the dominance of *S. cerevisiae*. The effect of MLF in Shiraz wines was inconclusive due to high ethanol levels providing an inhospitable environment for lactic acid bacteria. When compared to *S. cerevisiae* alone, *Kazachstania* spp. significantly increased acetate esters, particularly phenylethyl acetate and isoamyl acetate, in both Merlot and Shiraz. The Shiraz wines fermented with *Kazachstania* spp. had higher jammy and red fruit aroma/flavour compared to *S. cerevisiae* (monoculture) wines. No influence was observed on colour one-year post-bottling. Results from this study show the contribution of *Kazachstania* spp. to the aroma profile of red wines and demonstrate their potential as starter cultures for improving the aromatic complexity of wines.

1. Introduction

Traditional winemaking practises rely on un-inoculated fermentations, in which temporal succession of grape- and must- associated microbiota (eg. yeast, filamentous fungi, bacteria) are responsible for the bioconversion of grape juice to wine (Belda et al., 2017; Morrison-Whittle and Goddard, 2018; Padilla et al., 2016b). Whilst these 'wild' fermentations have become popular (with at least 3% of red and 6% of premium white wines in Australia made this way, Nordestgaard, 2019), issues of reliability and the production of undesirable wine traits/characteristics are not uncommon (Jolly et al., 2014). As such, most winemakers still rely on *Saccharomyces cerevisiae* strains as pure starter cultures to ensure the reliable and timely completion of alcoholic fermentation (AF). But in this case, the wines are sometimes regarded as lacking complexity and characteristic aroma and flavours (Padilla et al., 2016a). Whilst un-inoculated fermentations are essentially a "gamble" by winemakers due to their inherent unpredictability, this risk is tolerated in exchange for enhancement of the organoleptic properties of premium wines, whether undertaken on their own, or when blended with other wines (Navarrete-Bolaños, 2012).

To achieve the rewards of a diverse fermentation with less risk, winemakers are now employing non-*Saccharomyces* starter culture(s) in combination (co- and sequential-inoculation) with *S. cerevisiae* (Gobbi et al., 2013; Parapouli et al., 2020; Raynal et al., 2011). To date commercial non-*Saccharomyces* starter cultures include seven genera (8 species) sold as innovative biotechnological tools to help winemakers achieve 'tailored' wines: *Candida zemplinina*, *Kluyveromyces wickerhamii*, *Lachancea thermotolerans*, *Metschnikowia fructicola*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* (Benito et al., 2019; Roudil et al., 2020; Vejarano and Gil-

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Calderón, 2021). Some commercial non-*Saccharomyces* are promoted as alternatives to sulfur dioxide (SO_2) addition pre-fermentation, preventing microbial spoilage e.g. GAÏA™ (*M. fructicola*; Lallemand), ZYMAFLORE® ÉGIDE (*T. delbrueckii* and *M. pulcherrima*; Laffort). However, their primary use is to modulate the wine sensory profile in terms of enhanced aroma and/or mouthfeel (*T. delbrueckii* (BIODIVA™; Lallemand, PRELUDE™; Chr. Hansen), *M. pulcherrima* (FLAVIA™; Lallemand), increased acidity and freshness (*L. thermotolerans* (LAKTIA™; Lallemand, CONCERTO™; Chr. Hansen), and increased fruitiness (*P. kluyveri* (FROOTZEN®; Chr. Hansen). These are used in conjunction with *S. cerevisiae*, whilst some products are blends, for example Melody™ (*S. cerevisiae* (60%), *T. delbrueckii* (20%) and *L. thermotolerans* (20%); Chr. Hansen).

The contribution to wine aroma derived from non-*Saccharomyces* yeasts occurs through different mechanisms, but most importantly, via the direct biosynthesis of volatile aroma compounds (Godoy et al., 2020). Several flavour and aroma compounds (eg. monoterpenes, C_{13}-norisoprenoids, C_6 compounds, volatile phenols) are present in grapes as glycosidic precursors which are released by glycosidic enzymes produced by some non-*Saccharomyces* yeasts (Liu et al., 2017). Other metabolic products include esters, acetaldehyde, volatile fatty acids, terpenoids, higher alcohols, organic acids, carbonyl, and sulfur compounds (Capozzi et al., 2015; Carrau et al., 2020). These contribute to sensorial complexity, acidity and mouthfeel, which are highly sought-after attributes associated with premium wines (Carrau et al., 2020).

We have previously identified several *Kazachstania* spp. isolates from spontaneous Shiraz fermentations, which from genotypic comparison of the ITS1-5.8S rRNA-ITS2 gene sequence were identical (>99%) to *Kazachstania aerobia* and *Kazachstania servazzii* (Lin et al., 2020). The genus *Kazachstania* is part of the *Saccharomycetaceae* family (belonging to the *Saccharomyces sensu lato* complex) with *Kazachstania viticola* being the first discovered in 1971 from fermenting grapes (Vaughan-Martini et al., 2010). Whilst several species belonging to *Saccharomyces*, *Kluyveromyces*, *Arxiozyma* and *Pachytichospora* are now genetically reassigned to the *Kazachstania* genus (Kurtzman and Robnett, 2003), on an evolutionary scale, this genus is the most closely related to *Saccharomyces cerevisiae*. *Kazachstania aerobia* was first identified in an aerobically deteriorating corn silage in Japan (Lu et al., 2004) and *K. servazzii* (previously *Saccharomyces servazzii*) was first isolated from soil in Finland (Capriotti, 1967). Both closely related species are also associated with grapes and grape must (Bagheri et al., 2015; Jood et al., 2017; Lin et al., 2020).

The oenological potential of the *Kazachstania* genus is yet to be realised, with only a few reports, such as that for *K. aerobia* (in co-fermentation with *S. cerevisiae*) describing floral and dried fruit associated aromas in white wines (Beckner Whitener et al., 2016; Lin et al., 2020). Furthermore, a relatively unknown *Kazachstania* species, *K. gamospora* produces large quantities of esters in red (Syrah) and white wines, particularly ethyl proprionate (associated with floral aromas) (Beckner Whitener et al., 2015). We have also reported on production of high amounts of the ester isoamyl acetate with *K. servazzii* in Viognier; a fruity aroma that resembles banana (Lin et al., 2020). Whilst these reports are promising, gaps remain in terms of their dominance during fermentation, their effect on bacterial malolactic fermentation (MLF) (as in red wine production) and modulation of sensorial and chemical properties of the finished wine.

To date, research has focused on the use of 'mixed' cultures, and how *Saccharomyces* and non-*Saccharomyces* interact during fermentation, with little attention devoted to their potential effect on MLF (Bartle et al., 2019; Sumby et al., 2019), which is essential to red wine production and some white and sparkling based wines (Bartowsky et al., 2015). MLF reduces wine acidity, improves microbiological stability and is responsible for aroma and flavour changes (eg., diacetyl, esters, higher alcohols and volatile acids) (du Plessis et al., 2017). When in combination with non-*Saccharomyces*, MLF may increase wine colour intensity (total phenolics and anthocyanins), and reduce ethanol and astringency in red wines (du Plessis et al., 2017; Minnaar et al., 2019). To date, we are not aware of any reports on the use of *K. aerobia* or *K. servazzii* in red winemaking, which specifically answer some of these questions with regards to MLF.

This study describes the influence of *K. aerobia* and *K. servazzii* to red wine aroma and overall sensory perception when sequentially fermented with EC1118 in a non-sterile vinification setting. Small-scale (500 mL) sequential fermentations were conducted using Merlot grapes (the third most planted red variety in Australia (after Shiraz and Cabernet Sauvignon - 8000 ha; OIV – International Organisation of Vine and Wine (www.oiv.int) In both cases, MLF was conducted either as post-alcoholic fermentation (Merlot) and during alcoholic fermentation (Shiraz). From our earlier work with Viognier (Lin et al., 2020), the two *Kazachstania* species were hypothesised to increase acetates and alcohols (isoamyl- and phenylethyl-), resulting in a change in sensory perception. We report not only on the effect of the yeast but also the MLF bacteria on the wine composition and sensory properties of the resulting wines.

2. Materials and methods

2.1. Yeast isolates

Three isolates from two *Kazachstania* species (*K. aerobia* and *K. servazzii*) were used for this study (Table 1), which were selected because of their high enzymatic activities, ethanol tolerance and low H_2S production (Lin et al., 2020).

2.2. Laboratory-scale fermentation

Frozen Merlot must (12 kg, de-stemmed and crushed; 2015 vintage) was used for laboratory-scale sequential alcoholic fermentation and post-alcoholic MLF. Thawed must was processed and analysed for total soluble solids (TSS: 23°Brix), and pH (4.03), followed by the addition of dry ice and sulfur dioxide (SO_2) (as potassium metabisulfite (PMS; 40 mg/L)). The must was adjusted to pH 3.5 with tartaric acid prior to weighing out the pomace and measurement of the juice. The reconstituted must was aliquoted into sealed plastic bags, with a ratio of 1:1.125 (v/w) of juice to pomace, before transfer into 500 mL Bodum® French press coffee plungers (in triplicate). Nitrogen was supplemented in the form of diammonium phosphate (DAP) at 100 mg/L prior to fermentation, with a final concentration of 250 mg/L YAN.

Kazachstania spp. yeasts were grown in yeast extract-peptone-dextrose (YEPD) medium (Dymond, 2013) before transfer into Chemically Defined Grape Juice Medium (CDGJM) starter as described in Lin et al. (2020). The overnight starter culture was used to inoculate the must at a final concentration of 5×10^6 cells/mL. The commercial wine yeast strain *Saccharomyces cerevisiae* EC1118 (Lallemand, Australia), as activated dried yeast, was rehydrated according to the manufacturer's instructions prior to inoculating at 5×10^6 cells/mL after 72 h. Cell numbers were counted using a haemocytometer. Alcoholic fermentation was at 22 °C, with fermentation progress (as residual sugar) monitored daily using Megazyme's D-Glucose/D-Fructose Assay kit (K-FRUGL; Megazyme, USA). The Bodum® coffee plungers were covered with a loosely fitting lid and the must plunged twice daily. At the end of alcoholic fermentation (residual sugar <2 g/L), the wines were pressed

Table 1
List of species and isolates used in this study.

Isolate	Species	Accession number[a]	Source
PF_8_W29	*Kazachstania aerobia*	MN328365	Grape must
PF_9_W18	*Kazachstania aerobia*	MN328372	Grape must
PF_9_W20	*Kazachstania servazzii*	MN328373	Grape must

[a] ITS (fungi) sequences deposited in NCBI's GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

off the skins and cold settled at 4 °C for two weeks prior to MLF. Post alcoholic fermentation (AF) samples were transferred to 40 mL glass vials sparged with nitrogen and stored at 4 °C for later analysis.

Precultures of the commercial lactic acid bacteria strain *Oenococcus oeni* VP41 (Lallemand, Australia) from glycerol stocks were grown in a sterile (0.22 μm) mixture of 30% Merlot wine and 70% liquid MRSAJ (de Man, Rosa and Sharpe supplemented with 20% apple juice) broth medium and incubated for 7 days. Growth of bacterial starter cultures was measured by optical density at 600 nm (OD_{600}) on the Helios cuvette spectrophotometer (Fisher Scientific). Upon reaching an OD_{600} of 0.6, the cultures were inoculated at 3.8×10^8 CFU/mL into wine to start MLF. The cell density was confirmed by serial dilution of the culture and spot plating onto MRSAJ agar (see below).

MLF was conducted at 22 °C in 500 mL Schott bottles (each fitted with a bung and airlock). Glass marbles were added to reduce headspace/ullage. Malic acid consumption was measured weekly using a L-malic acid enzyme assay (K-LMALQR, Megazyme, USA). Viable cell numbers were determined by ten-fold serial dilution (10^{-1} - 10^{-5}) of samples in ultra-pure de-ionised water. Diluted samples (5 μL) were spot-plated on MRSAJ agar supplemented with cycloheximide to suppress yeast growth. The agar plates were incubated at 30 °C with 20% (v/v) CO_2 atmosphere for 7 days. Cell viability was enumerated as colony forming units (CFUs) per mL. Upon completion of MLF (< 0.05 g/L malic acid), samples were centrifuged and stored for subsequent volatile compound analysis.

2.3. Small-lot winemaking (sequential fermentation)

To evaluate the sensory effect of *Kazachstania* spp. it was necessary to scale up fermentation size up to 7 kg (allowing for sufficient replication and volume). Shiraz grapes (84 kg) (2020; McLaren Vale, South Australia) were mechanically crushed and de-stemmed, then randomly allocated (7 kg) to 10 L plastic buckets and processed (in triplicate) according to small-lot winemaking procedures (Holt et al., 2006). Sulfur dioxide was added (20 mg/L) using PMS (8% (w/v) solution). The pH of the must was adjusted with tartaric acid to 3.6, whilst DAP (100 mg/L, with an addition of 66.6 ml of water to each replicate) was added to yield 265 mg YAN/L.

The *Kazachstania* spp. cultures were grown in two steps: firstly, in YEPD overnight at 25 °C, and then in juice starter (50% filter sterilised Shiraz juice (2017), 40% sterilised RO water, 10% YEPD) overnight at 22 °C after inoculating at 2.5×10^6 cells/mL. The starter culture was inoculated in the must, at a rate of 5×10^6 cells/mL and grown 72 h prior to adding the *S. cerevisiae* strain EC1118 (Lallemand, Australia) as a sequential inoculation at 5×10^6 cells/mL. Cell numbers were counted using a haemocytometer. Fermentation was conducted on skins at ambient temperature (~25 °C), with the cap plunged twice daily, and DAP (100 mg/L) supplemented at 72 h. Fermentations were sampled once daily, and sugar and L-malic acid concentration were measured by enzymatic analysis. At 96 h, *O. oeni* VP41 (Lallemand, Australia), in active dried powder-form was rehydrated according to manufacturer's instructions and inoculated at 1 g/hL for MLF (0.9 mL per replicate).

At 2 g/L residual sugar, the wines were pressed and held at 25 °C until the residual sugars reached 0 g/L. Wines were racked from the yeast lees into glass flagons and cold stabilised (at 4 °C for 8 weeks). Wines were adjusted to pH 3.5 and 30 mg/L free SO_2 prior to bottling in 375 mL Claret bottles, with Novatwist™ screw cap closures (Plasdene Glass-Pak Pty Ltd., Adelaide, Australia). Bottles were stored at 15 °C for two months prior to sensory and chemical analyses.

2.4. Profiling of fungal diversity during alcoholic fermentation in Shiraz

Must samples (10 mL per replicate) were collected during alcoholic fermentation at 72, 95 and 164 h. Cell pellets were centrifuged at 5000 rpm (2236 ×*g*) for 5 min before cryopreservation at −20 °C. Frozen pellets (raw samples) were submitted to the Australian Genome Research Facility (AGRF) (Adelaide, South Australia) for genomic DNA extraction (DNeasy Powersoil Kit, #1288-50; QIAGEN) and deep-sequencing of internal transcribed spacer (ITS) 1 regions (amplicon sequencing) on the Illumina MiSeq platform; with amplicons obtained using the primer set ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCATCGATGAAGAACGCAGC-3′).

Operational taxonomic units (OTU) were assigned based on sequence comparison using the Basic Local Alignment Search Tool (BLAST), where the BLAST search is optimised for highly similar sequences (megaBLAST) at >95% similarity on the GenBank database (AGRF, Melbourne, Australia). OTU for each sample were classified taxonomically at different levels (from kingdom to family). Fungal diversity across all samples was visualised on GraphPad Prism 9.0.0 (GraphPad, USA).

2.5. Sensory evaluation of Shiraz wines

The replicate wines from each treatment were assessed for wine faults and similarities between replicates by a group of sensory experts from the University of Adelaide and CSIRO. As the replicates were deemed similar and without obvious faults, they were blended for the sensory analysis to prevent sensory fatigue. A list of wine attributes previously used in sensory trials at the University of Adelaide was also presented to the expert panel to select the most appropriate ones for use in this trial (Supplementary Table 1).

The sensory profile of the wines ($n = 4$) was assessed by participants ($n = 53$) aged 21 to 63 years (16 males, 37 females) recruited among the students and staff at the School of Agriculture, Food and Wine. The participants either were regular consumers of red wine and/or educated in wine science. Wines were evaluated using the Rate-All-That-Apply (RATA) method according to Danner et al. (2017), with assessments conducted during one session, in sensory booths at room temperature (22–23 °C) under white sodium lights. Panellists were presented with 30 mL of each wine in 4-digit coded, covered International Organisation for Standardisation (ISO) clear wine glasses (215 mL) in a randomised order. A seven-point intensity scale (1 = "extremely low". 4 = "moderate", 7 = "extremely high") was used to rate the applicable aroma attributes (ortonasally), flavour attributes (retronasally), and attributes related to taste, mouthfeel and length upon expectoration. Water and plain water crackers were also provided for participants as palate cleansers.

Sensory data were collected using the RedJade software (Redwood City, California, USA). All panellists provided their informed consent, with the study approved by the Human Research Ethics Committee of The University of Adelaide (approval number: H-2018-130). Differences in a range of sensory attributes (Supplementary Table 1) were determined by XLSTAT premium (ver. 2018.1.1.61323, Addinsoft, Paris, France), using one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) multiple comparison test at $p < 0.05$.

2.6. Profiling of secondary metabolites produced in wine

Wine samples (supernatants) were prepared and analysed as outlined in Lin et al. (2020), with minor modifications. High-performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1260 Infinity II system (Agilent Technologies) to quantify glycerol and ethanol. Organic acids (malic, lactic, succinic and acetic) were quantified enzymatically using the following test kits: L-malic acid (L-MALAF; Megazyme, USA), L-lactic acid (4A150; Vintessential Laboratories, Victoria, Australia), succinic acid (K-SUCC; Megazyme, USA) and acetic acid (4A105; Vintessential Laboratories, Victoria, Australia). Ethanol in Shiraz wines was additionally analysed using the Anton Parr DMA 4500 M Alcolyzer, according to the manufacturer's instructions. Wine composition data were analysed by one-way ANOVA (Tukey's multiple comparisons test) in GraphPad Prism 9.0 (GraphPad, USA).

2.7. Profiling of volatile compounds produced in wine

Headspace–solid phase micro extraction – gas chromatography with mass spectrometry (HS-SPME-GC–MS) was used to analyse the volatile compounds of Merlot and Shiraz wines, following the protocol described in Lin et al. (2020).

Statistical analysis of volatile constituents was carried out by one-way ANOVA (Tukey's multiple comparisons test) in GraphPad Prism 9.0 (GraphPad, USA). Differences across treatments were considered significant if the *p*-values were less than 0.01 ($p < 0.01$). Compounds with mean values statistically non-significant across treatments ($p > 0.01$) were excluded from Principal Component Analysis (PCA) using XLSTAT (version 22.5.1, Addinsoft, Paris, France).

2.8. Wine colour analysis (Shiraz)

Wine colour was analysed one-year after bottling. The modified Somer's method (Mercurio et al., 2007) was used to determine wine phenolic composition (colour density, hue, SO_2 resistant pigments, total phenolics and anthocyanins) by using an Infinite® 200 PRO spectrophotometer (TECAN, Switzerland). Wine colour was determined with the CIELAB colourspace model through using GBC Scientific Equipment Cintra 4040 (10 degree observer angle, in a wavelength range of 375 to 780 nm with 2 nm slit widths and at a scan speed of 1000 nm/min), and the results are expressed in terms of colour space L* (degree of lightness), a* (red/green opponent colours), b* (blue/yellow opponent colours). The hue angle or shade of colour (h°) was calculated from CIELAB values a* and b* in Excel as DEGREES(ATAN a*/b*). The chroma or colour saturation (C*) was calculated as $\sqrt{((a^*)^2 + (b^*)^2)}$. The difference (or spatial distance) between two colours or $\triangle$E was calculated from L*, a*, b* using $\sqrt{((L1^*\text{-}L2^*)^2 + (a1^*\text{-}a2^*)^2 + (b1^*\text{-}b2^*)^2)}$. Wine phenolic substances and colour parameters were analysed by one-way ANOVA (Tukey's multiple comparisons test) in GraphPad Prism 9.0 (GraphPad, USA).

3. Results

3.1. Sequential alcoholic fermentation and malolactic fermentation in Merlot

The three *Kazachstania* spp. isolates (Table 1) were first evaluated as sequential cultures with *S. cerevisiae* (EC1118) in non-sterile Merlot must, with the wines subsequently undergoing post-alcoholic MLF. EC1118 was chosen because of its neutrality in terms of aromatic contribution and extensive use in Australian red and white wine production (Nordestgaard, 2019). As such, the EC1118 treatment (monoculture) was considered a suitable control to determine the contribution of *Kazachstania* spp. to the finished wines which were analysed for chemical composition (metabolites and volatiles). All of the isolates/treatments completed alcoholic fermentation, with the wines considered dry (< 2 g/L) by 10 days (Supplementary Fig. 1A). Similarly, MLF finished 21 days after sequential inoculation of *O. oeni* VP41, with the L-malic acid (1.5 g/L) completely consumed (Supplementary Table 3a). As the fermentations took place in a non-sterile must, it is probable that MLF was partially completed by autochthonous lactic acid bacteria during AF and finished by VP41.

3.2. Sequential alcoholic fermentation in Shiraz

Small-lot winemaking was undertaken using Shiraz must, with the *Kazachstania* spp. isolates used in sequential culture with *S. cerevisiae* (EC1118), which was inoculated 3 days (72 h) later. Alcoholic fermentation duration ranged from 9 to 14 days, with EC1118 depleting sugar the quickest (9 days), followed by the sequential fermentations/treatments (Supplementary Fig. 1B). MLF was induced at 96 h (~1.95 g/L L-malic acid) during alcoholic fermentation in all treatments but was terminated after 30 days (~0.7 g/L L-malic acid) to prevent the potential wine spoilage, formation of off-flavours, and formation of amines (Supplementary Table 3b).

3.3. Fungal diversity profile in Shiraz must

Given that *S. cerevisiae* is well known for dominating spontaneous (Alonso-del-Real et al., 2019) and mixed-culture fermentations (Bagheri et al., 2017), the implantation and progression of inoculated yeasts and the fungal communities present in the fermenting Shiraz must were measured via diversity profiling. Individual samples (36) were taken from all replicates (of each treatment) at three time points (72, 95 and 164 h) during alcoholic fermentation. A total of 194,502 ITS high-quality sequences were generated from all samples, which were clustered into 95 fungal OTUs with a threshold of 97% pairwise identity. A total of 95 species were detected across all samples (Supplementary Table 2), and the species (10) which had a relative abundance of >1% are shown in Fig. 1, with the EC1118 (1) replicate having the most variation of species (Fig. 1A).

Several filamentous fungi and basidiomycetes (> 1% relative abundance) belonging to *Alternaria* (1%), *Aureobasidium* (5.6%), *Cladopsporium* (1.8%), *Epicoccum* (19.5%), *Seimatosporium* (1.9%), *Stemphylium* (1.1%) and *Vishniacozyma* (2%) were present at 72 h (Fig. 1A). At 164 h, *S. cerevisiae* had dominated ~70 to 90% of the community in *Kazachstania* spp. ferments, with the latter dramatically declining in relative abundance after 95 h (Fig. 1A and B). One of the *K. servazzii* replicates (PF_9_W20 (1)) showed an unusual result at 72 h (Fig. 1A), with low abundance of inoculated yeast and > 20% relative abundance for *Epicoccum* sp. This is probably not due to a failure of samples to implant and is most likely due to a PCR or sequencing error as the later timepoints align with the other replicates (Fig. 1B and C).

Kazachstania spp. persisted in the fermentation well beyond the fourth day (95 h) at levels ranging from 18 to 74% relative abundance (Fig. 1B). At such frequencies, the chemical and sensory profiles of the resultant wines were likely to be different from those with EC1118. To test this, the wines were analysed for their major metabolites derived from glycolysis and the TCA cycle, and volatiles (e.g. esters, acids and alcohols); all of which contribute the sensory profile and wine quality.

3.4. Wine composition analysis

Both wines were analysed after AF and MLF for organic acids, glycerol and ethanol concentrations via enzymatic assays and HPLC, listed in Supplementary Tables 3a (Merlot) and 3b (Shiraz). There were no significant differences ($p > 0.01$) in Merlot post AF for succinic and acetic acids and ethanol, although the *Kazachstania* spp. wines had approximately 1% less ethanol compared to EC1118 (*S. cerevisiae*) (Supplementary Table 3a). Malic and lactic acid concentrations varied slightly between treatments in post AF wines, as malic acid had not been completely consumed since ferments were not inoculated for MLF until post AF (Supplementary Table 3a). Whilst acetic acid levels were at the lower end of the average for young wines (0.1–0.4 g/L) (Vilela-Moura et al., 2011), the *Kazachstania* spp. concentrations were similar to the EC1118 wines. Glycerol concentration was significantly affected by the yeast inoculation, with *Kazachstania* spp. treatments resulting in higher concentrations (2.5 to 3.5 g/L more than EC1118) in both AF and MLF wines (Supplementary Table 3a). For the Shiraz wines, differences were also observed in glycerol concentrations, again with *Kazachstania* spp. treatments being 1.5 to 2.5 g/L higher than EC1118 (Supplementary Table 3b). Whilst MLF did not finish in the Shiraz fermentations, the residual malic acid was considered not statistically different between treatments (Supplementary Table 3b). MLF in this instance was considered to have minimal effect on aroma composition, with the yeast treatments being the major 'drivers' of any observed variation (see below).

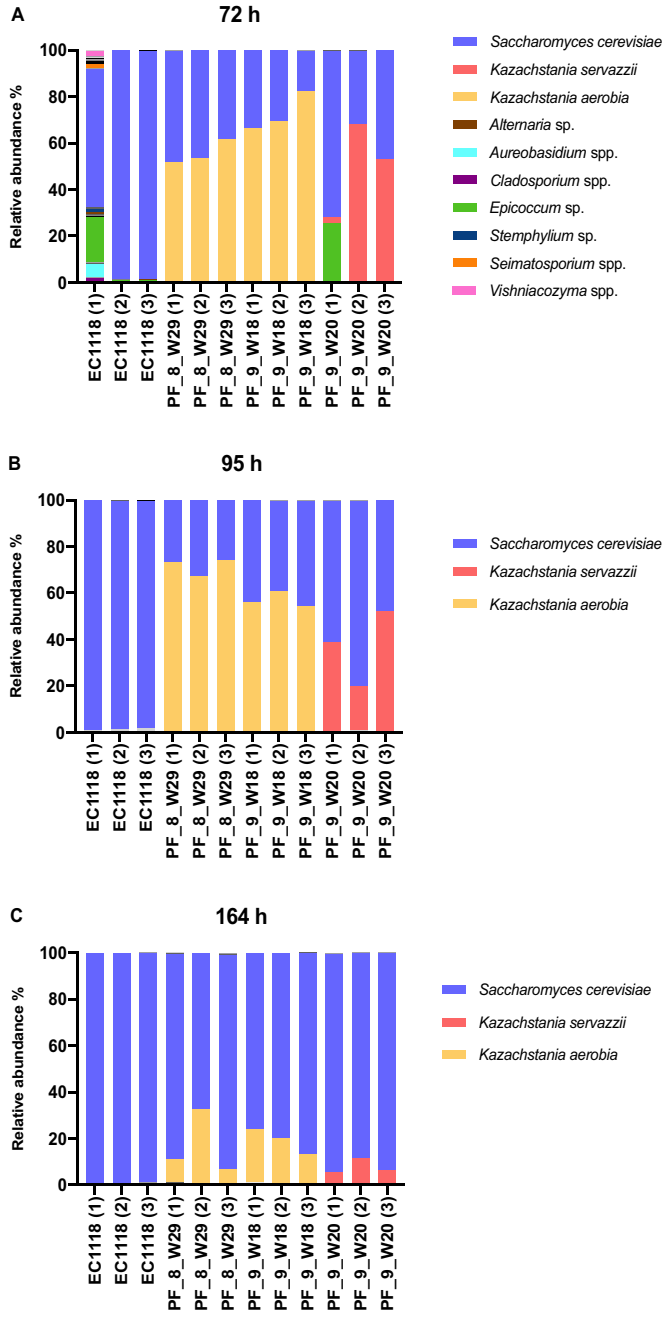


Fig. 1. Composition of fungal microbiota (relative abundance > 1 %) at species level in Shiraz must at 72 h (A), 95 h (B) and 164 h (C) during alcoholic fermentation. Samples (replicates of treatments) are on the x-axis and expressed as the relative operational taxonomic unit (OTUs) abundance for each sample/group.

3.5. Volatile compound analysis by GC–MS

3.5.1. Merlot wines

End of fermentation samples were analysed using SPME-GC–MS, with 74 compounds in total, identified and quantified. Testing of the compounds using one-way ANOVA at $p > 0.01$, identified compounds that were not significantly different and discounted from further analyses. There were 35 compounds from the AF and 33 compounds from the MLF that displayed significant differences ($p < 0.01$) when comparing means among the wines fermented with each isolate (Supplementary Tables 4a and 4b).

Significant differences were observed in esters quantified in the AF and MLF wines (Supplementary Tables 4a and 4b). Among the ethyl esters, there was a 2-fold increase in ethyl propanoate concentration in the wines produced with *K. servazzii* (KSW20). Wines made with *K. aerobia* (KAW18) had higher ethyl 2-methylbutanoate, isoamyl propanoate and ethyl 9-decenoate compared to the rest (Supplementary Table 4a). Overall all acetate esters and alcohols (except 1-nonanol) had the highest concentrations in *Kazachstania* AF wines compared to *S. cerevisiae* (Supplementary Table 4a). In particular, there was an 8- and 11-fold increase in phenylethyl acetate in the AF wines produced with *K. aerobia* and *K. servazzii* (Supplementary Tables 4a and 4b). Following MLF, the influence of VP41 had altered the concentration of esters, especially acetate esters as the concentrations had decreased drastically but remained higher in the *Kazachstania* spp. treatments compared to EC1118. Although MLF decreased the concentration of diethyl succinate in all treatments, this was an exception as the concentration was higher in EC1118 (in both AF and MLF) (Supplementary Tables 4a and 4b).

Principal component analyses (PCA) were used to visualise the relationship between the *Kazachstania* spp. sequential and *S. cerevisiae* (EC1118) monoculture treatments, with the chemical composition of Merlot wines. For the AF wines, the first two PCs accounted for 92.9% of the variation in the volatile compounds, with PC1 explaining 77.5% of the variance and PC2 at 15.4% (Fig. 2). Wine samples were separated along PC1 based on the volatile composition and the different isolates, with *Kazachstania* spp. in sequential culture (KAW18, KAW29 and KSW20) on the left-hand side of the plot and the *S. cerevisiae* (EC1118) located on the right-hand side of the plot (Fig. 2). The *Kazachstania* spp. were highly associated with acetate esters and alcohols. Separation along PC2 was driven by ethyl 9-decenoate and 2-methyl-1-propanol. A few ethyl esters (including diethyl succinate), alcohols, and acid were associated with the control wines (Fig. 2). Again, the PCA of the composition of MLF wines displayed a clear separation between *Kazachstania* spp. treatments and the control, where a total of 89% variance was explained by PC1 at 76.4% and PC2 at 12.6% (Fig. 2b). The *Kazachstania* spp. wines on the right of PC1 are again associated with acetate esters and alcohols (Fig. 2b). Towards the left-hand side of the plot, the control wines are characterised by higher concentrations of ethyl esters, with (*S*)-(+)-3-methyl-1-pentanol, 1-butanol, 1-propanol and diethyl succinate appear to be driving the separation along PC2 (Fig. 2b).

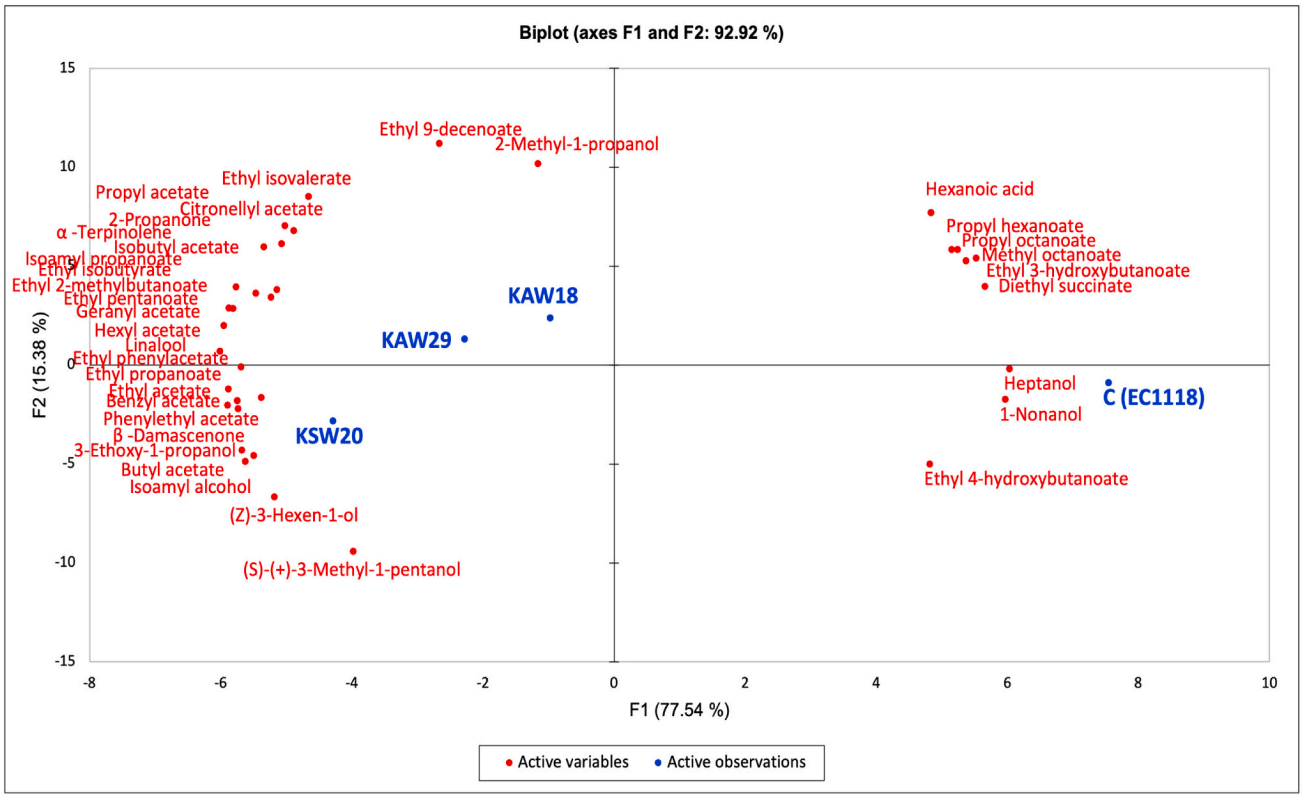
3.5.2. Shiraz wines

The Shiraz wines were bottled and stored at 15 °C for two months before chemical and sensory analysis. Volatile composition and sensory analyses were performed concurrently on the replicates and blended wines. Forty-one out of the 67 compounds quantified were significantly different ($p < 0.01$; Supplementary Table 5). The *Kazachstania* spp. wines had higher concentration of acetate esters, especially isoamyl and phenylethyl acetate which were 2.5 to 2.8 times and 3 to 4 times, respectively, higher than the control (Supplementary Table 5). Of the alcohols, isoamyl and phenylethyl alcohols were also higher in *Kazachstania* spp. wines (Supplementary Table 5). Acetaldehyde (or ethanal) was also slightly higher in *Kazachstania* spp. wines (13–14 mg/L) compared to EC1118 (10 mg/L) (Supplementary Table 5).

The PCA analysis of the composition of Shiraz wines (replicates and blended) displayed a distinct separation between *Kazachstania* spp. and the control (Supplementary Fig. 2). The total variance across all samples was 84.7%, with 1-octanol, 1-octen-3-ol, 1-propanol, isobutyl acetate, phenylethyl acetate and isoamyl isobutyrate driving the separation along PC1 (F1: 70%) (Supplementary Fig. 2). For PC2 (F2: 14.7%), ethyl decanoate and isopentyl octanoate were located towards the top of the plot, and α-terpinolene and heptanol towards the bottom of the plot. Higher concentrations of alcohols, acid, terpenes, lactones were associated with *S. cerevisiae* wines (Supplementary Fig. 2).

3.6. Sensory analysis of Shiraz wines

Prior to sensory analysis, an expert panel assessed that the replicate wines were without fault and similar within each treatment. The



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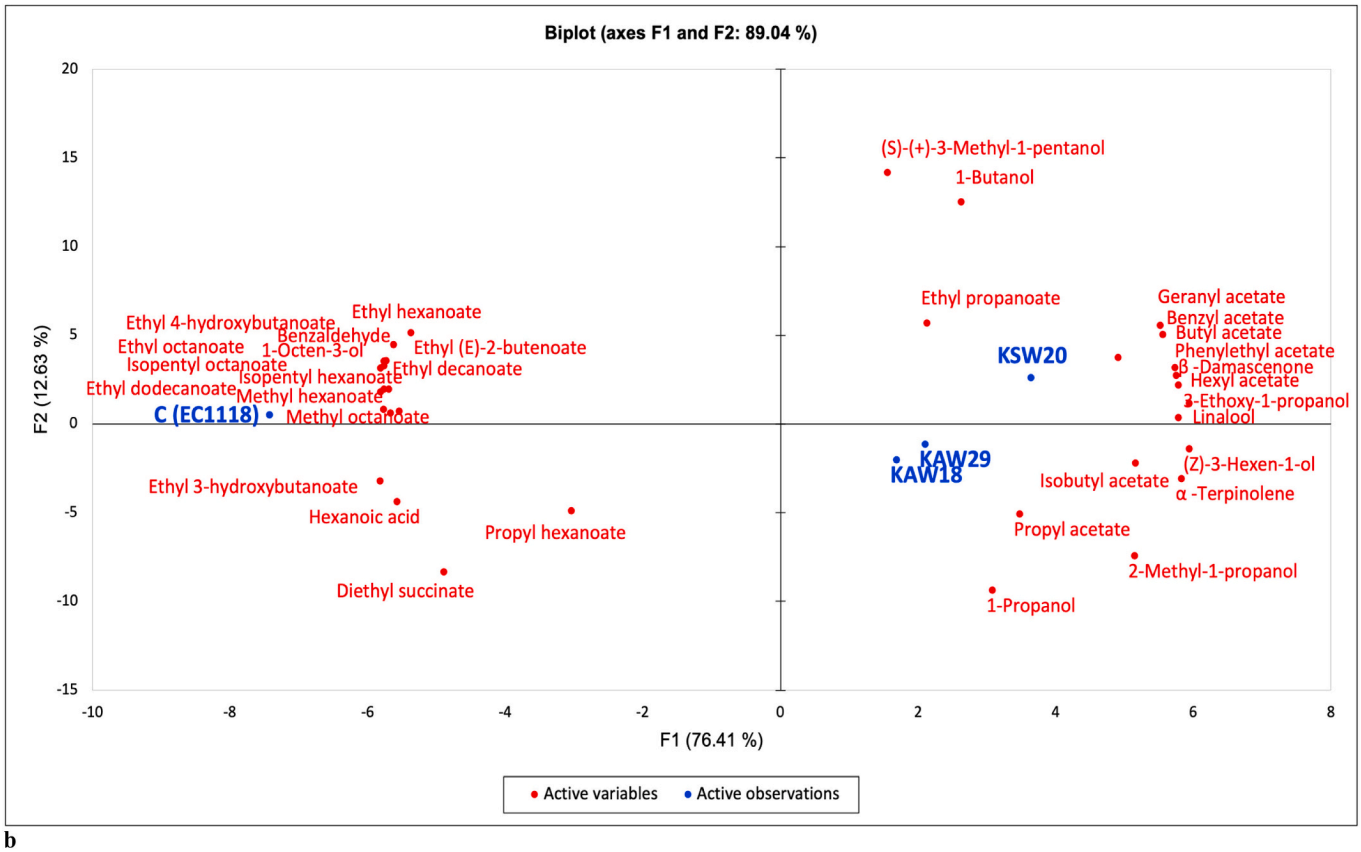

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Fig. 2. a. Principal component analysis of volatile profiles for alcoholic fermentation in Merlot wines. Yeast isolates (treatments) represented in blue and volatile compounds in red. C (EC1118) = *S. cerevisiae*, KAW18 = *K. aerobia*, KAW29 = *K. aerobia*, KSW20 = *K. servazzii*
b. Principal component analysis of volatile profiles for post-alcoholic malolactic fermentation in Merlot wines. Yeast isolates (treatments) represented in blue and volatile compounds in red. C (EC1118) = *S. cerevisiae*, KAW18 = *K. aerobia*, KAW29 = *K. aerobia*, KSW20 = *K. servazzii*. *(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

replicates were blended to prevent sensory fatigue during the evaluation. A list of 23 attributes characteristics of Shiraz varietal wine was collated (Supplementary Table 1). Out of a total of 23 aroma and flavour attributes tested, the participants perceived 11 attributes to be significantly different ($p < 0.05$) between the wines (Fig. 3). The *K. servazzii* (PF_9_W20) blended wine scored the highest overall aroma intensity, red fruit aroma and jammy flavour (Fig. 3). The *K. aerobia* derived wines were more similar to those fermented with *K. servazzii* compared to EC1118; the latter was characterised by earthy, forest floor, savoury and cooked vegetable aroma and flavours (Fig. 3).

Using the same 11 significantly different attributes, a principal component analysis (PCA) was performed with significantly different chemical/volatile compounds analysed by GC–MS to explore their underlying relationship. The first two principal components (PC) accounted for 93.6% of the variance in the data (Fig. 4). The first PC (F1: 74.4%) separated the wine samples fermented with *Kazachstania* spp. (8570, 4538 and 1483) from the blended wine made with *S. cerevisiae* (7254). The attributes driving the separation along PC1 were earthy, cooked vegetable, savoury and overall aroma intensity. The second PC (F2: 19.2%) was associated with separation of jammy and red fruit attributes with *Kazachstania* spp. wines on the bottom right-hand side, and forest floor, earthy, savoury and cooked vegetable attributes on the top left-hand side perceived in *S. cerevisiae* wines. These characteristics (jammy and red fruit) are correlated with fruity compounds such as methyl hexanoate (fruity), ethyl (E)-2-butenoate (sweet, caramel), phenylethyl acetate (floral), ethyl pentanoate (fruity, apple). The earthy, cooked vegetable, savoury and forest floor characteristics with benzaldehyde (almond, spice), α-terpinolene (pine), 1-octen-3-ol (mushroom), 3-(methylthio)-1-propanol (savoury, cooked vegetable).

3.7. Wine colour analysis

The effects of sequential inoculation of yeasts on anthocyanins, phenolics, colour and colour parameters in Shiraz wines were investigated one-year post-bottling. The colour and phenolic composition of the wines analysed by the modified Somer's method (Mercurio et al., 2007), were not significantly different ($p < 0.01$) between control and *Kazachstania* spp. treatments (Supplementary Table 6). Likewise, variation in colour was determined using the CIELAB colour space model, which expressed colour as perceived by human vision using the tristimulus values L* (perceptual lightness), a* (red-green) and b* (blue-yellow). There were no significant differences in chroma and hue values across all treatments, (Supplementary Table 6); although the hue angle (~ 12) indicated a purple to red hue (tint) to the wines. The measurement of colour variation between two wines (or △E) was calculated for control and *Kazachstania* spp. treatments: the value of ~ 3 (data not shown) was less than the values correlated with a significant colour difference (> 4 or 5; Witzel et al., 1973).

