IMPACT OF HIGH SUGAR CONTENT ON METABOLISM AND PHYSIOLOGY OF INDIGENOUS YEASTS

Federico Tondini

A thesis submitted for the degree of Doctor of Philosophy

School of Agriculture, Food and Wine

Faculty of Sciences

The University of Adelaide

July 2018

THE UNIVERSITY of ADELAIDE

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. I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research TrainingProgram Scholarship.

10/07/18

Abstract

This PhD project is part of an ARC Training Centre for Innovative Wine Production larger initiative to tackle the main challenges for the Australian wine industry. In particular, the aim is to address the implication of the increasing trend of sugar accumulation in ripe grapes that consequently results in high sugar musts and high ethanol wines. These increase the risk of sluggish and stuck fermentation, especially when only the indigenous microflora of yeast is exploited. At the beginning of fermentation, yeast cells must coordinate genome expression rapidly in response to external changes to maintain competitive fitness and cell survival. Understading how cells modulate their adaptation strategies can be the key to predicting their capacity to survive in a harsh environment and consequently be able to influence wine composition. This project aims to give strategic advice to deal with fermentations by studying non-conventional yeast physiology in response to high sugar must and correlating it with growth and metabolism.

Chapter 2 compares *T. delbrueckii* and *S. cerevisiae* oenological traits at a molecular level. The mechanisms behind the metabolic differences that exist between these two species were inspected using Next Generation Sequencing technology (ILLUMINA) and analysed by assembling RNA transcriptomes. In **Chapter 3** two Australian indigenous yeast species genomes were sequenced with the newest Next Generation Sequencing (NGS) technology, Nanopore MinION. **Chapter 4** further analyzed the global short-term stress adaptive response to grape must, implementing the technique previously used.

The results, discussed in **Chapter 5**, summarize the improvements in high-throughput data analysis and reveal the genomic and physiological differences of these wine-related species.

Table of Contents

1. Chapter 1: Non-conventional yeast *T. delbrueckii*

1.1 Introduction

Wine is a natural alcoholic beverage that has accompanied mankind since the dawn of history. It has been called 'the nectar of the gods', however it does not require a miracle but only human intervention to avoid vinegar being formed, the complete result of 'naturally' fermented grapes (Jolly et al. 2014). The resulting wine quality is determined by many elements including the grape variety, weather conditions, and the winemaking process (Styger et al. 2011). However, one of the major influences is microbial metabolism (Lambrechts and Pretorius 2000). To be transformed into wine, the grape must has to undergo complex biological and biochemical changes, driven by different microorganisms, mainly yeast and bacteria, which compete at different stages of winemaking for substrates (Faria-Oliveira et al. 2015). Alcoholic fermentation and malolactic fermentation are two of these major stages: while bacteria are usually responsible for malic acid consumption, primary or alcoholic fermentation is carried out by yeast (Querol and Bond 2009). During alcoholic fermentation, the primary metabolic activity of yeast consists of the consumption of the sugar in the grape must and the production of ethanol and carbon dioxide (Fleet 2003). However, the production of hundreds of secondary end-products, like organic acids, higher alcohols and esters, accompany the main production of ethanol, significantly impacting on flavour and aroma and determining the subtlety and individuality of the wine (Romano et al. 2003, Swiegers and Pretorius 2005). Malolactic fermentation occurs secondarily, as it is carried out by bacteria and consists of the conversion of L-malic acid into lactic acid and carbon dioxide, resulting in a deacidification of the wine (Liu 2002).