4. Discussion

This current work builds on our previous study (Lin et al., 2020), and evaluates the fermentative properties of yeast isolates of *Kazachstania* spp. and their contribution to the chemical and sensory profile of red wines. We investigated the fermentative traits of two *Kazachstania* species (*K. aerobia* and *K. servazzii*) in non-sterile red musts (Merlot and Shiraz). In the case of Shiraz must (and early stages of fermentation) the fungal communities are highly diverse and characterised by ubiquitous genera (*Alternaria* (1.05%), *Aureobasidium* (5.6%), *Hanseniaspora* (0.1%)) (Fig. 1, Supplementary Table 2). This diversity had diminished as fermentation (controlled through yeast inoculation) progressed, and fermentative yeasts reshaped the community, particularly due to the dominance of *S. cerevisiae*. The inoculated *Kazachstania* spp. established quickly during the first 95 h, and were outcompeted by *S. cerevisiae* sometime between 4 and 6 days after the initial inoculation (Fig. 1). This is reflected in the increase in some fruit-driven sensory attributes on the resulting wine aroma profile compared to the wines produced with EC1118 (Fig. 4). These findings allude to their potential as starter cultures for sequential fermentation, at least when used in a 1:1 ratio with *S. cerevisiae*. Maintenance of the *Kazachstania* spp. population may

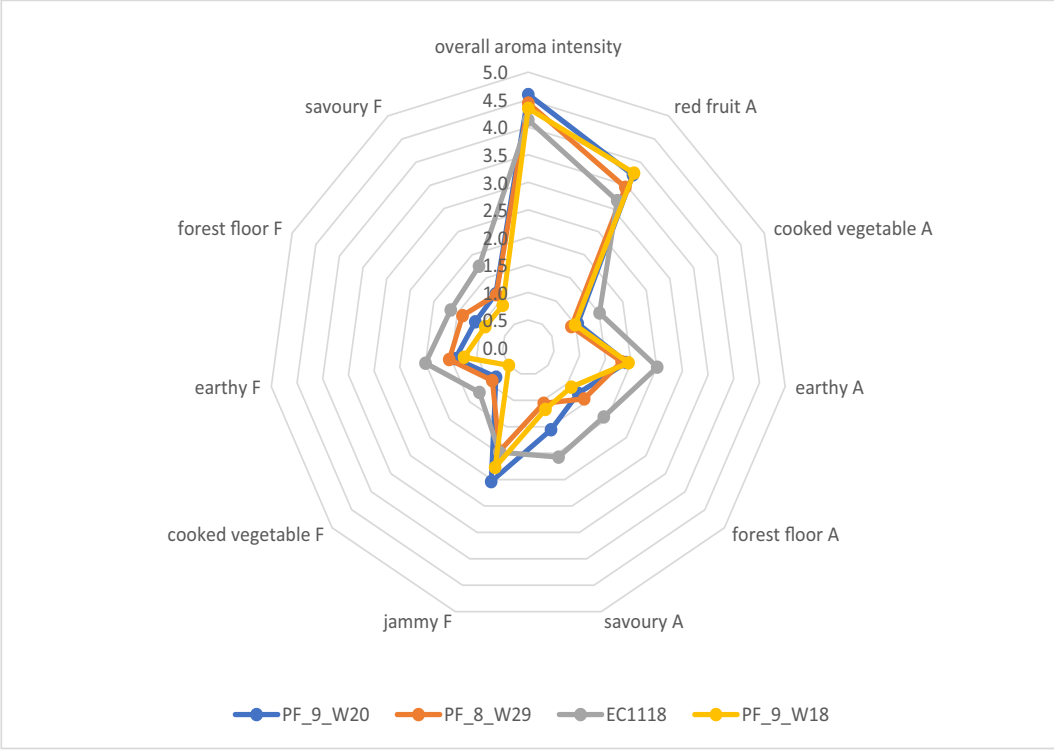

Fig. 3. Sensory attribute scores of Shiraz wines (blended replicates) obtained with *Kazachstania* spp. and *S. cerevisiae*. Sensory attributes were quantified by a 7-point intensity scale. Means in attribute intensities were significantly different at $p < 0.05$ (Fisher's LSD test). EC1118 = *S. cerevisiae*, PF_9_W18 = *K. aerobia*, PF_8_W29 = *K. aerobia*, PF_9_W20 = *K. servazzii*. F = Flavour, A = Aroma.

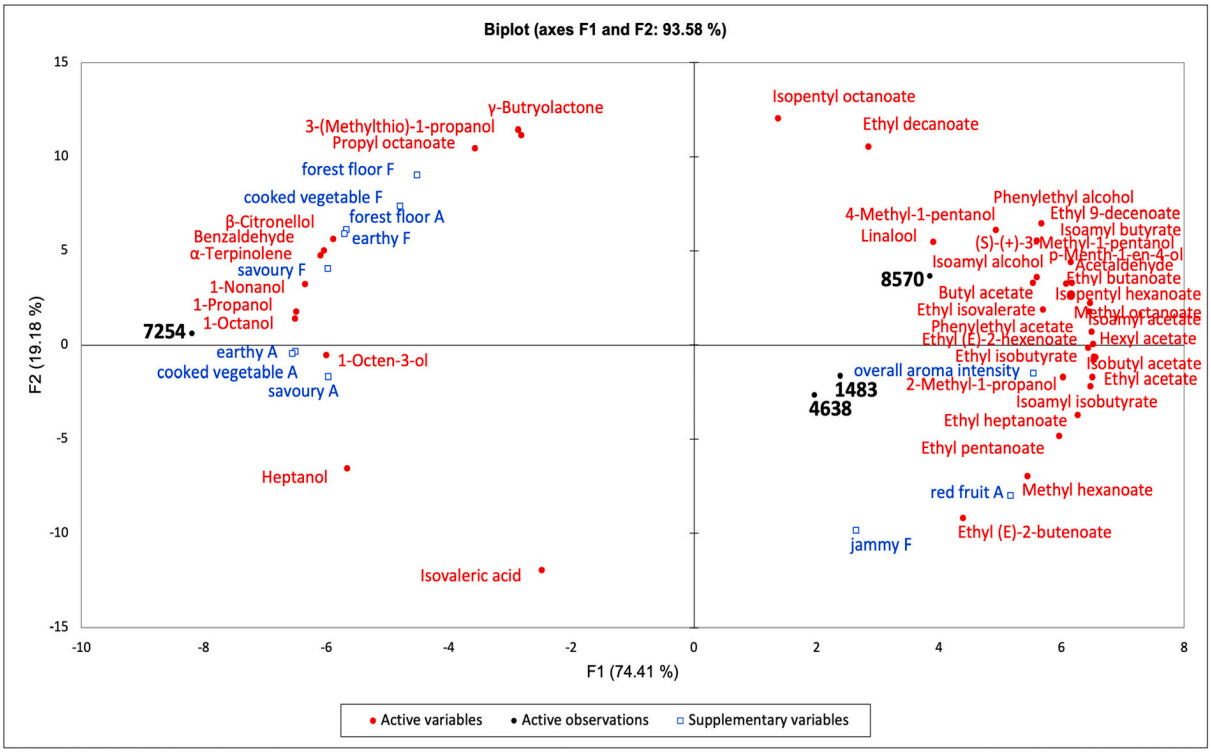

Fig. 4. Principal component analysis of volatile and sensory profiles in blended Shiraz wines. Yeast isolates (treatments) represented in black, volatile constituents represented in red and sensory attributes represented in blue. 7254 = *S. cerevisiae* (EC1118), 4638 = *K. aerobia* (PF_9_W18), 8570 = *K. aerobia* (PF_8_W29), 1483 = *K. servazzii* (PF_9_W20). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

require higher inoculum ratios to minimise competition with *S. cerevisiae*, as well as altered timing prior to inoculation of *S. cerevisiae* in order to enhance desirable compounds, although this is yet to be tested. Several studies have demonstrated the effect of inoculum ratio of non-*Saccharomyces* yeasts in sequential fermentations, from modulating phenolic compounds in red wines (*M. pulcherrima*, *T. delbrueckii*, *Zygosaccharomyces bailii* with *S. cerevisiae*) (Escribano-Viana et al., 2019) to ethanol reduction (*M. pulcherrima* with *Saccharomyces uvarum*) (Varela et al., 2021), and this could be applied to these *Kazachstania* spp. isolates to optimise their use in fermentations.

In recent years, the average ethanol concentration of wines has increased, in part due to climate change (Varela et al., 2015). This often lends to decreased complexity and suppressed overall aroma intensity (Varela et al., 2015). As consumer preference shifts from higher to lower alcohol wines, the inclusion of non-*Saccharomyces* yeasts has shown the potential to produce lower alcohol wines. In agreement with our previous findings in Viognier wines (Lin et al., 2020), *Kazachstania* spp. produced approximately 1% (v/v) less alcohol compared to *S. cerevisiae* when used to ferment the Merlot must in this study (Supplementary Table 3a). However, this was not the case in Shiraz as there were no differences in alcohol observed (~ 15 to 16% v/v) between the yeast treatments (Supplementary Table 3b). The results were not unexpected, given the Shiraz grapes grown in warmer climate regions such as Australia, ripen faster and accumulate more sugars, resulting in high alcohol wines. One reason for the lack of ethanol reduction in the Shiraz fermentations could be due to relative sugar consumption by *Kazachstania* spp. prior to the inoculation of EC1118 at 72 h. For example, *Kazachstania* spp. had consumed 30% (~ 69 g/L) of the sugar in the Merlot vs only 20% (~ 58 g/L) in the Shiraz. This left a relatively larger amount of sugar to be converted to ethanol by EC1118. One possible strategy to mitigate this in future experiments could be to delay *Saccharomyces* inoculation in high sugar juices and/or must allowing the non-*Saccharomyces* yeast more time to have an effect. It is also important to note although we did not measure fungal diversity at time 0, it is likely that there were indigenous *S. cerevisiae* present in the must, which could also have been one of the factors contributing to the increased ethanol concentration (being highly efficient in ethanol production). The timing and effect of *Kazachstania* spp. in high sugar non-sterile must requires further investigation using a range of alternate inoculation strategies.

Ethanol tolerance was not measured as monocultures, with the study only looking at sequential cultures versus EC1118. The *Saccharomyces* were included to complete the fermentation, so that the wines were dry and to specification. Glycerol content was measured, as it is the second major by-product after ethanol to influence flavour intensity by contributing body and fullness to wine (Gawel et al., 2008). In both the Merlot and Shiraz wines produced with *Kazachstania* spp., the glycerol yields were significantly higher than the *S. cerevisiae* wines (Supplementary Tables 3a and 3b). In the case of the Merlot wines, the increased glycerol content and corresponding lower ethanol content resulting from *Kazachstania* spp. sequential fermentation, corroborates the findings that non-*Saccharomyces* yeasts have the capacity to redirect sugar consumption away from ethanol by altering NAD^+/NADH balance during glycolysis and redirecting carbon flux towards glycerol (Goold et al., 2017; Hranilovic et al., 2020; Ivit et al., 2020). Non-*Saccharomyces* yeasts (eg. *Candida* spp., *Hanseniaspora* spp., *Kluyveromyces* spp., *Metschnikowia* spp., *Pichia* spp., *Torulaspora* spp., *Zygosaccharomyces* spp.) in mixed fermentation, in general produce more glycerol compared to pure cultures of *S. cerevisiae*, although the resulting glycerol concentration is species and strain-dependent (Romani et al., 2010).

The contribution of *Kazachstania* spp. to the volatile profile during sequential fermentation in Merlot and Shiraz wines were through an increased production of esters and higher alcohols. The two classes of flavour-active esters - acetate and ethyl esters produced during the initial stages of fermentation by non-*Saccharomyces* yeasts can contribute to overall aroma profile and this production is strain- and species-dependent (Gamero et al., 2016; Padilla et al., 2016a). In accord with our previous work (Lin et al., 2020), *Kazachstania* spp. sequential fermentations (treatments) yielded higher phenylethyl (floral aroma) and isoamyl acetates (fruity aroma) compared to *S. cerevisiae*

(Supplementary Tables 4a, 4b and 5). Additionally, higher concentrations of isoamyl and phenylethyl alcohol were observed in *Kazachstania* spp. wines (Supplementary Tables 4a, 4b and 5) and as well as contributing to wine flavour themselves, they can also act by serving as ester precursors (Padilla et al., 2016a). In particular, phenylethyl alcohol is regarded to be one of the most important aromatic alcohol, contributing to wine aroma. Furthermore, the aroma profile may be influenced by bacterial MLF, with ester modifications being dependent upon the bacterial strain used, as well as the influence of indigenous lactic acid bacteria present in the must during AF (Gámbaro et al., 2008; Sumby et al., 2010). In this case MLF conducted by *O. oeni* VP41 resulted in general decrease of esters in Merlot wines. Diethyl succinate, a fatty acid ester associated with MLF was higher in *S. cerevisiae* treatments in both AF and MLF Merlot wines (Supplementary Tables 4a and 4b). These results corroborate similar findings reported in comparison to other non-*Saccharomyces* in Shiraz wines that underwent sequential MLF (du Plessis et al., 2017), differences being their scale was larger (70 L replicates) and the LAB strain used (Viniflora® Oenos (Chr. Hansen)).

The effects of *O. oeni* during MLF have been shown to increase diethyl succinate in young red wines (Soufleros et al., 1998; Malherbe et al., 2012), which was not observed in the Merlot wines in this study (Supplementary Table 4b). One explanation could be the effects of different inoculation techniques to induce MLF, as Lasik-Kurdyś et al. (2018) reported that the wines that underwent sequential MLF resulted in lower concentrations of diethyl succinate compared to co-inoculated (simultaneous) wines. However, we cannot report the effects of MLF in our Shiraz fermentations as all the treatments became stuck/sluggish (EC1118 included). Additionally, no conclusions can be drawn regarding *Kazachstania* spp. and LAB compatibility, due to intrinsically harsh conditions in the Shiraz including high ethanol and the interaction with other possible inhibitors present in the must which meant that all fermentations did not finish MLF. The induction of sequential or simultaneous MLF can result in changes to wine flavour profiles, as well as the MLF strategy for each yeast strain to enhance wine sensory and quality, which appears to be strain-dependent (du Plessis et al., 2017), therefore the compatibility and interactions between *Saccharomyces*, non-*Saccharomyces* and LAB would require further investigation.

The effects of yeast-derived volatile compounds on sensory perception were also evident in the Shiraz wines, as there is a positive/strong correlation between perceived sensory attributes and aroma compounds (Fig. 4, Supplementary Fig. 2). In this first detailed study on the sensory impact of *Kazachstania* spp. in red wines, the volatile compounds produced in higher concentrations in fermentations using this non-*Saccharomyces* yeast are known to influence the fruit-driven sensory attributes and are predicted to be responsible for the differences in sensorial properties of the wines. The wines were characterised as having red fruit aroma and jammy flavour (Whitener et al., 2017), with *K. servazzii* derived wines being having the highest overall aroma intensity (Fig. 3). Jammy and red fruit attributes are stereotypical of ripe Shiraz grown in warm/hot climates (Herderich et al., 2012), which is intensified by the acetate esters produced by *Kazachstania* spp. (Fig. 4).

Yeasts inevitably produce acetaldehyde (or ethanal) during alcoholic fermentation, which is the most important occurring aldehyde in wine (Romano et al., 1994). Concentrations higher than 125 mg/L negatively affect the sensorial quality of wines, often imparting bruised apple and oxidation notes (Byrne and Howell, 2017). In the Shiraz wines, the *Kazachstania* spp. treatments produced approximately 13 to 14 mg/L and *S. cerevisiae* 10 mg/L of acetaldehyde (Supplementary Table 5). At lower concentrations (below 70 mg/L), acetaldehyde can impart a fruity flavour to wine (Coetzee et al., 2016). Other than its contribution to the aroma profile, acetaldehyde reacts with anthocyanins and tannins to affect wine astringency and colour stability. Colour is a vital sensory attribute perceived in red wines (Escot et al., 2001). Although there were no differences observed in colour parameters in the Shiraz wines (Supplementary Table 6), some non-*Saccharomyces* yeasts, when coupled with *S. cerevisiae* may contribute to the formation of stable pigments (pyranoanthocyanins and polymeric pigments) as observed by Hranilovic et al. (2018) with *L. thermotolerans*, *M. pulcherrima* and *T. delbrueckii* sequential fermentations. Hranilovic et al. (2018) suggested that the intraspecific diversity among *T. delbrueckii* strains influenced their effect on phenolic substances. Furthermore, *Schizosaccharomyces pombe* strains are reported to produce large amounts of pyranoanthocyanins and polymeric pigments, and have the potential to extend colour stability during aging (Morata et al., 2012). The prospects of *Kazachstania* spp. contribution to modulation of colour stability and astringency of wines through fermentation and aging remains to be explored.

In conclusion, this study was designed to assess the fermentation performance and sensory impact of *Kazachstania* spp. in non-sterile red wine fermentation with a view to their use as novel wine starter cultures. Our findings demonstrate the potential for *Kazachstania* spp. as means of increasing fruity aroma of wine and adding complexity. *Kazachstania* spp. consistently increased acetate ester concentration, more specifically phenylethyl acetate and isoamyl acetate, and produced wines that were distinct from those that were fermented with *S. cerevisiae* alone. Additionally, fermentation duration was not significantly affected and there was no negative effect on wine colour following one year of bottling. The findings demonstrate the potential that *Kazachstania* spp. have to alter wine style during red winemaking. In order to validate commercial potential of these isolates, studies at an industrial fermentation scale are required, and should incorporate chemical analysis including e.g. acetic acid yields.

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Declaration of competing interest

The authors declare there are no conflicts of interests regarding the study.

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Supplementary Material

Title: Influence of *Kazachstania* spp. on the chemical and sensory profile of red wines

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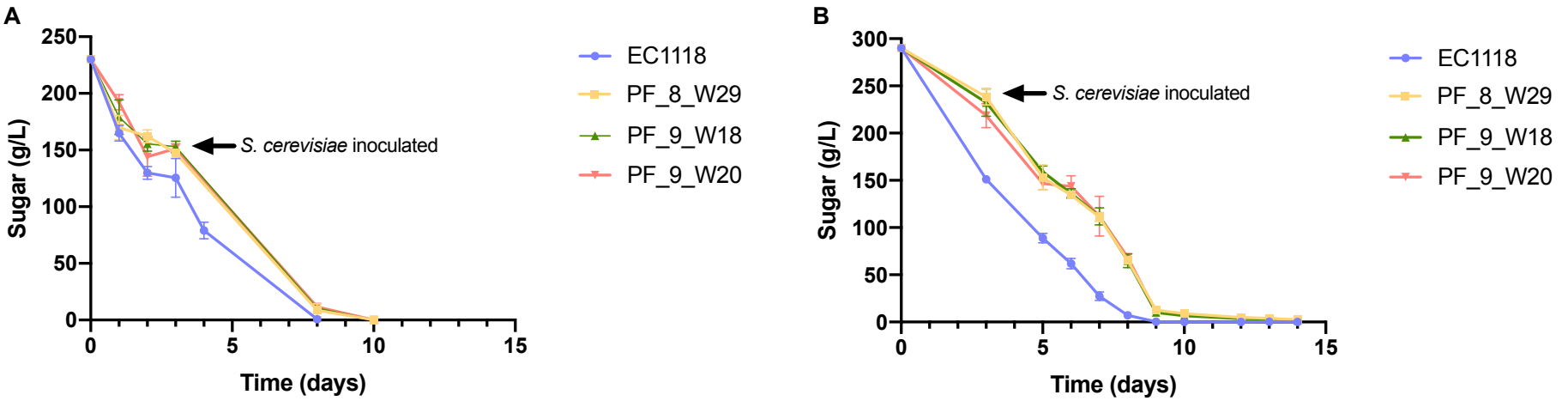


Supplementary Figure 1. Fermentation profile of *Kazachstania* spp. isolates in sequential fermentation with *S. cerevisiae* in Merlot (A) and Shiraz (B). EC1118 = *S. cerevisiae*, PF_8_W29 = *K. aerobia*, PF_9_W18 = *K. aerobia*, PF_9_W20 = *K. servazzii*

Supplementary Table 1. Attributes used to evaluate sensory properties in Shiraz wines in the Rate-All-That-Applies (RATA) analysis. Attributes were chosen by an expert panel when assessing replicate wines.

Sensory attribute	Description
Aroma/Flavour	
Confectionery	Lollies, bubblegum
Dark Fruit	Blackberry, blackcurrant, plum, dark cherry
Dried Fruit	Prune, raisin, fig, dried apricot
Red Fruit	Raspberry, strawberry, red cherry, redcurrants
Overall aroma/flavour intensity	Intensity ranging from low to high
Chocolate	Cocoa
Cooked vegetables	Cabbage, cauliflower, asparagus
Earthy	Mushroom, dusty
Eucalypt/mint	Herbal, camphor, medicinal
Floral/perfume/musk	Violet, rose
Forest floor/mushrooms	Musty, dirt
Herbaceous/stemmy/stalky	Grassy, leafy
Jammy	Preserved or cooked fruit
Pepper	Black, white
Savoury/meaty/gamey	Savoury, meaty, soy sauce
Spice	Clove, cinnamon, nutmeg
Vanilla	Sweet, toasty
Mouthfeel	
Acidity	Sourness, sharp taste, tart
Astringency	Dryness, puckering sensation
Bitterness	Unpleasant perception of tannins
Sweetness	Smooth, rich texture
Aftertaste	
Length of fruit flavours	Fruit flavours that linger in the mouth/left on the palate after wine is swallowed
Length of non-fruit flavours	Non-fruit flavours that linger in the mouth/left on the palate after wine is swallowed

Supplementary Table 2. OTU table (raw data) of 95 fungal species present in Shiraz must (74, 95 and 164 h). Sum of all values in each column (replicate) = 1. Values (relative abundance) of each species were converted to percentages for Figure 1.

Taxon	EC1118 (1) 7	EC1118 (2) 7	EC1118 (3) 7	PF_8_W29 (1	PF_8_W29 (2	PF_8_W29 (3	PF_9_W18 (1	PF_9_W18 (2	PF_9_W18 (3	PF_9_W20 (1	PF_9_W20 (2	PF_9_W20 (3	EC1118 (1) 9	EC1118 (2) 9	EC1118 (3) 9	PF_8_W29 (1	PF_8_W29 (2	PF_8_W29 (3	PF_9_W18 (1	PF_9_W18 (2	PF_9_W18 (3	PF_9_W20 (1	PF_9_W20 (2	PF_9_W20 (3	EC1118 (1) 1	EC1118 (2) 1	EC1118 (3) 1	PF_8_W29 (1	PF_8_W29 (2	PF_8_W29 (3	PF_9_W18 (1	PF_9_W18 (2	PF_9_W18 (3	PF_9_W20 (1	PF_9_W20 (2	PF_9_W20 (1) 164 h
s__Diplodia_seriata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00027503	0	0	0	0	0	0
s__Neofusicoccum_cryptoaustrale	0	0.00022202	0	0	0	0	0	0	0	0	0	0.00023354	0	0.00015211	0.00034459	0	0	0	0	0	0.00019001	0	0.00013314	0	0	0	0.00035575	0	0	0	0	0	0	0.0007165	0.00026582	0.00023935
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Botryosphaeriales;f__unidentified;g__unidentified;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00023935
s__Cladosporium_delicatulum	0.01797473	0	0.00029308	0	0	0	0	0	0.0002377	0	0	0	0.00056433	0.00015211	0.00017229	0	0.00011533	0	0.00015987	0	0	0	0.00013314	0.00057654	0	0	0.00017787	0	0	0.00082508	0	0.00048387	0.00033824	0	0	0
g__Cladosporium;s__Cladosporium_grevilleae	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
s__Mycosphaerella_tassiana	0.00373732	0	0.00029308	0	0	0	0	0	0.00011885	0.00035186	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00027503	0.00035398	0	0	0	0	0.00023935
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Neodevriesiaceae;g__Neodevriesia;s__Neodevriesia_capensis	0.00017797	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Neodevriesiaceae;g__Neodevriesia;s__Neodevriesia_lagerstroemiae	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00019001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Neodevriesiaceae;g__Neodevriesia;s__Neodevriesia_stirlingiae	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Teratosphaeriaceae;g__Devriesia;s__Devriesia_pseudoamericana	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017229	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00023883	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Teratosphaeriaceae;g__Lapidomyces;s__unidentified	0.00017797	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__Teratosphaeriaceae;g__Neocatenulostroma;s__Neocatenulostroma_abietis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00013314	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Capnodiales;f__unidentified;g__unidentified;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00023883	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Dothideales;f__Aureobasidiaceae;g__Aureobasidium;s__Aureobasidium_namibiae	0.05570386	0.00155417	0.00205158	0.00039085	0.00037573	0.00010898	0.00034423	0.00050454	0.00059425	0	0.00067249	0	0.001693	0.00060846	0.00155065	0.000212	0.00034598	0	0.00015987	0.00033411	0.00057002	0.00075514	0.00026628	0.00115307	0.00029095	0.00056465	0.00106724	0.00117536	0.00020404	0.00055006	0.00070796	0	0.00067648	0.00095534	0.00106326	0.00119674
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Dothideales;f__Dothioraceae;g__Hormonema;s__unidentified	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Myriangiales;f__Myriangiaceae;g__Myriangium;s__Myriangium_citri	0.00088984	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Ascochyta;s__Ascochyta_medicaginicola_var._macrospora	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017787	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Didymella;s__Didymella_glomerata	0.00017797	0	0.00014654	0	0	0	0	0	0	0	0.00016812	0	0.00018811	0	0	0	0.00023065	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Didymella;s__Didymella_rhei	0.00373732	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00029095	0	0	0	0	0	0	0	0	0	0.00026582	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Didymella;s__Didymella_viburnicola	0.00017797	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Epicoccum;s__Epicoccum_brasiliense	0.00195764	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Epicoccum;s__Epicoccum_nigrum	0.19523047	0.00666075	0.00556858	0.00312683	0.00075146	0.00119878	0.00137694	0.00050454	0.00166389	0.25334272	0.00184936	0.00256889	0.00470278	0.00973532	0.0101654	0.000636	0.00080729	0.0004356	0.00223821	0.00066823	0.00019001	0.00264301	0.00319531	0.00201787	0.00378237	0.00301148	0.00747065	0.00658204	0.00265252	0.00467547	0.00353982	0.00193548	0.00236766	0.00549319	0.00478469	0.00287219
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Macroventuria;s__Macroventuria_anomochaeta	0	0	0	0	0	0	0	0	0	0	0	0.00023354	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Neoascochyta;s__Neoascochyta_desmazieri	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00015211	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Neodidymelliopsis;s__Neodidymelliopsis_sp	0.0005339	0	0.00043962	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00070796	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Stagonosporopsis;s__Stagonosporopsis_dorenboschii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00037644	0	0	0	0	0	0	0.00016912	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__Xenodidymella;s__Xenodidymella_humicola	0	0.00022202	0	0	0	0	0	0	0	0	0	0	0	0	0.00017229	0	0	0	0	0	0	0	0.00013314	0	0	0.00018822	0	0.00023507	0	0	0	0.00016129	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymellaceae;g__unidentified;s__unidentified	0.00160171	0	0	0	0	0	0	0	0.00011885	0	0.00016812	0	0	0	0	0	0	0	0	0	0	0	0	0.00028827	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Didymosphaeriaceae;g__Didymosphaeria;s__Didymosphaeria_variabile	0.00373732	0.00066607	0.00073271	0.00019543	0	0	0	0	0.00011885	0	0.00016812	0	0.00056433	0.00030423	0.00034459	0.000212	0	0	0.00015987	0	0	0	0	0.00057654	0	0	0.00053362	0	0.00020404	0	0.00035398	0.00016129	0.00016912	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Lentitheciaceae;g__Keissleriella;s__Keissleriella_quadriseptata	0.00195764	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00023883	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Lophiostomataceae;g__Lophiostoma;s__Lophiostoma_semiliberum	0.00017797	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Periconiaceae;g__Periconia;s__Periconia_macrospinosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00019001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Phaeosphaeriaceae;g__Sclerostagonospora;s__Sclerostagonospora_lathyri	0	0	0.00014654	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Phaeosphaeriaceae;g__Setophoma;s__Setophoma_sacchari	0.00266951	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00033411	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Phaeosphaeriaceae;g__unidentified;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0.00018811	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Alternaria;s__Alternaria_betae-kenyensis	0.00569496	0	0.00131887	0.00019543	0	0	0	0	0.0002377	0.00035186	0.00016812	0.00023354	0.00094056	0.00076057	0.00051688	0	0.00011533	0.0001452	0.00015987	0	0.00019001	0	0.00119824	0	0.00029095	0	0.00088936	0	0	0.00027503	0.00106195	0	0.00033824	0.00047767	0.00053163	0.00071805
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Alternaria;s__Alternaria_botryospora	0.00177968	0	0	0	0	0	0	0	0	0	0	0	0	0.00045634	0.00034459	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Alternaria;s__Alternaria_metachromatica	0.00320342	0.00044405	0.00073271	0.00019543	0	0	0	0	0	0	0	0.00023354	0.00075245	0	0.00086147	0	0	0	0	0	0	0	0.00026628	0	0	0.00018822	0.00053362	0	0	0	0.00070796	0	0.00033824	0	0.00053163	0.0004787
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Comoclathris;s__unidentified	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporaceae;g__Stemphylium;s__Stemphylium_sp	0.01085602	0.00044405	0.00043962	0.00058628	0.00018786	0.00021796	0	0.00067272	0	0	0	0	0.00018811	0.00076057	0.00051688	0	0	0	0.00015987	0	0	0	0.00359473	0.00028827	0.00029095	0.00037644	0.00053362	0.00023507	0.00081616	0	0.00106195	0	0	0.00023883	0.00026582	0.0004787
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Pleosporales_fam_Incertae_sedis;g__Libertasomyces;s__Libertasomyces_myopori	0.00142374	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Sporormiaceae;g__Preussia;s__Preussia_persica	0.00071187	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__Sporormiaceae;g__unidentified;s__unidentified	0.00213561	0	0	0	0	0.00010898	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Dothideomycetes;o__Pleosporales;f__unidentified;g__unidentified;s__unidentified	0.00231358	0.00044405	0.00029308	0	0	0	0	0	0.00011885	0	0	0	0.00018811	0	0.00034459	0.000424	0	0	0	0	0	0	0.00026628	0	0	0.00018822	0.00017787	0	0.00020404	0	0.00070796	0	0	0.00023883	0	0.0004787
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Herpotrichiellaceae;g__Coniosporium;s__Coniosporium_apollinis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017229	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Herpotrichiellaceae;g__Rhinocladiella;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0001452	0	0	0	0	0	0	0	0	0	0	0.00020404	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Chaetothyriales;f__Trichomeriaceae;g__Knufia;s__Knufia_mediterranea	0	0	0	0	0	0	0	0	0	0	0	0	0.00018811	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Eurotiales;f__Aspergillaceae;g__Penicillium;s__Penicillium_brevicompactum	0	0.00022202	0	0	0	0	0	0	0.00011885	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Eurotiomycetes;o__Eurotiales;f__Aspergillaceae;g__Penicillium;s__Penicillium_corylophilum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017229	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Erysiphales;f__Erysiphaceae;g__Podosphaera;s__Podosphaera_sp	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Helotiaceae;g__Articulospora;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00019001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Leotiomycetes;o__Helotiales;f__Sclerotiniaceae;g__Botrytis;s__Botrytis_caroliniana	0.00213561	0.00022202	0	0	0	0.00010898	0	0.00016818	0.0002377	0	0.00033625	0	0	0	0	0	0	0	0	0	0	0	0.00013314	0	0	0.00018822	0	0.00023507	0	0	0	0	0	0	0.00026582	0
k__Fungi;p__Ascomycota;c__Pezizomycetes;o__Pezizales;f__Pyronemataceae;g__Desertella;s__unidentified	0	0.00044405	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Saccharomycetes;o__Saccharomycetales;f__Phaffomycetaceae;g__Wickerhamomyces;s__Wickerhamomyces_anomalus	0.00017797	0	0	0	0	0.00010898	0	0	0	0	0	0	0	0	0	0.000212	0.00011533	0	0	0	0	0	0.00026628	0.00028827	0	0	0	0	0	0	0	0	0.00016912	0	0.00026582	0
k__Fungi;p__Ascomycota;c__Saccharomycetes;o__Saccharomycetales;f__Pichiaceae;g__Pichia;s__unidentified	0.00017797	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00016706	0	0	0	0	0	0	0	0	0	0	0	0	0.00033824	0.00023883	0	0
s__Citeromyces_matritensis	0.00017797	0	0.00014654	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00013314	0	0	0	0	0	0	0	0	0	0	0	0	0
s__Kazachstania_aerobia	0	0	0	0.51416846	0.53240654	0.61355711	0.6636833	0.69172553	0.8212503	0	0	0	0	0	0	0.73139707	0.66970361	0.74067083	0.55827338	0.60457735	0.54094623	0	0	0	0	0	0	0.10014104	0.31952663	0.06353135	0.23185841	0.19903226	0.12633181	0	0	0
s__Kazachstania_servazzii	0	0	0	0	0	0	0	0	0	0.02814919	0.67837929	0.52989257	0	0	0	0	0	0	0	0	0	0.38738909	0.18759153	0.51484578	0	0	0	0	0	0	0	0	0	0.04585622	0.10712387	0.05744375
s__Lachancea_quebecensis	0	0	0	0	0	0	0	0	0.00011885	0	0.00033625	0	0	0	0	0	0.00011533	0	0	0.00016706	0.00019001	0	0	0	0	0	0	0.00047015	0.00061212	0.00027503	0	0.00016129	0	0	0.00053163	0.00023935
s__Saccharomyces_cerevisiae	0.59530166	0.98645648	0.98388042	0.47860074	0.46552696	0.38415432	0.33390706	0.30608813	0.16888519	0.71428571	0.31523201	0.46520318	0.9883371	0.98494068	0.97966919	0.26584694	0.32741322	0.25802236	0.4372502	0.39107918	0.45487365	0.60675854	0.80069232	0.47852407	0.99389002	0.99303595	0.98577019	0.8864598	0.67516833	0.92161716	0.75539823	0.79548387	0.86605784	0.94196322	0.8825093	0.93441838
s__Torulaspora_delbrueckii	0.00177968	0	0	0	0.00018786	0	0	0.00016818	0.00035655	0.00246305	0.00033625	0	0	0.00015211	0	0	0.00023065	0	0	0	0.00057002	0.00037757	0.00013314	0.00028827	0	0	0	0.00047015	0.00020404	0.00082508	0	0.00032258	0.00016912	0.00023883	0	0
s__Hanseniaspora_uvarum	0	0	0	0	0	0	0	0	0	0.00105559	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
s__Hanseniaspora_vineae	0.00142374	0	0	0.00039085	0.00018786	0	0.00068847	0	0.00546708	0	0.00067249	0	0	0	0	0.000212	0.00069196	0	0.00047962	0.00250585	0.00133004	0.00056636	0.00079883	0	0	0	0	0.00305595	0	0.00687569	0.00106195	0.00177419	0.00118383	0.00119417	0.00026582	0.00071805
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Diaporthales;f__Diaporthaceae;g__Diaporthe;s__Diaporthe_foeniculina	0	0	0	0	0	0	0	0	0.00011885	0	0	0	0.00018811	0	0	0	0	0	0	0	0.00019001	0.00037757	0	0	0	0	0	0	0	0	0	0	0	0.00023883	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Diaporthales;f__Valsaceae;g__Cytospora;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00026628	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Cordycipitaceae;g__Beauveria;s__Beauveria_australis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.000424	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Hypocreales_fam_Incertae_sedis;g__Acremonium;s__Acremonium_furcatum	0	0	0.00029308	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Hypocreales;f__Nectriaceae;g__unidentified;s__unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00035398	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Sordariales;f__Sordariaceae;g__Neurospora;s__Neurospora_terricola	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00013314	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Xylariales;f__Amphisphaeriaceae;g__Seimatosporium;s__Seimatosporium_pistaciae	0.01939847	0.00044405	0.00058617	0.00175884	0	0.00010898	0	0	0	0	0.00033625	0.00070061	0.00037622	0.00030423	0.00051688	0	0	0.0001452	0.00031974	0	0	0.00018879	0.00013314	0.00028827	0.00087285	0.00056465	0.00071149	0.00023507	0	0	0	0	0.00016912	0.00023883	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Xylariales;f__Apiosporaceae;g__Arthrinium;s__unidentified	0	0.00022202	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__Sordariomycetes;o__Xylariales;f__Diatrypaceae;g__Cryptovalsa;s__Cryptovalsa_ampelina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00018879	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Ascomycota;c__unidentified;o__unidentified;f__unidentified;g__unidentified;s__unidentified	0.00943228	0.00022202	0.00058617	0.00019543	0	0	0	0.00016818	0	0	0.00050437	0.00046707	0.00037622	0	0.00068918	0	0	0.0001452	0	0	0	0	0	0.00028827	0	0.00018822	0.00017787	0.00023507	0	0	0	0	0	0.00023883	0.00053163	0
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Russulales;f__Peniophoraceae;g__Peniophora;s__Peniophora_nuda	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017229	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Agaricomycetes;o__Sebacinales;f__Sebacinaceae;g__Sebacina;s__unidentified	0.00765261	0	0.00029308	0	0	0	0	0	0	0	0	0	0	0.00015211	0	0	0	0	0	0	0	0	0	0	0	0	0.00017787	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Cystobasidiomycetes;o__Cystobasidiomycetes_ord_Incertae_sedis;f__Buckleyzymaceae;g__Buckleyzyma;s__Buckleyzyma_aurantiaca	0	0.00022202	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00047015	0	0	0	0	0.00016912	0	0	0
k__Fungi;p__Basidiomycota;c__Cystobasidiomycetes;o__Cystobasidiomycetes_ord_Incertae_sedis;f__Buckleyzymaceae;g__Buckleyzyma;s__Buckleyzyma_phyllomatis	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00015211	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Exobasidiomycetes;o__Entylomatales;f__Entylomataceae;g__Entyloma;s__Entyloma_ranunculorum	0.00231358	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Exobasidiomycetes;o__Tilletiales;f__Tilletiaceae;g__Tilletia;s__Tilletia_puccinelliae	0	0	0	0	0	0.00021796	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Cystofilobasidiales;f__Cystofilobasidiaceae;g__Cystofilobasidium;s__Cystofilobasidium_macerans	0.00195764	0	0.00014654	0	0	0	0	0	0	0	0	0	0.00018811	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00032258	0.00033824	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Cystofilobasidiales;f__Mrakiaceae;g__Udeniomyces;s__Udeniomyces_puniceus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00035398	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Filobasidiales;f__Filobasidiaceae;g__Filobasidium;s__Filobasidium_floriforme	0.00231358	0	0	0	0	0	0	0	0.00011885	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017787	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Filobasidiales;f__Filobasidiaceae;g__Filobasidium;s__Filobasidium_magnum	0.00124577	0	0	0	0	0	0	0	0	0	0.00016812	0	0	0.00015211	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00016912	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Filobasidiales;f__Filobasidiaceae;g__Filobasidium;s__Filobasidium_oeirense	0.00035594	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Filobasidiales;f__Filobasidiaceae;g__Filobasidium;s__Filobasidium_sp	0.0005339	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00017229	0	0	0	0	0	0	0	0	0.00028827	0	0	0	0	0	0	0	0	0	0	0	0
s__Naganishia_antarctica	0	0	0	0	0.00018786	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
s__Piskurozyma_capsuligena	0	0	0	0	0.00018786	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
s__Solicoccozyma_aeria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00070796	0	0	0	0	0
s__Holtermanniella_wattica	0.00088984	0	0	0	0	0	0	0	0	0	0.00016812	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00018822	0	0	0	0	0	0	0	0	0.00026582	0
s__Vishniacozyma_carnescens	0.00836448	0.00022202	0.00058617	0	0	0	0	0	0.00011885	0	0	0	0	0.00030423	0.00068918	0	0	0	0.00015987	0	0	0.00056636	0	0	0	0	0.00035575	0	0	0	0	0.00016129	0	0.00047767	0.00026582	0
s__Vishniacozyma_dimennae	0.00177968	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00023883	0	0
s__Vishniacozyma_victoriae	0.01886457	0.00066607	0.00087925	0	0	0	0	0	0	0	0.00033625	0.00023354	0.00037622	0.00060846	0.00206754	0.000424	0.00011533	0.0002904	0.00047962	0.00016706	0.00019001	0.00018879	0.00039941	0.00028827	0.00029095	0.00094109	0.00071149	0	0.00020404	0	0.00106195	0	0.00033824	0.00023883	0.00026582	0.00023935
s__Papiliotrema_terrestris	0	0	0	0.00019543	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__Basidiomycota;c__Tremellomycetes;o__Tremellales;f__unidentified;g__unidentified;s__unidentified	0.00088984	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
k__Fungi;p__unidentified;c__unidentified;o__unidentified;f__unidentified;g__unidentified;s__unidentified	0.00160171	0	0.00014654	0	0	0.00010898	0	0	0	0	0	0	0	0.00015211	0.00017229	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00016912	0	0	0

Supplementary Table 2 (OTU raw data) is available at Figshare:

https://adelaide.figshare.com/articles/dataset/Influence_of_Kazachstania_spp_on_the_chemical_and_sensory_profile_of_red_wines/19297562

Supplementary Table 3a. Composition of Merlot wines from alcoholic (AF) and malolactic fermentation (MLF) by yeast isolates. Organic acids (malic, lactic, succinic and acetic) were measured by enzymatic assays, glycerol and ethanol were measured by HPLC in terminal wine samples. EC1118 = *S. cerevisiae*, PF_9_W18 = *K. aerobia*, PF_8_W29 = *K. aerobia*, PF_9_W20 = *K. servazzii*.

Treatment	Yeast strain/isolate	Malic acid (g/L)	Lactic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (g/L)
AF	EC1118	0.81 ± 0.03[a]	1.02 ± 0.15[a]	0.45 ± 0.05	0.04 ± 0.008	9.94 ± 0.21[b]	114.1 ± 4.38
	PF_9_W18	0.79 ± 0.08[a]	0.88± 0.17[ab]	0.62 ± 0.21	0.04 ± 0.002	13.68 ± 0.4[a]	106.1 ± 5.8
	PF_8_W29	0.95 ± 0.26[a]	0.52 ± 0.24[bc]	0.38 ± 0.02	0.05 ± 0.007	12.45 ± 0.14[ab]	110.2 ± 5.42
	PF_9_W20	0.38 ± 0.16[b]	0.08 ± 0.07[c]	0.53 ± 0.08	0.04 ± 0.002	13.83 ± 0.38[a]	108.9 ± 9.2
				ns	ns		ns
MLF	EC1118	0	1.92 ± 0.45	0.87 ± 0.49	0.07 ± 0.009	9.61 ± 0.1[b]	115.4 ± 2.58[a]
	PF_9_W18	0	3.58 ± 0.64	0.86 ± 0.33	0.06 ± 0.002	13.1 ± 0.18[a]	108.7 ± 0.51[b]
	PF_8_W29	0	2.36 ± 1.3	0.54 ± 0.06	0.09 ± 0.03	12.25 ± 0.29[a]	111.2 ± 1.74[ab]
	PF_9_W20	0	2.02 ± 0.26	0.54 ± 0.12	0.06 ± 0.01	13.03 ± 0.39[a]	110.7 ± 1.36[ab]
			ns	ns	ns		

All values are expressed as means of three replicates ± standard deviation. [a] Values within the same column with different letters are significantly different at $p < 0.01$. "ns" indicates not significant ($p > 0.01$). Tukey's multiple comparison test.
Concentration of alcohol/ethanol by volume (% v/v) = [g/L]/7.8924 (density of ethanol).

Supplementary Table 3b. Composition of Shiraz wines by yeast isolates. Organic acids (malic, lactic, succinic and acetic) were measured by enzymatic assays, glycerol was measured by HPLC and ethanol measured by HPLC and alcolyser in terminal wine samples. EC1118 = *S. cerevisiae*, PF_9_W18 = *K. aerobia*, PF_8_W29 = *K. aerobia*, PF_9_W20 = *K. servazzii*.

Yeast strain/isolate	Malic acid (g/L)	Lactic acid (g/L)	Succinic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (g/L)
EC1118	0.67 ± 0.38	0.53 ± 0.5	1 ± 0.12	0.08 ± 0.02	13.35 ± 0.14[c]	125.9 ± 4.92
PF_9_W18	0.53 ± 0.04	0.52 ± 0.4	0.86 ± 0.12	0.06 ± 0.02	15.93 ± 0.07[a]	127 ± 0.67
PF_8_W29	0.71 ± 0.13	0.31 ± 0.11	0.92 ± 0.09	0.08 ± 0.02	14.87 ± 0.12[b]	128.8 ± 1.33
PF_9_W20	0.92 ± 0.25	0.68 ± 0.25	0.89 ± 0.15	0.07 ± 0.004	15.25 ± 0.18[ab]	126.6 ± 2.51
	ns	ns	ns	ns		ns

All values are expressed as means of three replicates ± standard deviation. [a] Values within the same column with different letters are significantly different at $p < 0.01$. "ns" indicates not significant ($p > 0.01$). Tukey's multiple comparison test.
Concentration of alcohol/ethanol by volume (% v/v) = [g/L]/7.8924 (density of ethanol).

Supplementary Table 4a. Mean concentrations (µg/L) of volatile compounds in sequential alcoholic fermentation by yeast isolates and *S. cerevisiae* (EC1118) in Merlot. Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated.

	Yeast isolate[a]			
Compound	**C (EC1118)**	**KAW18**	**KAW29**	**KSW20**
Esters				
Ethyl propanoate	270.93[b]	491.33[a]	397.63[ab]	554.37[a]
Ethyl isobutyrate	1.45[b]	2.54[a]	3.19[a]	2.7[a]
Ethyl 2-methylbutanoate	3.18[b]	5.21[a]	5.23[a]	5.14[a]
Ethyl isovalerate	0.33[b]	0.54[a]	0.51[a]	0.45[a]
Ethyl pentanoate[1]	1.28[b]	1.75[a]	1.74[a]	1.77[a]
Isoamyl propanoate	8.25[b]	19.91[a]	14.99[a]	17.77[a]
Propyl hexanoate[2]	38.99[a]	33.78[a]	20.84[b]	13.73[b]
Methyl octanoate	16.22[a]	13.85[ab]	10.13[ab]	7.71[b]
Ethyl 3-hydroxybutanoate	0.49[a]	0.4[ab]	0.34[b]	0.23[c]
Propyl octanoate	30.48[a]	26.19[ab]	18.02[bc]	12.36[c]
Diethyl succinate[3]	514[a]	364[b]	372[b]	238[b]
Ethyl 9-decenoate[4]	456.12[b]	1350[a]	579.93[b]	484.36[b]
Ethyl phenylacetate	0.66[c]	0.94[b]	1.2[a]	1.15[ab]
Ethyl 4-hydroxybutanoate[1]	24.52[a]	19.86[ab]	15.62[b]	19.73[ab]
Acetates				
Ethyl acetate	13197[b]	21597[a]	20602[a]	24883[a]
Propyl acetate[5]	137.01[b]	343.02[a]	299.88[a]	273.58[a]
Isobutyl acetate	8.92[c]	40.85[b]	68.64[a]	48.36[ab]
Butyl acetate	3.54[c]	10.91[b]	9.88[b]	17.55[a]
Hexyl acetate	3.77[b]	26.02[a]	30.07[a]	30.33[a]

Citronellyl acetate	2.32[b]	6.63[a]	5.26[a]	5.12[a]
Benzyl acetate	4.26[c]	18.64[b]	15.64[b]	24.6[a]
Geranyl acetate[6]	0.17[b]	1.16[a]	1.04[a]	1.17[a]
Phenylethyl acetate	11.96[c]	89.12[b]	86.57[b]	130.78[a]
Acids				
Hexanoic acid	2766[a]	2564[a]	1939[b]	1238[c]
Alcohols				
2-Methyl-1-propanol	38220[c]	74074 [ab]	87193[a]	42559[bc]
(S)-(+)-3-Methyl-1-pentanol	1501[b]	1683[b]	1599[b]	2440[a]
3-Ethoxy-1-propanol	4.98[c]	15.16[b]	16[b]	25.29[a]
(Z)-3-Hexen-1-ol	12.03[b]	14.80[ab]	15.12[ab]	19.7[a]
Heptanol	49.48[a]	35.92[b]	34[b]	31[b]
1-Nonanol	26.98[a]	17.6[b]	15.55[b]	15.43[b]
Isoamyl alcohol	158581[c]	180190[b]	186439[b]	208002[a]
Ketones				
2-Propanone[7]	0.02[b]	0.03[a]	0.03[a]	0.025[ab]
β-Damascenone	0.41[b]	0.64[b]	0.87[a]	0.92[a]
Terpenes				
α-Terpinolene	0.31[b]	0.60[a]	0.66[a]	0.55[a]
Linalool	0.96[b]	1.57[a]	1.6[a]	1.73[a]

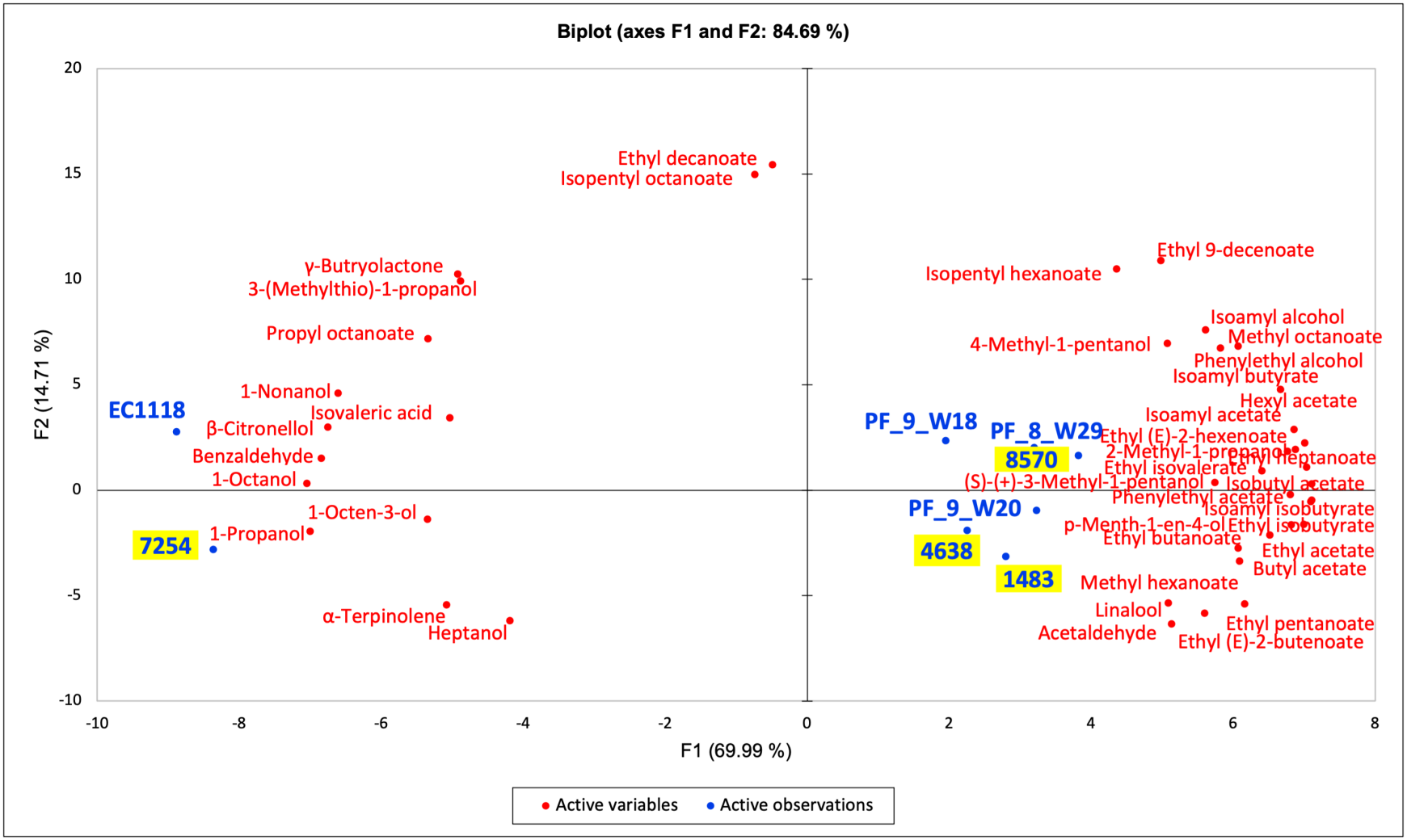
[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Ethyl butanoate, [2]Propyl octanoate, [3]Ethyl lactate, [4]Ethyl hexanoate, [5]Ethyl acetate, [6]Phenyl acetate, [7]2-Heptanone.

Supplementary Table 4b. Mean concentrations (µg/L) of volatile compounds in sequential (post-alcoholic) malolactic fermentation by yeast isolates and *S. cerevisiae* (EC1118) in Merlot. Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated.

	Yeast isolate[a]			
Compound	**C (EC1118)**	**KAW18**	**KAW29**	**KSW20**
Esters				
Ethyl propanoate	165.36^{bc}	207.18^{ab}	136.02^{c}	219.81^{a}
Ethyl (E)-2-butenoate[1]	1.32^{a}	0.72^{b}	0.65^{b}	0.75^{b}
Methyl hexanoate[2]	2.79^{a}	2.06^{ab}	1.56^{b}	1.77^{b}
Propyl hexanoate[3]	6.27^{ab}	6.74^{a}	3.86^{c}	4.54^{bc}
Methyl octanoate	11.14^{a}	7.15^{ab}	4.92^{b}	5.69^{b}
Isopentyl hexanoate	0.57^{a}	0.32^{b}	0.27^{b}	0.28^{b}
Ethyl 3-hydroxybutanoate	0.50^{a}	0.35^{b}	0.34^{b}	0.27^{b}
Ethyl decanoate	162^{a}	70.43^{b}	40.29^{b}	56.69^{b}
Isopentyl octanoate	3.53^{a}	1.77^{b}	1.03^{b}	1.45^{b}
Diethyl succinate[4]	369^{a}	336^{ab}	332^{ab}	279^{b}
Ethyl 4-hydroxybutanoate[1]	24.71^{a}	15.53^{b}	17.05^{b}	16.76^{b}
Ethyl dodecanoate	12.78^{a}	5.1^{b}	2.93^{b}	3.96^{b}
Ethyl hexanoate	81.06^{a}	56.13^{b}	48.62^{b}	59.46^{b}
Ethyl octanoate	54.05^{a}	27.76^{b}	25.86^{b}	28.54^{b}
Acetates				
Propyl acetate[5]	49.11^{b}	127^{a}	63.09^{b}	87.54^{ab}
Isobutyl acetate	2.73^{c}	11.77^{a}	7.2^{b}	10.19^{a}
Butyl acetate	1.04^{c}	2.492^{a}	1.61^{b}	2.90^{a}
Hexyl acetate	0.45^{b}	2.61^{a}	2.21^{a}	3.28^{a}
Benzyl acetate	3.81^{b}	11.53^{a}	11.1^{ab}	17.27^{a}

Geranyl acetate[6]	0.08[b]	0.24[a]	0.25[a]	0.39[a]
Phenylethyl acetate	13.08[b]	58.24[a]	51.43[ab]	77.92[a]
Alcohols				
1-Propanol[7]	468[b]	777[a]	565[b]	551[b]
2-Methyl-1-propanol	24322[c]	39196[a]	38992[a]	33808[b]
1-Butanol	1261[b]	1266[b]	1234[b]	1500[a]
(S)-(+)-3-Methyl-1-pentanol	1428[b]	1277[b]	1369[b]	1756[a]
3-Ethoxy-1-propanol	3.19[c]	13.69[b]	15.38[ab]	17.66[a]
(Z)-3-Hexen-1-ol	10.76[b]	15.01[a]	15.23[a]	15.44[a]
1-Octen-3-ol	1.34[a]	0.9[b]	0.97[b]	0.95[b]
Acids				
Hexanoic acid	2626[a]	2106[ab]	1785[b]	1541[b]
Aldehydes				
Benzaldehyde	7.38[a]	4.15[b]	4.71[b]	4.79[b]
Ketones				
β-Damascenone	0.48[b]	0.66[a]	0.75[a]	0.79[a]
Terpenes				
α-Terpinolene	0.54[b]	0.77[a]	0.75[a]	0.75[a]
Linalool	0.99[b]	1.32[a]	1.47[a]	1.46[a]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Ethyl butanoate, [2]Ethyl hexanoate, [3]Propyl octanoate, [4]Ethyl lactate, [5]Ethyl acetate, [6]Phenyl acetate, [7]1-Hexanol.



Supplementary Figure 2. Principal component analysis of volatile profile in Shiraz wines. Yeast isolates (treatments) are represented in blue font, and blended wines (numerals) are as blue font highlighted in yellow, and volatile compounds in red. 7254 = *S. cerevisiae* (EC1118), 4638 = *K. aerobia* (PF_9_W18), 8570 = *K. aerobia* (PF_8_W29), 1483 = *K. servazzii* (PF_9_W20).

Supplementary Table 5. Mean concentrations (µg/L) of volatile compounds in sequential alcoholic fermentation by yeast isolates and *S. cerevisiae* (EC1118) and their blended wines. Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated. 7254 = *S. cerevisiae* (EC1118), 4638 = *K. aerobia* (PF_9_W18), 8570 = *K. aerobia* (PF_8_W29), 1483 = *K. servazzii* (PF_9_W20).

	Yeast isolate/Treatment							
Compound	**EC1118**	**PF_9_W18**	**PF_8_W29**	**PF_9_W20**	**7254**	**4638**	**8570**	**1483**
Esters								
Ethyl butanoate	84.83^{c}	123.87^{abc}	134.64^{ab}	142.68^{a}	96.24^{bc}	122.42^{abc}	145.32^{a}	140.26^{a}
Ethyl pentanoate[1]	2.81^{b}	3.69^{ab}	4.53^{ab}	5.55^{a}	2.85^{b}	4.90^{ab}	4.50^{ab}	5.32^{a}
Ethyl (E)-2-butenoate[2]	12.12^{c}	15.63^{abc}	20.31^{ab}	21.87^{a}	13.24^{bc}	19.74^{ab}	15.51^{abc}	20.88^{a}
Methyl hexanoate	2.66^{c}	3.59^{abc}	5.21^{a}	4.84^{abc}	2.83^{bc}	5.11^{ab}	4.06^{abc}	4.62^{abc}
Ethyl heptanoate	7.90^{ab}	9.53^{ab}	10.89^{ab}	11.43^{a}	6.03^{b}	10.24^{ab}	9.72^{ab}	10.62^{ab}
Ethyl (E)-2-hexenoate	62.79^{b}	126.61^{a}	145.77^{a}	114.30^{a}	61.80^{b}	130.48^{a}	135.41^{a}	124.39^{a}
Methyl octanoate	6.51^{ab}	7.97^{ab}	9.38^{a}	7.91^{ab}	5.34^{b}	7.66^{ab}	8.35^{ab}	7.43^{ab}
Isopentyl hexanoate	1.36^{ab}	1.38^{ab}	1.61^{a}	1.41^{ab}	1.02^{b}	1.35^{ab}	1.44^{ab}	1.25^{ab}
Propyl octanoate	28.98^{a}	18.61^{b}	21.24^{ab}	20.05^{b}	22.12^{ab}	17.46^{b}	21.36^{ab}	19.12^{b}
Ethyl decanoate	361.03^{a}	326.80^{ab}	342.64^{ab}	270.74^{abcd}	228.02^{cd}	241.35^{bcd}	320.41^{abcd}	212.57^{d}
Isopentyl octanoate[3]	0.65^{a}	0.58^{abc}	0.64^{a}	0.48^{abcd}	0.43^{bcd}	0.39^{cd}	0.61^{ab}	0.35^{d}
Ethyl 9-decenoate	252.31^{cd}	369.94^{ab}	395.55^{a}	297.01^{abc}	161.41^{d}	283.01^{bc}	369.01^{ab}	239.49^{cd}
Acetates								
Ethyl acetate	27761^{c}	49551^{ab}	60020^{a}	61514^{a}	31058^{bc}	56291^{a}	57047^{a}	57235^{a}
Ethyl isobutyrate	117.88^{c}	227.20^{ab}	265.72^{a}	269.93^{a}	124.86^{bc}	246.52^{a}	261.93^{a}	252.09^{a}
Isobutyl acetate	24.24^{b}	67.80^{a}	80.76^{a}	75.03^{a}	26.45^{b}	74.80^{a}	77.72^{a}	70.89^{a}
Ethyl isovalerate	9.16^{b}	13.84^{ab}	15.38^{a}	15.15^{a}	9.59^{b}	13.74^{ab}	15.62^{a}	14.11^{ab}
Butyl acetate	8.54^{c}	13.33^{abc}	13.13^{abc}	17.99^{a}	9.31^{c}	12.12^{bc}	15.44^{ab}	16.13^{ab}

Isoamyl acetate	306.60[b]	770.69[a]	870.57[a]	744.93[a]	307.27[b]	768.83[a]	849.88[a]	709.72[a]
Isoamyl isobutyrate[4]	18.36[bc]	29.82[abc]	39.18[ab]	41.41[a]	13.25[c]	36.83[ab]	36.27[ab]	36.88[ab]
Isoamyl butyrate[5]	2.21[bc]	4.06[a]	4.13[a]	3.65[ab]	2.12[c]	3.45[abc]	4.41[a]	3.46[abc]
Hexyl acetate	14.48[b]	29.69[a]	33.95[a]	27.54[a]	13.43[b]	30.23[a]	31.31[a]	26.86[a]
Phenylethyl acetate	78.65[b]	270.07[a]	291.66[a]	311.26[a]	79.30[b]	274.51[a]	327.20[a]	316.69[a]
Alcohols								
1-Propanol	1795[a]	1235[b]	1196[b]	1236[b]	1952[a]	1253[b]	1251[b]	1265[b]
2-Methyl-1-propanol	41125[c]	65211[ab]	67904[ab]	62547[ab]	40608[c]	69765[a]	65669[ab]	58381[b]
Isoamyl alcohol	154691[ab]	179716[a]	176577[ab]	164544[ab]	150299[b]	172093[ab]	177463[ab]	160002[ab]
4-Methyl-1-pentanol	947[d]	1881[a]	1625[b]	1211[c]	990.8[d]	1543[b]	1915[a]	1179[c]
(S)-(+)-3-Methyl-1-pentanol	1220[b]	1708[a]	1395[b]	1651[a]	1231[b]	1396[b]	1686[a]	1669[a]
1-Octen-3-ol	0.81[ab]	0.75[b]	0.80[ab]	0.76[b]	0.86[a]	0.79[ab]	0.75[b]	0.73[b]
Heptanol	49.02[a]	43.01[c]	48.77[a]	47.59[ab]	50.30[a]	47.79[ab]	44.22[bc]	47.21[abc]
1-Octanol	63.04[a]	39.22[b]	42.70[b]	41.16[b]	62.52[a]	40.62[b]	39.17[b]	39.11[b]
p-Menth-1-en-4-ol[6]	73.58[b]	96.75[ab]	94.32[ab]	100.34[ab]	79.71[ab]	94.86[ab]	110.60[a]	106.60[ab]
1-Nonanol	14.05[a]	11.15[ab]	11.12[ab]	11.12[ab]	12.88[ab]	10.07[b]	10.41[b]	10.15[b]
3-(Methylthio)-1-propanol	4632[a]	3590[ab]	3012[ab]	2838[b]	3397[ab]	2262[b]	3365[ab]	2763[b]
Phenylethyl alcohol	103727[ab]	116381[ab]	114672[ab]	116911[ab]	94016[b]	106852[ab]	126576[a]	111763[ab]
Acids								
Isovaleric acid	7983[a]	3226[b]	3950[ab]	4166[ab]	4242[ab]	4462[ab]	3338[b]	4213[ab]
Aldehydes								
Acetaldehyde	10724[b]	14341[ab]	13855[ab]	14756[ab]	13800[ab]	16160[ab]	18373[a]	17603[a]
Benzaldehyde	39.91[a]	26.47[b]	23.21[b]	22.73[b]	42.12[a]	22.27[b]	27.28[b]	22.10[b]

Terpenes								
α-Terpinolene	109.43[b]	81[e]	94.13[cd]	86.10[de]	128.06[a]	99.91[bc]	106.75[bc]	101.09[bc]
Linalool	41.56[b]	48.24[ab]	48.48[ab]	49.04[ab]	47.43[ab]	46.83[ab]	53.09[a]	53.38[a]
β-Citronellol	4[a]	2.53[b]	2.41[b]	2.04[b]	4.07[a]	2.06[b]	2.66[b]	1.99[b]
Lactones								
γ-Butryolactone	3912[a]	2576[ab]	2058[b]	1836[b]	2272[ab]	1443[b]	2261[ab]	1722[b]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Ethyl butanoate, [2]Ethyl (E)-2-hexenoate, [3]Isopentyl hexanoate, [4]Ethyl isobutyrate, [5]Isoamyl acetate, [6]α-terpineol

Supplementary Table 6. Colour measurements of Shiraz wines (replicates and blends) by modified Somer's assay (total anthocyanins, colour density, hue, total phenolics and SO_2 resistant pigments) and CIELAB (chroma and hue angle). Measurements were taken one year post-bottling. Wine colour parameters are expressed in absorbance units unless stated. 7254 = *S. cerevisiae* (EC1118), 4638 = *K. aerobia* (PF_9_W18), 8570 = *K. aerobia* (PF_8_W29), 1483 = *K. servazzii* (PF_9_W20).

Yeast isolate/blend	Total anthocyanins (mg/L)	Colour density (SO_2 corrected)	Hue	Total phenolics	SO_2 resistant pigments	Chroma (C*)	Hue angle (h°)
EC1118	269.8 ± 11.17	10.54 ± 0.67	0.75 ± 0.03	34.39 ± 2.15	2.62 ± 0.35	42.02 ± 2.33	14.49 ± 0.96
PF_9_W18	268.8 ± 19.23	10.58 ± 0.18	0.72 ± 0.001	32.09 ± 2.23	2.73 ± 0.1	43.61 ± 0.80	12.1 ± 0.17
PF_8_W29	277.3 ± 19.23	11.06 ± 1.63	0.71 ± 0.005	32.44 ± 2.16	2.93 ± 0.09	45.42 ± 0.45	11.76 ± 0.30
PF_9_W20	269.6 ± 13.75	10.08 ± 0.67	0.72 ± 0.02	31.92 ± 1.6	2.75 ± 0.3	43.18 ± 2.01	12.07 ± 0.32
7254	291.2 ± 8.89	10.91 ± 0.09	0.72 ± 0.005	35.63 ± 1.88	2.98 ± 0.04	44.28 ± 0.47	13.54 ± 0.62
4638	294.7 ± 2.40	11.11 ± 0.11	0.71 ± 0.006	34.99 ± 0.44	3.02 ± 0.1	45.19 ± 0.44	12.37 ± 0.40
8570	278.1 ± 4.17	10.35 ± 0.22	0.72 ± 0.006	32.91 ± 0.76	2.85 ± 0.09	43.85 ± 0.11	12.26 ± 0.17
1483	265.8 ± 8.53	9.75 ± 0.21	0.73 ± 0.006	32.68 ± 0.71	2.79 ± 0.06	42.62 ± 0.45	13.06 ± 0.46
	ns	ns	ns	ns	ns	ns	ns

All values are expressed as means of three replicates ± standard deviation. “ns” indicates not significant ($p > 0.01$). Tukey’s multiple comparison test.

CHAPTER 4

"Modification of terpenes in white wines by *Hanseniaspora uvarum*"

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Contribution to the Paper	Conceptualisation of the study/experimental design Experimental work/data collection Data collation, interpretation and analysis Manuscript preparation and editing		
Overall percentage (%)	75		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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

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

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Modification of terpenes in white wines by *Hanseniaspora uvarum*

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Abstract

Terpenes are grape-derived, and are predominantly glycosidically linked and odourless precursors. They can be enzymatically liberated by yeast during fermentation whereby they are responsible for characteristic floral and fragrant aromas. This study reports the impact of *Hanseniaspora uvarum* isolates on white wine terpene content. Five *H. uvarum* isolates were inoculated into Viognier juice as monocultures, or in sequential culture with *S. cerevisiae* (EC1118). Fermentation efficiency and their contribution to wine aroma was evaluated against the *S. cerevisiae* monoculture. Contrary to the literature, the wines from sequential treatments had reduced terpene content. To better understand this, the sequential fermentations were conducted in Chemically Defined Grape Juice Medium (CDGJM) spiked with linalool, and in Muscat and Riesling and repeated in Viognier. Whilst the experimental methodology was not changed, terpene concentrations increased in sequential treatments all three aromatic wines, highlighting the need for robust evaluation of results and the inherent variability of fermentations.

Highlights

- Five *Hanseniaspora uvarum* isolates were evaluated for fermentation performance.
- *H. uvarum*'s effect on wine aroma composition is dependent upon grape variety.
- Sequential cultures of *H. uvarum* and *S. cerevisiae* reduced terpenes in Viognier.
- No significant changes in linalool concentrations were observed in 'spiked' CDGJM.

Keywords: *Hanseniaspora uvarum*, aroma, flavour, volatiles, terpenes

1. Introduction

Hanseniaspora uvarum (*Kloeckera apiculata*) is one of the predominant non-*Saccharomyces* yeasts found on grapes and in grape musts worldwide (Albertin et al., 2016). It is frequently isolated from industrial fermented beverages such as beer (Spitaels et al., 2014), cider (Lachance 1995) and tequila (Bilbao et al., 1997), and plays a major role during food fermentation processes e.g., coffee (Masoud et al., 2004) and cocoa (Batista et al., 2015). *H. uvarum* is one of the main apiculate species found during spontaneous grape juice fermentations (Ciani et al., 2010) and is often considered as a spoilage species. Certain *H. uvarum* strains produce high levels of volatile acidity and esters (e.g., ethyl acetate; Coulon et al., 2019), which can negatively impact wine sensory profile when levels are greater than the odour threshold (Moreira et al., 2011).

Whilst non-*Saccharomyces* are traditionally associated with wine spoilage (e.g., *Candida* spp., *Kluyveromyces* spp., *Metschnikowia* spp., *Torulaspora* spp.), their role as positive contributors to fermentation has been re-examined because of their specific flavour-active characteristics which can enhance sensory complexity in wines (Ciani et al., 2010; Ciani and Comitini 2011; Valera et al., 2021; Windholtz et al., 2021). Several *H. uvarum* strains, for example, are reported to exhibit β-glucosidase activity, with up to 6.6-fold higher activity than that of indigenous *Saccharomyces cerevisiae* strains (Martin et al., 2018). This β-glucosidase activity plays an important role in releasing varietal aroma compounds, such as C_{13}-norisoprenoids, non-flavanoid phenols and terpenes from non-volatile precursors (Liu et al., 2017). The effects of mixed-starter cultures of selected *H. uvarum* strains with *S. cerevisiae* (both co- and sequential inoculation) have been shown to increase the quantity of desirable compounds, such as esters, higher alcohols and terpenes, thus enhancing the fruity and floral characters of wine bouquet (Maicas et al., 2015; Moireira et al., 2005; Pietrafesa et al., 2020; Tristezza et al., 2016).

Terpenes are important contributors to grape and wine aroma, and are characterised by floral and fruity aromas. Terpene-rich varieties include Muscat (Muscat of Alexandria, Muscat de Frontignan and Muscat Hamburg), Gewürtztraminer, Riesling, Torrontés and Viognier (Mateo and Jiménez 2000; Marais 1983; Song et al., 2018). The concentration in grapes and wine is dependent upon factors, such as cultivar, region and winemaking techniques (Baron et al., 2017). Terpenes exist in grapes and wines predominantly as glycosidically bound precursors or intermediates, but when unbound as a volatile aglycone, they are responsible for the aroma/flavour characteristic of the finished wine (Black et al., 2015). Of particular importance are monoterpenes (the focus of this study), having two isoprene units, sesquiterpenes (having three) and the terpenoid class, C_{13}-noisoprenoids (Tufariello et al., 2021). Monoterpenes are secondary metabolites of wine grape varieties of *Vitis vinifera*, and are widely studied and used analytically to determine and characterise varietal typicality (Carrau et al., 2008).

De novo terpene biosynthesis occurs in several non-*Saccharomyces* species (*Candida stellata, Kloeckera apiculata, Kluyveromyces lactis, Metschnikowia pulcherrima, Toluraspora delbrueckii*)as well as *Saccharomyces cerevisiae* (Carrau et al., 2005). *K. lactis* has been reported to produce up to 50 μg/L of citronellol and linalool, and geraniol in trace amounts (Drawert and Barton, 1978). The comparatively small concentrations produced during fermentation can be increased through high nitrogen content and micro-aeration (Carrau et al., 2005). *Hanseniaspora*/*Kloeckera* spp. have been explored for their hydrolytic capability in cleaving terpenes from sugars in Muscat wines (Lopez et al., 2015). Thirty-one strains, including *H. guilliermondii, H. osmophila, H. vineae* and *H. uvarum* exhibited β-glucosidase and β-xylosidase activities in Muscat; with *H. uvarum* producing notable amounts of β-glucosidase (Lopez et al., 2015). *H. vineae* strains grown as co-cultured fermentations were

also demonstrated to produce monoterpenes and sesquiterpenes at levels (5–60 μg/L) which exceed the threshold values, and higher concentrations when compared to *S. cerevisiae* alone.

Liberation of terpenes by *S. cerevisiae* has been well documented (Carrau et al., 2004; Zhu et al., 2021), where strategies to synthesise terpenes have been explored through metabolic engineering of *S. cerevisiae* strains (Takahashi et al., 2007; Zhang et al., 2017) for commercial applications. As *S. cerevisiae* yeasts are capable of modifying/metabolising terpenes in wine, the concentrations are strain-dependent, for example that of citronellol from nerol and geraniol (Dugelay et al., 1992). More complex pathways have been proposed to occur in *S. cerevisiae*, including the transformation of geraniol to (i) geranyl acetate and citronellol, and nerol to neryl acetate, (ii) nerol to geraniol, linalool and α-terpineol (cyclised), (iii) linalool to α-terpineol and (iv) citronellol to citronellyl acetate (Pardo et al., 2015).

The ability of *H. uvarum* to enhance varietal aroma in wines by increasing terpenes is well documented (Hu et al., 2018; Tristezza et al., 2016). However, to the best of our knowledge, there are no reports describing the effect of this species in relation to reducing terpene concentrations, thereby negatively impact aroma. This study focuses on the influence of *H. uvarum* isolates on monoterpenes, particularly linalool, one of the most abudant terpene compounds found in in aromatic grapes and wine. Fermentations were conducted with five *H. uvarum* isolates both in mono- and sequential cultures with *S. cerevisiae* in sterile Viognier juice. One isolate was chosen for further evaluation in Chemically Defined Grape Juice Medium spiked with linalool, as well as three aromatic white varietals – Viognier, Muscat and Riesling. The aromatic profiles of the finished wines were measured using GC-MS to investigate the effect of *H. uvarum* on terpene concentrations.

2. Materials and methods

2.1 Yeast isolation and culture from grape must

Uninoculated Pecorino (12.9 Bé) and Malvesia must (11.1 Bé) were sourced from Heathcote, Victoria (Chalmer's vineyard; 2018). Must samples were serially diluted using 10-fold volumes of sterile MilliQ (ultra-pure) water and cultured on Wallerstein laboratory (WL) agar using the spread plate technique. After incubation, individual colonies with different morphologies (colour and topography) were picked and streaked on fresh WL agar to obtain single colonies. Cell morphology was microscopically determined on Nikon Eclipse 50i at 40X magnification (bright-field). Pure cultures of yeast isolates were cultured in liquid YEPD medium overnight at 28 °C for cryopreservation (in 40% glycerol) at -80 °C, and species identified by rDNA sequencing (Hutzler et al., 2018).

2.2 Yeast identification by sequencing of the D1/D2 region of 26S rDNA gene

Genomic DNA was isolated from yeast according to the method of Adams et al. (1998). The primer pair NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) was used to amplify the 26S rDNA gene of the D1/D2 region (Hutzler et al., 2018). PCR amplifications were performed in 25 μL reactions using MangoTaq™ DNA polymerase (Bioline, USA) according to the manufacturer's instructions, with 50 pmol of each primer and ~200 ng of genomic DNA used as template. PCR was initiated at 95 °C for 5 min, followed by 35 cycles of 95 °C for 2 min, 60 °C for 2 min, and 72 °C for 2 min before a final extension of 72 °C for 10 min.

Amplicons were separated on 1.5 % agarose gels stained with GelRed® (Biotium, California, USA) and removed as gel slices under UV light before being purified (Lin et al., 2020). DNA samples were submitted to the Australian Genome Research Facility (Adelaide, Australia) for sequencing. The resulting 26S rDNA were analysed using the Basic Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) in the Genbank nucleotide

collection. The *Hanseniaspora uvarum* consensus sequences were deposited into the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/), under accession numbers listed in **Table 1**. *Saccharomyces cerevisiae* isolates (cream-coloured colonies) were also identified by D1/D2 26S rDNA sequencing, but not used in this study.

Table 1. List of *Hanseniaspora uvarum* isolates used in this study

Isolate	Accession number*	Source
H11_G1_1	MT712212	Grape (Malvesia) must
H11_G1_2	MT712213	Grape (Malvesia) must
H11_G1_3	MT712214	Grape (Malvesia) must
A11_G1_1	MT712215	Grape (Pecorino) must
A11_G1_3	MT712216	Grape (Pecorino) must

*GenBank (https://www.ncbi.nlm.nih.gov/genbank/)

2.3 Fermentation of isolates in filter-sterile Viognier juice

Monoculture and sequential fermentations of the *H. uvarum* isolates were carried out in sterile Viognier juice (2017, Waite vineyard, South Australia), which was frozen (20 L) at -20 °C. Before use the juice was thawed and mixed prior to sterile filtration. Specifically, solids were removed from a 5 L aliquot using a 0.45 µm 'in-line' filter (Waterra FHT-45; https://www.airmet.com.au/) and the clarified juice sterilised using a nitrocellulose 0.22 μm membrane (https://www.vintessential.com.au/). The remainder was refrozen at -20 °C. The sugar content (23 °Brix) was measured with a refractometer. The initial pH (3.74) was adjusted to 3.3 with tartaric acid. Initial yeast assimilable nitrogen (YAN; 251 mg N/L) was determined by spectrophotometer (TECAN, Switzerland) using the Primary Amino Nitrogen (K-PANOPA) and Ammonia (K-AMIAR) enzymatic kits (Megazyme, USA). Nitrogen was added as diammonium phosphate (DAP; 100 mg/L) to increase the YAN content to 351 mg/L.

Fermentations (100 mL) were conducted in triplicate in 250 mL Erlenmeyer flasks fitted with sampling ports and airlocks.

The *H. uvarum* yeasts were grown in YEPD medium before transferring into CDGJM starter medium as described in Lin et al. (2020). The starter cultures were inoculated at 5 x 10^6 cells/mL into grape juice at the start of fermentation. The commercial wine yeast strain *Saccharomyces cerevisiae* EC1118 (Lallemand, Australia) was rehydrated as per manufacturer's instructions, and (sequentially) inoculated at 5 x 10^6 cells/mL after 72 h. Cell numbers were counted using a haemocytometer. As a control, EC1118 was fermented on its own as a monoculture (C). Fermentations were conducted at 22 °C, and were monitored daily (residual sugars) using the D-Glucose/D-Fructose enzymatic assay kit (Megazyme, USA). At the end of alcoholic fermentation, samples were collected in glass vials sparged with nitrogen gas and stored at 4 °C for one week prior to volatile analysis.

2.4 Spiking of linalool in Chemically Defined Grape Juice Medium and in Muscat juice

Linalool, a major monoterpenol in aromatic white varietals (e.g., Gewürztraminer, Muscat, Riesling, Sauvignon Blanc and Viognier) was used in a 'spiking' experiment to determine the effect of *H. uvarum* on its metabolism. Chemically Defined Grape Juice Medium (Henschke and Jiranek 1993), was used as a base medium as it does not contain the compound. 500 µg/L linalool was added to CDGJM (200 g/L sugar, 450 mg N/L) prior to alcoholic fermentation. The amount represented an average concentration based on published data (Marais 1983; Rusjan et al., 2009).

The H11_G1_1 isolate was chosen for this experiment, based on its activity in Viognier, to investigate the changes in terpenes in CDGJM as well as in unmodified filter-sterilised Muscat juice (frozen and thawed). Preparation of yeast cultures, and the inoculation strategy (monoculture and sequential (with EC1118), cell density and ratio) are described in Lin et al.

(2020) and above (Section 2.3). Alcoholic fermentations (100 mL) were conducted in triplicate, in flasks fitted with sampling ports and airlocks. Fermentation conditions and monitoring progress were the same as described in Section 2.3. The pH was adjusted from 3.43 to 3.3 with tartaric acid. The initial YAN of the Muscat juice (167 mg/L) was supplemented with 187 mg/L of DAP to give a final concentration of 354 mg/L. A schematic diagram of the experimental setup is presented in **Fig. 1**.

For comparison, unmodified CDGJM (- linalool) was also used for fermentation, and uninoculated CDGJM (+/- linalool) and Muscat served as negative controls (**Fig. 1**) to monitor the linalool levels over the experimental duration. At < 2 g/L residual sugar, terminal samples were stored at 4 °C prior to volatile analysis by GC-MS.

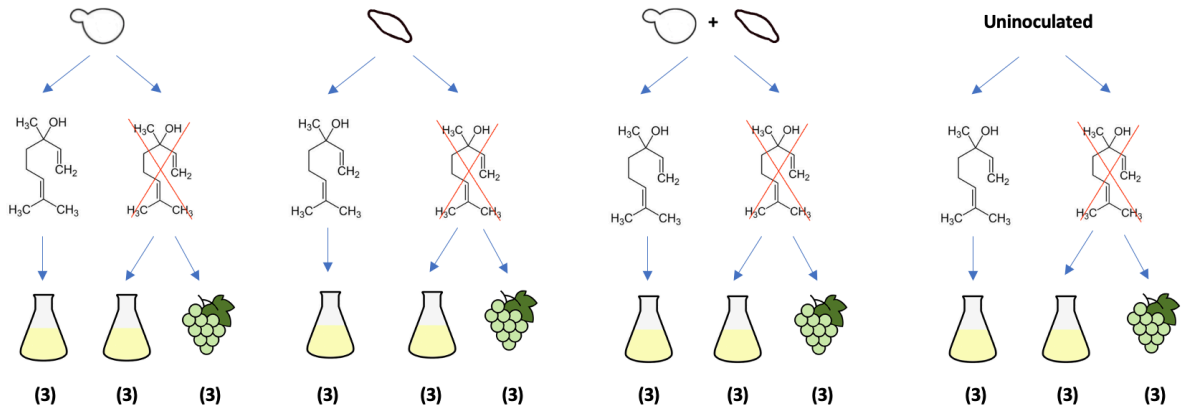

Figure 1. Schematic diagram of the experimental setup of pure and sequential culture fermentations of *H. uvarum* (H11_G1_1) ( ) and *S. cerevisiae* (EC1118) ( ) in CDGJM ( ) (unspiked (X) and spiked with linalool ($C_{10}H_{18}O$)) and sterile Muscat juice ( ). All experiments were conducted in triplicate. Numbers in brackets indicate total number of replicates/ferments for each independent variable (treatment conditions).

2.5 *H. uvarum* performance in 3 different white juices

H11_G1_1 isolate was used in juice fermentations (as monoculture and sequential cultures) with three aromatic white varietals (Muscat, Riesling and Viognier) to see the impact of *H. uvarum* on terpene compounds in white wines. After thawing, the initial YAN of Riesling was 147 mg/L and was supplemented with DAP to adjust to a final concentration of 350 mg/L. The pH was adjusted from 2.82 to 3.3 with calcium carbonate. The Muscat and Viognier used was from the same batch as in Sections 2.3 and 2.4, the remainder of the juice having been refrozen. The thawed juice was briefly mixed and then filter sterilised as before. The fermentation conditions, inoculation rates and fermentation monitoring are described in Section 2.3.

2.6 GC-MS profiling of volatile components

Headspace solid-phase microextraction-gas chromatography with mass spectrometry (HS-SPME-GC-MS) was used to identify volatile compounds in terminal fermentation samples and wines, following the protocol outlined in Lin et al. (2020). Volatile compounds produced by yeast isolates were subjected to one-way analysis of variance (ANOVA), and the significance of difference between means was determined by Tukey's multiple comparisons test ($p < 0.01$) using GraphPad Prism 9.0 software (GraphPad, USA). Compounds with mean values statistically non-significant across treatments ($p > 0.01$) were excluded from the collated data presented in the results. Volatile compounds (with significant differences) were analysed by Principal Component Analysis (PCA) using XLSTAT (version 22.5.1, Addinsoft, Paris, France).