1.2. The good, the bad and the ugly yeast

Saccharomyces cerevisiae is the yeast that is primarily responsible for wine alcoholic fermentation, given its possession of a combination of several 'oenological' traits: rapid and complete sugar consumption, ethanol production, transformation of aromatic precursors, tolerance to initial and final harsh conditions, and anaerobic metabolism (Marsit and Dequin 2015, Steensels and Verstrepen 2014). The application of genomic technologies to the analysis of wine strains of *Saccharomyces cerevisiae*, followed by the analysis of the impacts and characteristics of wines fermented by these has greatly enhanced our understanding of fermentation. Coupled with improved molecular tools, microbiological research has led to the rational development of improved strains and consequently the commercialization of countless *S. cerevisiae*. On the other hand, spoilage yeast *Brettanomyces bruxellensis* has attracted researchers' attention for its production of unpleasant aromas, such as ethyl-phenols (Conterno 2006). In between, undesired initially having being regarded as spoilage until recently, many other yeast species are found in wine fermentation (Bokulich et al. 2014). These are commonly referred to as non-*Saccharomyces* yeast, and play multiple roles in wine production, contributing to the organoleptic characteristics of a final wine (Fleet 2008). It is now widely accepted that they may positively impact on the winemaking process with the production of a broad range of aromatic compounds (Suárez-Lepe and Morata 2012, Padilla et al. 2016). Their effects on the sensory characteristics of wine are highly dependent on yeast population diversity and succession during fermentation (Romano et al. 2003, Pizarro et al. 2007, Jolly et al. 2014, Grangeteau et al. 2017). Therefore, to maintain the desirable natural variability of wine, high aroma and flavour complexity, the optimisation of fermentation techniques involving non-*Saccharomyces* yeast has become an important approach to achieving improved wine quality in modern winemaking (Jolly et al. 2014). Consequently, recent research has widened the

analysis of the wine chemical and sensory outcomes resulting from the use of different non-*Saccharomyces* species.

Non-*Saccharomyces* use has proven to be helpful to modulate aroma and flavour and to re-direct the style of wine, while the size of the contribution to the final wine sensory profile is influenced by the growth rate and metabolic activity of these yeasts (Romano et al. 2003). Typically, non-*Saccharomyces* are unable to complete fermentation due to their lower ethanol tolerance compared to *S. cerevisiae* (Jolly et al. 2014). Thus, in most cases, sequential fermentation is reported to be the only option (Azzolini et al. 2012, Loira et al. 2014, Belda et al. 2015)). Such studies have rarely been validated through industrial scale trials (Jolly et al. 2014), after which only a couple of non-*Saccharomyces* yeasts made it to the market. As has been done with *S. cerevisiae*, in which physiology, gene expression and metabolic networks have been well defined, understanding factors that influence the growth and development of non- *Saccharomyces* species would be important to modulate their contribution (Jolly et al. 2014, Steensels and Verstrepen 2014). Such factors include grape juice chemical composition (e.g. pH and sugar concentration), winemaking techniques (e.g. clarification, sulfur addition), fermentation conditions (temperature, oxygenation) and the effect of the ethanol produced. In order to evaluate their potential applicability better, the investigation of molecular and cellular processes of non-*Saccharomyces* has to be a central focus of future oenological research (Rossouw et al. 2012) to promote the passage from the laboratory to the industry and help deliver yeast strains more suited to future winemaking challenges.

1.3. Into the yeast: investigation of molecular and cellular processes

Future research aimed at uncovering the role of non-*Saccharomyces* yeasts in wine fermentation could not forbear a functional comparison with *S. cerevisiae* to evaluate the

impact on wine making outcomes. The yeast *S. cerevisiae* became the most important industrial and experimental organism, starting a new era in biotechnology research after the completion of the genome sequence (Bisson et al. 2007). The complete sequence allowed the development of new laboratory tools, such as deleting and barcoding genes, which brought the investigation of the physiology of this yeast to a new molecular depth (Goffeau 2000). A new systematic approach was developed, which elucidated the function of any individual gene. 6,604 ORFs were found in the *Saccharomyces* genome, of which 80% of the gene products have a characterized function. Further sequencing efforts advanced our understanding of genome evolution in yeasts and permitted modification of the phenotypic characteristics of yeast based only on their genome (Hittinger et al. 2015). The *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/) assembled all of the molecular biology and genetic information of the yeast *S. cerevisiae* into a scientific database, allowing genome-wide comparison, sequence similarity searching for individual genes but also determination of common features of groups of genes between or across species (Christie et al. 2004). Taking advantage of these complete inventories of data, comprehensive (-omics) methods have analysed gene function in different contexts (Oliver 2002), included winemaking.