3. Results

3.1 Evaluation of *H. uvarum* isolates as potential wine starter cultures

3.1.1 Fermentation performance of five *H. uvarum* isolates as mono- and sequential cultures in Viognier

Five *H. uvarum* isolates (**Table 1**) were evaluated for fermentation efficiency and their effect on wine composition as mono- or sequential cultures with *S. cerevisiae*, EC1118 in Viognier juice. The fermentation dynamics of the *H. uvarum* mono- and sequential culture fermentations were compared to the EC1118 control (*S. cerevisiae*) (**Figs. 2A and B**). None of the *H. uvarum* isolates could complete fermentation on their own whilst EC1118 completely consumed sugars in 6 days (**Fig. 2A**). Sugar consumption was similar for all sequentially fermented treatments, which progressed rapidly after the inoculation of EC1118; with all fermentations finishing by day 10 (7 days after the inoculation of EC1118; **Fig. 2B**)

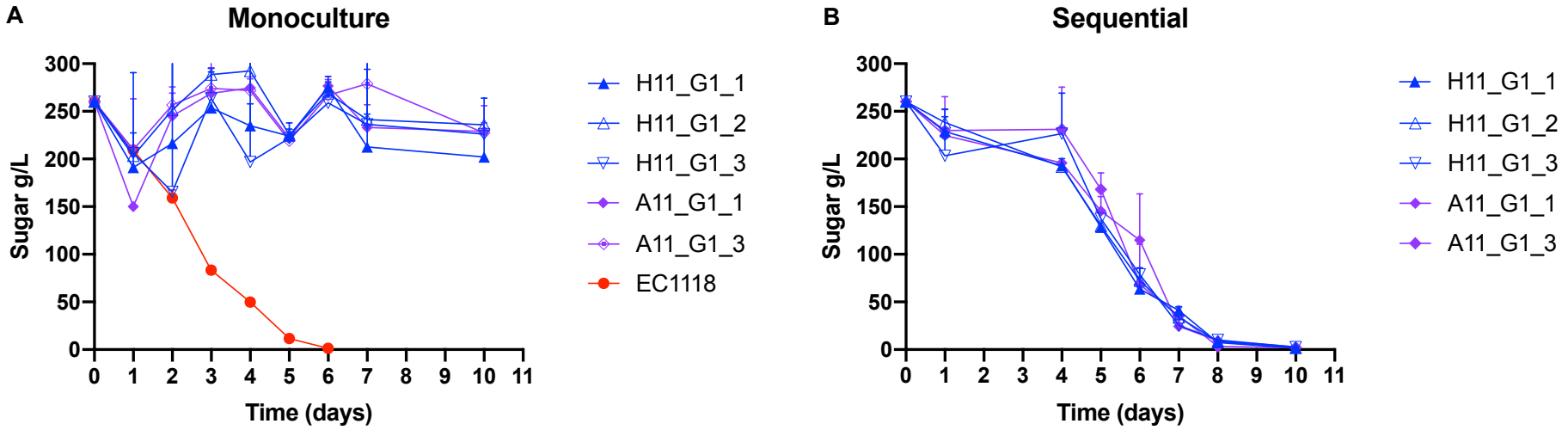

Figure 2. Fermentation profile of *H. uvarum* isolates in pure culture (monoculture) (A) and sequential culture (B) with *S. cerevisiae* (EC1118) in Viognier juice. Results are the average of three biological replicates ± standard deviation.

3.1.2 Viognier wines produced by sequential cultures of *S. cerevisiae* (EC1118) and *H. uvarum* isolates

Terminal wine samples were analysed using SPME-GC-MS, with a total of 76 compounds identified and quantified. One-way ANOVA identified 49 compounds that were not significantly different ($p > 0.01$) and were excluded from the subsequent quantitation of volatile concentrations. There were 27 compounds that were significantly different ($p < 0.01$) in the sequentially fermented Viognier wines when mean values were compared using Tukey's multiple comparisons test (**Table 2**). The concentrations of terpenes in the sequential wines were overall one- to 10-times less than those quantified in the EC1118 wines (**Table 2**). Major differences were observed between the H11_G1_1 sequential wines and that of the control (EC1118); with large reductions in linalool, α-humulene, α-terpineol, geraniol, α-calacorene and caladene in the wines produced with sequential cultures (**Table 2**). Of the esters identified, significant differences was only observed between H11_G1_1 treatment and EC1118 for ethyl 4-hydroxybutanoate and linalyl acetate (**Table 2**). The concentrations of ethyl phenyl acetate and linalyl acetate were overall lower in the sequential wines compared to the EC1118 control, while the opposite was observed in ethyl 4-hydroxybuanoate, as the H11_G1_1 (5.5 μg/L), H11_G1_2 (3.65 μg/L) and A11_G1_3 treatments (2.49 μg/L) produced more than EC1118 (2.39 μg/L) (**Table 2**). In addition, the concentration of linalyl acetate (acetate ester of linalool) was 2 to 6 times lower in the sequential wines compared to EC1118 (**Table 2**). Ethyl 4-hydroxybutanoate, ethyl phenylacetate and linalyl acetate are associated with caramel, rose/sweet blossom and floral/citrus/mint nuances respectively.

The volatile/chemical content of the sequential wines were subjected to PCA to outline the differences among the treatments in relation to the yeast isolates. All samples were mapped in the spaces shared by the first two principal components (PC1 and PC2), with a total variance

of 92% and separation of samples fermented with *H. uvarum* isolates from those made with *S. cerevisiae* (EC1118; **Fig. 3**). The majority of the volatile compounds were clustered at the right-hand side of the plot, towards the EC1118 control (C) (**Fig. 3**). Ethyl 4-hydroxybutanoate, and 1-nonanol (citrus odour) were located towards the left-hand upper corner of the plot, and were more associated with H11_G1_1 wines (**Fig. 3**). This result was expected, as H11_G1_1 sequential wines produced higher concentrations of ethyl 4-hydroxybutanoate and 1-nonanol compared to the other treatments (**Table 2**).

Table 2. Mean concentrations (µg/L) of volatile compounds in sequential fermentation by *H. uvarum* isolates and *S. cerevisiae* (EC1118) in Viognier. Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated.

	Yeast isolate/strain					
Compound	**C(EC1118)**	**H11_G1_1**	**H11_G1_2**	**H11_G1_3**	**A11_G1_1**	**A11_G1_3**
Esters						
Ethyl 4-hydroxybutanoate[1]	2.39^{b}	5.50^{a}	3.64^{ab}	2.05^{b}	2.18^{b}	2.49^{b}
Ethyl phenylacetate	0.84^{a}	0.72^{ab}	0.64^{b}	0.70^{ab}	0.71^{ab}	0.65^{b}
Acetates						
Linalyl acetate[2]	4.27^{a}	0.71^{d}	1.29^{c}	2.02^{bc}	2.29^{b}	2.00^{bc}
Alcohols						
1-Hexanol	1209.49^{a}	1094.12^{b}	1115.34^{b}	1100.19^{b}	1121.29^{b}	1101.16^{b}
1-Nonanol	2.43^{b}	6.51^{a}	3.55^{b}	3.45^{b}	3.37^{b}	3.06^{b}
Aldehydes						
3-Methyl-butanal	0.99^{a}	0.28^{c}	0.33^{bc}	0.27^{c}	0.36^{bc}	0.65^{ab}
Benzaldehyde	12.88^{a}	3.41^{c}	6.50^{bc}	8.37^{b}	8.28^{b}	12.88^{a}
Ketones						
2-Propanone[3]	0.0267^{a}	0.0072^{c}	0.0144^{c}	0.0141^{c}	0.0168^{bc}	0.0259^{ab}
β-damascenone	4.13^{a}	0.29^{d}	0.75^{cd}	0.89^{bcd}	1.34^{bc}	1.55^{b}
Terpenes						
β-myrcene	3.52^{a}	1.73^{b}	2.24^{b}	2.89^{ab}	3.52^{a}	1.85^{b}
Limonene	3.83^{a}	0.41^{b}	0.75^{b}	1.26^{b}	1.58^{b}	1.55^{b}

α-Pinene[4]	2.30[a]	0.27[b]	0.38[b]	0.74[b]	1.10[b]	1.30[ab]
β-trans-Ocimene[4]	3.68[a]	0.41[d]	0.71[cd]	1.20[bc]	1.56[b]	1.75[b]
α-humulene	12.61[a]	0.94[d]	2.23[cd]	3.99[bc]	4.87[b]	6.33[b]
Linalool	28.79[a]	9.70[d]	12.63[cd]	16.27[bc]	18.29[bc]	20.35[b]
Hotrienol[2]	0.72[a]	0.14[c]	0.22[c]	0.34[bc]	0.36[bc]	0.47[b]
β-Farnesene[5]	0.12[a]	0.01[b]	0.02[b]	0.01[b]	0.03[b]	0.01[b]
α-Terpineol	28.67[a]	5.56[d]	7.91[cd]	12.07[bcd]	13.74[bc]	15.99[b]
α-Bergamotene[6]	3.59[a]	1.22[b]	1.48[b]	1.65[b]	1.82[b]	1.01[b]
β-Citronellol	3.26[a]	2.59[b]	2.50[b]	2.45[b]	2.56[b]	2.40[b]
Geraniol	13.01[a]	4.84[c]	7.12[b]	7.95[b]	7.30[b]	7.96[b]
α-Calacorene[6]	15.80[a]	1.57[d]	2.85[cd]	4.35[bc]	5.45[b]	6.34[b]
Nerolidol	0.23[a]	0.02[b]	0.03[b]	0.03[b]	0.05[b]	0.03[b]
Cadalene[6]	5.10[a]	0.54[d]	0.93[cd]	1.55[bc]	1.80[bc]	2.11[b]
Lactones						
γ-Butyrolactone[7]	0.22[a]	0.12[b]	0.12[b]	0.07[b]	0.11[b]	0.15[ab]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Ethyl butanoate, [2]Linalool, [3]2-hexanone, [4]Limonene, [5]Nerolidol, [6]α-terpineol, [7]2-heptanone.

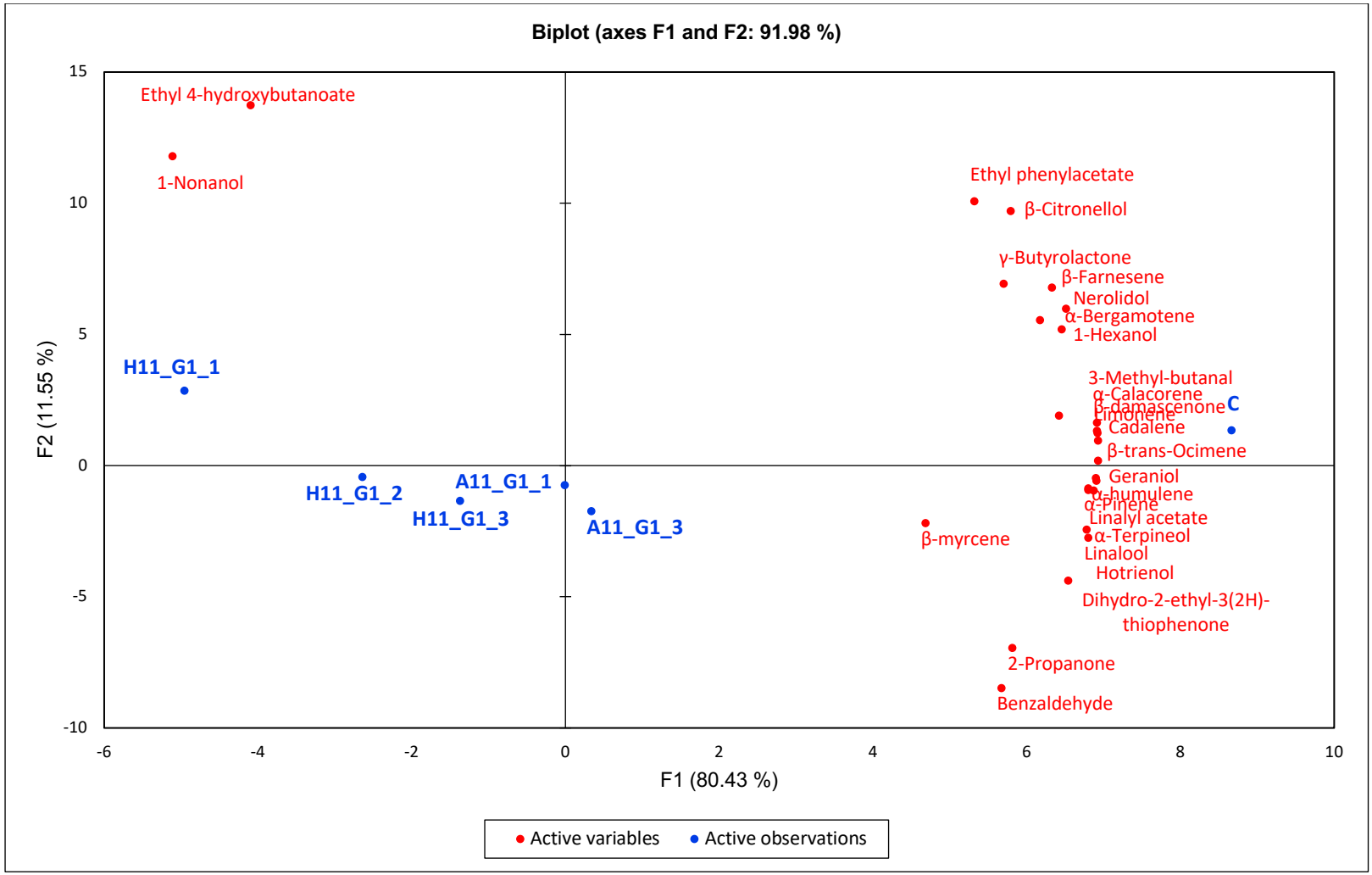

Figure 3. Principal component analysis of volatile profiles for sequential fermentation of *H. uvarum* and *S. cerevisiae* in Viognier. Yeast isolates (treatments) represented in blue and volatile compounds in red. C = *S. cerevisiae* (EC1118), H11_G1_1, H11_G1_2, H11_G1_3, A11_G1_1, and A11_G1_3 = *H. uvarum*.

3.2. Terpene profiles of wines produced from linalool-spiked CDGJM and aromatic varietals

3.2.1 Fermentation performance of H11_G1_1 in mono- and sequential cultures

To assess the impact of *H. uvarum* on the concentration of terpenes in wine, the H11_G1_1 isolate was selected based on the results of the quantification of volatile compounds (Section 3.1.2), as it had the lowest concentration of terpenes in all treatments (sequential and EC1118; **Table 2**). In particular, the amount of linalool quantified in H11_G1_1 sequential wines (9.7 μg/L) was nearly 3-times lower than that of EC1118 wines (28.8 μg/L) (**Table 2**).

Mono- and sequential culture fermentations of H11_G1_1 and EC1118 were carried out in Muscat (known to have the highest monoterpene (linalool, geraniol, nerol) content) and CDGJM spiked with linalool, to assess its impact on the concentration of terpene compounds. Alcoholic fermentation duration ranged from 6 to 11 days, with EC1118 finishing the quickest, followed by the sequential ferments at day 11 (**Fig. 4**). The H11_G1_1 monoculture fermentations in CDGJM (200 g/L sugar) and Muscat (198 g/L sugar) were incomplete, with residual sugars ranging from ~57 to 73 g/L (**Fig. 4**).

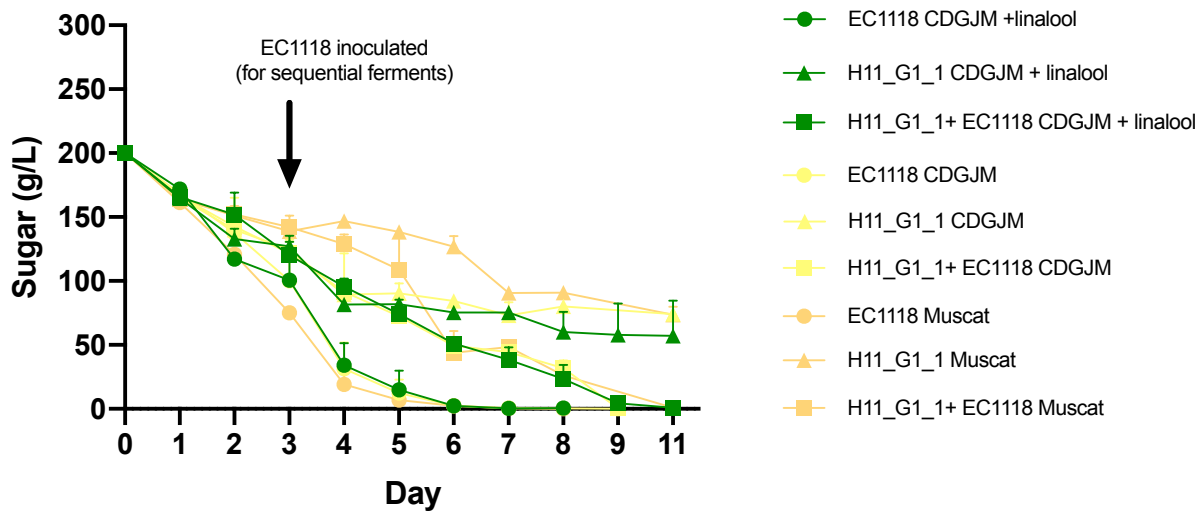

Figure 4. Fermentation profile of *H. uvarum* (H11_G1_1) in pure (mono-) and sequential culture with *S. cerevisiae* (EC1118) in chemically defined grape juice medium and Muscat juice. CDGJM (+ linalool) was spiked with 500 µg/L linalool. Uninoculated CDGJM (+/- linalool) and Muscat juice served as negative controls. Results are the average of three biological replicates ± standard deviation.

3.2.2 Terpene profiles in linalool-spiked CDGJM and unmodified Muscat wines

The analysis of aroma compounds in monoculture and sequential wines/ferments produced by H11_G1_1 and EC1118 in CDGJM (+/- linalool) and Muscat focused on terpenes, to evaluate the possible effect of *H. uvarum* on reducing terpenes as observed in the results from the previous experiment in Section 3.1.2. Since the initial terpene concentrations were undetermined in the Viognier juice (Section 3.1.2), uninoculated treatments (juice and CDGJM) served as negative controls for comparison. In general the terpene concentrations in Muscat were higher in the sequential wines compared to EC1118, but were overall reduced during alcoholic fermentation when compared to the uninoculated juice (**Table 3a**). Geranic oxide (camphor, citrus, floral, woody; 40.5 μg/L) and dehydroxylinalool oxide (herbal, minty; 48.1 μg/L) were two times higher in the sequential wines compared to the control, EC1118 (geranic oxide (21.3 μg/L) and dehydroxylinalool oxide (24.7 μg/L)). The opposite was observed in rose oxide (9.51 μg/L) which was significantly lower than with EC1118 ((rose oxide; 13.5 μg/L); **Table 3a**). The overall terpene content in mono- and sequential culture ferments/wines was reduced when compared with the uninoculated juice (**Table 3a**). Though there were no significant differences in linalool between EC1118, H11_G1_1 mono- and sequential culture treatments, significant differences were shown when compared to the uninoculated, as the three other treatments had been reduced by 11 to 17 μg/L (**Table 3b**). Linalool was the only terpene quantified in CDGJM, as no significant differences were observed in the linalool-spiked CDGJM between H11_G1_1, EC1118 and uninoculated treatments (**Table 3b**).

PCA was performed with the 22 significantly different volatile compounds analysed in Muscat wines in relation to the treatments – positive control (EC1118), *H. uvarum* monoculture (H11_G1_1), sequential culture (H11_G1_1 + EC1118) and the negative control (uninoculated). The PCA applied to the mean concentrations of volatiles (**Fig. 5**) explained

95.7% of the variability between wine samples. The two principal components (PC1: 80.4%, PC2: 11.6%) separated wine samples (EC1118, sequential and monoculture) and uninoculated treatment. On the left-hand side of the plot, β-farnesene, ethyl 4-hydroxybutanoate, citronellyl acetate, nerolidol, β-citronellol, ethyl geranate, (Z)-rose oxide, (E)-rose oxide, nerol and caladene were closely associated with EC1118 and sequential wines (**Fig. 5**). Towards the right-hand side of the plot, β-myrcene, sabinene, (Z)-ocimene, (E)- β-ocimene, terpinolene, limonene, linalool, hotrienol, α-terpineol, dehydroxylinalool oxide, epoxylinalool and β-damascenone were more associated with the uninoculated treatment – the majority of terpene concentrations were higher in uninoculated (**Fig. 5**).

Table 3a. Mean concentrations (µg/L) of volatile compounds in monoculture and sequential fermentation by *H. uvarum* (H11_G1_1) and *S. cerevisiae* (EC1118) in Muscat. UI = uninoculated juice which served as negative control. Concentration of volatile compounds were quantified against their respective standard compound or equivalents as indicated.

	Yeast strain/isolate			
Compound	**EC1118**	**H11_G1_1**	**H11_G1_1 + EC1118**	**UI**
Esters				
Ethyl geranate[1]	0.79[a]	0.01[c]	0.38[b]	0[c]
Ethyl 4-hydroxybutanoate	29.40[a]	1.03[b]	7.78[b]	0.06[b]
Acetates				
Citronellyl acetate[2]	639.08[a]	68.42[b]	323.89[ab]	0.98[bc]
Alcohols				
Nerol	320[a]	186.25[c]	273.25[ab]	204.83[bc]
Ketones				
β-damascenone	6.73[b]	7.39[b]	6.93[b]	11.58[a]
Terpenes				
β-myrcene	6.22[c]	11.77[b]	8.71[bc]	18.81[a]
Terpinoline[3]	0.51[bc]	0.71[b]	0.86[b]	1.38[a]
Limonene	2.26[c]	2.89[c]	3.86[b]	5.47[a]
Sabinene[3]	0.41[c]	0.65[b]	0.58[bc]	1.07[a]
(Z)-Ocimene[3]	1.86[c]	3.38[b]	2.78[bc]	5.91[a]
(E)-β-Ocimene[3]	2.71[b]	4.01[b]	4.28[b]	7.85[a]
Linalool	50.17[b]	50.94[b]	56.63[b]	67.94[a]

Hotrienol[4]	9.91[b]	7.40[c]	12.18[a]	12.42[a]
α-Terpineol	32.72[b]	25.66[bc]	40.32[a]	47.10[a]
Cadalene[5]	0.67[b]	0.26[c]	1.04[a]	0.55[b]
β-Citronellol	17.11[a]	2.77[b]	13.61[a]	0.18[b]
Nerolidol	0.12[a]	0.004[b]	0.05[b]	0.0002[b]
Oxides				
Geranic oxide[6]	21.29[c]	28.72[c]	40.47[b]	66.24[a]
Dehydroxylinalool oxide[6]	24.71[c]	29.41[c]	48.06[b]	66.20[a]
(E)-Rose oxide[7]	13.48[a]	1.11[b]	9.51[a]	0.29[b]
(Z)-Rose oxide	13.48[a]	1.11[b]	9.51[a]	0.29[b]
Epoxylinalool oxide[6]	18.71[b]	18.07[b]	19.35[b]	25.37[a]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Ethyl phenylacetate, [2]Phenyl acetate, [3]Limonene, [4]Linalool, [5]Benzyl alcohol, [6]Linalool oxide, [7](Z)-Rose oxide.

Table 3b. Mean concentrations (µg/L) of linalool in mono- and sequential culture fermentations by *H. uvarum* (H11_G1_1) and *S. cerevisiae* (EC1118) in CDGJM (+/- linalool). UI = uninoculated CDGJM (+/- linalool) which served as negative control. CDGJM (+ linalool) was spiked with 500 µg/L linalool prior to the inoculation of yeasts.

	Yeast strain/isolate							
Compound	**EC1118 (+ linalool)**	**H11_G1_1 (+ linalool)**	**H11_G1_1 + EC1118 (+ linalool)**	**EC1118**	**H11_G1_1**	**EC1118 + H11_G1_1**	**UI (+ linalool)**	**UI**
Linalool	2.97	2.28	2.65	0.06	0.03	0.05	2.14	0.007

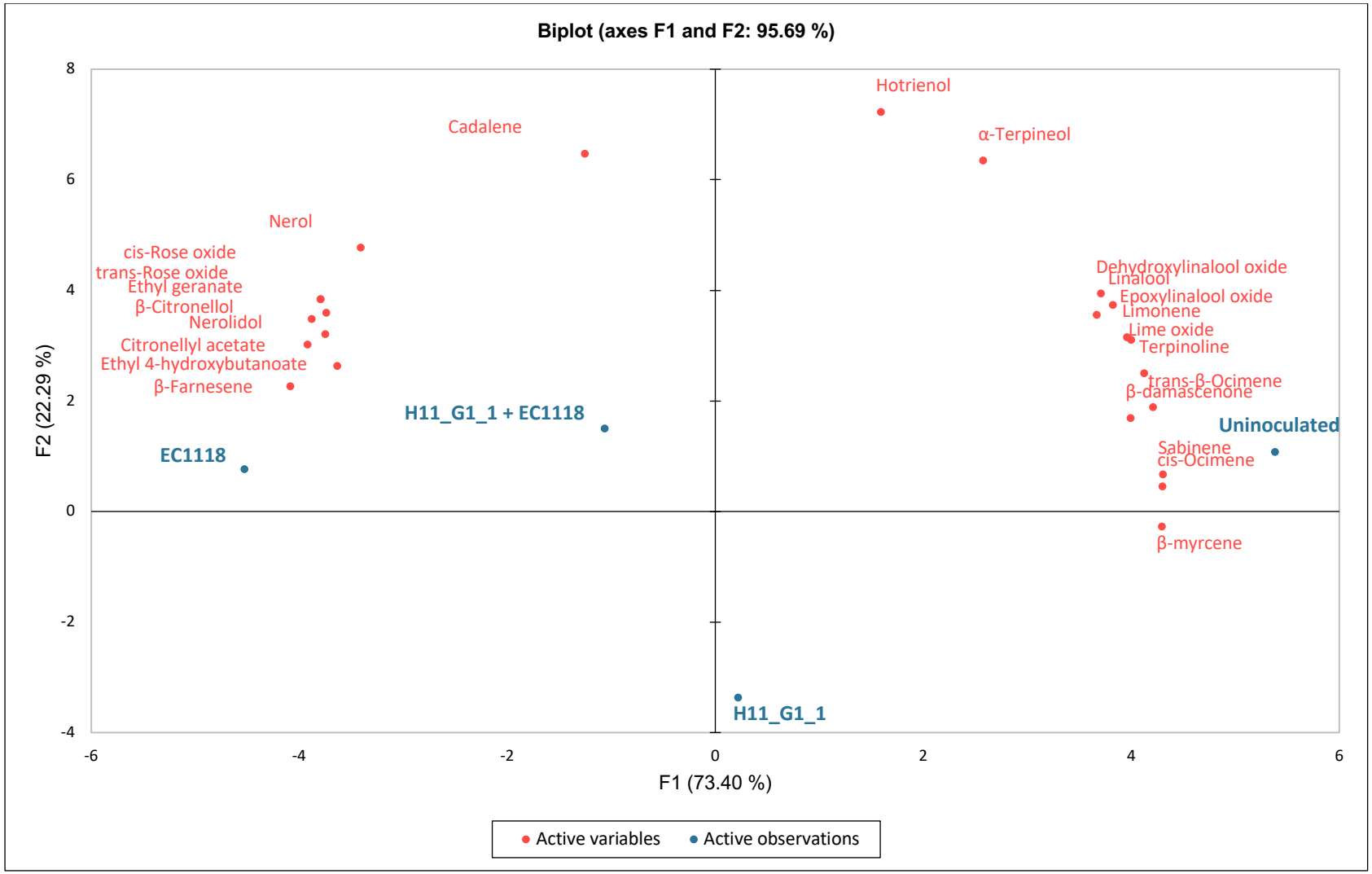

Figure 5. Principal component analysis of volatile profiles for monoculture and sequential fermentation of *H. uvarum* and *S. cerevisiae* in Muscat. Yeast isolates (treatments) represented in blue and volatile compounds in red. C = *S. cerevisiae* (EC1118), H11_G1_1 = *H. uvarum*. Uninoculated juice served as negative control.

3.3 Terpene profiles in three aromatic white wines

3.3.1 Fermentation dynamics/performance of H11_G1_1 in Muscat, Riesling and Viognier

In the interests of comparing terpene profiles in white wines influenced by *H. uvarum*, the H11_G1_1 isolate was selected to conduct monoculture and sequential fermentations (with EC1118) using three aromatic varietals (Muscat, Riesling and Viognier). Differences in sugar consumption were observed between the two species, dependent upon the juice and fermentation type (**Fig. 6**). The *S. cerevisiae* (EC1118) monoculture fermentation consumed sugars the fastest (4 to 6 days); fermentation was complete at day 4 in Muscat, day 5 in Riesling, and day 6 in Viognier (**Fig. 6**). This was expected since Muscat and Riesling had a lower starting °Brix (19.8 °B and 19.9 °B) respectively (**Fig. 6**). Sequential fermentations in all varietals was rapid after the inoculation of EC1118 (at day 3) and ultimately finished at day 11 (**Fig. 6**). None of the H11_G1_1 monocultures completed fermentation, having high amounts of residual sugars (~97 to 145 g/L; **Fig. 6**)

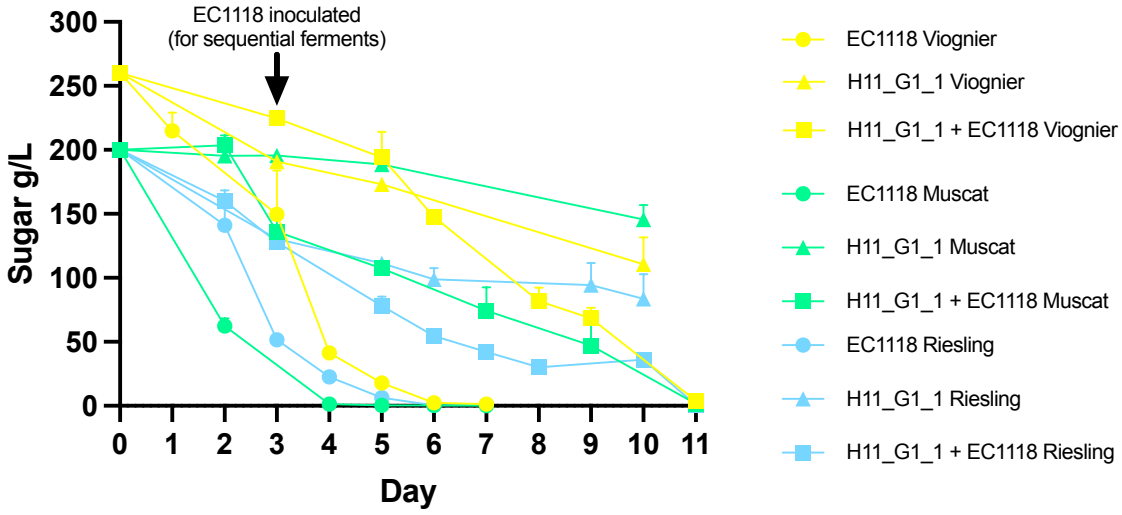

Figure 6. Fermentation profile of *H. uvarum* (H11_G1_1) in pure (mono-) and sequential culture with *S. cerevisiae* (EC1118) in Muscat, Riesling and Viognier juice. Results are the average of three biological replicates ± standard deviation.

3.3.2 Terpene profiles in Muscat, Riesling and Viognier wines

The terpene aromatic profiles of the three aromatic white wines produced by H11_G1_1 and EC1118, as well as the uninoculated treatments (juice) are shown in **Tables 4a** (Riesling), **4b** (Viognier) and **4c** (Muscat). In total 25 terpene compounds were identified and quantified by GC-MS, which all displayed significant differences at $p < 0.01$. In the Riesling wines/treatments, the majority of terpene concentrations were higher in the sequential wines, except (E)-β-farnesene (784.1 μg/L), α-terpineol (2.2 μg/L), citronellol (1.3 μg/L), nerol (1.5 μg/L), geraniol (6.6 μg/L), nerolidol (0.03 μg/L) and (Z)-rose oxide (1.8 μg/L), where the concentrations were lower than in the EC1118 wines (**Table 4a**). Linalool concentration was higher in the EC1118 (3.16 μg/L) and sequential (3.19 μg/L) treatment compared to the uninoculated (0.31 μg/L), and the concentration was lower in the monoculture (2.38 μg/L) compared to EC1118 (**Table 4a**). The acetate ester derivatives of geraniol and citronellol also varied. Geranyl acetate (fruity/rose) was higher in the sequential wines, whilst for citronellol acetate (floral-rosy, fruity, slightly citrus), EC1118 had over two-times as much as the H11_G1_1 sequential wines (28.7 μg/L vs 12.77 μg/L; **Table 4a**). An alkyl ether of geraniol, geranyl ethyl ether (fruity) was higher in EC1118 wines (16.1 μg/L) compared to the sequential wines (14 μg/L), but no significant differences were observed between the treatments (**Table 4a**). The volatile compounds in Riesling wines/treatments were analysed by PCA, displaying a total variance of 98% (PC1: 87.1%, PC2: 10.9%). All compounds were clustered at the right-hand side of the plot, and were more strongly associated with EC1118 and sequential treatments compared to the monoculture and uninoculated treatments (left-hand side) (**Fig. 7**).

In the Viognier wines/treatments, there appeared a similar trend in the terpene concentrations in the sequential wines, except for (E)-β-farnesene (748.6 μg/L), geraniol (25.1 μg/L), nerolidol (0.08 μg/L) and (Z)-rose oxide (10.7 μg/L) which were lower compared to the EC1118 wines (**Table 4b**). Both geranyl acetate and citronellol acetates had higher

concentrations in the EC1118 wines (**Table 4b**). Although there is a higher concentration of geranyl ethyl ether in the sequential treatment (85.9 μg/L), there were no significant differences between the sequential and EC1118 (78 μg/L). Comparing to the linalool concentrations observed in the first experiment (Section 3.1.2), there were less linalool quantified in this experiment, and the cause of this is likely due to the volatiles possibly been volatilised or degraded during freeze thawing of the juice. Additionally, the initial terpene concentrations cannot be compared between the two experiments, as the first experiment did not include negative controls. The PCA for the volatile compounds in Viognier wines/treatments displayed 99.9% variance (PC1: 96%, PC2: 3.9%), with all compounds more closely associated with EC1118 and sequential treatments at the right-hand side of the plot (**Fig. 8**)

The overall terpene concentrations in the H11_G1_1 sequential and EC1118 Muscat wines were relatively the same, as no significant differences were observed in most of the terpenes between the treatments (**Table 4c**). However, there were significant differences in limonene, geranic oxide, dehydroxylinalool oxide, and geranyl ethyl ether when comparing between sequential and EC1118 wines, as the latter produced higher levels of these compounds (**Table 4c**). While no significant differences were observed in acetate esters between EC1118 and sequential wines, both citronellol acetate and geranyl acetate had higher values in EC1118 (**Table 4c**). Both acetates and most terpenes in the uninoculated and the H11_G1_1 monoculture treatment had remained relatively the same, with only insignificantly small reductions with the *H. uvarum*. However, the monoculture appears to reduce oxides when compared to the uninoculated treatment, for example, nerol oxide (11 μg/L vs 17.6 μg/L; **Table 4c**). For the PCA of volatile compounds in Muscat treatments/wines, the results showed 99.8% total variance (PC1: 96.9%, PC2: 2.9%) across all samples, as all volatile compounds were closely associated with EC1118 and sequential wines at the right-hand side of the plot (**Fig. 9**).

Table 4a. Mean concentrations (µg/L) of volatile compounds in monoculture and sequential fermentation by *H. uvarum* (H11_G1_1) and *S. cerevisiae* (EC1118) in Riesling.

	Yeast isolate/treatment			
Compound	**Uninoculated**	**EC1118**	**H11_G1_1**	**H11_G1_1 + EC1118**
Acetates				
Citronellol acetate[1]	0.01[b]	28.69[a]	0.11[b]	12.77[a]
Geranyl acetate	2.19[b]	19.74[a]	8.27[a]	21.44[a]
Ethers				
Geranyl ethyl ether[2]	0.12[c]	16.09[a]	3.20[b]	13.97[a]
Ketones				
β-Damascenone	0.81[c]	3.69[a]	2.33[b]	3.60[a]
Terpenes				
β-myrcene	8.87[c]	9.22[b]	9.22[b]	9.31[a]
Terpinoline[3]	0.03[b]	0.26[a]	0.17[a]	0.34[a]
Limonene	0.21[c]	2.59[a]	1.50[b]	2.92[a]
Sabinene[3]	0.03[b]	0.46[a]	0.46[a]	0.56[a]
(Z)-ocimene[3]	0.17[c]	2.16[a]	1.37[b]	2.43[a]
γ-terpinene[3]	0.03[b]	0.77[a]	0.30[b]	0.69[a]
(E)-β-ocimene[3]	0.09[c]	1.33[a]	0.75[b]	1.47[a]
Linalool	0.31[c]	3.16[a]	2.38[b]	3.19[a]
Hotrienol[2]	0.13[c]	1.86[a]	1.18[b]	1.87[a]
(E)-β-farnesene	0.38[c]	971.80[a]	95.33[b]	784.10[a]
α-Terpineol	0.16[c]	2.44[a]	1.55[b]	2.20[a]

α-Calacorene	0.001[d]	0.31[b]	0.13[c]	0.39[a]
Citronellol	0.003[c]	2.51[a]	0.15[c]	1.31[b]
Nerol	0.04[b]	4.19[a]	0.70[b]	1.54[b]
Geraniol	0.66[b]	10.71[a]	2.95[a]	6.58[a]
Nerolidol	0.00005[b]	0.15[a]	0.003[b]	0.03[b]
Oxides				
Geranic oxide/linalool-3,7-oxide[3]	0.10[c]	1.76[b]	1.29[b]	2.39[a]
Dehydroxylinalool oxide[4]	0.43[b]	2.48[a]	2.20[a]	4.34[a]
(Z)-Rose oxide	0.07[b]	2.13[a]	0.35[b]	1.78[a]
(E)-Rose oxide[5]	0.02[b]	0.47[a]	0.04[b]	0.37[a]
Nerol oxide[4]	0.81[c]	24.30[a]	16.36[b]	28.68[a]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Benzyl acetate, [2]Linalool, [3]Limonene, [4]Linalool oxide, [5](Z)-Rose oxide.

Table 4b. Mean concentrations (µg/L) of volatile compounds in monoculture and sequential fermentation by *H. uvarum* (H11_G1_1) and *S. cerevisiae* (EC1118) in Viognier.

	Yeast isolate/treatment			
Compound	**Uninoculated**	**EC1118**	**H11_G1_1**	**H11_G1_1 + EC1118**
Acetates				
Citronellol acetate[1]	0[b]	336.10[a]	0.01[b]	252.30[a]
Geranyl acetate	2.34[b]	31.85[a]	2.89[b]	24.16[a]
Ethers				
Geranyl ethyl ether[2]	0.64[b]	77.95[a]	0.72[b]	85.92[a]
Ketones				
β-Damascenone	0.57[b]	2.25[a]	0.66[b]	2.23[a]
Terpenes				
β-myrcene	9.18[b]	9.65[b]	9.35[b]	10.42[a]
Terpinoline[3]	0.08[b]	0.44[a]	0.11[b]	0.56[a]
Limonene	1.85[b]	9.33[a]	2.44[b]	9.97[a]
Sabinene[3]	0.30[b]	1.40[a]	0.42[b]	1.52[a]
(Z)-ocimene[3]	1.60[b]	8.18[a]	2.59[b]	8.92[a]
γ-terpinene[3]	0.11[b]	0.90[a]	0.16[b]	1.23[a]
(E)-β-ocimene[3]	1.18[b]	5.28[a]	1.61[b]	6.20[a]
Linalool	2.19[b]	10.29[a]	2.66[b]	11.07[a]
Hotrienol[2]	0.04[b]	0.20[a]	0.05[b]	0.26[a]
(E)-β-farnesene	1.03[c]	1045[a]	0.91[c]	748.60[b]
α-Terpineol	1.32[b]	7.11[a]	1.94[b]	9.07[a]

α-Calacorene	0.03[c]	0.52[b]	0.04[c]	0.62[a]
Citronellol	0.008[b]	3.89[a]	0.005[b]	3.88[a]
Nerol	1.23[b]	11.72[a]	1.40[b]	12.13[a]
Geraniol	5.10[b]	29.24[a]	5.27[b]	25.08[a]
Nerolidol	0[b]	0.15[a]	0[b]	0.08[a]
Oxides				
Geranic oxide/linalool-3,7-oxide[3]	1.19[b]	5.81[a]	1.82[b]	8.07[a]
Dehydroxylinalool oxide[4]	0.94[b]	5.41[a]	1.23[b]	5.17[a]
(Z)-Rose oxide	0.04[b]	11.08[a]	0.01[b]	10.69[a]
(E)-Rose oxide[5]	0.008[b]	2.81[a]	0.006[b]	2.50[a]
Nerol oxide[4]	0.71[c]	3.82[b]	0.96[bc]	5.27[a]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Benzyl acetate, [2]Linalool, [3]Limonene, [4]Linalool oxide, [5](Z)-Rose oxide.

Table 4c. Mean concentrations of (µg/L) of volatile compounds in monoculture and sequential fermentation by *H. uvarum* (H11_G1_1) and *S. cerevisiae* (EC1118) in Muscat.

	Yeast isolate/treatment			
Compound	**Uninoculated**	**EC1118**	**H11_G1_1**	**H11_G1_1 + EC1118**
Acetates				
Citronellol acetate[1]	0.09[b]	303.40[a]	0.07[b]	233.20[a]
Geranyl acetate	7.50[b]	46.67[a]	5.25[b]	35.51[a]
Ethers				
Geranyl ethyl ether[2]	6.76[c]	296.70[a]	4.56[c]	160.80[b]
Ketones				
β-Damascenone	2.04[b]	5.97[a]	1.38[b]	4.05[a]
Terpenes				
β-myrcene	10.59[b]	13.68[a]	10.27[b]	13.40[a]
Terpinoline[3]	0.41[c]	1.90[a]	0.24[c]	1.34[b]
Limonene	8.06[c]	38.89[a]	5.61[c]	26.84[b]
Sabinene[3]	1.86[b]	5.35[a]	1.16[b]	5.37[a]
(Z)-ocimene[3]	9.96[b]	28.87[a]	7.06[b]	23.83[a]
γ-terpinene[3]	0.68[b]	3.76[a]	0.57[c]	2.28[b]
(E)-β-ocimene[3]	6.59[b]	23.80[a]	5.01[b]	18.35[a]
Linalool	9.52[b]	42.15[a]	8.64[b]	37.77[a]
Hotrienol[2]	1.45[b]	11.22[a]	1.74[b]	8.88[a]
(E)-β-farnesene	0.36[b]	562.40[a]	1.01[b]	473.70[a]

α-Terpineol	5.92[b]	34.37[a]	5.31[b]	25.38[a]
α-Calacorene	0.09[c]	1.41[a]	0.04[c]	0.82[b]
Citronellol	0.08[b]	10.75[a]	0.05[b]	11.37[a]
Nerol	14.77[b]	61.13[a]	13.87[b]	59.87[a]
Geraniol	67.27[b]	132.20[a]	73.85[b]	123.50[a]
Nerolidol	0.00004[b]	0.12[a]	0.0002[b]	0.07[a]
Oxides				
Geranic oxide/linalool-3,7-oxide[3]	10.32[c]	59.79[a]	5.07[c]	34.86[b]
Dehydroxylinalool oxide[4]	10.82[c]	59.48[a]	4.06[c]	31.05[b]
(Z)-Rose oxide	1.07[b]	30.38[a]	0.10[b]	29.75[a]
(E)-Rose oxide[5]	0.27[b]	5.18[a]	0.03[b]	7.27[a]
Nerol oxide[4]	17.58[b]	147.60[a]	11.04[b]	98.36[a]

[a] Means within rows with different letters are significantly different at $p < 0.01$. Tukey's multiple comparison test.
Concentration values of compounds are expressed in equivalents of: [1]Benzyl acetate, [2]Linalool, [3]Limonene, [4]Linalool oxide, [5](Z)-Rose oxide.

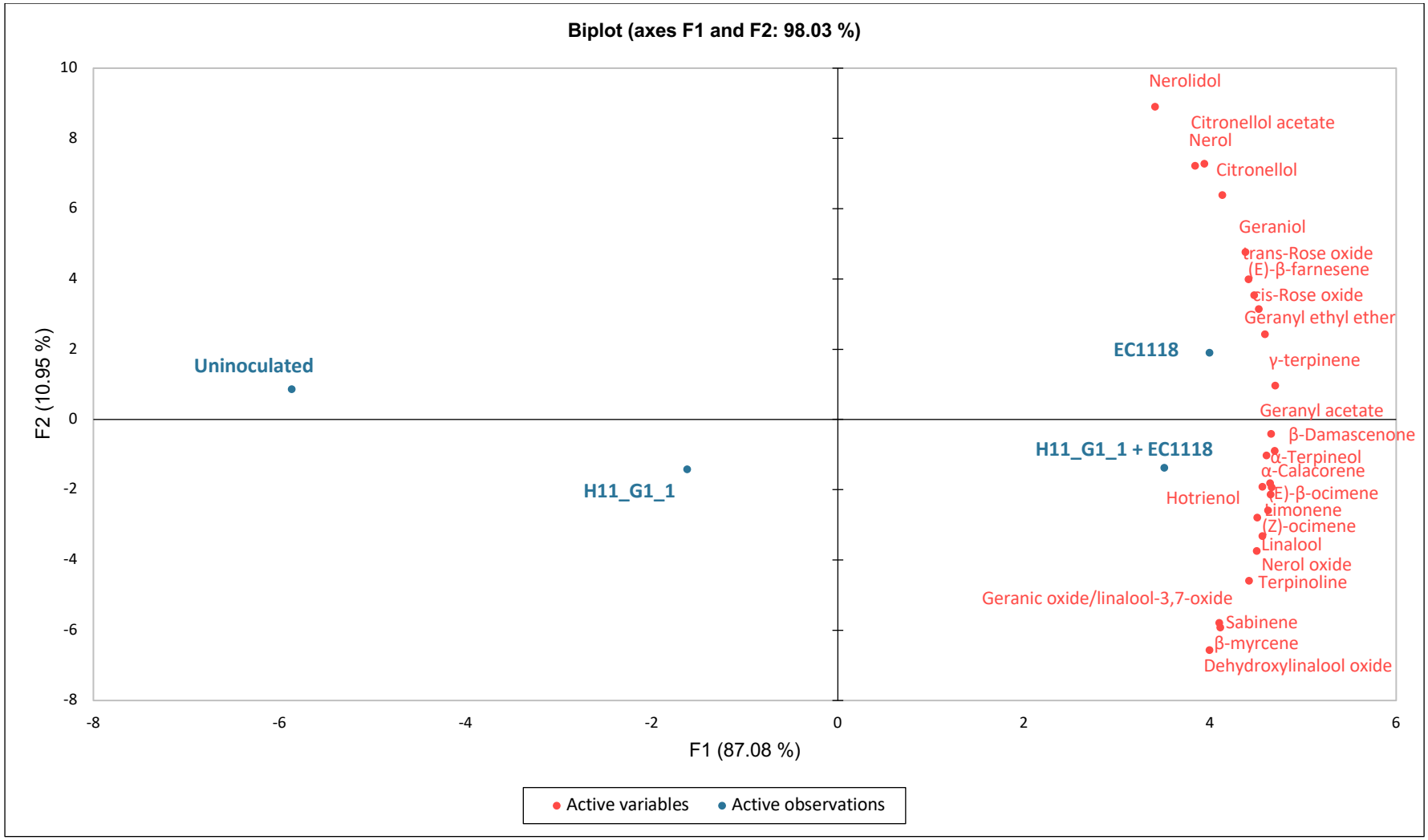

Figure 7. Principal component analysis of volatile profiles for monoculture and sequential fermentation of *H. uvarum* and *S. cerevisiae* in Riesling. Yeast isolates (treatments) represented in blue and volatile compounds in red. C = *S. cerevisiae* (EC1118), H11_G1_1 = *H. uvarum*. Uninoculated juice served as negative control.

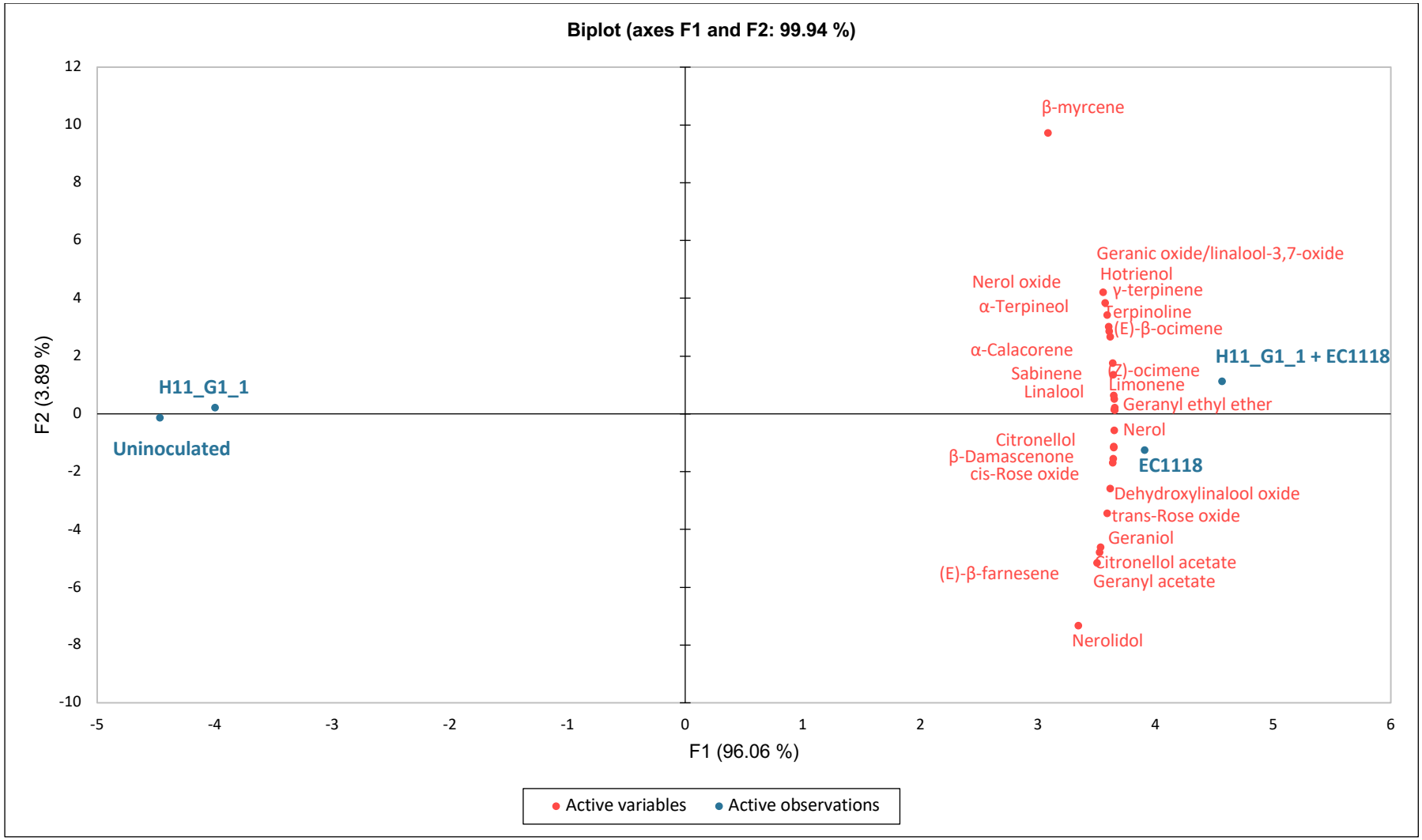

Figure 8. Principal component analysis of volatile profiles for monoculture and sequential fermentation of *H. uvarum* and *S. cerevisiae* in Viognier. Yeast isolates (treatments) represented in blue and volatile compounds in red. C = *S. cerevisiae* (EC1118), H11_G1_1 = *H. uvarum*. Uninoculated juice served as negative control.

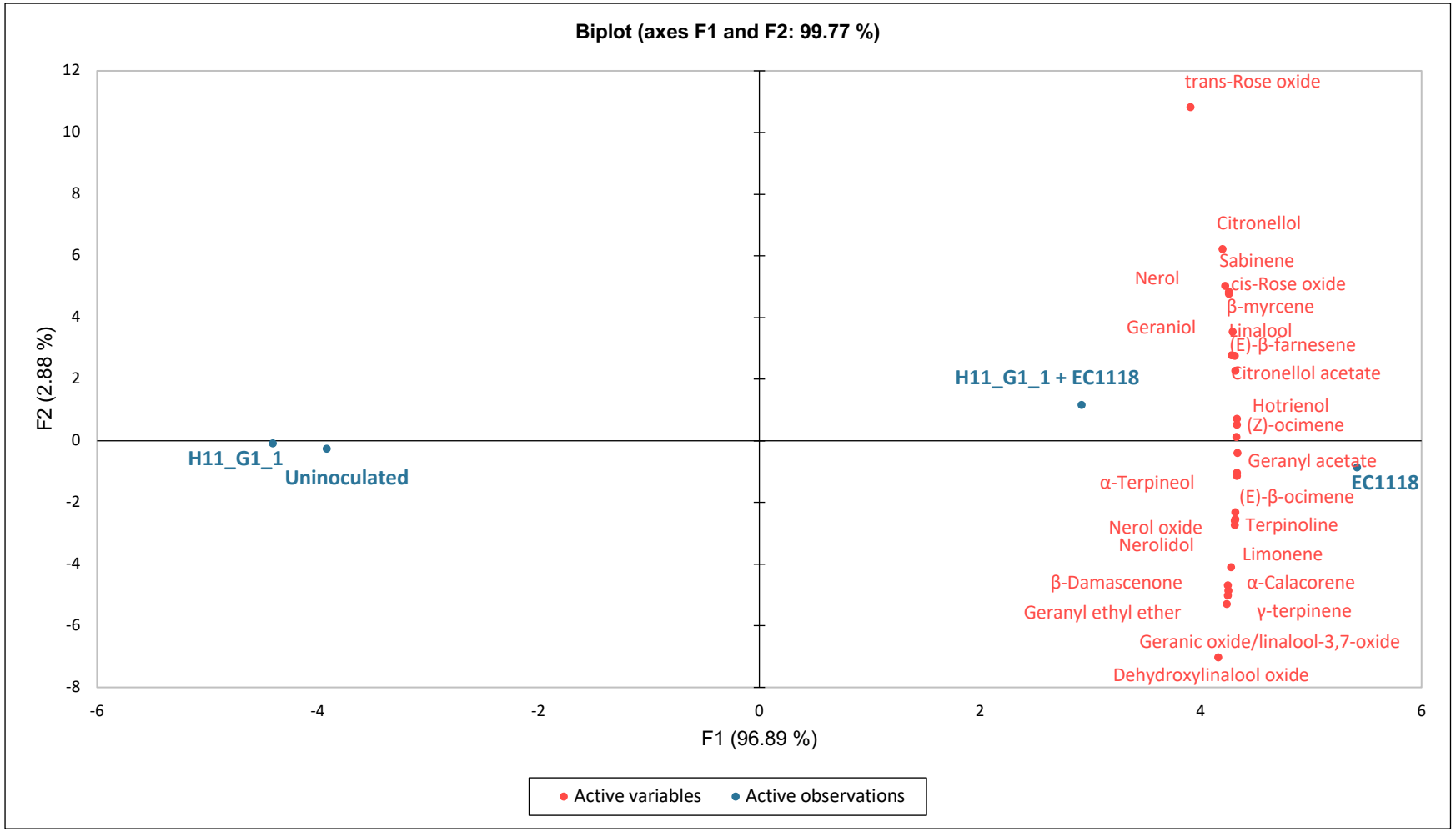

Figure 9. Principal component analysis of volatile profiles for monoculture and sequential fermentation of *H. uvarum* and *S. cerevisiae* in Muscat. Yeast isolates (treatments) represented in blue and volatile compounds in red. C = *S. cerevisiae* (EC1118), H11_G1_1 = *H. uvarum*. Uninoculated juice served as negative control.

4. Discussion

In this study we assessed the impact of *H. uvarum* isolates (sourced from grape must) on the terpene content of white wines. Initial experiments were conducted with five *H. uvarum* isolates in both mono- and sequential culture fermentations with EC1118 in Viognier (**Fig. 2**). A reduction in terpene and acetate ester concentrations was observed in the sequential wines, when compared to the EC1118 control (**Table 2, Fig. 3**). This phenomenon was unexpected, as multiple studies have reported that wines fermented with *Hanseniaspora* spp. have increased terpene concentrations (Lòpez et al., 2014; Hu et al., 2018, Mendes-Ferreira et al., 2001). Strains belonging to *H. uvarum* and *H. vineae* have been reported to contribute to an overall increase in terpenes (1.1 – 1.3-fold) in Muscat (Lòpez et al., 2014). Other studies have shown that *H. uvarum* contributed to the increase of free terpene, C_{13}-norisoprenoids and monoterpenols, which was directly correlated with the high β-glucosidase activity (Hu et al., 2018; Mendes-Ferreira et al., 2001).

To test the assumption that the reduction of terpenes was directly affected by *H. uvarum*, the H11_G1_1 isolate was selected to assess its capability to reduce linalool 'spiked' CDGJM alongside Muscat. Negative controls (uninoculated CDGJM and juice) were included for comparison of terpene concentrations (before and after fermentation). A slight increase in terpene concentrations was observed in the sequential Muscat wines (compared to EC1118) (**Table 3a**), as opposed to initial trial in Viognier (**Table 2**). Interestingly, there was a reduction in linalool in the monoculture, sequential and EC1118 treatments compared with the uninoculated treatment (**Table 3a**). In contrast, no differences were observed between the treatments in the CDGJM (spiked and unspiked); neither *H. uvarum* nor EC1118 metabolised or degraded the linalool. This was expected as there were no glycosidic aroma precursors present in CDGJM for the yeast to liberate terpenes. When the H11_G1_1 isolate was again used to ferment in three aromatic varieties, the linalool concentration was higher in both the

EC1118 and sequential wines (compared to the uninoculated). It appears that H11_G1_1 is neither metabolising or degrading linalool, the timing of the second inoculation could have delayed terpene biosynthesis in EC1118, as the linalool amount had slightly lowered in the sequential wines (in comparison with EC1118; **Tables 4a, b and c**). Although there is no literature to support this observation, one explanation could be that the release of monoterpenes from glycosides may be less than the losses of free monoterpenes in the juice (Delcroix et al., 1994). Delcroix et al. (1994) reported on the substantial decrease of linalool, geraniol, and α-terpineol during fermentation of Muscat de Frontignan with *S. cerevisiae* strains. The authors also noted that enzymatic activities (β-glucosidase, α-arabinosidase and α-rhamnosidase) measured in juice and yeast biomass, which reached a maximum during exponential growth and declined quickly afterwards. As the most abundant glycoside, β-glucosidase exhibited poor stability at wine pH (Delcroix et al., 1994). Loss of monoterpenes in wines could also be caused by lees binding, volatilisation, yeast metabolism or chemical reactions (Waterhouse et al., 2016).

There are several factors that could influence the terpene profile in wine during alcoholic fermentation. Nitrogen and oxygen availability has been demonstrated to influence monoterpene formation (Carrau et al., 2005; Carrau et al., 2008). High compared to low YAN (400 mg/L vs 180 mg/L) stimulated fermentation rate, as well as monoterpenes (but not sesquiterpenes (nerolidol and farnesol)) of *S. cerevisiae* and *H. uvarum* (Carrau et al., 2005). Additionally, microaerobic conditions favoured terpene accumulation in ferments over anaerobic conditions, as the latter were suggested to be an inhibitor of sterol biosynthesis (Carrau et al., 2008). The mevalonic acid (MVA) pathway, which is involved in the biosynthesis of sterols is known to occur in the cytosol, through isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) as intermediates to synthesise monoterpenes in plants and fungi (Carrau et al., 2005; Carrau et al., 2008). However, there is another proposed

alternative pathway through which monoterpenes might be derived from – the leucine catabolism 'MCC' pathway, which is known to occur in the yeast mitochondria (Carrau et al., 2005; Carrau et al., 2008). This pathway involves the conversion of leucine to mevalonic acid, which is regulated by assimilable nitrogen and oxygen. Geranyl diphosphate (GPP), also regulated by assimilable nitrogen and oxygen is speculated to be an intermediate in the MCC pathway, acting as a precursor for terpene biosynthesis (Carrau et al., 2008). The mechanism of this pathway is still under investigation, as no putative genes were found for the formation of linalool and α-terpineol.

Temperature can also influence the synthesis of yeast-derived volatile aroma compounds during wine fermentation (Lambrechts and Pretorius, 2000; Molina et al., 2008). Generally white wines are produced at lower temperatures (7–16 °C) than red wines (20–30 °C) to retain the fruit- and floral-driven aromas. The experiments in this study were conducted at ambient temperature. Additional variables such as amino acid composition, grape variety, yeast strain and acidity (pH, affects enzyme activity) affects volatile compounds (Burin et al., 2015; Ilc et al., 2016). As such, further experiments are warranted as to these factors on the influence of wine aroma composition.

The differences in monoterpene content in grape varieties may also be linked to winemaking practices and known chemical stability at wine conditions (Song et al., 2018). Other than being synthesised by yeasts and during aging of wines, the majority of monoterpenes occur in grape skins and are usually released by maceration of grape must (Baron et al., 2017). In a study done by Radeka et al. (2008), maceration at temperatures of 20–25 °C released phenolic compounds, which in turn can lead to oxidation and browning in juice. Conversely, cryomaceration (5–8 °C) resulted in increased concentrations of terpenic compounds in Malvesia must, with suppressed level of extracted phenolic compounds (Radeka et al., 2008). Storage method (fresh and frozen) can also affect the extraction of components in

the juice (Ouellet and Pedneault, 2016), as the juices utilised in our experiments were defrosted/thawed which would have altered the composition/matrix.

Additionally, acidity (pH) can also cause changes/alteration in monoterpenes, as polyols such as 3,7-dimethylocta-l,5-diene-3,7-diol, 3,7-dimethylocta-l,7-diene-3,6-diol, 3,7-dimethyloct-l-ene-3,7-diol, and 3,7-dimethyloct-lene-3,6,7-triol can be chemically rearranged under pH of 3.2 (Song et al., 2018). This chemical rearrangement results in the formation of, such as nerol oxide (and other oxides) which is highly reactive (Song et al., 2018).

In conclusion, we assessed the influence of *H. uvarum* on terpene profiles during fermentation in both juice and CDGJM. *H. uvarum* appeared to initially negatively impact aroma composition in Viognier wines depending on the fermentations, but this finding was not reproduced in more comprehensive, subsequent trials. While the reasons behind such an occurrence have not been resolved and could not be validated by experiments in linalool-spiked CDGJM other aromatic white wines, future studies should include testing under different conditions, which would include negative controls (uninoculated juice/must/synthetic media) (with other variables excluded). It is also noteworthy to mention that the supporting literature evidence on terpene biosynthesis by yeasts did not include negative controls; thus the true effect of the ability of yeasts to modulate terpenes may have been missed. Although β-glucosidase activity was not investigated in detail in our study, from the preliminary screening process on plate-based assays, none of the isolates possessed the ability to hydrolyse arbutin (data not shown) as reported by Strauss et al. (2001). Expression of genes involved in the terpene biosynthesis pathway (encoding glucosidase activity) should be explored and will lead to a better understanding of the contribution of abiotic factors in biosynthesis of yeast-derived terpene compounds during wine fermentation.

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Conflicts of interest

The authors declare there are no conflicts of interest regarding the study.

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CHAPTER 5

"Whole-genome analysis of two *Kazachstania* spp. isolates from grape must fermentations: identification of genes related to acetate ester production "

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Whole-genome analysis of two *Kazachstania* spp. isolates from grape must fermentations: identification of genes related to acetate ester production

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Abstract

Kazachstania aerobia and *Kazachstania servazzii* can affect the wine aroma profile by increasing acetate ester concentrations, most remarkably phenylethyl acetate and isoamyl acetate. The genetic basis of this is unknown, there being little to no sequence data available on the genome architecture. We report for the first time the near complete genome sequence of the two species using long-read (PacBio) sequencing platform (15 contigs, one scaffold). The annotated genomes of *K. aerobia* (12.5 Mb) and *K. servazzii* (12.3 Mb) were compared to *Saccharomyces cerevisiae* genomes (laboratory strain S288C and wine strain EC1118). Whilst a comparison of the two *Kazachstania* spp. genomes revealed only few differences between them, divergence was evident in relation to the genes involved in ester biosynthesis as alluded to by gene duplications and absences. The annotations of these genomes are valuable resources for future research into the evolutionary biology of *Kazachstania* species (comparative genomics) as well as understanding the metabolic processes associated with alcoholic fermentation and the production of secondary 'aromatic' metabolites (transcriptomics, proteomics, and metabolomics).

Keywords: PacBio, whole genome sequencing, *Kazachstania aerobia*, *Kazachstania servazzii*, ester genes, wine aroma

Author Notes

All supporting data and protocols have been provided within the article or through supplementary data files. Fourteen supplementary tables and twelve supplementary figures are available with the online version of this article.

Data Summary

All sequencing data generated in this study are available in the National Center for Biotechnology Information under BioSample SAMN25820612 (genome; PF_8_W29) and SAMN25820613 (genome; PF_9_W20), which are both under the NCBI BioProject accession number PRJNA799447. Genomic data used in this study can be retrieved from NCBI Genome accession numbers JAKOOU000000000 and JAKOOT000000000, as all accession numbers are indicated in Table 1. Genome sequences of the reference *S. cerevisiae* strains S288C and EC1118 were downloaded from NCBI and *Saccharomyces* Genome Database (SGD, https://www.yeastgenome.org). All software used in the analyses of the genome sequences are publicly available (except for Geneious), and the sources have been provided in the article.

Abbreviations

AATase, alcohol acetyltransferase; ADH, alcohol dehydrogenase; GO, gene ontology; QTL, quantitative trait loci; WGD, whole genome duplication

Impact statement

Kazachstania aerobia and *Kazachstania servazzii* are members of the *Kazachstania* genus. Only two members are fully sequenced – *K. africana* and *K. naganishii*, together with the closely-related species in the *Saccharomycetaceae* family (*Naumovozyma castellii* and *Nauvomozyma dairenensis*). In this study, the genomes of *K. aerobia* (PF_8_W29) and *K. servazzii* (PF_9_W20) were sequenced and assembled as 15 contigs, 1 scaffold (including 1 gap) and the mitochondrial genome. Gene orthologs were identified by sequence comparison to *Saccharomyces cerevisiae* for proteins related to ester production. These were compared for sequence similarities to the four species above to identify any gene divergence between these members of the *Saccharomycetaceae* family. These results provide valuable information necessary for studying the variation in ester formation during fermentations.

1. Introduction

Yeast play an essential role in the fermentation of alcoholic beverages transforming sugars to ethanol, carbon dioxide and other metabolites [1–3]. Many of these secondary metabolites contribute to the unique aroma and flavour of fermented beverages. *Saccharomyces cerevisiae*, the primary yeast involved in alcoholic fermentation has received wide attention in research and in the beverage (and food) industry due to its fast growth rate and ability to complete fermentation. Inoculation with *S. cerevisiae* starters is considered lower risk of off-flavours or stuck fermentation [4]. However, *S. cerevisiae* strains (with a few exceptions) do not contribute significantly to the sensory properties of the final product, which often lacks complexity [5]. The demand for new wine styles, greater complexity and reduced alcohol has led to bioprospecting for novel yeasts capable of enhancing beverage flavour or limiting alcohol content [6, 7]. Non-*Saccharomyces* yeasts, once considered undesirable as a source of spoilage, are of particular interest as potential wine starters, due to their ability to secrete enzymes (e.g., β-glucosidase to release glycosidically-bound aroma compounds), reduce ethanol concentration and produce secondary metabolites such as esters [8, 9]. Their sensitivity to ethanol, lends non-*Saccharomyces* yeasts to be used in mixed- or co-culture fermentations with *S. cerevisiae*, allowing for complete sugar utilisation as well as modulation of positive volatile compounds [10]. In addition, some species have antimicrobial activity towards wine spoilage organisms [11], which also lends these yeasts to potential use as starter cultures to preserve (as a bioprotectant; [12, 13]) and improve the sensory quality of wine and beers [14].

Whilst the application of non-*Saccharomyces* in wine production is becoming more common place [15], there has only recently has there been an intense focus on the genetics and the physiology of these organisms. High-throughput whole genome sequencing [16] has led to the repository of mostly draft genomes [17–19], with only a few complete assemblies to allow

the prediction of functional genes, gene annotation and genome architecture [20–22]. Additionally, recent studies have reported on specific flavour gene duplications and absence in genes putatively involved in the ester production in the non-*Saccharomyces* yeast species *Hanseniaspora uvarum*, *Hansenaspora osmophila*, and *Hanseniaspora vineae* [23, 24]. The increasing availability of these genome sequences and others is important in allowing a better understanding of the genomic and metabolic features of non-*Saccharomyces* yeasts and how these relates to the fermentation of foods and beverages.

Kazachstania is a non-*Saccharomyces* yeast genus belonging to the *Saccharomycetaceae* family [25, 26]. In 1971, Zubkova first proposed the genus *Kazachstania* with the description of *Kazachstania viticola*, which was first isolated in Kazakhstan from fermenting grapes [27]. It was later considered to be a synonym of *Saccharomyces dairenensis* [28]; but it was not until 2003 when several species belonging to *Arxiozyma*, *Kluyveromyces*, *Pachytichospora* and *Saccharomyces* (*sensu lato*) were reassigned and reclassified into the *Kazachstania* genus [25] based on the multigene sequence analysis of the '*Saccharomyces* complex' (~ 80 species into 14 clades). The phylogenetic relationships (using the D1/D2 LSU rRNA gene sequences) resulted in over 32 species being assigned to this genus [26]; this number is still increasing. Several species of this genus (including *K. aerobia*, *K. gamospora* and *K. servazzii*) are now reported to produce high amounts of floral and fruity compounds in white and red wines when sequentially fermented with *S. cerevisiae* [29–32]. The phylogenetic diversity of the *Kazachstania* genus; [25, 26, 33–36] makes useful assembly and annotation arduous.

Data on the genetic features and physiological properties of the *Kazachstania* genus is scarce in comparison to its closest relative, *S. cerevisiae*; the latter being well characterised as an eukaryotic model organism. Studies by Gordon et al. [20] and Wolfe et al. [21] have reported briefly on the whole-genome approaches of *K. africana* and *K. naganishii*, but with

limited information regarding their genome annotation. With regards to *K. servazzii*, the mitochondrial genome (30.8 kb) was reported by Langkjær et al. [37], from a soil isolate (strain CBS4311; NCBI BioProject accession no.: PRJNA12156). To date, there are only 4 draft (incomplete) genomes publicly available: two isolates from kimchi strain CBA6004 (36 contigs (12.5 Mb); NCBI BioProject accession no.: PRJNA434537), and strain SRCM102023, (91 contigs (12.8 Mb); NCBI BioProject accession no.: PRJNA390859; [38]) and two isolates from soil - UCD13 (12 Mb) and UCD335 (11.8 Mb) (assembled at scaffold level, both under NCBI BioProject accession no.: PRJNA564535) [18].

In our previous studies [31, 32], we explored the fermentative traits and characteristics of *K. aerobia* and *K. servazzii* isolates in both sterile and non-sterile red and white wines. Wines fermented with *Kazachstania* spp. were chemically and sensorially distinct from those that were fermented with *S. cerevisiae* alone. Whilst further evaluation is required in winery-scale fermentations, these species appear to be ideal as potential starter cultures partnered with *S. cerevisiae* as they produce high levels of acetate esters, such as 2-phenylethyl acetate and isoamyl acetate [39, 40]. Sensory analysis of Shiraz wines showed that these compounds were perceived as jammy and fruity flavours when compared with the *S. cerevisiae* fermented wines [32]. Other non-*Saccharomyces* species associated with increased levels of 2-phenylethyl acetate in wines include *H. guillermondii* and *H. osmophila* [41, 42]. Additionally, *Pichia anomala* and *H. guillermondii* increased isoamyl acetate concentrations in mixed fermentations [41]. More recently the increased formation of 2-phenylethyl acetate in *H. vineae* was suggested to be caused by gene duplications of aromatic amino acid aminotransferases (*ARO8* and *ARO9*) and phenylpyruvate decarboxylases (*ARO10*) [23].

The first genes identified as being involved in acetate ester synthesis were alcohol acetyltransferase (AATase) catalysing the formation of esters from acetyl coenzyme A (CoA) and their corresponding alcohols [43]. For example, during alcoholic fermentation 2-phenyl

ethanol and CoA condense to phenylethyl acetate to produce a floral aroma that is reminiscent of roses [44]. It is known that the AATase acetyl coA encoding genes *ATF1* and *ATF2* (paralog of *ATF1*) are responsible for the majority of acetate ester biosynthesis in *S. cerevisiae* [45]. The overexpression of *ATF1* in *S. cerevisiae* significantly increases ester production (10–200 fold) [46]. In contrast the deletion of *ATF1* resulted in a large decrease in ester production [46]. The double deletion of *ATF1* and *ATF2* in *S. cerevisiae* did not form any isoamyl acetate, while lower amounts of 2-phenylethyl acetate were still produced (11% of the parent strain) [45]. When *ATF1* was constitutively expressed in three commercial wine yeasts, the levels of acetate esters increased, including 2-phenylethyl acetate which increased 2–10 fold [46]. For additional information on AATase in *S. cerevisiae* see Sumby et al. [47]. Since the discovery of AATase genes in *S. cerevisiae* orthologues have been identified in several non-*Saccharomyces* species including *Candida glabrata*, *Kluyveromyces lactis*, *Kluyveromyces waltii* [48], and *Hanseniaspora vineae* [23]. Each species has a single AATase orthologue, which is similar to *S. cerevisiae* Atf2 based on the pairwise alignment of AATase orthologous amino acid sequences [48].

Focusing on 2-phenylethyl acetate and isoamyl acetate, two pathways lead to their synthesis in *S. cerevisiae* during fermentation (Fig. 1a). *De novo* synthesis from sugar substrates or the catabolism of branch amino acids. The latter is via the Ehrlich pathway to form fusel alcohols which are then esterified by alcohol acetyltransferase (Fig. 1a). In the case of 2-phenylethyl acetate, L-phenylalanine is the precursor to phenylethyl alcohol and isoleucine and valine, which are precursors to amyl alcohol and isobutanol. The first and the second steps of the Ehrlich pathway are catalysed by amino acid transaminases (Aro8, Aro9, Bat1 and Bat2) and thiamine pyrophosphate (TPP)-dependent decarboxylases (Aro10, Pdc1, Pdc5 and Pdc6) [49, 50]. The final step of the Ehrlich pathway (higher alcohol formation) may

be catalysed by any of the ethanol dehydrogenases (Adh1–5) or by Sfa1 (a formaldehyde dehydrogenase) [51].

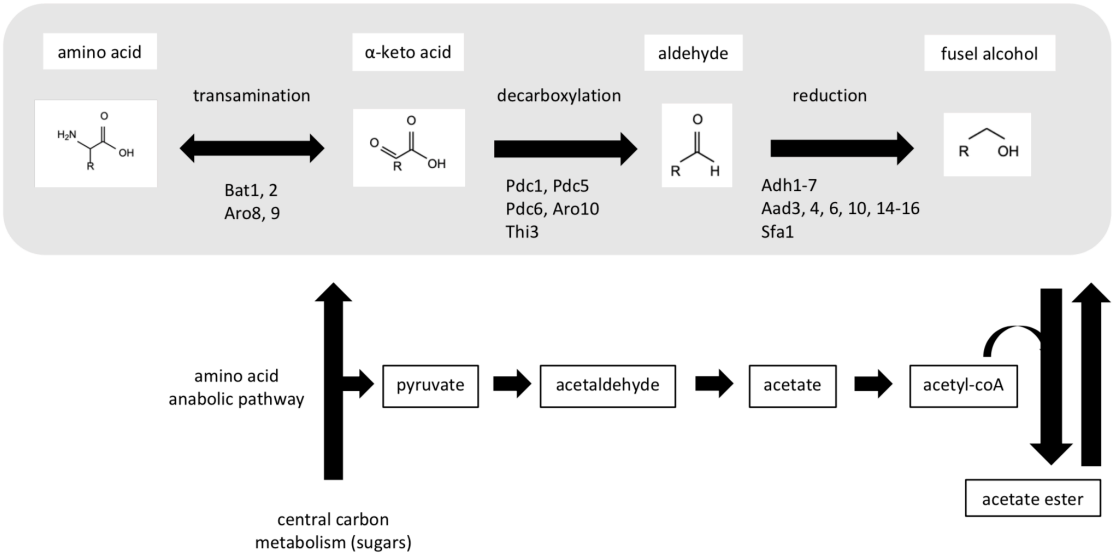


Figure 1a. Enzymes involved in ester production in *S. cerevisiae*. The Ehrlich pathway reactions are shaded grey. Once fusel (higher) alcohols are formed, they can be esterified to the corresponding esters (acetates).

The high levels of acetate ester production (nominally; phenylethyl acetate and isoamyl acetate) by *Kazachstania* spp. could be explained in two ways: 1) species-specific differences in the genes involved in aroma formation, 2) resistance or lack of negative feedback of high levels of phenylethyl acetate and isoamyl acetate. For example, yeasts which are resistant to toxic analogues of phenylalanine show increased production of aromatic alcohols and their corresponding esters [52–54]. Researchers have used toxic analogues of phenylalanine to engineer yeasts with increase phenylalanine metabolism to increase the production of 2-phenylethanol and 2-phenylethlacetate by yeast during saké production [53, 54]. Yeasts that are resistant to toxic analogues of phenylalanine, such as o-fluoro-DL-phenylalanine or p-

fluoro-DL-phenylalanine, displayed changes in the action of phenylalanine-dependent 3-deoxy-D-arabino-heptulosonate-7 phosphate (DAHP) synthase, [53], which catalyses the first step in the production of aromatic amino acids (tryptophan, tyrosine and phenylalanine) via the Shikimate pathway.

3-deoxy-D-arabino-heptulosonate-7 phosphate (DAHP) synthase is now known to be encoded by two genes gene *ARO3* and *ARO4* [55]. The gene *ARO3* is regulated feedback inhibition by phenylalanine with the GCN4 activator protein implicated in both the activation and the basal control of *ARO3* [55]. More recent efforts to understand the genes involves in production of phenylethyl acetate have focused on quantitative trait loci (QTL) analysis [56]. High 2-phenylethyl acetate production was linked to four QTLs and upon further investigation the two causative genes were identified. The first gene *FAS2*, encodes the alpha subunit of the fatty acid synthetase complex and the second was the mutant allele of *TOR1*, which is involved in nitrogen regulation [56]. CRISPR-Cas9 mediated allele exchange of the superior alleles of *TOR1* and *FAS2* in the parent strain increased 2-phenyethyl acetate production by 70% [56].

Other major genes involved in ester biosynthesis in *S. cerevisiae* include the paralogs *EEB1*/*EHT1* [57], which encode an acyl-coA: ethanol O-acyltransferase (an enzyme required to produce medium-chain fatty acid (MCFA) ethyl esters (e.g., ethyl hexanoate, ethyl octanoate, ethyl decanoate)) (Fig. 1b). Ethyl hexanoate, which imparts a fruity flavour (apple-like aroma) in alcoholic beverages is formed by enzyme-catalysed condensation reaction of hexanoic acid and ethanol. Formation of ethyl hexanoate is dependent upon the substrate concentrations and enzymatic activity, as the Eht1 enzyme has the largest contribution to the formation of MCFA ethyl esters [58]. In *Pichia pastoris*, the esterase activity of *EHT1* knockout and overexpression strains were either significantly lower or higher, respectively, which demonstrates the importance of *EHT1* in regulating esterase activity in fermentation products [58]. The major esterase, isoamyl acetate-hydrolysing esterase encoded by *IAH1* (YOR126C) has been cloned

and characterised in *S. cerevisiae* [59]. The authors reported a loss of function of this increased isoamyl acetate (banana aroma) in saké, and concluded that isoamyl acetate accumulation was dependent on the ratio of the esterase and alcohol acetyltransferase [59]. More recently, the alcohol transferase Eat1 was discovered in *Cyberlindnera fabianii*, *K. lactis*, *K. marixanus* and *Wickerhamomyces anomalus*, which was responsible for bulk ethyl acetate production [60, 61]. Two putative homologs were then identified in *S. cerevisiae* (*EAT1* and *IMO32*), with evidence that *EAT1* is responsible for 50% of ethyl acetate production. Thought to be like other AATases that are located in the cytosol, Eat1 is instead located in the yeast mitochondria [62], with the coding region predicted to have a mitochondrial targeting sequence. The mechanism which promotes AATase activity in Eat1 remains elusive, as the crystal structure of the enzyme and its acetyl-coA intermediate are required to study this.

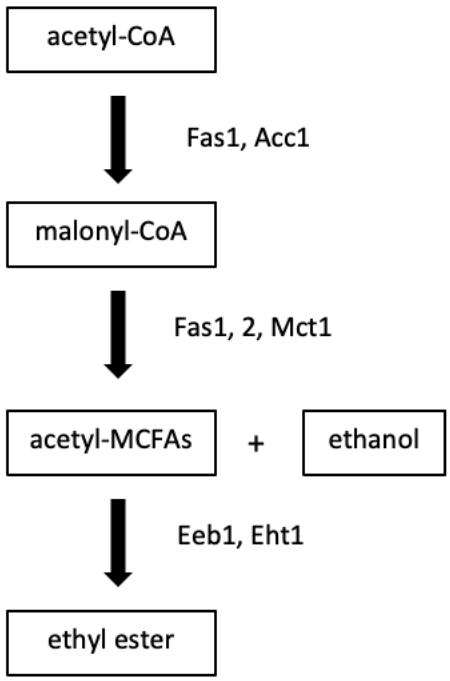


Figure 1b. Enzymes involved in the synthesis of fatty acid ethyl esters.

In this study, we sequenced the genomes of two *Kazachstania* spp. isolates, with the aim of providing an insight into the genomic and metabolic features of *K. aerobia* and *K. servazzii* using data that is readily available on the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) database and the *Saccharomyces* Genome Database (SGD; https://www.yeastgenome.org/). We present the *de novo* sequences and assembly (at contig level) of both isolates using PacBio technology, as well as the analysis of orthologous genes responsible for flavour compounds. Sequencing of wine yeast genomes is the first step towards understand the genetic differences and phenotypic variation between the different *Kazachstania* species suited to winemaking.

2. Materials and methods

2.1 Yeast isolates

Isolates used in this study (Table 1) were grown in YEPD (1% yeast extract, 2% bactopeptone, 2% dextrose (glucose)) from glycerol stocks.

Table 1. *Kazachstania* spp. isolates used in this study. NCBI GenBank accession numbers (ITS sequences) were obtained in a previous study [31].

Species (isolate)	NCBI GenBank accession number (ITS)	NCBI Genome accession number	NCBI BioSample accession number	NCBI BioProject accession number
K. aerobia (PF_8_W29)	MN328365	JAKOOU000000000	SAMN25820612	PRJNA799447
K. servazzii (PF_9_W20)	MN328373	JAKOOT000000000	SAMN25820613	

2.2 DNA extraction

Genomic DNA (gDNA) was extracted using Qiagen Genomic-tip 100/G kit (cat. no. 10243; Qiagen Pty Ltd, Victoria, Australia) according to the manufacturer's instructions for yeast, with minor modifications: 500 µL of Zymolyase™ 20T (MP Biomedicals LLC, Ohio, USA; 1000 U mL^{-1} in distilled water) was added to *Kazachstania* cells (~ 2 x 10^9 cells). The yield and concentration of the eluted DNA was assessed by the NanoDrop™ One spectrophotometer (Thermo Fisher Scientific). DNA purity and integrity were based on calculation of the A_{260}/A_{280} and A_{260}/A_{230} ratios, as well as visually after electrophoresis (0.75% agarose in tris acetate EDTA (TAE) buffer). gDNA samples with an A_{260}/A_{280} ratio of 1.8 – 2.0 (final concentration of 7.5 µg (413.9 (PF_8_W29) and 56.2 (PF_9_W20) ng μL^{-1}) for each sample) were submitted to the South Australia Genomics Centre (SAGC) (Adelaide, Australia) for PacBio sequencing.

2.3 Library preparation and PacBio sequencing

Library preparation and sequencing were performed by the Central Analytical Research Facility (CARF) at the Queensland University of Technology, Australia (subcontracted by SAGC). gDNA samples were checked for sugars (N-acetyl-D-glucosamine) by HPLC using a Shodex OHpak SB-806M HQ (8.0mmI.D. x 300mm) column (https://www.shodex.com/en/dc/03/06/05.html#!). Chitosan, a carbohydrate found in certain fungi, crustaceans and insects can inhibit PacBio sequencing and reduce the number and quality of the reads. 1 µg of each gDNA sample was sheared using a Covaris g-Tube to produce sheared library sizes of 9.5–10 kb. Femto Pulse (Agilent Technologies) was used to confirm the size fragments and concentrations were measured on a Qubit 4.0 fluorometer (Thermo Fisher Scientific). Libraries were then prepared following the protocol in the PacBio Procedure & Checklist – Preparing Multiplexed Microbial Libraries Using SMRTbell® Express Template Prep Kit 2.0 (Pacific Biosciences), with double standard volumes throughout the procedure due

to having a 2-plex library. Beads were used to remove <3 kb SMRTbell templates and the final library size and concentration was measured by Femto Pulse and Qubit for equimolar pooling.

The PacBio Sequel® Binding kit 3.0 was used to bind prepared DNA libraries to the Sequel I system, with the calculations obtained from SMRT Link software (v8.0), sequencing primer v4, 1 h polymerase binding time and 1.2X AMPure® PB beads complex clean-up. Libraries were sequenced on a SMRT Cell 1M v3 LR for 20 h in Continuous Long Read (CLR) mode with 2 h pre-extension, as recommended by PacBio for De Novo Assembly – Microbial Multiplexing.

2.4 *De novo* genome assembly, annotation, gene orthology analysis (flavour compounds)

De novo genome assembly was performed by SAGC (Adelaide, Australia) following these procedures: the raw sequences (subreads.bam (in PacBio BAM files, from zero-mode waveguide (ZMW) hole)) were split into BAM files by sample which includes demultiplexing of barcoded data by using Lima (v.2.0.0). The demultiplexed reads was then converted to CCS/HiFi reads with a minimum predicted accuracy read of 0.99 (default) using pbccs (v6.0.0), and finally converted to fasta format using bam2fastx (v1.3.1). The long read assembler – Flye (v2.8.3) was used to perform *de novo* assembly on the CCS reads with the --pacbio-hifi command line argument.

Gene features were annotated in the genome sequences using *S. cerevisiae* S288C as the reference genome. Protein coding gene models were predicted using both AUGUSTUS (ver. 3.4.0) [63] and the Yeast Genome Annotation Pipeline (YGAP) [64]. For homology-based prediction of transcripts/genes, the S288C open reading frames (ORF) were downloaded from the *Saccharomyces* Genome Database (SGD; https://www.yeastgenome.org/) and Geneious Prime (ver. 2021.0.3) was employed to align the annotated ORFs with the hypothetical protein ORFs. Protein sequences were functionally assigned using InterPro (ver. 87.0) (https://www.ebi.ac.uk/interpro/). A protein BLAST (BLASTp) analysis search (E-value ≤

0.01, gapped alignments, W value = 3) was performed with amino acid sequences of *K. aerobia* and *K. servazzii*, which resulted in the best hit with the two members of the *Kazachstania* genus (*K. africana*, *K. naganishii*) as well as those closely-related species in the *Saccharomycetaceae* family (*Naumovozyma castellii* and *Naumovozyma dairenensis*).