Functional analysis of yeast genes during fermentation allowed a better understanding of their activities and assisted in the development of well-characterized industrial processes (Bisson et al. 2007, Bisson et al. 2010, Walker et al. 2014). Furthermore, transcriptome analyses elucidate the yeast cell responses to fermentation stresses and, consequently, changes in metabolic activity/metabolite production (Marks et al. 2008). The behaviour of *S. cerevisiae* in an industrial environment is largely predictable nowadays (Pretorius 2003). With the development of Next Generation Sequence analyses, implementing microarray technologies and allowing billions of DNA strands to be sequenced in parallel (Buermans and Den Dunnen 2014), functional genomics information of non–conventional yeast cells is now more easily

accessible. Sequencing "omics" approaches (i.e. genomics, transcriptomics, and metagenomics) have provided a powerful opportunity to explore a wider range of wine yeast species habitats, functions and metabolism (Bokulich and Mills 2012). Alongside this, bioinformatics pipelines have been developed to provide the tools for high throughput data analysis, which require high processing and computing capability, a real bottleneck and limiting factor for these techniques. Whole-genome analysis can predict some biochemical traits more and more precisely, and new biotechnological applications of lesser-known yeast species can now be fully exploited with comparative genomics and transcriptomic (Riley et al. 2016). Nevertheless, complicated intracellular networks controlling growth, stress responses and metabolic characteristics of non-*Saccharomyces* have to be investigated and compared with the well-known *Saccharomyces* in order to fully uncover their potential and industrial use.

1.3.1. Yeast growth

Sugars and nitrogen compounds are key nutrients for yeast in wine fermentation. *S. cerevisiae* cells finely tune their cell cycle and growth, depending on the availability of these nutrients, with multiple signalling networks: PKA in presence of sugar and TORC1 for nitrogen (Broach 2012). These networks fuel cell growth and are able to reprogram metabolism and the proliferative capacity of cells, primarily supporting ribosome biogenesis and protein synthesis. The propagation and establishment of numerical dominance is fundamental in the early phase of wine fermentation. However, the environment tightly controls the internal balance of the yeast, not always supporting optimal growth and metabolism (Gasch 2003). Under starvation conditions or through a large number of environmental assaults, the yeast cells respond with a complex pattern of gene expression changes, referred to as the stress response, in which the predominant component of genes repressed are those responsible for biomass accumulation

(Broach 2012). Extreme starvation or stress results in a quiescent state, autophagy or even cell death.

Grape must is not an optimal environment for either inoculated or indigenous yeasts. *Saccharomyces* has evolved a particularly effective mechanism for adapting its physiology to unfavourable growing conditions, such as the harsh conditions of the fermentation process (Bauer and Pretorius 2000). To ferment, the cell population must be able to grow whilst being vulnerable to high osmotic strength due to sugars, rapid exhaustion of oxygen, depletion of nutrients (especially nitrogenous) and finally, high ethanol concentrations (Walker et al. 2014). The chemical changes in the external medium have to be sensed by cells to allow successful adaptation to the environment and this information must be accurately transmitted by a network of dedicated pathways to relevant cellular compartments (Bauer and Pretorius 2000). Cell growth is therefore modulated by a complex network of intracellular signals that implement cellular adaption processes (Chen and Thorner 2007).

Non-*Saccharomyces* can be divided in three groups depending on their metabolism and growth profile during fermentation: largely aerobic, yeast with low fermentative activity, or yeast with fermentative metabolism (Jolly et al. 2014). The metabolic activity of non-*Saccharomyces* yeasts is usually strong at the beginning of the fermentation and can continue for several days before the fermentation is normally dominated by *S. cerevisiae* (Cocolin et al. 2000). With increasing ethanol concentration, the diversity of the yeast community tends to narrow, and toward the end of fermentation it is dominated by *Saccharomyces* spp, which are highly ethanol tolerant compared to the other yeasts species (Steensels and Verstrepen 2014). However, how non-*Saccharomyces* yeasts effect nutrient sensing during fermentation remains mostly unclear. In general, growth rate influences the population dynamics of each wine yeast species, determining specific metabolic activity and the size of the contribution to the final wine sensory profile (Romano et al. 2003). Therefore, to elucidate their fermentation predictability and

impact on the overall wine quality, the link between cell proliferation and adaptation capacity to the harsh environment has to be fully discovered.

1.3.2. Stress responses

Many factors can directly impact on the contribution that yeasts have on wine, as their metabolic activity can be prematurely reduced. High sugar concentrations represent a challenging, stressful environment for yeast due to a very high initial osmotic pressure (Fleet 2003). The hyperosmotic pressure results in a water efflux from the cell and subsequently diminished turgor pressure, which leads to rapid cell shrinkage (Pratt et al. 2003, Jimenez-Marti et al. 2011). The sugar content of grape juice (over 200 g/l) generates such osmotic pressure (Nishino et al. 1985). These stressful conditions influence the growth rate of *Saccharomyces* yeasts and consequently fermentation progress, potentially resulting in slow fermentations prone to premature arrest and spoilage (Ishmayana et al. 2011). This problem is becoming more important for Australian winemakers, given the trend of increasing grape sugar content due to recent climatic changes and human selection and management of grape varieties (Godden and Muhlack 2010).

The response of *S. cerevisiae* to a high sugar medium is well known in laboratory and fermentation conditions (Hohmann 2009, Erasmus et al. 2003, Landolfo et al. 2008, Li et al. 2010, Saxena and Sitaraman 2016). After experiencing osmotic stress, *S. cerevisiae* starts a complex adaptive program that involves the temporary arrest of cell-cycle progression, changing transcription and translation rates, and adjustment of metabolism by enhancing the production of intracellular glycerol in order to counter-balance the osmotic pressure (Erasmus et al. 2003, Varela et al. 2005, Jimenez-Marti et al. 2011). The HOG pathway seems to guide these processes, during which long-term adaptation involves the transcriptional and

translational regulation of the genome, whereas short-term adaptation is accomplished by changes in accumulation of glycerol and other osmolytes (Hohmann 2009, Gonzalez et al. 2016). The HOG pathway seems to be highly conserved across fungal species (Krantz et al. 2006). It is well know that it can modulate metabolism in *S. cerevisiae* but its effects on other yeasts remain unknown (Saito and Posas 2012, Steensels and Verstrepen 2014). For non-*Saccharomyces* yeasts there is a lack of knowledge about their behaviour in an environment such as wine making, whereas the response of only a few species has been characterized under laboratory conditions. To understand the effect of high sugar concentration in winemaking, a thorough examination could provide interesting information on how and which metabolites are produced in this condition. At the same time, RNA expression analysis could highlight the activation of the yeast stress response pathway and its repercussions for metabolic pathways.

While the fermentation moves on, nutrients are consumed and ethanol produced. The cellular response to high ethanol content is very similar to other water-associated stresses, with increased production of trehalose and the induction of heat shock proteins (Querol et al. 2003). Aside from ethanol toxicity during fermentation, nutrient starvation is an inducer of the stress-associated response (Bauer and Pretorius 2000). Yeast cells exhibit cross-protection against different stresses, using a general mechanism of cellular protection called the environmental stress response (ESR; Gasch 2003). According to Marks et al (2008), under oenological conditions *S. cerevisiae* responds transcriptionally to stress by general and or stimuli-specific response mechanisms. These genes, named fermentation stress response genes (FSR), overlap with the ESR genes or the specific stress genes related to osmotic stress, nitrogen depletion, or ethanol toxicity (Marks et al. 2008). In terms of wine fermentation, understanding how yeast gene expression changes in the adaptation to multiple stresses and nutrient availabilities is critical to evaluate the risk of stuck and sluggish fermentations (Bauer and Pretorius 2000, Fuchs and Mylonakis 2009). Other than *S. cerevisiae*, the ESR has been investigated in

different non-*Saccharomyces* yeasts, such as the opportunistic pathogenic yeast, *Candida albicans* (Enjalbert et al. 2003, Gasch 2007), the fission yeast *Schizosaccharomyces pombe* (Gasch 2007), *Lachancea kluyveri* (Brion et al. 2016) and *Torulaspora delbrueckii* (Tronchoni et al. 2017).