Amino acid sequences of orthologs were used to generate a multiple sequence alignment with Clustal Omega ([65]; https://www.ebi.ac.uk/Tools/msa/clustalo), in order to find conserved regions and important sequences. Orthologous relationships with *S. cerevisiae* strain S288C and the wine strain EC1118 sequences was analysed on OrthoVenn2 (https://orthovenn2.bioinfotoolkits.net/home) [66].

3. Results

3.1 High-quality *de novo* sequencing and genome assemblies of *K. aerobia* and *K. servazzii*

High-quality genome assemblies for *K. aerobia* PF_8_W29 and *K. servazzii* PF_9_W20 were generated from the PacBio Sequel I platform. A total of 16.45 Gbp of raw reads was generated for both isolates, which was subsequently demultiplexed. Out of the initial 612,011 productive ZMWs, 40.9% (250,078) contained reads with one or two barcodes used for the isolates. Following the demultiplexing step, subreads (≥ 470x coverage) were collapsed to generate higher accuracy (≥ 99% base accuracy) Hi-Fi reads, which was subsequently assembled into 12.5 Mb and 12.3 Mb genome for *K. aerobia* PF_8_W29 and *K. servazzii* PF_9_W20 respectively. The sequencing results and assembled contigs (and scaffolds) are summarised in Table 2. The genomes of *K. aerobia* (12.5 Mb) and *K. servazzii* (12.3 Mb) were comparable to the previously published genomes for *K. africana* (11.13 Mb; [20]) and *K. naganishii* (10.84 Mb) and other members of *Saccharomycetaceae* [21, 22].

Table 2. Summary of *K. aerobia* PF_8_W29 and *K. servazzii* PF_9_W20 genome assembly using PacBio (Sequel I) platform.

Metric	*K. aerobia* PF_8_W29	*K. servazzii* PF_9_W20
Scaffold	1 (one gap)	1 (one gap)
Contigs	15	15
Contig N50 (bp)	572,088	578,717
Maximum contig length (bp)	1,231,885	1,131,086
Mitochondrial genome size (kbp)	29.6	29.4
GC (%)	35.8	34.4
Total length (Mb)	12.5	12.3

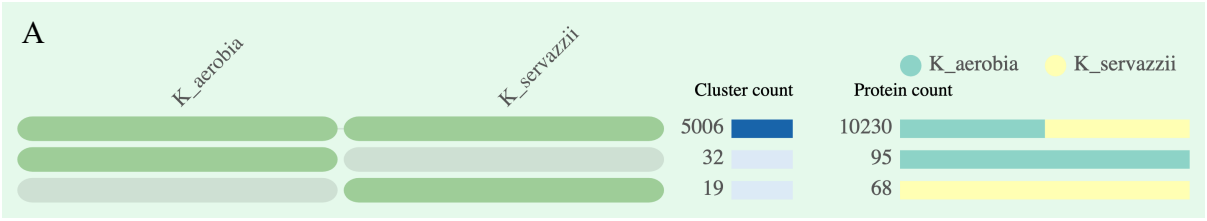
3.2 *K. aerobia* and *K. servazzii* genome prediction and annotation

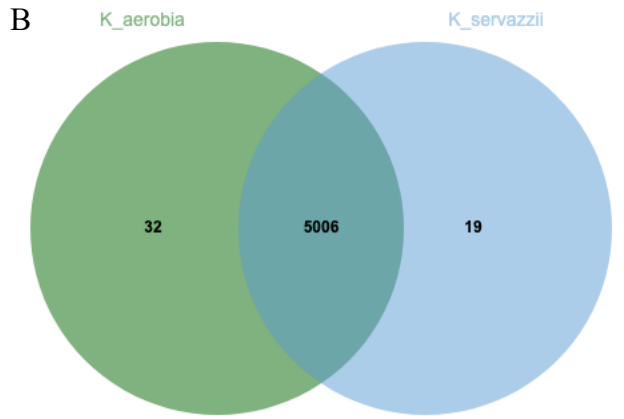
Based on the reference genome of a closely-related species and well annotated *S. cerevisiae* (https://www.yeastgenome.org/), the high-quality *de novo* assembly of *Kazachstania* spp. genomes enabled the prediction of 5425 protein-coding genes for *K. aerobia* PF_8_W29 and 5335 for *K. servazzii* PF_9_W20 using the AUGUSTUS and YGAP programs, of which 4621 and 4550, respectively, were *S. cerevisia*e (S288C) homologues and 804 and 785, respectively, were unique genes. The number of gene annotations are among the highest reported for species of the *Kazachstania* genus, and are only comparable to the annotated *K. africana* and *K. naganishii*, for which 5378 and 5321 protein-coding genes, respectively, were predicted [21].

3.3 Genome comparison (orthologous relationships) between *K. aerobia*, *K. servazzii* and *S. cerevisiae* (S288C and EC1118)

The predicted proteome of *K. aerobia* and *K. servazzii* was assigned into orthologous clusters (along with *S. cerevisiae* (S288C and EC1118)) in an attempt to identify shared and/or unique characteristics between the species. The OrthoVenn2 web server generated comparison

results in tables showing the occurrence of cluster groups between species (left), the number of clusters shared between the species (middle) and the number of protein members (protein count) in the shared clusters (right) (Figs. 1A, 2A and 3A). The OrthoVenn2 software also generated Venn diagrams indicating the number of orthologues shared between the species. In the case of *K. aerobia* (having 5038 clusters) 5006 were shared with *K. servazzii* (5025 total clusters). Thirty-two clusters were unique to *K. aerobia* and 19 clusters to *K. servazzii* (Fig. 1B). When compared to the S288C and EC1118 strains, 4192 clusters were shared between *K. aerobia*, *K. servazzii* and S288C (Fig. 2B), and 4009 clusters shared between *K. aerobia*, *K. servazzii* and EC1118 (Fig. 3B). There were less unique gene clusters identified in *Kazachstania* spp. compared to *S. cerevisiae* (both S288C and EC1118 have 62) (Figs 2B and 3B). The results from Orthovenn2 (Figs. 1C, 2C and 3C) reflected the genome size differences between the two *Kazachstania* spp. as the predicted 5425 proteins and 5038 clusters in *K. aerobia* was higher than that of *K. servazzii*. Similarly, the two *Saccharomyces* strains varied in protein number, with S288C having 5997 proteins (Fig. 2C) and EC1118 having 6017 proteins (Fig. 3C). The additional 20 proteins most likely originate from horizontal gene transfer, as EC1118 has an additional 120 kb sequence not found in S288C [67]. Additionally, the bar plots/graphs revealed the total number of orthologous gene clusters in each species (Figs. 1B, 2B and 3B).





C

Summary

Species	Proteins	Clusters	Singletons
K_aerobia	5425	5038	210
K_servazzii	5335	5025	157

The species form 5057 clusters, 133 orthologous clusters (at least contains two species) and 4924 single-copy gene clusters.

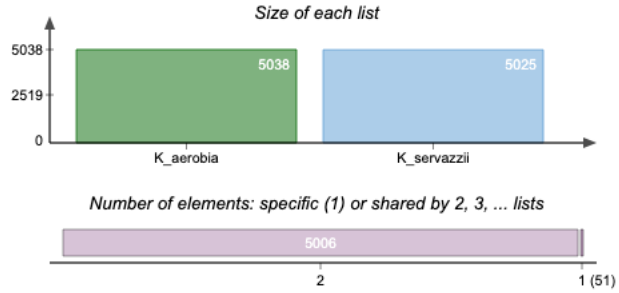
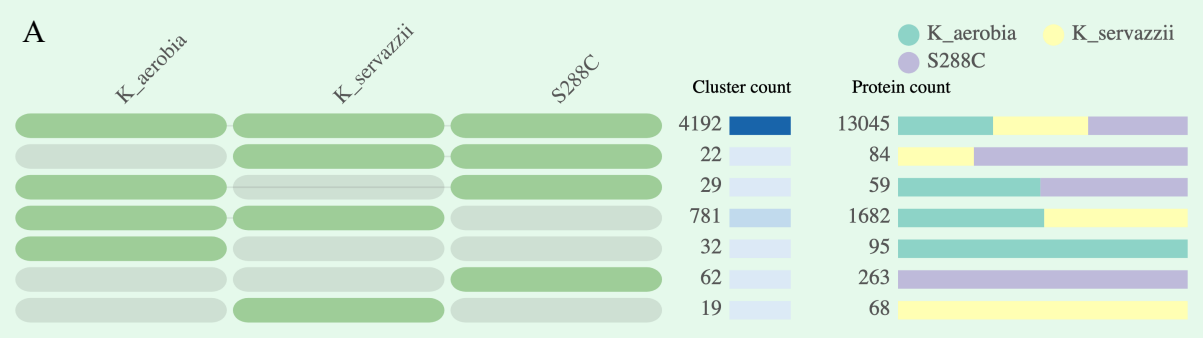
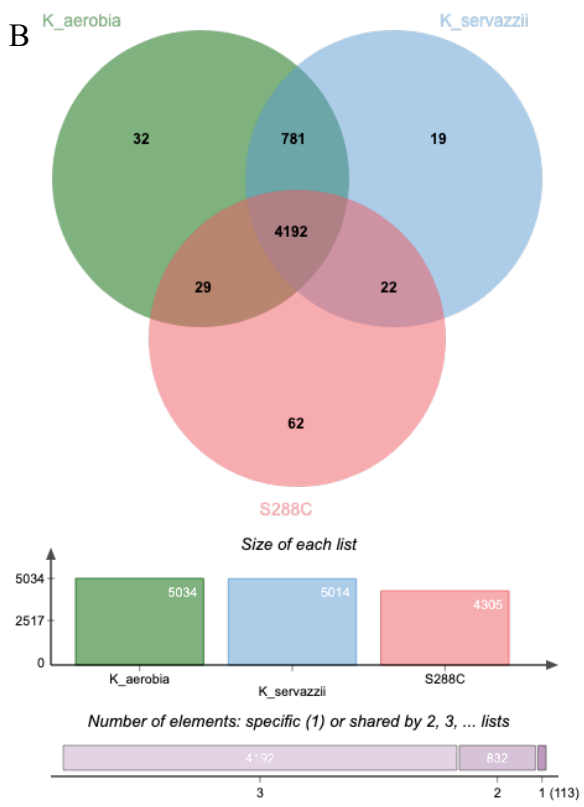


Figure 2. (A) Occurrence table indicating shared orthologous group patterns between *K. aerobia* (PF_8_W29) and *K. servazzii* (PF_9_W20) (B) Venn diagram displaying the shared orthologous cluster distributions among the species (C) Cluster count in each genome. Singletons describe those genes for which no orthologs can be found in other species.





C

Summary

Species	Proteins	Clusters	Singletons
K_aerobia	5425	5034	170
K_servazzii	5335	5014	131
S288C	5997	4305	1160

The species form 5137 clusters, 1217 orthologous clusters (at least contains two species) and 3920 single-copy gene clusters.

Figure 3. (A) Occurrence table indicating shared orthologous group patterns between *K. aerobia* (PF_8_W29), *K. servazzii* (PF_9_W20) and *S. cerevisiae* (S288C) (B) Venn diagram displaying the shared orthologous cluster distributions among the species (C) Cluster count in each genome. Singletons describe those genes for which no orthologs can be found in other species.

Figure 4. (A) Occurrence table indicating shared orthologous group patterns between *K. aerobia* (PF_8_W29), *K. servazzii* (PF_9_W20) and *S. cerevisiae* (EC1118) (B) Venn diagram displaying the shared orthologous cluster distributions among the species (C) Cluster count in each genome. Singletons describe those genes for which no orthologs can be found in other species.

The orthologous clusters of *Kazachstania* spp. and *S. cerevisiae* were also annotated, which assigned the clusters to three main categories in gene ontology (GO): 1) biological process, 2) molecular function and 3) cellular component. The GO analysis/functional information associated with each cluster is provided in Supplementary Table 14. Among the three main categories, GO terms for core orthologous gene clusters were mainly distributed in biological processes in both *Kazachstania* spp., as the most abundant number was associated with enriched biological and metabolic processes (Supplementary Table 14). For the unique genes found in both *Kazachstania* spp., the majority of GO terms were not assigned to *K. aerobia*, and the majority of GO terms of *K. servazzii* was for helicase activity and SRP-dependent co-translational protein targeting to membrane (Supplementary Table 14).

3.4 *In silico* analysis of yeast genes involved in ester (and higher alcohol) biosynthesis

Since there is no information on the genes involved in ester production in *Kazachstania* spp., a list of genes based on *S. cerevisiae* was compiled, with the gene sequences used to search for their orthologues in *Kazachstania* spp. (Table 3). All orthologous amino acid sequences (putative proteins) were identified, except for those of Eat1, Adh2 and Adh4 (Supplementary Tables 1–13; Supplementary Figs 1–12). Two genes encoding for alcohol dehydrogenases (ADHs), *ADH1* and *ADH5* had similar sequences (Supplementary Fig. 4b). Additionally there was only one ortholog (sequence) identified for Eht1 and Eeb1 in *K. aerobia* and *K. servazzii*, which was referred to as Eht1/Eeb1 (Supplementary Table 2; Supplementary Fig. 2).

Table 3. Genes of interest involved/related to flavour compound biosynthesis (esters and higher alcohols.

Gene name	Related flavour compounds	Major function	Cellular compartment
ATF1	Acetate esters	Alcohol acetyl-coA transferase	Lipid droplets
ATF2	Acetate esters	Alcohol acetyl-coA transferase	Endoplasmic reticulum
ARO10	2-Phenylethanol	Phenylpyruvate decarboxylase	Cytoplasm
ARO3	2-Phenylethanol	3-Deoxy-D-arabino-heptulosonate-7-phosphase (DAHP) synthase	Cytoplasm
ARO4	2-Phenylethanol	DAHP synthase	Cytoplasm
ARO7	2-Phenylethanol	Chorismate mutase	Cytoplasm
EAT1	Ethyl acetate	Ethanol acetyl-coA transferase	Mitochondrion
EEB1	Ethyl hexanoate	Ethanol acyl-coA transferase	Unknown
EHT1	Ethyl hexanoate	Ethanol acyl-coA transferase	Lipid droplets/mitochondrion
IAH1	Acetate esters (isoamyl acetate)	Isoamyl acetate-hydrolysing esterase	Cytoplasm
ADH1	Higher alcohols	Alcohol dehydrogenase	Cytoplasm
ADH2	Higher alcohols	Alcohol dehydrogenase	Cytoplasm
ADH3	Higher alcohols	Alcohol dehydrogenase	Mitochondrion
ADH4	Higher alcohols	Alcohol dehydrogenase	Mitochondrion
ADH5	Higher alcohols	Alcohol dehydrogenase	Cytoplasm/nucleus
BAT1	Higher alcohols and other aroma compounds	Branched-chain amino acid transferase	Mitochondrion
BAT2	Higher alcohols and other aroma compounds	Branched-chain amino acid transferase	Cytoplasm
FAS2	Phenylethyl acetate	Fatty acid synthetase	Mitochondrion/cytoplasm
TOR1	Phenylethyl acetate	Phosphatidylinositol kinase (PIK)-related protein kinase	Nucleus/cytoplasm

The identified putative protein sequences were used as queries in BLASTp, where the highest alignment scores showed similarities with orthologues in closely related species within the *Saccharomycetaceae* family (*S. cerevisiae*, *K. africana, K. naganishii*, *N. castellii,* and *N. dairenensis)*. The percentages of the sequence similarity and identity are shown in Supplementary Tables 1 to 13. Of the AATase family (Atf1 and Atf2), both *K. aerobia* and *K. servazzii* revealed only one AATase orthologue, and only showed similarities with *S. cerevisiae* Atf2 (Supplementary Table 1). The AATase of *K. aerobia* and *K. servazzii* have 38.98 and 39.96 % amino acid identity with *S. cerevisiae* Atf2, which also showed less homology when compared to orthologues of *Kazachstania* spp. and *Naumovozyma* spp. (Supplementary Table 1). The rest of the protein sequences displayed relatively similar identities with higher homology across all species (including *S. cerevisiae*), ranging from 50.78 to 88.73% (Supplementary Tables 2 to 13). Interestingly, no hits were found for the Tor1 (PIK family) orthologue in *N. dairenensis* (Supplementary Table 13).

The amino acid sequences of the identified flavour (ester and higher alcohol) orthologues were aligned using Clustal Omega with default parameters, and revealed highly conserved regions between *K. aerobia*, *K. servazzii* and *S. cerevisiae* (Supplementary Figs. 1 to 12). The *Kazachstania* spp. Atf and the *S. cerevisiae* Atf1 and Atf2 orthologs shares two conserved motifs: 1) an H-X-X-X-D catalytic (active) site (*S. cerevisiae* Atf1 residue 19 to 198) and 2) the WRLICLP region (*S. cerevisiae* Atf1 residue 169 to 175) (Supplementary Fig. 1). The conserved GHARRMG sequence was used to search for similar sequences in *Kazachstania* spp. (Supplementary Fig. 1).

4. Discussion

In recent years there has been growing interest in the application of non-*Saccharomyces* yeasts in winemaking, due to their substantial role in producing volatile aroma/flavour compounds that contribute to the uniqueness of the wine. Thus, the positive characteristics and the biodiversity of non-*Saccharomyces* yeasts have been explored, with potential biotechnological and industrial application. Among these yeasts, several fairly novel species belonging to the *Kazachstania* genus, found to modulate the aroma profile in wines, through their metabolic activities (particularly ester biosynthesis), are attracting significant interest [26–29]. However, contrary to *Saccharomyces*, where most genomics studies are conducted in *S. cerevisiae*, and to a lesser extent other members of the *Saccharomycotina*, there is a lack of gene knowledge related to *Kazachstania* spp. genes. Likewise, from a phenotypic perspective, there are no physiological or morphological traits that can accurately describe the *Kazachstania* genus [68]. In this regard, genomic studies allowing for the linkage of genes to traits would be a valuable resource for future biotechnological application [69].

In this study we present the *de novo* whole-genome sequencing of two *Kazachstania* spp. isolates (*K. aerobia* and *K. servazzii*) from spontaneous Shiraz fermentations from the McLaren Vale region of South Australia [31]. Comparison of the whole-genome assemblies of the two species showed similar numbers/values for genome size (~12.4 Mb), GC content (~35 %) and the number of predicted genes (~5380 (average)) (Table 2). These values were expected given the properties of other fully annotated members of the *Kazachstania* genus [20–22]. In general, the predicted protein-coding genes in *K. aerobia* and *K. servazzii* were also comparable to the those reported by Wolfe et al. [21] in *K. africana* and *K. naganishii* (all > 5000), with *K. aerobia* displaying the highest number. Comparative analyses of the inferred proteins among the two species showed that there were more unique gene clusters in *K. aerobia* than *K. servazzii* (Fig. 2). Additionally, when compared with both *S. cerevisiae* strains (S288C

and EC1118) *K. aerobia* shared more orthologous gene clusters than *K. servazzii* (Figs. 2B and 3B). Lastly, *K. aerobia* exhibited the highest diversity of orthologous gene clusters out of the three species, which could be explained as the result of genetic divergence and domestication events (Figs. 2B and 3B).

Amino acid metabolism in yeasts during alcoholic fermentation is responsible for 80% of flavour-active compounds, as their catabolism leads to the production of higher alcohols and their corresponding esters [70]. Of the putative alcohol acetyltransferases (AATase) involved in ester biosynthesis, only one orthologue in the AATase family was found in *Kazachstania* spp., which only had ~38 to 39 % identity to *S. cerevisiae* Atf2 (Supplementary Table 1). As mentioned earlier, *S. cerevisiae* AATase is encoded by two genes, as opposed to distantly related yeast species *C. glabrata*, *K. lactis*, *K. waltii*, *S. castellii* (now *Nauvomozyma castellii*) and *P. anomala* (now *Wickerhamomyces anomalus*) which have only one [48, 71]. The presence of two genes in *Saccharomyces* (*sensu stricto*) species and only one in closely and distantly related species (noted above) may be the result of whole genome duplication (WGD) during the evolution of ascomycete yeasts. The *Kazachstania* genus, along with several genera in the *Saccharomycetaceae* family (*Saccharomyces*, *Nakaseomyces*, and *Tetrapisispora*) went through WGD event (known as the post-WGD clade), which resulted in differential gene loss and gene duplications (the latter being referred to as ohnologs) [72–74]. Though *ATF1* and *ATF2* have similar functions, it is expected that only one orthologue is in pre-WGD species (*K. waltii* and *K. lactis*) as van Laere et al. [48] had suggested that *ATF2* in *S. cerevisiae* had retained its initial function of AATase pre-WGD, while *ATF1* had developed a new function, most likely in anaerobic lipid metabolism. Moreover, the existence of one AATase gene in some post-WGD species could also be explained by reciprocal gene loss after speciation [48].

In the alcohol dehydrogenase family (ADH), seven genes have been identified and characterised in *S. cerevisiae* [75]. Almost every species has at least two *ADH* genes, although numbers vary and are diversified across species. In this study, only two putative genes encoding for ADHs were found in *K. aerobia* and *K. servazzii* (*ADH1* and *ADH3*), with *S. cerevisiae ADH5* sequence being the same as *ADH1* in both *Kazachstania* spp., suggesting gene duplication in the latter species. Crabtree-negative yeasts such as *K. lactis*, being a poor fermentative species [76] have four *ADH* genes: *ADH1* and *ADH2* (which has a similar function to *ADH1* in *S. cerevisiae*), and *ADH3*- and *ADH4*-mitochondrially encoded alcohol dehydrogenase, which possesses reciprocal regulation properties. Recently, ethanol metabolism has been investigated in *Pichia pastoris*, with four ADH genes being identified (*ADH2*, *ADH6*, *ADH7*, *ADH900*) [77]. *ADH900* is the main gene responsible for ethanol production in *P. pastoris*, as *ADH2* plays a minor role in the absence of *ADH900* [77]. In contrast, the duplication of ADH encoding genes and WGD was suggested to be the origin of the Crabtree effect in *Saccharomycetaceae*, which had occurred after the split of WGD yeasts from the *Kluyveromyces* lineage. Species belonging to the post-WGD lineage have a more pronounced Crabtree effect, with increased carbon metabolism at both anaerobic and aerobic conditions [78].

Regarding the biosynthesis of higher alcohols, the branched chain amino acid transaminases (BCAATases) catalyse the transfer of amino groups to α-keto acids, the precursors of higher (fusel) alcohols which influence the aroma and flavour of yeast-derived fermentation products [44, 79]. In *S. cerevisiae*, BCAATases are encoded by two paralogous genes: *BAT1* and *BAT2* that arose through a WGD event, as each perform different functions since Bat1 is mitochondrially located while Bat2 is cytosolic [79]. Both Bat1 and Bat2 orthologs were identified in *K. aerobia* and *K. servazzii*, as they both have high sequence similarity with the orthologues in closely-related species (Supplementary Tables 10 and 11).

As *Kazachstania* spp. are high producers of acetate esters, in particular phenylethyl acetate [31, 32], the *in silico* analysis for the set of *ARO* genes (*ARO3*, *4*, *7* and *10*) involved in the Ehrlich pathway and the biosynthesis of 2-phenylethanol showed highly conserved sequences between *Kazachstania* spp. and *S. cerevisiae* (Supplementary Figs. 6 – 9). The putative orthologues for *FAS2* and *TOR1* involved in the production of phenylethyl acetate (esterified from phenylethyl alcohol) in *S. cerevisiae* were also identified in *Kazachstania* spp., with high sequence similarity (Supplementary Tables 12 and 13). Though AATases are primarily responsible for the production of acetate esters, the high production of phenylethyl acetate in *Kazachstania* spp. could be explained by the presence of the *TOR1* and *FAS2* genes.

In conclusion, these data contribute to and provide a good starting point to better understand the *Kazachstania* spp. genomes and their potential usefulness in winemaking and other applications (transcriptomic and metabolomic studies). While the exact function of these putative orthologous genes is unknown, further comparative functional genomics studies is required to characterise these genes and their genetic context.

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Conflicts of interest

The authors declare there are no conflicts of interest regarding the study

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Supplementary Material

Title: Whole-genome analysis of two *Kazachstania* spp. isolates from grape must fermentations: identification of genes related to acetate ester production

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Supplementary Table 1. Protein sequence similarity and identity of *K. aerobia* Atf and *K. servazzii* Atf and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Atf		*K. servazzii* Atf	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Atf2 YGR177c	60	39.96	60	38.78
K. africana hypothetical protein KAFR_0D01730	68	47.40	71	50.60
K. naganishii hypothetical protein KNAG_0H02650	67	47.57	66	47.09
N. castellii (CBS 4309) hypothetical protein NCAS_0A06920	61	42.80	61	43.03
N. dairenensis (CBS 421) hypothetical protein NDA_0D01980	59	39.52	60	39.51

Supplementary Table 2. Protein sequence similarity and identity of *K. aerobia* Eht1/Eeb1 and *K. servazzii* Eht1/Eeb1 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Eht1/Eeb1		*K. servazzii* Eht1/Eeb1	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Eht1 YBR177c	77	60.73	78	59.66
S. cerevisiae Eeb1 YPL095c	68	50.78	69	54.78
K. africana hypothetical protein KAFR_0I000680	76	61.74	77	60.43
K. naganishii hypothetical protein KNAG_0A03490	78	65.30	78	64.84
N. castellii (CBS 4309) hypothetical protein NCAS_0A03490	76	61.59	76	60.65
N. dairenensis (CBS 421) hypothetical protein NDA_0D01980	76	60.56	76	60.34

Supplementary Table 3. Protein sequence similarity and identity of *K. aerobia* Iah1 and *K. servazzii* Iah1 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Iah1		*K. servazzii* Iah1	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Iah1 YOR126C	70	50	71	46.67
K. africana hypothetical protein KAFR_0D05100	71	51.29	71	47.41
K. naganishii hypothetical protein KNAG_0B04160	73	52.56	72	50
N. castellii (CBS 4309) hypothetical protein NCAS_0H02130	73	46.81	69	44.68
N. dairenensis (CBS 421) hypothetical protein NDAI_0C01520	70	47.60	68	45.41

Supplementary Table 4. Protein sequence similarity and identity of *K. aerobia* Adh1 and *K. servazzii* Adh1 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Adh1		*K. servazzii* Adh1	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Adh1 YOL068C	91	84.64	90	84.64
K. africana hypothetical protein KAFR_0G01420	94	88.73	94	89.31
K. naganishii hypothetical protein KNAG_0K02000	94	87.50	93	87.79
N. castellii (CBS 4309) hypothetical protein NCAS_0I02350	93	86.05	93	86.63
N. dairenensis (CBS 421) hypothetical protein NDAI_0A06930	92	86.30	92	86.59

Supplementary Table 5. Protein sequence similarity and identity of *K. aerobia* Adh3 and *K. servazzii* Adh3 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Adh3		*K. servazzii* Adh3	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Adh3 YMR083W	89	81.94	88	82.50
K. africana hypothetical protein KAFR_0A00790	89	79.72	89	80
K. naganishii hypothetical protein KNAG_0E02590	92	86.97	92	88.03
N. castellii (CBS 4309) hypothetical protein NCAS_0A07780	88	81.38	88	82.18
N. dairenensis (CBS 421) hypothetical protein NDAI_0H02160	90	83.20	91	83.20

Supplementary Table 6. Protein sequence similarity and identity of *K. aerobia* Aro3 and *K. servazzii* Aro3 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Aro3		*K. servazzii* Aro3	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Aro3 YDR035W	92	84.41	93	84.41
K. africana hypothetical protein KAFR_0A01020	91	84.45	91	84.45
K. naganishii hypothetical protein KNAG_0H01240	91	84.14	91	83.33
N. castellii (CBS 4309) hypothetical protein NCAS_0A10560	93	87.63	93	87.10
N. dairenensis (CBS 421) hypothetical protein NDAI_0H05730	93	86.56	94	87.10

Supplementary Table 7. Protein sequence similarity and identity of *K. aerobia* Aro4 and *K. servazzii* Aro4 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Aro4		*K. servazzii* Aro4	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Aro4 YBR249C	91	85.41	91	83.42
K. africana hypothetical protein KAFR_0AG03630	92	86.03	92	84.43
K. naganishii hypothetical protein KNAG_0M00510	90	83.83	89	82.11
N. castellii (CBS 4309) hypothetical protein NCAS_0H01150	93	85.48	93	83.61
N. dairenensis (CBS 421) hypothetical protein NDAI_0F02150	92	86.68	92	84.93

Supplementary Table 8. Protein sequence similarity and identity of *K. aerobia* Aro7 and *K. servazzii* Aro7 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Aro7		*K. servazzii* Aro7	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Aro7 YPR060C	87	76.26	87	76.26
K. africana hypothetical protein KAFR_0H00990	88	76.06	89	75.68
K. naganishii hypothetical protein KNAG_0H00740	87	71.60	87	70.82
N. castellii (CBS 4309) hypothetical protein NCAS_0A11070	88	76.17	88	75.78
N. dairenensis (CBS 421) hypothetical protein NDAI_0A05230	88	75.42	88	75.85

Supplementary Table 9. Protein sequence similarity and identity of *K. aerobia* Aro10 and *K. servazzii* Aro10 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Aro10		*K. servazzii* Aro10	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Aro10 YDR380W	75	58.71	76	57.49
K. africana hypothetical protein KAFR_0E03730	77	61.75	77	60.65
K. naganishii hypothetical protein KNAG_0C04790	79	62.34	79	62.46
N. castellii (CBS 4309) hypothetical protein NCAS_0A11830	76	60	76	60.16
N. dairenensis (CBS 421) hypothetical protein NDAI_0A04450	78	59.96	78	59.75

Supplementary Table 10. Protein sequence similarity and identity of *K. aerobia* Bat1 and *K. servazzii* Bat1 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Bat1		*K. servazzii* Bat1	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Bat1 YHR208W	84	76.34	86	79.26
K. africana hypothetical protein KAFR_0B07030	87	79.59	88	80.53
K. naganishii hypothetical protein KNAG_0M00140	88	79.79	88	79.69
N. castellii (CBS 4309) hypothetical protein NCAS_0J02240	88	78.44	89	78.34
N. dairenensis (CBS 421) hypothetical protein NDAI_0D03470	90	81.40	89	80.11

Supplementary Table 11. Protein sequence similarity and identity of *K. aerobia* Bat2 and *K. servazzii* Bat2 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Bat2		*K. servazzii* Bat2	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Bat2 YJR148W	86	76.08	85	75.40
K. africana hypothetical protein KAFR_0E04480	89	79.41	89	79.62
K. naganishii hypothetical protein KNAG_0M00140	86	77.96	85	77.27
N. castellii (CBS 4309) hypothetical protein NCAS_0J02240	89	81.02	89	79.89
N. dairenensis (CBS 421) hypothetical protein NDAI_0D03470	89	78.02	89	77.87

Supplementary Table 12. Protein sequence similarity and identity of *K. aerobia* Fas2 and *K. servazzii* Fas2 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Fas2		*K. servazzii* Fas2	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Fas2 YPL231W	89	81.16	89	80.96
K. africana hypothetical protein KAFR_0F01200	90	81.54	89	81.39
K. naganishii hypothetical protein KNAG_0D00890	88	81.46	88	81.25
N. castellii (CBS 4309) hypothetical protein NCAS_0G01260	90	81.67	90	81.47
N. dairenensis (CBS 421) hypothetical protein NDAI_0F01390	89	81.66	89	81.34

Supplementary Table 13. Protein sequence similarity and identity of *K. aerobia* Tor1 and *K. servazzii* Tor1 and orthologous proteins found in closely related species of the *Saccharomycetaceae* family.

	K. aerobia Tor1		*K. servazzii* Tor1	
	% similarity	% identity	% similarity	% identity
S. cerevisiae Tor1 YJR066W	77	61.19	77	61.41
K. africana hypothetical protein KAFR_0A08100	80	63.99	80	64.39
K. naganishii hypothetical protein KNAG_0B00160	82	69.06	82	68.48
N. castellii (CBS 4309) hypothetical protein NCAS_0G03440	96	60.71	77	60.62
N. dairenensis (CBS 421)	0	0	0	0

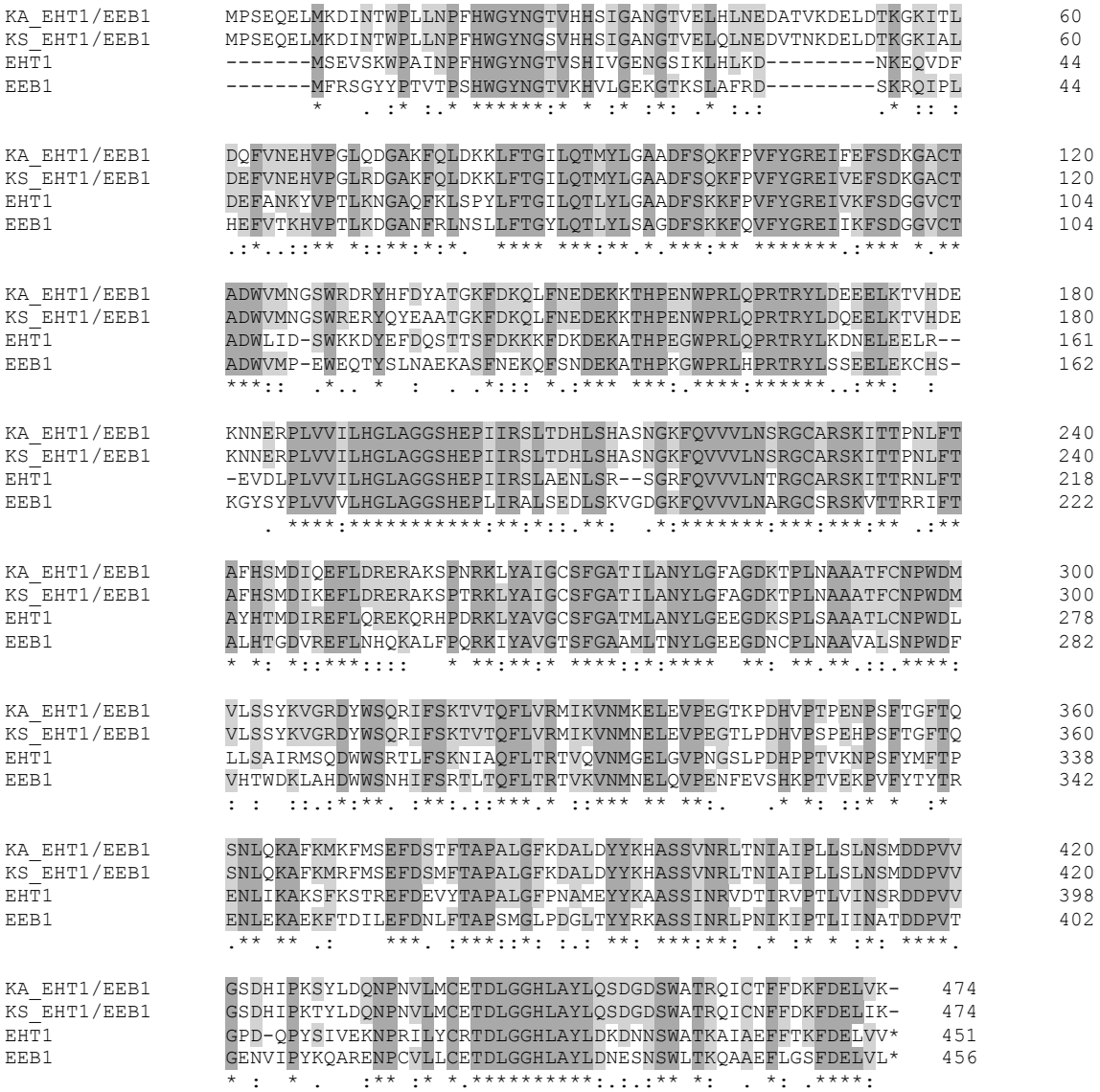
Supplementary Table 14 is available at Figshare:
https://figshare.com/s/190769c1660b8b224faa

Supplementary Figure 1. Multiple alignment of the amino acid sequences of Atf1 and Atf2 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background. The conserved residues (H-X-X-X-D), a possible catalytic site is highlighted in light blue. The conserved region of Atf1 and Atf2 WRLICLP is highlighted in yellow.

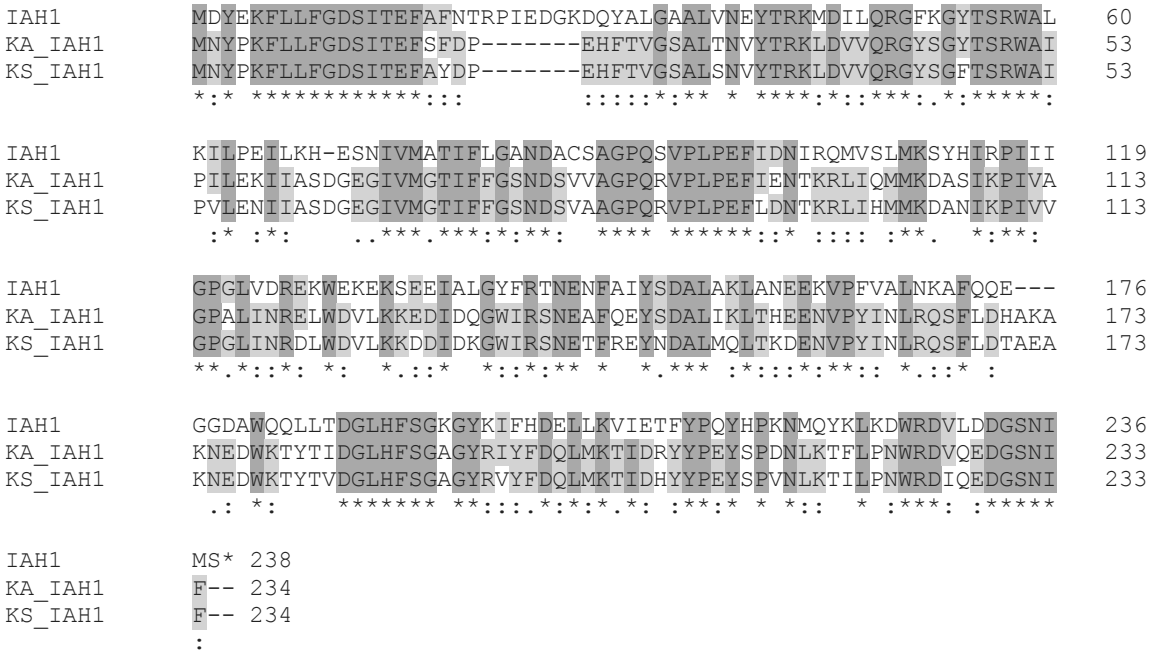
```
ATF1        MNEIDE------KNQAPVQQECLKEMIQNGHARRMGSVEDLYVALNRQNLYRNFCTYGEL    54
ATF2        ----------MEDIEGYEPHITQELIDRGHARRMGHLENYFAVLSRQKMYSNFTVYAEL    49
KA_ATF      MSVKDLEQENVIEHLLNESDGIDESMLERGHARRMGHLENYFALLQRQDLYGNFSCYCEY    60
KS_ATF      MSVKVHKQEEVVERLLKETDGIDDNMLERGHARRMGHLENYFALLQRQDLYGNFSCYCEY    60
                       :          ..::::******* :*: :. *.**.:* **  * *

ATF1        SDYCTRDQLTLALREICLKNPTLLHIVLPTRWPNHENYYRSSEYYSRPHPVHDYISVLQE    114
ATF2        NKGVNKRQLMLVLKVLLQKYSTLAHTIIPKHYPHHEAYYSSEEYLSKPFPQHDFIKVISH    109
KA_ATF      DSSISVDRLAPVLREIFFKHPILVHTIIPKNYPNHESFYLDKEYLEQPYPEHDFIKVIPK    120
KS_ATF      DSSIDVNKLAPILREIFFKHPILVHTIIPKNYPNHESFYLDKEYLEQPYPEHDFIKVIPK    120
            ..     :*   *: :  *   * * ::*..:*:** :* ..** .:*.* **:*.*: .

ATF1        LKLSGVVLNEQPEYSAVMKQILEEFKNSKGSYTAKIFKLTTTLTIPYFGPTGPSWRLICL    174
ATF2        LEFDDLIMNNQPEYREVMEKISEQFKKDDFKVTNRLIELISPVIIPLGNPKRPNWRLICL    169
KA_ATF      LHLDDIIINNQEEYKDIISSIVEQFQNDKFEITEQLTEKVSKIRIPVCHPTKPNWRLLLL    180
KS_ATF      LHLNDIVINNQEEYKDIVSSIMDQFQKDKFEITEQLTEKVSQIRIPVCHSTKPNWRLLLL    180
            *.:..:::*:* **  ::..* ::*::.. . * :: :  : : **    . *.***: *

ATF1        PEEH---TEKWKKFIFVSNHCMSDGRSSIHFFHDLRDELNNIKTP-------PKKLDYIF    224
ATF2        PGKDTDGFETWKNFVYVTNHCGSDGVSGSNFFKDLALLFCKIEEKGFDYDEEFIEDQVII    229
KA_ATF      PEGD--DQTKLKHIVYISNHCSSDATSGINLFKDIAEGLSQEDVA-----SSDANTSLIY    233
KS_ATF      PEND--DYSKLMHIVYISNHCSSDATSGINLFKDIAEGLSFEDIT-----PSDGNDPLIY    233
            *   .     .  ::::::*** **. *. ::*:*:   :   .          :   *

ATF1        KYEEDYQLLRKLPEPIEKVIDFRPPYLFIPKSLLSGFIYNHLRFSSKGVCMR---MDDVE    281
ATF2        DYDRDYTEISKLPKPITDRIDYKPALTSLPKFFLTTFIYEHCNFKTSSESTLTARYSPSS    289
KA_ATF      DYEIDYEKFVRIPIPITERIDYRPGMVAMGKFIGTTMIMNYLTFKFKDSQTAKI--KE--    289
KS_ATF      DYELDHEKFSRIPVPITERIDYRPGMVAMGKFIGTTMVMNYLTFKFKDSQTAKI--KE--    289
            .*: *:  : ::* ** . **::*    : * : : :: ::  *. ..        .

ATF1        KTDDVVTEIINISPTEFQAIKANIKSNIQGKCTITPFLHVCWFVSLHKWGKFFKPLNFEW    341
ATF2        NANASYNYLLHFSTKQVEQIRAQIKKNVHDGCTLTPFIQACFLVALYRLDKLFTKSLLEY    349
KA_ATF      DLRENFHYNLNISWEELTNLKLV---LLQHQSSITGFLQACLFIVLAEQGIFKEKKWNEM    346
KS_ATF      DLRQNYHYNLNITWDELTSLKLI---LLKHESSITGFLQACLFITLTEQGIFKDKKWNEM    346
            .        ::::  :.  ::      ::  .::* *::.* :: * . . :      *

ATF1        LTDIFIPADCRSQLPDDDEMRQMYRYGANVGFIDFTPWISEFDMND-NKENFWPLIEHYH    400
ATF2        GFDVAIPSNARRFLPNDEELRDSYKYGSNVGGSHYAYLISSFDIPEGDNDKFWSLVEYYY    409
KA_ATF      GFDMSIPNDNRKNLPAE-LVEEQYKYGSNVGGSHYSFLLSSFK-----RDQLWELSKYYT    400
KS_ATF      GFDMSIPNDNRKNLPSE-LVEQQYKYGSNVGGSHYSFLLSSFK-----RDQLWELSKYYT    400
              *: ** : *  ** :  :.: *:**:***  .::  :*.*.     .:::* * ::*

ATF1        EVISEALRNKKHLHGLGFNIQGFVQKYVNIDKVMCDRAIGKRRGGTLLSNVGLFNQLEEP    460
ATF2        DRFLESYDNGDHLIGLGVLQLDFIVENKNIDSLLANSYLHQQRGGAIISNTGLVSQDT--    467
KA_ATF      SV----IRNADYNVGLGTLMLDMVYKKQNVDKIISESYLGNQRGGIILSNIGLHEHKG--    454
KS_ATF      NV----IKNADYNVGLGTLMLDMVYKKQNVDKIISESYLGNKRGGIILSNIGLHQHKG--    454
            .       * .:  ***    .:: :  *:*.::.:  : ::*** ::** ** .:

ATF1        DAKYSICDLAFGQFQGSWHQAFSLGVCSTNVKGMNIVVASTKNVVGSQESLEELCSIYKA    520
ATF2        TKPYYVRDLIFSQSAGALRFAFGLNVCSTNVNGMNMDMSVVQGTLRDRGEWESFCKLFYQ    527
KA_ATF      --GIGIKDLKFVQDVGALNFALVVNACSTKTKGMNICISGVEGTIGDREQFTSTGDALKS    512
KS_ATF      --GIGIQDLKFVQDVGALNFALVVNACSTKMKGMNICMSGIEGTIGDREQFNSTGDALKA    512
                 : ** * *  *: . *: :..***: :***: ::  :..: .: .  .  .

ATF1        LLLGP*---     525
ATF2        TIGEFASL*     535
```

```
KA_ATF      LIQEYCK--     519
KS_ATF      LIHEYCK--     519
             :
```

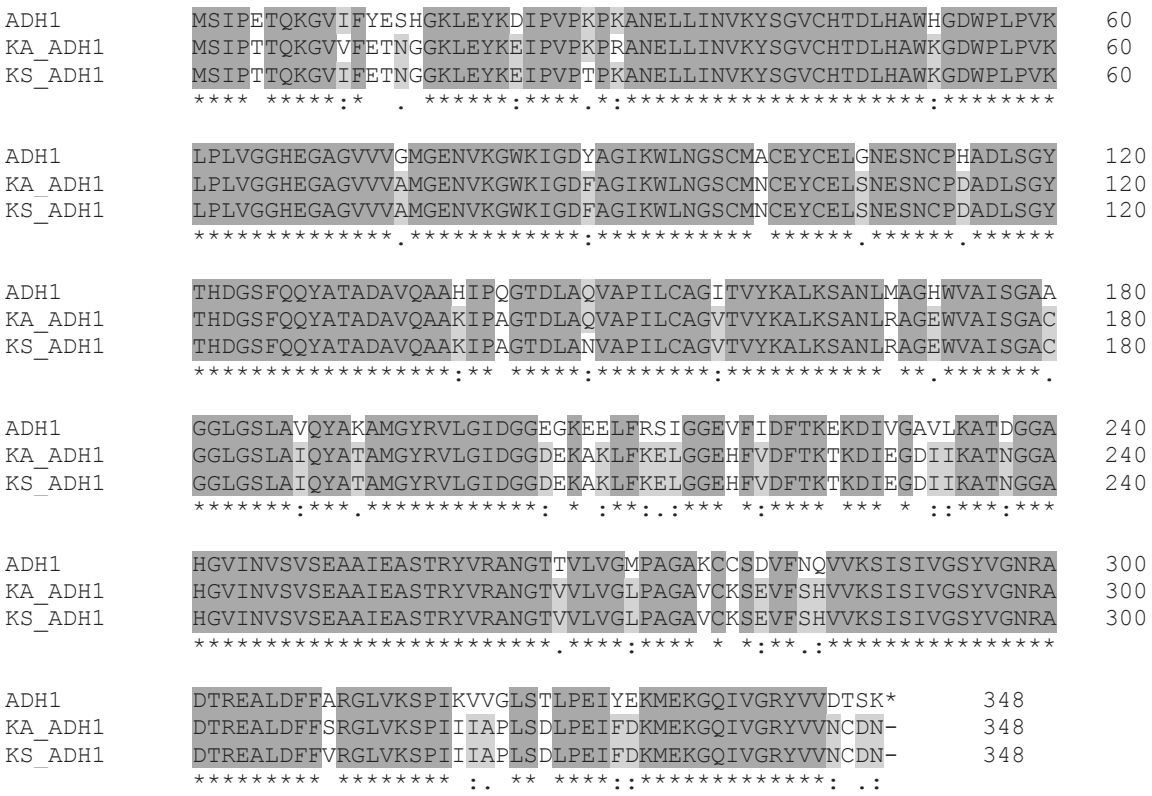
Supplementary Figure 2. Multiple alignment of the amino acid sequences of Eht1 and Eeb1 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.



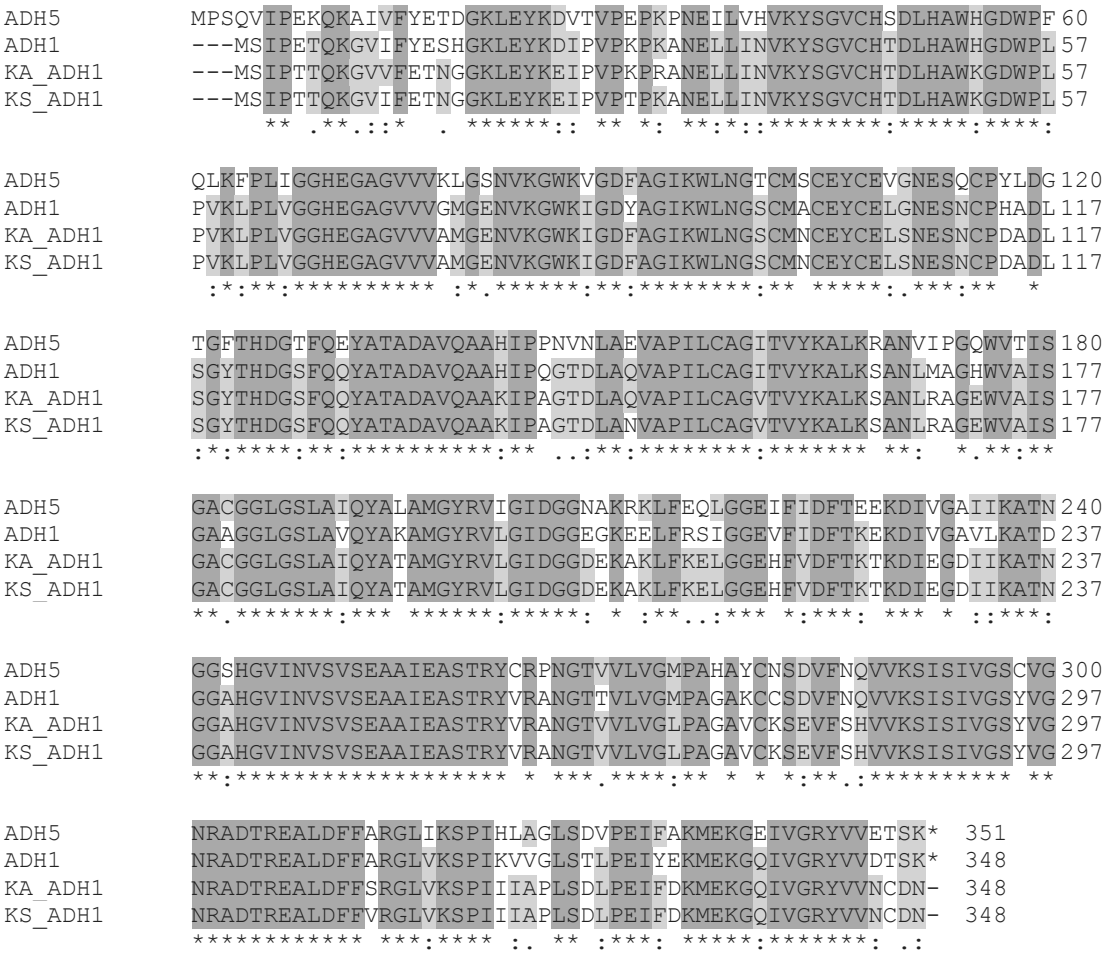
Supplementary Figure 3. Multiple alignment of the amino acid sequences of Iah1 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. ((*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.



Supplementary Figure 4a. Multiple alignment of the amino acid sequences of Adh1 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.



Supplementary Figure 4b. Multiple alignment of the amino acid sequences of Adh1 and Adh5 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.



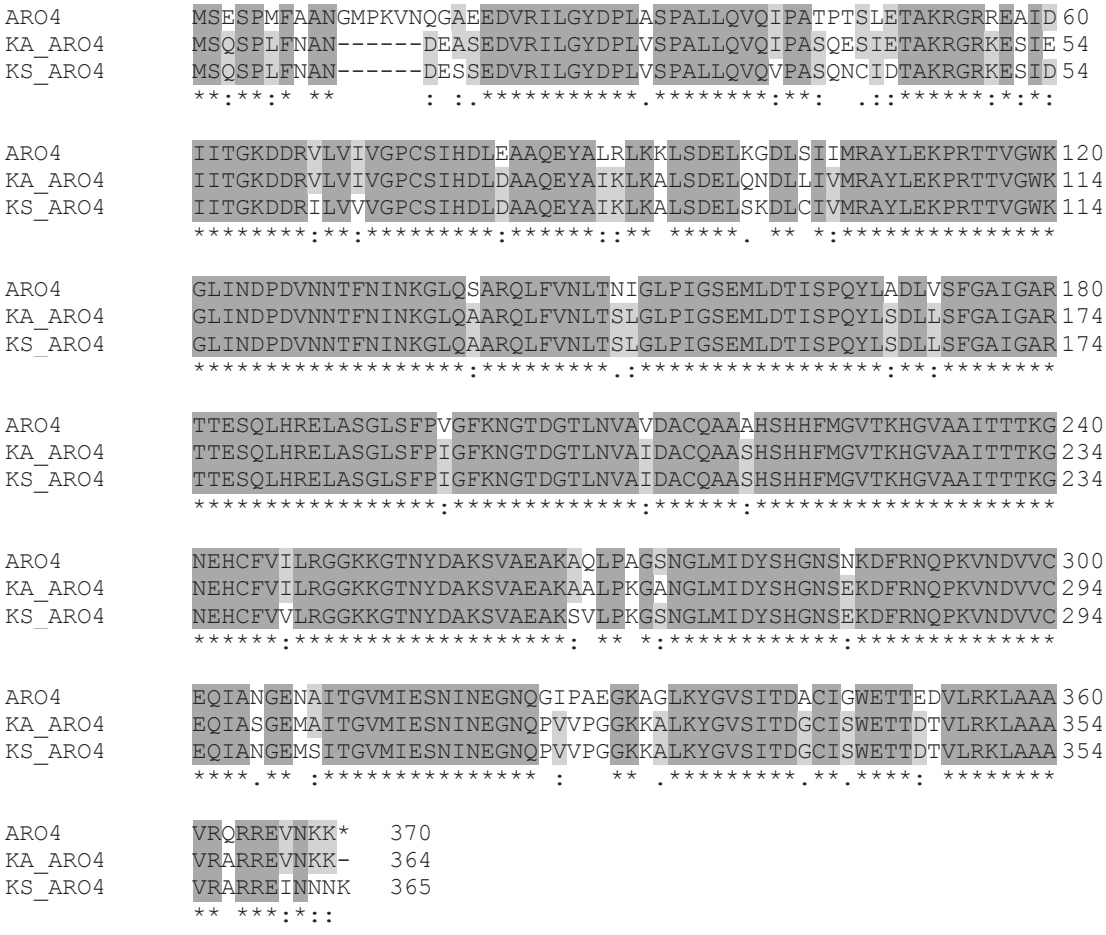
Supplementary Figure 5. Multiple alignment of the amino acid sequences of Adh3 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.

```
ADH3            MLRTSTLFTRRVQPSLFSRNILRLQSTAAIPKTQKGVIFYENKGKLHYKDIPVPEPKPNE   60
KA_ADH3         MLKLITQH--QTVARAGFKQFSRLQSTFTIPSTQKGVVFYEHGGELHYKDIPVPKPKPNE   58
KS_ADH3         MLKLITQH--QTVARSGFKQFSRLQSTFTIPSTQKGVIFYEHGGQLHYKDIPVPKPKPNE   58
                **:  * .  :.      ::: ***** :**.*****:***: *:*********:*****

ADH3            ILINVKYSGVCHTDLHAWHGDWPLPVKLPLVGGHEGAGVVVKLGSNVKGWKVGDLAGIKW   120
KA_ADH3         ILINVKYSGVCHTDLHAWKGDWPLPVKLPLVGGHEGAGVVVAKGENVKNFKIGDLAGIKW   118
KS_ADH3         ILINVKYSGVCHTDLHAWKGDWPLPVKLPLVGGHEGAGVVVAKGENVTNFKIGDLAGIKW   118
                ******************:**********************  *.**..:*:********

ADH3            LNGSCMTCEFCESGHESNCPDADLSGYTHDGSFQQFATADAIQAAKIQQGTDLAEVAPIL   180
KA_ADH3         LNGSCMSCELCESGHESNCKQADLSGYTHDGSFQQYATADAVQAAKIQPGTNLAEVAPVL   178
KS_ADH3         LNGSCMSCELCESGHESNCEHADLSGYTHDGSFQQYATADAVQAAKIQPGTNLAEVAPVL   178
                ******:**:********* .**************:*****:****** **:******:*

ADH3            CAGVTVYKALKEADLKAGDWVAISGAAGGLGSLAVQYATAMGYRVLGIDAGEEKEKLFKK   240
KA_ADH3         CAGITVYKAIKEANLRPGQWICISGAAGGLGSLAVQYAKCMGLRVIGIDGGPGKKELFES   238
KS_ADH3         CAGITVYKAIKEANLRPGQWICISGAAGGLGSLAVQYAKCMGLRVLGIDGGPGKKELFES   238
                ***:*****:***:*: *:*:.***************..** **:***.*  *::**:.

ADH3            LGGEVFIDFTKT---KNMVSDIQEATKGGPHGVINVSVSEAAISLSTEYVRPCGTVVLVG   297
KA_ADH3         LGGETFIDFTKYKEPKDMVRAIQDATKGGPHGVVNVSVSEAAISLSTEYVRACGTVVLVG   298
KS_ADH3         LGGECFIDFTKHKDPKDMVRAIQDATKGGPHGVINVSVSEAAISLSTEYVRACGTVVLVG   298
                **** ******    *:**  **:*********:***************** ********

ADH3            LPANAYVKSEVFSHVVKSINIKGSYVGNRADTREALDFFSRGLIKSPIKIVGLSELPKVY   357
KA_ADH3         LPAHSVVKSNVFSHVVKSINIKGSYVGNRADTREALDFFSRGLIKSPIKVVGLSELPKVY   358
KS_ADH3         LPAHSIVKSDVFSHVVKSINIKGSYVGNRADTREALDFFSRGLIKSPIKIVGLSELPKVY   358
                ***:: ***:**************************************:**********

ADH3            DLMEKGKILGRYVVDTSK* 375
KA_ADH3         ELMEAGKILGRYVVDTSK- 376
KS_ADH3         ELMEAGKILGRYVVDTAK- 376
                :*** ***********:*
```

Supplementary Figure 6. Multiple alignment of the amino acid sequences of Aro3 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.

```
ARO3        MFIKNDHAGDRKRLEDWRIKGYDPLTPPDLLQHEFPISAKGEENIIKARDSVCDILNGKD   60
KA_ARO3     MFIKNEHAGNRKRLEDWRIKGYDPLTPPDLLQHEYPISEQGEKNIVEAREGVCKVLNGED   60
KS_ARO3     MFIKNEHAGNRKRLEDWRIKGYDPLTPPDLLQHEYPISEQGEKHIVEAREGVCKVLNGED   60
            *****:***:*************************:*** :**::*::**:.**.:***:*

ARO3        DRLVIVIGPCSLHDPKAAYDYADRLAKISEKLSKDLLIIMRAYLEKPRTTVGWKGLINDP   120
KA_ARO3     DRLVIVIGPCSIHDPQAAYEYCDRLQKISQKLSGDLLIIMRAYLEKPRTTVGWKGLINDP   120
KS_ARO3     DRLVIVIGPCSIHDPQAAYEYCDRLQKISQKLSGDLLIIMRAYLEKPRTTVGWKGLINDP   120
            ***********:***:***:*.*** ***:*** *************************

ARO3        DMNNSFQINKGLRISREMFIKLVEKLPIAGEMLDTISPQFLSDCFSLGAIGARTTESQLH   180
KA_ARO3     DIDNSFQINKGLRISREMFTKLVEKLPIAGEMLDTISPQFLSDCFSLGAIGARTTESQLH   180
KS_ARO3     DIDNSFQINKGLRISREMFTKLVEKLPIAGEMLDTISPQFLSDCFSLGAIGARTTESQLH   180
            *::**************** ****************************************

ARO3        RELASGLSFPIGFKNGTDGGLQVAIDAMRAAAHEHYFLSVTKPGVTAIVGTEGNKDTFLI   240
KA_ARO3     RELASGLSFPIGFKNGTDGGLQVAIDAMRAAAHEHYFLSVTKPGITAIVGTEGNADTFII   240
KS_ARO3     RELASGLSFPIGFKNGTDGGLQVAIDAMRAAAHEHYFLSVTKPGITAIVGTEGNADTFII   240
            ********************************************:********* ***:*

ARO3        LRGGKNGTNFDKESVQNTKKQLEKAGLTDD--SQKRIMIDCSHGNSNKDFKNQPKVAKCI   298
KA_ARO3     LRGGKNGTNFDAESVKSAKDQLAKANLLDAEGKKRRIMIDCSHGNSNKDYRNQPKVAQTI   300
KS_ARO3     LRGGKNGTNFDAESVKSAKEQLLKANLLDTEGKKRRIMIDCSHGNSDKDFRNQPKVAQTI   300
            *********** ***:.:*.** **.* *   .::***********:**::******: *

ARO3        YDQLTEGENSLCGVMIESNINEGRQDIPKEGGREGLKYGCSVTDACIGWESTEQVLELLA   358
KA_ARO3     YDQLVAGENSLCGVMIESNLVEGRQDVPPEGGRAGLKYGCSITDACIGWDSTEDVLELLA   360
KS_ARO3     YDQLVAGENSLCGVMIESNLVEGRQDVPPEGGRAGLKYGCSITDACIGWESTEDVLELLA   360
            ****. *************: *****:* **** *******:*******:***:******

ARO3        EGVRNRRKALKK*       370
KA_ARO3     EGVRKRRTILQK-       372
KS_ARO3     EGVRKRRSILQK-       372
            ****:**. *:*
```

Supplementary Figure 7. Multiple alignment of the amino acid sequences of Aro4 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.



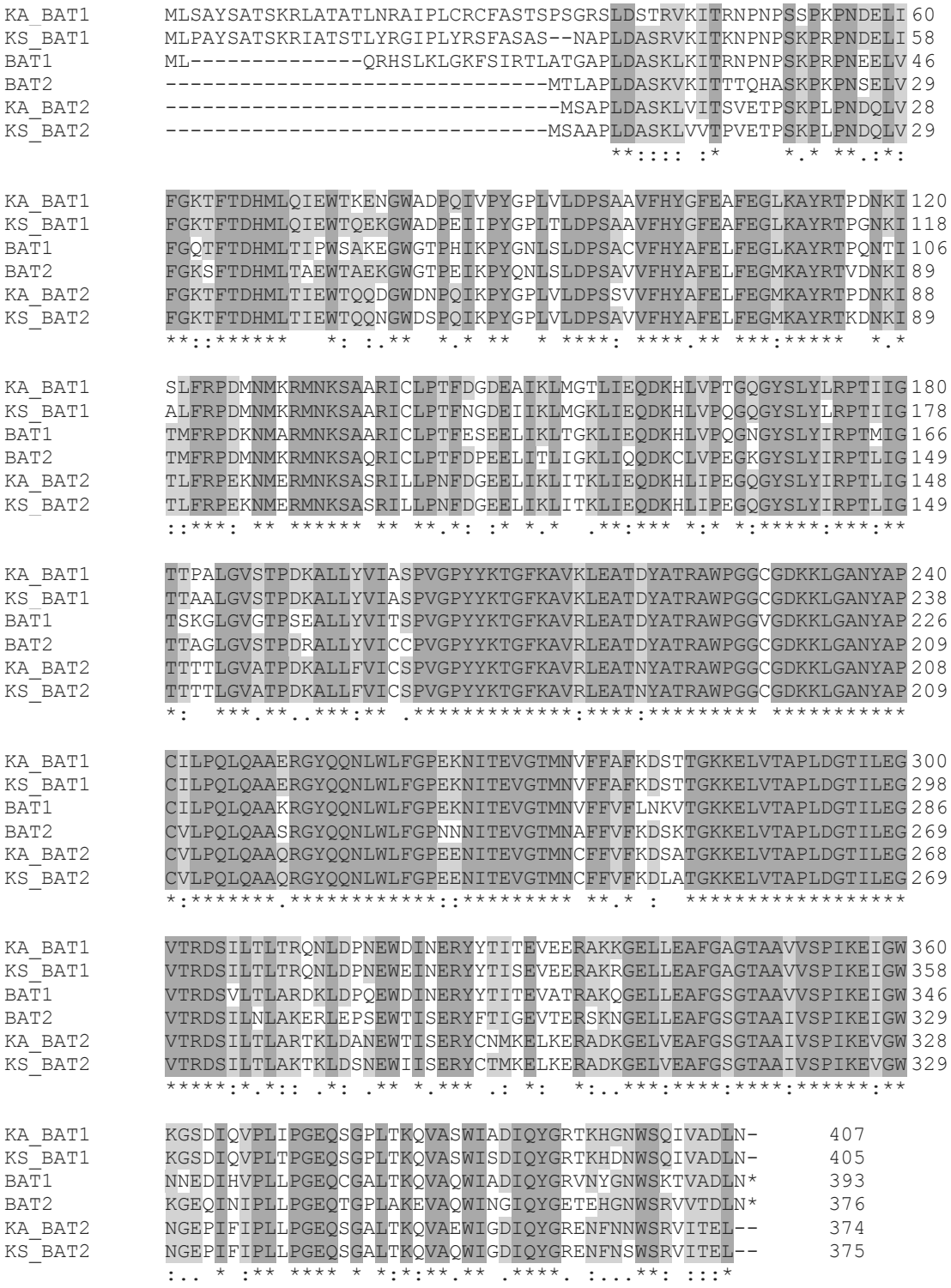
Supplementary Figure 8. Multiple alignment of the amino acid sequences of Aro7 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.

```
ARO7        MDFTKPETVLNLQNIRDELVRMEDSIIFKFIERSHFATCPSVYEANHPGLEI-PNFKGSF 59
KA_ARO7     MDFTKPETVLNLQNIRDELIKMEDSIIFKFIERSHFPTCNGVYQIDNPNITLGSDFHGSF 60
KS_ARO7     MDFTKPETVLNLQNIRDELIKMEDSIIFKFIERSHFPTCNGVYQIDHPNITLGQDFNGSF 60
            ********************::*************** ** .**: ::*.: :  :*:***

ARO7        LDWALSNLEIAHSRIRRFESPDETPFFPDKIQKSFLPSINYPQILAPYAPEVNYNDKIKK 119
KA_ARO7     LDWALMQLEITHSQLRRFESPDQTPFFPNSLKKSILPSINYPKLLSNYSNEVNYNDKIKK 120
KS_ARO7     LDWALMQLEITHSQLRRFESPDQTPFFPNNLKKSILPSINYPKLLSNYSNEVNYNDKIKK 120
            ***** :***:**::*******:*****:.::**:*******::*: *: **********

ARO7        VYIEKIIPLISKRDGDDKNNFGSVATRDIECLQSLSRRIHFGKFVAEAKFQSDIPLYTKL 179
KA_ARO7     IYIEQMVPLISKIDKDDPNNYGSIASCDIECLQSLSRRIHFGKFVAEAKYQSNKELYNKL 180
KS_ARO7     IYIEQMVPLISKIDKDDPNNYGSIASCDIECLQSLSRRIHFGKFVAEAKYQSNKQLYNKL 180
            :***:::***** * ** **:**:*: ***********************:**:  **.**

ARO7        IKSKDVEGIMKNITNSAVEEKILERLTKKAEVYGVDPTNESGERRITPEYLVKIYKEIVI 239
KA_ARO7     IQDKNVEGIMHEITNSAVEAKILERLTTKAEVYGVDPTNAEGERRITPEYLVKIYKEFVI 240
KS_ARO7     IQDKNIEGIMHEITNSAVEAKILERLTTKAEVYGVDPTNAEGERRITPEYLVKIYKEFVI 240
            *:.*::****::******* *******.*********** .****************:**

ARO7        PITKEVEVEYLLRRLEE*- 256
KA_ARO7     PITKEVEVEYLLRRLEDPN 259
KS_ARO7     PITKEVEVEYLLRRLEDPN 259
            ****************:
```

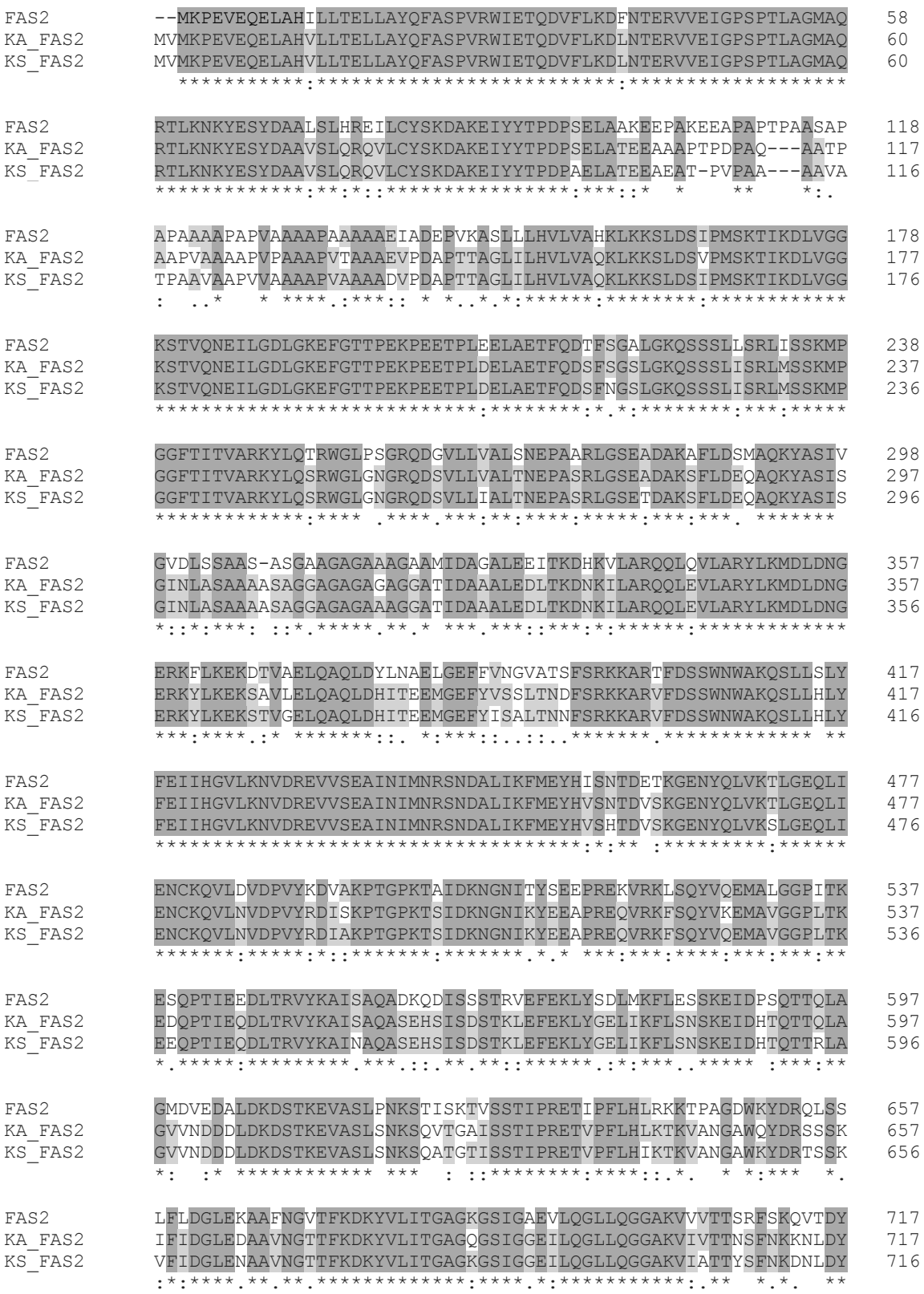
Supplementary Figure 9. Multiple alignment of the amino acid sequences of Aro10 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.

```
ARO10          ---------------MAPVTIEKFV---NQEERHLVSNRSATIPFGEYIFKRLLSIDTKS      42
KA_ARO10       MILSSEDKETYINNLPNSSILDTFGEKQAPVPSTAVATLSSQIPLGQYIFQRIITAGTKS      60
KS_ARO10       MILSSEDKETYIGNFTNSAILDTFGEKQAPAPSNTVTTLSSQIPLGQYLFQRIITAGTKS      60
                                ::.*           *:. *: **:*:*:*:*::: .***

ARO10          VFGVPGDFNLSLLEYLYSPSVESAGLRWVGTCNELNAAYAADGYSRYSNKIGCLITTYGV      102
KA_ARO10       IFGVPGDFNLPLLEYLYEPTLLKQGLRWIGNCNELNSAYSADGFSRYSNKIGVIITTYGV      120
KS_ARO10       IFGVPGDFNLPLLEYLYEPGLLKQGLRWIGNCNELNSAYSADGFSRYSNKIGVVVTTYGV      120
               :********* ******.* : . ****:*.*****:**:***:******** ::*****

ARO10          GELSALNGIAGSFAENVKVLHIVGVAKSIDSRSSN-FSDRNLHHLVPQLHDSNFKGPNHK      161
KA_ARO10       GELSAMNGVAGAFAENVKLLHIVGVAKETDSRPGSLNKDKNIHHLIPNLKDSNFVGPNHK      180
KS_ARO10       GELSAMNGIAGAFAENVKLLHIVGVAKETDSRPDSTNANKNIHHLIPNLKNSNFVGPNHK      180
               *****:**:**:******:********. *** ..   ::*:***:*:*::*** *****

ARO10          VYHDMVKDRVACSVAYLEDIETACDQVDNVIRDIYKYSKPGYIFVPADFADMSVTCDNLV      221
KA_ARO10       VYYEMIKDKISCSSEWLDDINTACDQIDKVIKDIYKYSKPGYIFVPVDFVNQLVSITNLI      240
KS_ARO10       VYYEMIKDKLSCSSEWLDDINTACDQIDKVIKDIYKFSKPGYIFVPVDFVNKLVNIKNLI      240
               **::*:**:::**  :*:**:*****:*:**:****:*********.**.:  *.  **:

ARO10          NVPRISQQDCIVYPSENQLSDIINKITSWIYSSKTPAILGDVLTDRYGVSNFLNKLICKT      281
KA_ARO10       QEPHISMDTCLSEPSQQVSTEITDLILKWMYESKTPGLIGDVLVDRYGANNSLNHFIKQT      300
KS_ARO10       QEPRISMDTCLSKPSSQVTTEITDLICKWVYESKTPALIGDVLVDRYGANKNLNQFIKQT      300
               : *:** : *:  **.:  ::* : * .*:*.****.::****.****..: **::* :*

ARO10          GIWNFSTVMGKSVIDESNPTYMGQYNGKEGLKQVYEHFELCDLVLHFGVDINEINNGHYT      341
KA_ARO10       KMWNFSTVNGKSIINESNPYYMGLYNGKEGASVVIERFLQCDLILNFGLDINEINHGHYT      360
KS_ARO10       KMWNFSTVNGKSIVDESNPYYMGLYNGKEGVSVVIERFLQCDLILNFGLDINEINHGHYT      360
                :****** ***:::**** *** ****** . * *:*  ***:*:**:******:****

ARO10          FTYKPNAKIIQFHPNYIRLVDTRQGNEQMFKGINFAPILKELYKR-IDVSKLSLQYDSNV      400
KA_ARO10       FNYRKDVNIVEFHPDYIRFVETSTGKEKIFKDVNFVFILQELLEKTLDNDQLNFQYDPNV      420
KS_ARO10       FNYKKDANIVEFHPDYIRFVETSTGKEKIFNDVNFVFILQELLKKTSNNNRLNFKYDPKV      420
               *.*: :.:*::***:***:*:*  *:*::*:.:**. **:** ::  : .:*.::** :*

ARO10          TQYTNETMRLE-----DPTNGQSSIITQVHLQKTMPKFLNPGDVVVCETGSFQFSVRDFA      455
KA_ARO10       KAYSSDEVYPDVVDTDSKEKPQQQNITVDFLSKSLPNLLNPGDVCFSETGAIYFAIRDMV      480
KS_ARO10       KAYSSDEVYPDVIDTDLKG---KQNITVDYLSKSLPNLFNPGDVCFSETGAIYFAIRDMV      477
               . *:.: :  :           .. **  .*.*::*:::***** ..***:: *::**:.

ARO10          FPSQLKYISQGFFLSIGMALPAALGVGIAMQDHSNAHING---GNVKEDYKPRLILFEGD      512
KA_ARO10       FPSQVKYMSQGFYLSIGTALPAAFGVGIAMQDYPRCHITDEDDDSIPIDYKPRLILFEGD      540
KS_ARO10       FPNQFKYMSQGFYLSIGTALPAAFGVGIAMQDYPRCHITD--DESIPINYKPRLILFEGD      535
               **.*.**:****:**** *****:********: ..**..    .:  :***********

ARO10          GAAQMTIQELSTILKCNIPLEVIIWNNNGYTIERAIMGPTRSYNDVMSWKWTKLFEAFGD      572
KA_ARO10       GAAQMTIQEMTSMIRYQVPIELFIWNNDGYTVERAICGPTRSYNDIMPWNWTKLFEAFGD      600
KS_ARO10       GAAQMTVQEMTSMLRYKVPIELFIWNNDGYTVERAICGPTRSYNDIMPWNWTKLFEAFGD      595
               ******:**:::::: ::*:*::****:***:**** ********:* *:**********

ARO10          FDGKYTNSTLIQCPSKLALKLEELKNSNKRSGIELLEVKLGELDFPEQLKCMVEAAALKR      632
KA_ARO10       TDGKYSINTFIDTKTKLTDKFEQLKKKKERNIIELIEVKLGIMDYPKQLQSMVTAMQSQD      660
KS_ARO10       TGGEYSINTFIDTKAKLADKFEQLRKKKERNVIELMEVKLGVLDYPKQLQSMVTAMQSKH      655
                .*:*: .*:*:  :**: *:*:*::.::*. ***:***** :*:*:**:.** *   :

ARO10          NKK*  635
KA_ARO10       ----  660
KS_ARO10       ----  655
```

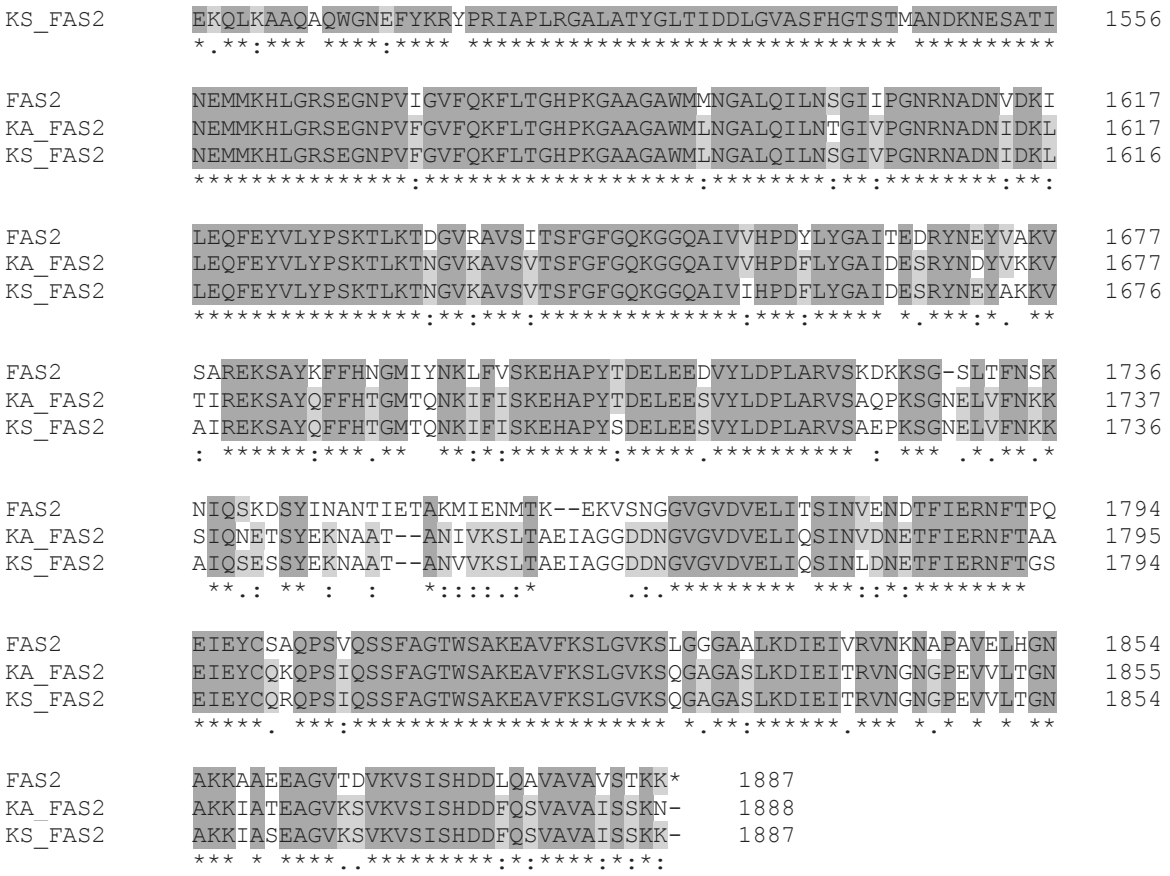
Supplementary Figure 10. Multiple alignment of the amino acid sequences of Bat1 and Bat2 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.



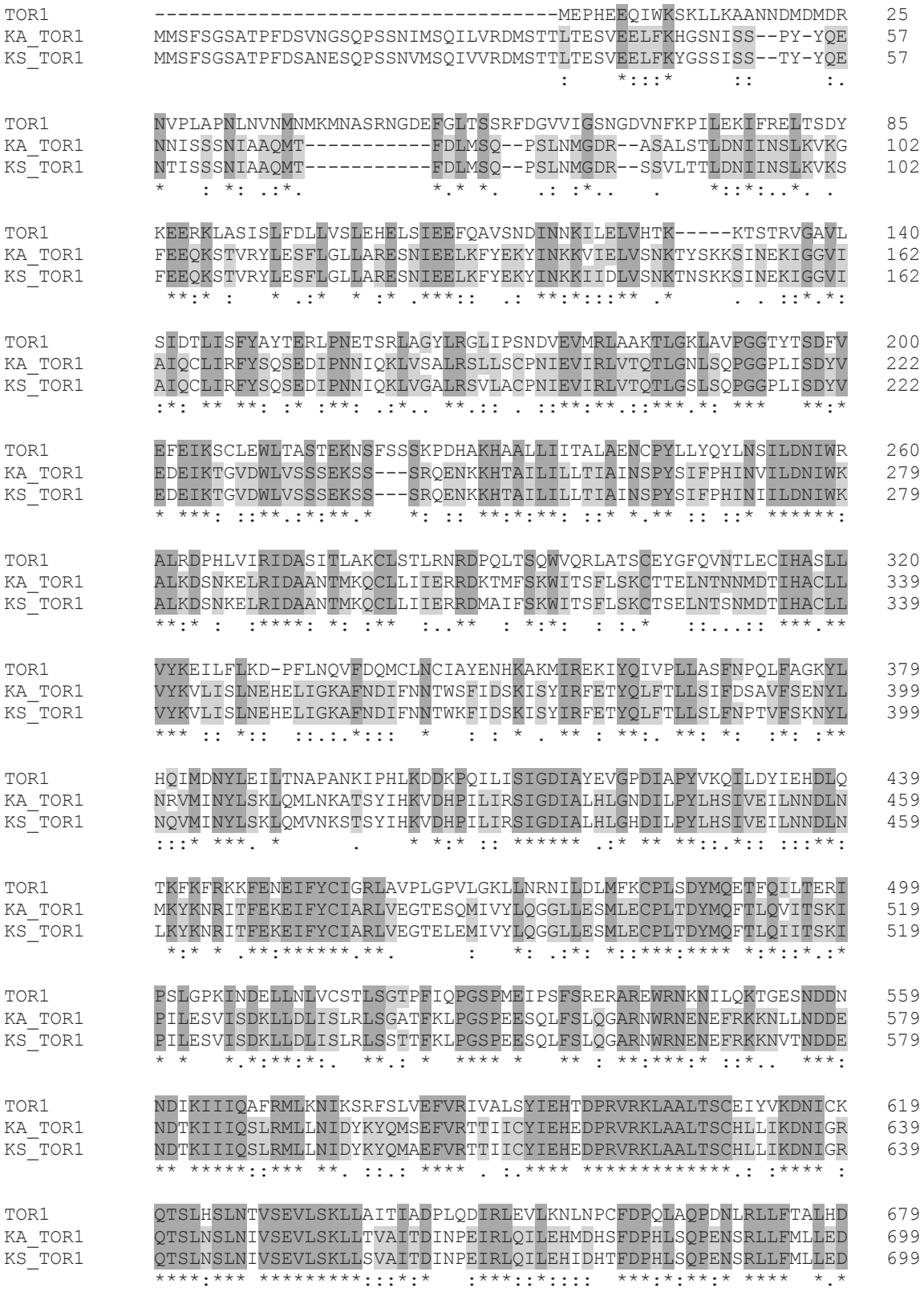
Supplementary Figure 11. Multiple alignment of the amino acid sequences of Fas2 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.