1.3.3. Metabolic pathways impacting on wine quality

Wine attributes are mostly derived from grapes, fermentation, maturation and ageing (Swiegers and Pretorius 2005). Indeed, yeast metabolism during fermentation impacts on all of the attributes of a wine (i.e. aroma, flavour, mouth feel, colour and chemical complexity), extracting and modifying grape compounds and producing a substantial amount of yeast metabolites (Lambrechts and Pretorius 2000), with a significant influence on the sensory profile (Swiegers et al. 2005, Bisson and Karpel 2010). *S. cerevisiae* produces multiple compounds with an impact on the aroma and flavour of wine (Bartowsky and Pretorius 2009). These metabolites, which can be volatile or non-volatile, are synthesized predominantly via sugar and amino acid metabolism (Swiegers al. 2005, Bisson and Karpel 2010) and include acids, esters, glycerol, alcohols, carbonyls, fatty acids, phenols, sulfur compounds and monoterpenoids (Swiegers et al. 2005). Aside from the well-known glycolysis and fermentation reactions (Puig and Pérez-Ortín 2000, Rossignol et al. 2003), with their main secondary metabolites (i.e. glycerol and acetic acid), other pathways are involved in the production of three classes of odour-impact compounds, which are commonly found at levels above their thresholds of detection: higher alcohols, esters, and hydrogen sulfide (H_2S; Swiegers et al. 2005, Bisson and Karpel 2010). Several studies allowed identification of the genetic bases and enzymatic reactions of the formation of these flavours in yeast.

S. cerevisiae produces a wide range of higher alcohols, which characterise its wine sensory properties (Bisson and Karpel 2010). These alcohols are produced starting from intermediates of the amino acid and sugar catabolism and then processed in the so-called Ehrlich pathway (Hazelwood et al. 2008). Production of these compounds has been studied extensively in *S. cerevisiae* wine fermentations (Fraile et al. 2000) with supplementation of amino acids having a marked influence (Hernández-Orte et al. 2006, Garde-Cerdán and Ancín-Azpilicueta 2008). Esters, instead, are made from the condensation of one alcohol and an organic acid, such as Coenzyme A for ethyl esters or Acetyl-CoA for acetate esters (Bartowsky and Pretorius 2009). Their production is regulated by two classes of enzymes, esterases and lipases, which have been identified in yeast (Saerens et al 2010). Finally, H_2S, characterized by an unpleasant rotten egg aroma, is related to both sulfur-compound and sulfur amino acid metabolism by yeasts, through the so-called sulfate reduction sequence (Lambrechts and Pretorius 2000, Bisson and Karpel 2010, Huang et al. 2016).

During the progress of fermentation, non-*Saccharomyces* produce a variety of metabolites that lead to the formation of a wide range of aromatic compounds (Pizarro et al. 2007). Terpenoids, esters, higher alcohols, glycerol, acetaldehyde, polysaccharides, and organic acids are the main known metabolites differing from fermentation with non-*Saccharomyces* yeasts (Jolly et al. 2014). Furthermore other microbial properties such as the production of extracellular enzymes (e.g. esterases, proteases, pectinases) could also be of significant interest in winemaking (Jolly et al. 2014). Non-*Saccharomyces* use is proving helpful in modulating the aroma and flavour and to re-direct the style of wine, depending on different strain combinations and fermentation strategies. However, a better understanding of their physiological properties would allow winemakers to make better use of such microbial tools to achieve the desired wine sensory outcome.