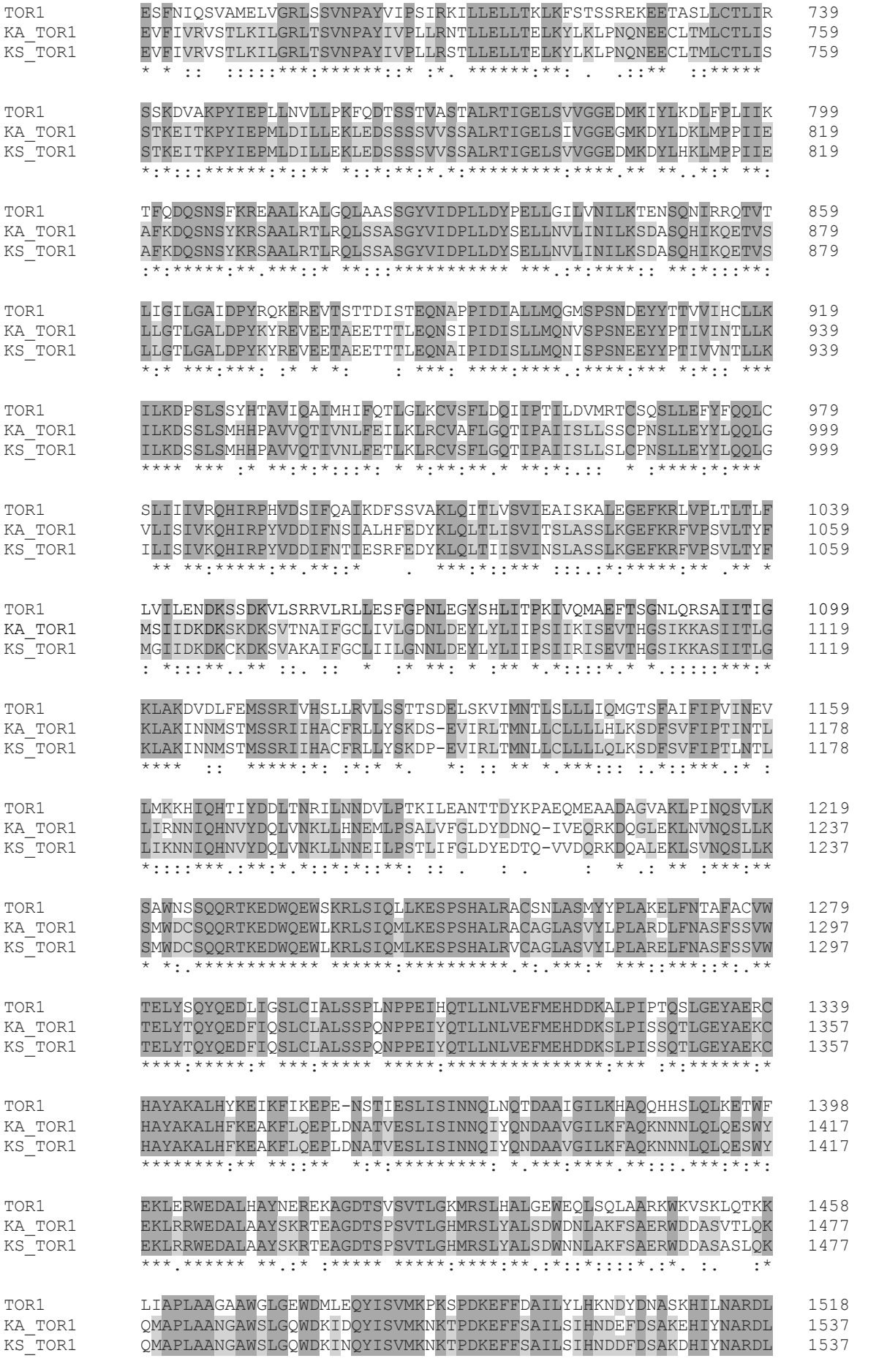


```
FAS2        YQSIYAKYGAKGSTLIVVPFNQGSKQDVEALIEFIYDTEKNGGLGWDLDAIIPFAAIPEQ   777
KA_FAS2     FQSQYAKYGAKGSTLIVAPFNQGSKNDVEALIDYIYDDEKNGGLGWDLDAIIPFAAIPEN   777
KS_FAS2     FQSQYAKYGAKGSTLIVAPFNQGSKNDVVALVDYIYDDEKNGGLGWDLDAIIPFAAIPEN   776
            :** ************.*******:** **:::*** **********************:

FAS2        GIELEHIDSKSEFAHRIMLTNILRMMGCVKKQKSARGIETRPAQVILPMSPNHGTFGGDG   837
KA_FAS2     GIELEDIDSKSEFAHRIMLTNIYRIMGCVKKQKSAKGIETRPAQVILPMSPNHGTFGGDG   837
KS_FAS2     GIELEDIDSKSEFAHRIMLTNIYRIMGCVKKQKTAKGIETRPAQVILPMSPNHGTFGGDG   836
            *****.**************** *:********:*:************************

FAS2        MYSESKLSLETLFNRWHSESWANQLTVCGAIIGWTRGTGLMSANNIIAEGIEKMGVRTFS   897
KA_FAS2     LYSESKLSLETLFNRWHSESWANQLTVCGAIIGWTRGTGLMSGNNIIAEGIEKMGVRTFS   897
KS_FAS2     LYSESKLSLETLFNRWHSESWANQLTVCGAIIGWTRGTGLMSGNNIIAEGIEKMGVRTFS   896
            :****************************************.*****************

FAS2        QKEMAFNLLGLLTPEVVELCQKSPVMADLNGGLQFVPELKEFTAKLRKELVETSEVRKAV   957
KA_FAS2     QKEMAFNLLGLLTPEVTQLCQKSPVMADLNGGLQYLKDLKSFTAKLRRELTETSEIRKAV   957
KS_FAS2     QKEMAFNLLGLLTPEVTQLCQKSPVMADLNGGLQYLKDLKNFTAKLRRELTETSEIRKAV   956
            ****************.:*****************:: :**.******:**.****:****

FAS2        SIETALEHKVVNGNSADAAYAQVEIQPRANIQLDFPELKPYKQVKQIAPAELEGLLDLER   1017
KA_FAS2     SIETALEHKAVNGDKADAAYAEVEVQPRANIQLDFPELKPYKQVKELAAPELEGMLDLEK   1017
KS_FAS2     SIETALEHKAVNGDKADAAYAEVEVQPRANIQLDFPELKPYKQVKQLAAPELEGMLDLER   1016
            *********.***:.******:**:**********************::*  ****:****:

FAS2        VIVVTGFAEVGPWGSARTRWEMEAFGEFSLEGCVEMAWIMGFISYHNGNLKGRPYTGWVD   1077
KA_FAS2     VIVVTGFSEVGPWGSSRTRWQMEAFGEFSLEGCVEMAWMMNLIKYHNGNLKGRPYTGWVD   1077
KS_FAS2     VIVVTGFSEVGPWGSSRTRWQMEAFGEFSLEGCVEMAWMMNLIKYHNGNLKGRPYTGWVD   1076
            *******:*******:****:*****************:*.:*.****************

FAS2        SKTKEPVDDKDVKAKYETSILEHSGIRLIEPELFNGYNPEKKEMIQEVIVEEDLEPFEAS   1137
KA_FAS2     AKTNEPVEDKDVKPMYEKYILDHAGIRLIEPELFNGYDPKKKQLVQEVIVEEDMEPFEAS   1137
KS_FAS2     AKTNEPVEDKDVKPMYEKYILDHAGIRLIEPELFNGYDPKKKQMVQEIIVEEDMEPFEAS   1136
            :**:***:*****  **. **:*:*************:*:**:::**:*****:******

FAS2        KETAEQFKHQHGDKVDIFEIPETGEYSVKLLKGATLYIPKALRFDRLVAGQIPTGWNAKT   1197
KA_FAS2     KETAEQFKHEHGDKVDIFEIPETGEFSVRLLKGATLFVPKALRFDRLVAGQVPTGWDAKT   1197
KS_FAS2     KETAEQFKHEHGDRVDIFEIPETGEFSVKLLKGATLFVPKALRFDRLVAGQVPTGWDAKT   1196
            *********:***:**********:**:********::*************:****:***

FAS2        YGISDDIISQVDPITLFVLVSVVEAFIASGITDPYEMYKYVHVSEVGNCSGSGMGGVSAL   1257
KA_FAS2     YGISEDTISQVDPITLFVLVSVAEAFIAAGITDPYEMYEYVHVSEVGNCSGSGMGGVSAL   1257
KS_FAS2     YGISEDTISQVDPITLFVLVSVAEAFIAAGITDPYEMYEYVHVSEVGNCSGSGMGGVSAL   1256
            ****:* ***************.*****:*********:**********************

FAS2        RGMFKDRFKDEPVQNDILQESFINTMSAWVNMLLISSSGPIKTPVGACATSVESVDIGVE   1317
KA_FAS2     RGMFKDRYKDLPVQNDILQESFINTMSAWVNMLLISSSGPIKTPVGACATAVESVDIGAE   1317
KS_FAS2     RGMFKDRYKDLPVQNDILQESFINTMSAWVNMLLISSSGPIKTPVGACATAVESVDIGAE   1316
            *******:** ****************************************:*******.*

FAS2        TILSGKARICIVGGYDDFQEEGSFEFGNMKATSNTLEEFEHGRTPAEMSRPATTTRNGFM   1377
KA_FAS2     TILSGKAKICIVGGYDDFQEEGSYEFANMKATSNTLEEYEHGRTPAEMSRPATTTRSGFM   1377
KS_FAS2     TILSGKAKICIVGGYDDFQEEGSYEFANMKATSNTLEEYEHGRTPAEMSRPATTTRSGFM   1376
            *******:****************:**.***********:*****************.***

FAS2        EAQGAGIQIIMQADLALKMGVPIYGIVAMAATATDKIGRSVPAPGKGILTTAREHHSSVK   1437
KA_FAS2     EAQGSGIQVIMTAELALKMGVPIYGIVALTATATDKIGRSVPAPGKGILTTAREHHGSLK   1437
KS_FAS2     EAQGSGIQVIMTADLALKMGVPIYGIVALTATATDKIGRSVPAPGKGILTTAREHHGSLK   1436
            ****:***:** *:***************::**************************.*:*

FAS2        YASPNLNMKYRKRQLVTREAQIKDWVENELEALKLEAEEIPSEDQNEFLLERTREIHNEA   1497
KA_FAS2     YASPLLDIKYRRRQLTKREVQIKQWVEDELELLQVEIEEIPEADQAEFVAERTKEIKLEG   1497
KS_FAS2     YASPLLDIKYRRRQLSNREVQIKQWVEDELELLQVEIEEIPEVDQAEFIAERTKEIKLEG   1496
            **** *::***:*** .**.***:***:*** *::* ****. ** **: ***:**: *.

FAS2        ESQLRAAQQQWGNDFYKRDPRIAPLRGALATYGLTIDDLGVASFHGTSTKANDKNESATI   1557
KA_FAS2     EKQLKAAQAQWGNEFYKRYPRIAPLRGALATYGLTIDDLGVASFHGTSTKANDKNESATI   1557
```


KS_FAS2 EKQLKAAQAQWGNEFYKRYPRIAPLRGALATYGLTIDDLGVASFHGTSTMANDKNESATI 1556
FAS2 NEMMKHLGRSEGNPVIGVFQKFLTGHPKGAAGAWMMNGALQILNSGIIPGNRNADNVDKI 1617
KA_FAS2 NEMMKHLGRSEGNPVFGVFQKFLTGHPKGAAGAWMLNGALQILNTGIVPGNRNADNIDKL 1617
KS_FAS2 NEMMKHLGRSEGNPVFGVFQKFLTGHPKGAAGAWMLNGALQILNSGIVPGNRNADNIDKL 1616
FAS2 LEQFEYVLYPSKTLKTDGVRAVSITSFGFGQKGGQAIVVHPDYLYGAITEDRYNEYVAKV 1677
KA_FAS2 LEQFEYVLYPSKTLKTNGVKAVSVTSFGFGQKGGQAIVVHPDFLYGAIDESRYNDYVKKV 1677
KS_FAS2 LEQFEYVLYPSKTLKTNGVKAVSVTSFGFGQKGGQAIVIHPDFLYGAIDESRYNEYAKKV 1676
FAS2 SAREKSAYKFFHNGMIYNKLFVSKEHAPYTDELEEDVYLDPLARVSKDKKSG-SLTFNSK 1736
KA_FAS2 TIREKSAYQFFHTGMTQNKIFISKEHAPYTDELEESVYLDPLARVSAQPKSGNELVFNKK 1737
KS_FAS2 AIREKSAYQFFHTGMTQNKIFISKEHAPYSDELEESVYLDPLARVSAEPKSGNELVFNKK 1736
FAS2 NIQSKDSYINANTIETAKMIENMTK--EKVSNGGVGVDVELITSINVENDTFIERNFTPQ 1794
KA_FAS2 SIQNETSYEKNAAT--ANIVKSLTAEIAGGDDNGVGVDVELIQSINVDNETFIERNFTAA 1795
KS_FAS2 AIQSESSYEKNAAT--ANVVKSLTAEIAGGDDNGVGVDVELIQSINLDNETFIERNFTGS 1794
FAS2 EIEYCSAQPSVQSSFAGTWSAKEAVFKSLGVKSLGGGAALKDIEIVRVNKNAPAVELHGN 1854
KA_FAS2 EIEYCQKQPSIQSSFAGTWSAKEAVFKSLGVKSQGAGASLKDIEITRVNGNGPEVVLTGN 1855
KS_FAS2 EIEYCQRQPSIQSSFAGTWSAKEAVFKSLGVKSQGAGASLKDIEITRVNGNGPEVVLTGN 1854
FAS2 AKKAAEEAGVTDVKVSISHDDLQAVAVAVSTKK* 1887
KA_FAS2 AKKIATEAGVKSVKVSISHDDFQSVAVAISSKN- 1888
KS_FAS2 AKKIASEAGVKSVKVSISHDDFQSVAVAISSKK- 1887

Supplementary Figure 12. Multiple alignment of the amino acid sequences of Tor1 from *S. cerevisiae* (S288C) and their *Kazachstania* spp. (*K. aerobia* (KA) and *K. servazzii* (KS)) orthologues. The alignments were performed using Clustal Omega software. Non-conserved residues are black text on white background, identical residues are black text on light grey background, and conserved residues in all sequences are black text on dark grey background.




TOR1 ESFNIQSVAMELVGRLSSVNPAYVIPSIRKILLELLTKLKFSTSSREKEETASLLCTLIR 739
KA_TOR1 EVFIVRVSTLKILGRLTSVNPAYIVPLLRNTLLELLTELKYLKLPNQNEECLTMLCTLIS 759
KS_TOR1 EVFIVRVSTLKILGRLTSVNPAYIVPLLRSTLLELLTELKYLKLPNQNEECLTMLCTLIS 759
TOR1 SSKDVAKPYIEPLLNVLLPKFQDTSSTVASTALRTIGELSVVGGEDMKIYLKDLFPLIIK 799
KA_TOR1 STKEITKPYIEPMLDILLEKLEDSSSSVVSSALRTIGELSIVGGEGMKDYLDKLMPPIIE 819
KS_TOR1 STKEITKPYIEPMLDILLEKLEDSSSSVVSSALRTIGELSVVGGEDMKDYLHKLMPPIIE 819
TOR1 TFQDQSNSFKREAALKALGQLAASSGYVIDPLLDYPELLGILVNILKTENSQNIRRQTVT 859
KA_TOR1 AFKDQSNSYKRSAALRTLRQLSSASGYVIDPLLDYSELLNVLINILKSDASQHIKQETVS 879
KS_TOR1 AFKDQSNSYKRSAALRTLRQLSSASGYVIDPLLDYSELLNVLINILKSDASQHIKQETVS 879
TOR1 LIGILGAIDPYRQKEREVTSTTDISTEQNAPPIDIALLMQGMSPSNDEYYTTVVIHCLLK 919
KA_TOR1 LLGTLGALDPYKYREVEETAEETTTLEQNSIPIDISLLMQNVSPSNEEYYPTIVINTLLK 939
KS_TOR1 LLGTLGALDPYKYREVEETAEETTTLEQNAIPIDISLLMQNISPSNEEYYPTIVVNTLLK 939
TOR1 ILKDPSLSSYHTAVIQAIMHIFQTLGLKCVSFLDQIIPTILDVMRTCSQSLLEFYFQQLC 979
KA_TOR1 ILKDSSLSMHHPAVVQTIVNLFEILKLRCVAFLGQTIPAIISLLSSCPNSLLEYYLQQLG 999
KS_TOR1 ILKDSSLSMHHPAVVQTIVNLFETLKLRCVSFLGQTIPAIISLLSLCPNSLLEYYLQQLG 999
TOR1 SLIIIVRQHIRPHVDSIFQAIKDFSSVAKLQITLVSVIEAISKALEGEFKRLVPLTLTLF 1039
KA_TOR1 VLISIVKQHIRPYVDDIFNSIALHFEDYKLQLTLISVITSLASSLKGEFKRFVPSVLTYF 1059
KS_TOR1 ILISIVKQHIRPYVDDIFNTIESRFEDYKLQLTIISVINSLASSLKGEFKRFVPSVLTYF 1059
TOR1 LVILENDKSSDKVLSRRVLRLLESFGPNLEGYSHLITPKIVQMAEFTSGNLQRSAIITIG 1099
KA_TOR1 MSIIDKDKSKDKSVTNAIFGCLIVLGDNLDEYLYLIIPSIIKISEVTHGSIKKASIITLG 1119
KS_TOR1 MGIIDKDKCKDKSVAKAIFGCLIILGNNLDEYLYLIIPSIIRISEVTHGSIKKASIITLG 1119
TOR1 KLAKDVDLFEMSSRIVHSLLRVLSSTTSDELSKVIMNTLSLLLIQMGTSFAIFIPVINEV 1159
KA_TOR1 KLAKINNMSTMSSRIIHACFRLLYSKDS-EVIRLTMNLLCLLLLHLKSDFSVFIPTINTL 1178
KS_TOR1 KLAKINNMSTMSSRIIHACFRLLYSKDP-EVIRLTMNLLCLLLLQLKSDFSVFIPTLNTL 1178
TOR1 LMKKHIQHTIYDDLTNRILNNDVLPTKILEANTTDYKPAEQMEAADAGVAKLPINQSVLK 1219
KA_TOR1 LIRNNIQHNVYDQLVNKLLHNEMLPSALVFGLDYDDNQ-IVEQRKDQGLEKLNVNQSLLK 1237
KS_TOR1 LIKNNIQHNVYDQLVNKLLNNEILPSTLIFGLDYEDTQ-VVDQRKDQALEKLSVNQSLLK 1237
TOR1 SAWNSSQQRTKEDWQEWSKRLSIQLLKESPSHALRACSNLASMYYPLAKELFNTAFACVW 1279
KA_TOR1 SMWDCSQQRTKEDWQEWLKRLSIQMLKESPSHALRACAGLASVYLPLARDLFNASFSSVW 1297
KS_TOR1 SMWDCSQQRTKEDWQEWLKRLSIQMLKESPSHALRVCAGLASVYLPLARELFNASFSSVW 1297
TOR1 TELYSQYQEDLIGSLCIALSSPLNPPEIHQTLLNLVEFMEHDDKALPIPTQSLGEYAERC 1339
KA_TOR1 TELYTQYQEDFIQSLCLALSSPQNPPEIYQTLLNLVEFMEHDDKSLPISSQTLGEYAEKC 1357
KS_TOR1 TELYTQYQEDFIQSLCLALSSPQNPPEIYQTLLNLVEFMEHDDKSLPISSQTLGEYAEKC 1357
TOR1 HAYAKALHYKEIKFIKEPE-NSTIESLISINNQLNQTDAAIGILKHAQQHHSLQLKETWF 1398
KA_TOR1 HAYAKALHFKEAKFLQEPLDNATVESLISINNQIYQNDAAVGILKFAQKNNNLQLQESWY 1417
KS_TOR1 HAYAKALHFKEAKFLQEPLDNATVESLISINNQIYQNDAAVGILKFAQKNNNLQLQESWY 1417
TOR1 EKLERWEDALHAYNEREKAGDTSVSVTLGKMRSLHALGEWEQLSQLAARKWKVSKLQTKK 1458
KA_TOR1 EKLRRWEDALAAYSKRTEAGDTSPSVTLGHMRSLYALSDWDNLAKFSAERWDDASVTLQK 1477
KS_TOR1 EKLRRWEDALAAYSKRTEAGDTSPSVTLGHMRSLYALSDWNNLAKFSAERWDDASASLQK 1477
TOR1 LIAPLAAGAAWGLGEWDMLEQYISVMKPKSPDKEFFDAILYLHKNDYDNASKHILNARDL 1518
KA_TOR1 QMAPLAANGAWSLGQWDKIDQYISVMKNKTPDKEFFSAILSIHNDEFDSAKEHIYNARDL 1537
KS_TOR1 QMAPLAANGAWSLGQWDKINQYISVMKNKTPDKEFFSAILSIHNDDFDSAKDHIYNARDL 1537

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                :*****..**.**:** ::******* *:******.*** :*::::*.*..** *****

TOR1        LVTEISALINESYNRAYSVIVRTQITEFEEIIKYKQLPPNSEKKLHYQNLWTKRLLGCQ   1578
KA_TOR1     LVTELSALVNESYNRAYNVIVRTQLITELEEVIEYKRLPANSEKRAVYNSAWNKRLLGCQ   1597
KS_TOR1     LVTEISALVNESYNRAYNVIVRTQLITELEEVIEYKKLPPNSDKRAVYNSTWNKRLLGCQ   1597
            ****:***:********.******:***:**:*:**:** **:*:  *:. *.*******

TOR1        KNVDLWQRVLRVRSLVIKPKQDLQIWIKFANLCRKSGRMRLANKALNMLLEGGNDPSLPN   1638
KA_TOR1     KNVDIWQRALKVRSLVISPKQDMHIWIKFANLCRKSGKLNLAEAALSALLEDTSDISPSL   1657
KS_TOR1     KNVDIWQRALKVRSLVISPKQDMHIWIKFANLCRKSGKLNLAEAALSALLEDTTDISPAL   1657
            ****:***.*:******.****::**************::.**: **. ***. .* *

TOR1        TFKAPPPVVYAQLKYIWATGAYKEALNHLIGFTSRLAHDLGLDPNNMIAQSVKLSSASTA   1698
KA_TOR1     PAKAPPPVVYSQLKYLWATGSRQEALRYLIGFTSRMAHDLGLDPSNMIAQSVSQNQSLSP   1717
KS_TOR1     PAKAPPPVVYSQLKYLWATGSRQEALRYLIGFTSRMAHDLGLDPSNMIAQSVSQNQSLSP   1717
              ********:****:****: :***.:*******:********.*******. ..: :

TOR1        PYVEEYTKLLARCFLKQGEWRIATQPNWRNTNPDAILGSYLLATHFDKNWYKAWHNWALA   1758
KA_TOR1     SNIEEYTKLLARCFLKQGEWRVTMQPNWREENPDAILGSYLLATHFDNKWYKAWHNWALV   1777
KS_TOR1     SNIEEYTKLLARCFLKQGEWRVTMQPNWREENPDAILGSYLLATHFDNRWYKAWHNWALV   1777
              :******************:: *****: ****************:.**********.

TOR1        NFEVISMVQEETKLNGGKNDDDDTAVNNDNVRIDGSILGSGSLTINGNRYPLELIQRHV   1818
KA_TOR1     NFEVISAVTSGKKPDNGTSTDVSKSNQ-------KHGNIQSDILKIEGREYSMELIQRHV   1830
KS_TOR1     NFEVISAVTSGKKPDNAASNDTSKPNQ-------KHGSIQNDIIKIEGREYSMELIQRHV   1830
            ****** * . .* :.. . * ..          . . : .. :.*:*..* :*******

TOR1        VPAIKGFFHSISLLETSCLQDTLRLLTLLFNFGGIKEVSQAMYEGFNLMKIENWLEVLPQ   1878
KA_TOR1     IPAIKGFFHSISLSKTSSLQDTLRLLTLWFTFGGTSEAGQAMHAGFNLIKIDSWLEVLPQ   1890
KS_TOR1     IPAIKGFFHSISLSKTSSLQDTLRLLTLWFTFGGTSEAGQAMYAGFNLIKIDSWLEVLPQ   1890
            :************ :**.********** *.*** .*..***: ****:**:.*******

TOR1        LISRIHQPDPTVSNSLLSLLSDLGKAHPQALVYPLTVAIKSESVSRQKAALSIIEKIRIH   1938
KA_TOR1     LISRIHQPDEAVSRSLLALLSDIGKAHPQVMVNPLIVAIKSESVSRQKAALSIIDKMKIH   1950
KS_TOR1     LISRIHQPDEAVSRSLLALLSDLGKAHPQVMVNPLIVAIKSESVSRQKAALSIIDKMKIH   1950
            ********* :**.***:****:******.:* ** ******************:*::**

TOR1        SPVLVNQAELVSHELIRVAVLWHELWYEGLEDASRQFFVEHNIEKMFSTLEPLHKHLGNE   1998
KA_TOR1     STKLVEQAELVSNELIRVAVLWHELWYEGLEDASRQFFGEHNTEKMFATLEPLHELLKRT   2010
KS_TOR1     STRLVEQAELVSNELIRVAVLWHELWYEGLEDASRQFFGEHNTEKMFATLEPLHELLKKS   2010
            *  **:******:************************* *** ****:******: * .

TOR1        PQTLSEVSFQKSFGRDLNDAYEWLNNYKKSKDINNLNQAWDIYYNVFRKITRQIPQLQTL   2058
KA_TOR1     PETIRELSFQNAFGRDLTDAYEWVLNFKRSKDVSNLNQAWDIYYNVFRRISRQLPQLQTL   2070
KS_TOR1     PETIRELSFQNAFGRDLTDAYEWVLNFKRSKDVSNLNQAWDIYYNVFRRISRQLPQLQTL   2070
            *:*: *:***::*****.*****: *:*:***:.**************:*:**:******

TOR1        DLQHVSPQLLATHDLELAVPGTYFPGKPTIRIAKFEPLFSVISSKQRPRKFSIKGSDGKD   2118
KA_TOR1     NLHHVSPKLLDARDLEMAVPGSYSVGKPIITISYFDPIFSVISSKQRPRKFTIRGSDGKD   2130
KS_TOR1     NLRHVSPKLLDARDLEMAVPGSYSAGKPIIRISYFDPIFSVISSKQRPRKFTIRGSDGKD   2130
            :*:****:** ::***:****:*  *** * *: *:*:*************:*:******

TOR1        YKYVLKGHEDIRQDSLVMQLFGLVNTLLKNDSECFKRHLDIQQYPAIPLSPKSGLLGWVP   2178
KA_TOR1     YQYVLKGHEDIRQDSLVMQLFGLVNTLLEHDSECFKRHLDIQKYPAIPLSPKSGLLGWVP   2190
KS_TOR1     YQYVLKGHEDIRQDSLVMQLFGLVNTLLEHDSECFKRHLDIQKYPAIPLSPKSGLLGWVP   2190
            *:**************************::************:*****************

TOR1        NSDTFHVLIREHRDAKKIPLNIEHWVMLQMAPDYENLTLLQKIEVFTYALDNTKGQDLYK   2238
KA_TOR1     NSDTFHVLIRDHRDANKVPLNIEHWVMLQMAPDYDNLTLLQKIEVFQYAMDNTKGQDLAQ   2250
KS_TOR1     NSDTFHVLIRDHRDANKVPLNIEHWVMLQMAPDYDNLTLLQKIEVFQYAMDNTKGQDLAQ   2250
            **********:****:*:****************:*********** **:******** :

TOR1        ILWLKSRSSETWLERRTTYTRSLAVMSMTGYILGLGDRHPSNLMLDRITGKVIHIDFGDC   2298
KA_TOR1     VLWLKSRSSETWLDRRTVYSRSLAVMSMVGYILGLGDRHPSNLMLDRITGKVIHIDFGDC   2310
KS_TOR1     VLWLKSRSSETWLDRRTVYSRSLAVMSMVGYILGLGDRHPSNLMLDRITGKVIHIDFGDC   2310
            :************:***.*:********.******************************

TOR1        FEAAILREKYPEKVPFRLTRMLTYAMEVSGIEGSFRITCENVMRVLRDNKESLMAILEAF   2358
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KA_TOR1       FEAAILREKFPEKVPFRLTRMLIKALEVSGIEGSFRITCEHVMRVLRNNKESLMAILEAF   2370
KS_TOR1       FEAAILREKFPEKVPFRLTRMLIKALEVSGIEGSFRITCEHVMRVLRNNKESLMAILEAF   2370
              *********:***********  *:**************:******:************

TOR1          ALDPLIHWGFDLPPQKLTEQTGIPLPLINPSELLRKGAITVEEAANMEAEQQNETKNARA   2418
KA_TOR1       AFDPLIHWGFDFPTDKIMEETGIRLPMVNPSELLRKGAITVSEANKMEEEQQIEIRNARA   2430
KS_TOR1       AFDPLIHWGFDFPTDKIMEETGIQLPMVNPSELLRKGAITVSEANKMEEQQQIEIRNARA   2430
              *:**********:* :*: *:*** **::**************.** :** :** * :****

TOR1          MLVLRRITDKLTGNDIKRFNELDVPEQVDKLIQQATSIERLCQHYIGWCPFW*  2470
KA_TOR1       LLVLKRITDKLTGNDIPRFKNLDIPDQVDKLTKEAMSIENLCQHYVGWCPFW-  2482
KS_TOR1       LLVLKRITDKLTGNDIPRFESLDIPDQVDKLTKEAMSIENLCQHYVGWCPFW-  2482
              :***:*********** **:.**:*:***** ::* ***.*****:******
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CHAPTER 6

General conclusions and future directions

6.1 Conclusions

The use of non-*Saccharomyces* yeast is a topic of current interest in the wine industry, as the market demand for newer styles and improved quality of wines continues to rise. This project focused on the potential impact of different non-*Saccharomyces* yeasts on the aroma/flavour profile and sensory of wines, as well as understanding the metabolic pathways that lead to the production of flavour compounds. Four individual studies were designed, specifically to address the aims of this research.

6.1.1 Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures

The purpose of this study was to identify and characterise non-*Saccharomyces* isolates from several time-points (pre-, mid- and end) during un-inoculated fermentations. A total of 77 yeast isolates were identified, belonging to 7 species: *Aureobasidium pullulans*, *Kazachstania aerobia*, *Kazachstania servazzii*, *Meyerozyma guillermondii*, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus*. To test these isolates to be considered as potential wine starter cultures, they were evaluated for attributes of oenological interest and their fermentative capability, where representative isolates from each species were assessed in pure cultures in Chemically Defined Grape Juice Medium (CDGJM). Although non-*Saccharomyces* yeast in general, are incapable of completing alcoholic fermentation, the representative isolates from *K. aerobia*, *K. servazzii*, *W. anomalus* and *T. delbrueckii* were assessed as sequential cultures (with *Saccharomyces cerevisiae*) in sterile white (Viognier) wine fermentations to see their effect on the volatile composition. The results showed that the wines fermented with non-*Saccharomyces* produced 1% (v/v) less alcohol compared to the wines fermented with *S. cerevisiae*, along with increased glycerol concentrations (~2 g/L). Moreover, the non-*Saccharomyces* isolates had a significant impact on the wine volatile

composition, particularly the *Kazachstania* spp. isolates as both *K. aerobia* and *K. servazzii* produced higher phenylethyl alcohol (honey, floral aroma) and isoamyl alcohols (marzipan aroma) and their corresponding esters in the monoculture and sequentially fermented wines. Whilst similar positive attribute contributions to wines have been reported for by other species belonging to *Kazachstania*, these earlier studies have yet to explore their sensory effects in wines produced in larger volume to allow for this. Therefore, the following study (Chapter 3) was designed to assess the influence of *K. aerobia* and *K. servazzii* in a larger-scale vinification setting, to allow for sensory evaluation of the wines to determine their key aroma/flavour attributes.

6.1.2 Influence of *Kazachstania* spp. on the chemical and sensory profile of red wines

As the *Kazachstania* spp. (*K. aerobia* and *K. servazzii*) isolates were screened for oenological properties in the previous study, three isolates (PF_8_W29, PF_9_W18, PF_9_W20) were chosen for this study based on their high enzymatic activities and low H_2S production. The *Kazachstania* spp. isolates were sequentially fermented with *S. cerevisiae* in non-sterile red musts (Merlot and Shiraz). The fermentations were monitored daily for sugar consumption kinetics, and the resulting wines were subjected to basic wine composition analysis, volatile analysis, phenolic composition (Shiraz) and sensory analysis (Shiraz). In the Merlot wines, alcoholic fermentations took place in the laboratory in 500 mL coffee plungers, followed by sequential malolactic fermentation (MLF) by *Oenococcus oeni*. The resultant wines fermented with *Kazachstania* spp. produced 1% (v/v) less alcohol and more glycerol compared to *S. cerevisiae* controls. Whilst the *Kazachstania* spp. contributed to the volatile profile through an increased production of higher alcohol and esters, the aroma profile was also influenced by MLF in Merlot, which resulted in wines with lower concentrations of esters. The incomplete conversion of L-malic to L-lactic acid by MLF in all Shiraz treatments is likely due

to the high initial sugar content in musts; this did not inhibit the effect the yeasts had on the wines. For the Shiraz wines, the interest was in the sensory responses of wine consumers. Wines made with *Kazachstania* spp. were perceived as 'jammy', 'red fruit' and overall higher aroma intensity, while the *S. cerevisiae* treatments were associated with less favourable attributes (i.e., 'cooked vegetable', 'earthy', 'forest floor' and 'savoury'). Although there were no differences observed in phenolic composition between all treatments, the contribution of non-*Saccharomyces* to the colour stability in red wines has been reported (Morata et al., 2020), which remains to be further studied during the aging of wine.

6.1.3 Modification of terpenes in white wines by *Hanseniaspora uvarum*

Apart from the collection of 77 isolates mentioned above (Chapter 2), 5 isolates belonging to *Hanseniaspora uvarum* from uninoculated musts sourced from Heathcote (Victoria) were included for characterisation. This yeast is one of the most abundant yeast species and typically dominates the early stages of fermentation. *H. uvarum* is reported to enhance varietal aroma content through the production of terpenes, C_{13}-norisoprenoids, esters (acetate and ethyl) and fatty acids (and often strain-dependent manner) (Tristezza et al., 2016). The *H. uvarum* isolates were initially evaluated in Viognier to test their fermentation efficiency and influence on the aroma profile. The sequential wines exhibited lower terpene profiles, when compared to the *S. cerevisiae* control. To further validate this observation, experiments were conducted in synthetic medium (CDGJM) spiked with linalool (as the most abundant terpene in wines), as well as and two more aromatic varietals (Muscat and Riesling) with uninoculated (negative) controls included. The analysis of the terpene profiles showed that *H. uvarum* neither synthesised or degraded the linalool in the wines. It appeared that the *S. cerevisiae* was altering the terpene content, which was higher in the sequential and control wines compared to the monoculture and uninoculated treatments. Whilst there is no relevant

literature on terpene degradation by yeasts in wine, it appears that there is a matrix- and/or temperature-effect on the formation of terpenes, which is yet to be investigated.

6.1.4 Whole-genome analysis of two *Kazachstania* spp. isolates from grape must fermentations: identification of genes related to acetate ester production

This final study was included to better understand *Kazachstania* spp. at a molecular level, as both *K. aerobia* and *K. servazzii* had consistently elevated acetate esters, specifically phenylethyl acetate (floral aroma) and isoamyl acetate (fruity, pear, banana aroma) as well as their corresponding alcohols in both red and white wines (Chapters 2 and 3). The genomic features and metabolic traits involved in acetate ester biosynthesis were investigated, as near-full genome sequences (at contig level, *de novo* assembly) for both *K. aerobia* (PF_8_W29) and *K. servazzii* (PF_9_W20) were obtained with PacBio long-read sequencing. The genome size and GC content for *K. aerobia* and *K. servazzii* were 12.5 Mb and 35.8%, and 12.3 Mb and 34.4%, respectively. This is comparable to the fully annotated genomes of *K. africana* (11.1 Mb) and *K. naganishii* (10.8 Mb), as all species have >5000 predicted protein-coding genes. Because there is no prior knowledge about the genes involved in the acetate ester metabolic pathways in these species, putative orthologs (with *S. cerevisiae* as reference genome) involved in higher alcohols and acetate ester formation were identified (except for Eat1, Adh2 and Adh4). In *S. cerevisiae*, the alcohol acetyltransferase (AATase) genes *ATF1* and *ATF2* are responsible for the formation of esters. Both *Kazachstania* spp. revealed only one AATase orthologue, which had a 38.98% to 39.96% consensus identity with *S. cerevisiae* Atf2. This may be the result of the whole genome duplication event which occurred in the *Saccharomycetaceae* family, leading up to gene duplications and differential gene losses (van Laere et al., 2008). Furthermore, genes encoding for alcohol dehydrogenase (ADH) differed across various species, as only two putative genes (*ADH1* and *ADH3*) were found in

Kazachstania spp. In addition, the *S. cerevisiae ADH5* sequence was identical to *ADH1* in *Kazachstania* spp., which alludes to this gene family undergoing gene duplication in the latter species. The exploration of the *Kazachstania* spp. genome has not only given a better insight into the important (putative) functions of proteins encoded by genes, but also laid the foundation for multi-omics approaches (e.g., proteomics, transcriptomics, metabolomics).

6.2 Future directions

In accordance with the above-mentioned studies, future research should consider the following aspects.

6.2.1 Other biotechnological applications of non-*Saccharomyces* yeasts

The yeast-like *A. pullulans* is a common saprophyte found in a diverse range of habitats. It is one of the most abundant microorganisms found on grapevines and during initial stages of fermentation. Within the collection of yeast isolates characterised in Chapter 2, 37 were *A. pullulans*, which accounted for 48% of the total isolates. Though they were proven to be poor fermenter, *A. pullulans* exhibited high extracellular β-glucosidase, lipase and proteolytic activities. Microbial enzymes have an essential role as metabolic catalysts, leading to their use in various biotechnological applications. Commercial enzyme preparations (e.g., proteases, pectinases, glucanases, xylanases) are frequently used in wine production to improve clarity and colour stability in wines (Merín and Morata de Ambrosini, 2020). *A. pullulans* increases the volatile aroma content, in particular monoterpenes, through the production of β-glucosidase (Baffi et al., 2013). For these reasons *A. pullulans* should be further tested for these enzymatic activities, by testing their crude and/or purified extracts under winemaking temperature, pH and SO_2. In addition, *A. pullulans*, along with *M. guillermondii* (which represented 1% of total isolates, Chapter 2) have been reported to show antagonistic activity against postharvest

pathogens (e.g., *Penicillium digitatum* and *Penicillium expansum*). The secretion of cell wall lytic enzymes (i.e., chitinase, pectinase, β-1,3 glucanase, protease and gelatinase) is a desirable biocontrol agent which inhibits fungal spore germination (Agirman and Erten, 2020). Strains of *H. uvarum* have also been reported to not only increase floral aroma through the action of β-glucosidases (Hu et al., 2016), but also possesses antagonistic activity against *Botrytis cinerea* on grape berries (Liu et al., 2010). Employing such microbial agents could reduce the use of chemical fungicides as well as concerns over residues. The antagonistic effects of these yeasts should not only be tested *in vitro*, but *in vivo* as well, as other factors (biological and environmental) can affect the activity and survival of biocontrol agents.

The heterothallic ascomycete yeast, *W. anomalus* predominates in the middle stages of fermentation, when ethanol is at about 3–4%. In Chapter 2, the majority of the *W. anomalus* isolates were recovered from a later stage of fermentation, with one isolate tolerant of 12% ethanol. Although the high production of ethyl acetate, by these isolates is not desirable as it can lead to serious wine fault. Nevertheless, their role as biocontrol agents is therefore an aspect for further investigation. *Hanseniaspora* spp. has been widely investigated with regards to the broad-spectrum killing ability through the production of killer toxins (Mehlomakulu et al., 2014; Radler et al., 1990). Killer toxins from *W. anomalus* have been studied for the control of *Brettanomyces*/*Dekkera* in wines (Comitini et al., 2004). Future studies on the isolates identified in this study could include screening and characterisation of novel killer proteins which would have potential application to reduce ethyl phenol production (by *B. bruxellensis*) during wine maturation and storage.

6.2.2 *Kazachstania* spp. – the path towards commercialisation

The contribution of *Kazachstania* spp. (i.e., *K. aerobia* and *K. servazzii*) to wine aroma/flavour profile through increased floral and fruity aromas should be considered for industrial wine production. Further validation is required to assess the effective implantation of *Kazachstania* spp. in such fermentations. It would also be of interest to determine whether ethanol reduction observed in sequential *Kazachstania* spp. and *S. cerevisiae* fermentations in the laboratory is repeated in non-sterile, large-scale fermentations where there may be differences in population dynamics and succession. Another approach to reducing alcohol in wines may therefore be the utilisation of pure cultures of *Kazachstania* spp., which would also maximise the production of important flavour compounds. At present, there is only one commercially available multi-starter containing *Kazachstania* spp. – a *K. servazzii* and *Pichia kluyveri* combination (Trillyeast, BioEnologia; https://www.bioenologia.com/vino/trillyeast) which produces 15 to 28 times more rose, peach, pear, and apple aromas and higher glycerol. *Kazachstania* spp. also have the potential for wider application to other beverages, as Trillyeast is also suitable for brewing, producing highly aromatic and fruity beers.

The biocompatibility of *Kazachstania* spp. with *S. cerevisiae* and other non-*Saccharomyces* yeasts should also be considered, in order to exploit their ability to improve the aromatic profile of wine, as well as inhibit spoilage microorganisms, thereby reducing SO_2 usage. While the interactions between *S. cerevisiae* and LAB in wine have been evaluated (Bartle et al., 2019; Mendoza et al., 2010; Nehme et al., 2008), the interactions between non-*Saccharomyces* and lactic acid bacteria (LAB) are poorly studied, even though LAB use for MLF is common practice in red wine production to reduce acidity and improve microbial stability during long-term storage. In order for *Kazachstania* spp. to be considered for commercial use, their compatibility with LAB should be closely evaluated, as high ethanol, SO_2, medium-chain fatty acids are strong inhibitors of LAB growth. A mass spectrometry-

based metabolomics approach should be used to explore the metabolic change arising from interactions between non-*Saccharomyces* yeasts and LAB.

6.2.3 Comparative multi-omics between *Kazachstania* spp. and *S. cerevisiae*

Whole-genome sequencing of *Kazachstania* spp. not only can unveil the genetic and molecular basis of evolutionary adaptations, but also opens up to further applications such as transcriptomic and metabolome analyses. Comparative transcriptomics can be used to investigate the differences in the physiology and the metabolism of *Kazachstania* spp. versus *S. cerevisiae* during fermentation. Transcriptome profiles can be obtained at different time points throughout fermentation, in order to compare the key genes expressed in relation to flavour compounds, including the duplicated genes which are unique to *K. aerobia* and *K. servazzii*. Additionally, transcriptome assembly could allow the identification of additional/differentially expressed genes in *Kazachstania* spp. These data could be integrated/combined with metabolomics to unravel the association between gene expression and the production of aromatic compounds. The overexpression or deletion of the genes involved in the synthesis of isoamyl acetate and phenylethyl acetate would also help with characterising gene function and understanding ester metabolism in the context of wine alcoholic fermentation.

6.2.4 Final concluding remarks

This thesis demonstrates the ability of non-*Saccharomyces* yeasts, particularly those belonging to *Kazachstania* spp. in winemaking to influence wine aroma profile. The *Kazachstania* spp. investigated in this project have potential application as starter cultures in the industry, as they contribute and enhance desirable fruity and floral aromas in red and white wines. The considerable knowledge this study has provided on the diversity of wine aroma profiles generated by the non-*Saccharomyces* isolates is particularly important given the growing trend in industry to use non-*Saccharomyces* to improve wine sensory characteristics. Furthermore, the exploitation of the *K. aerobia* and *K. servazzii* genomes will not only provide new insights into their genomic and metabolic features, but offer an excellent opportunity for further studies to unravel the presence of distinct traits.

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