1.4. Non-*Saccharomyces* yeast: *Torulaspora delbrueckii*

Non-*Saccharomyces* yeasts have been seen as options to address modern winemaking challenges together with wine quality improvement (Jolly et al. 2014). Of these, *Torulaspora delbrueckii* can be found naturally on grapes, must and wine or other fermented food and beverages (Kurtzman 2011), and has attracted the attention of food and beverage industry. Its metabolism couples good biomass production in aerobic conditions with high ethanol yield (up to 9% (v/v); Ciani and Maccarelli 1997) in anaerobiosis; an unusual but desired behaviour for a pre whole-genome duplication species (Albertin et al 2014). Generally, *T. delbrueckii* cannot complete alcoholic fermentation, which means it has to be coupled with a compatible *S. cerevisiae* strain. Sequential fermentations have been reported to increase fruity (Minnaar et al. 2015) and flowery descriptors (Cordero Bueso et al. 2013) with an enhanced overall aroma complexity (Renault et al. 2015, Cus and Jenko 2013); to increase the concentrations of varietal thiols (Renault et al. 2016, Belda et al. 2017), and to increase levels of mannoproteins and polysaccharides (Belda et al. 2015). However, a valued property of *T. delbrueckii* is low acetic acid production (0.2 g/L; Bely et al. 2008, Taillandier et al. 2014), especially in high sugar grape juice, which is contrary to *S. cerevisiae.* For all these qualities, *T. delbrueckii* has become the most studied, commercialised and used non-*Saccharomyces* in the wine industry (Benito 2018)

Based on the above, selection parameters for future *T. delbrueckii* strains appear to be (i) optimized fermentation without reliance on *S. cerevisiae*, (ii) consistent lower acetic acid production and (iii) increased varietal character expression (Benito 2018). None of these are fully achievable without a better understanding of the genomic and physiological parameters that differ between *T. delbrueckii* and *S. cerevisiae*, which therefore require further study, such as nitrogen nutrient use, the response to stress and oxygen, and regulation of fermentative metabolism. Importantly, oenological characteristics and interaction with *S. cerevisiae* seem to

be strain-specific (van Breda et al. 2013), so a deeper understanding of enzyme expression and interaction in the broader wine fermentation environment is needed. Unfortunately, the genetics and physiology of *T. delbrueckii* in wine fermentation remains poorly characterized compared to *S. cerevisiae.* The first genome of *T. delbrueckii* to be sequenced was strain CBS 1146 (unknown origin, Gordon et al. 2001), followed by mescal isolate NRRL Y-50541 (Gomez-Angulo et al. 2015), providing a reference to investigate biotechnological features of interest and gene expression during fermentation. Only a few studies have analysed the transcriptional reprogramming after exposure to stress (Hernandez-Lopez et al. 2006a, Hernandez-Lopez et al. 2006b) and during a synthetic wine fermentation (Tronchoni et al. 2017). Despite this, no *T. delbrueckii* genomes are reported from wine isolates, and its metabolic pathways are still not well characterized at a molecular level. For a better understanding of *T. delbrueckii* oenological traits, RNA sequencing (RNA-seq) studies under wine-like conditions are required. In the absence of a closely related reference sequence, significant challenges remain when it comes to assembling short reads into full-length gene and transcript models.

1.5. Summary and scope of the thesis

Many factors throughout fermentation influence yeast metabolic pathways (Bauer and Pretorius 2000, Fleet 2003), and in *Saccharomyces cerevisiae* the effect of the yeast stress response on metabolic gene expression has been characterized (Gasch 2003). This knowledge of *S. cerevisiae* physiology increases fermentation predictability, which relates to wine quality (Bauer and Pretorius 2000). In particular, during fermentation a direct correlation has been made between fermentation efficiency and a proper cell adaptation to environmental stresses (Bauer and Pretorius 2000, Marks et al. 2008). However, while significant information has led to the selection and development of *S. cerevisiae* strains with desired oenological features

(Marsit and Dequin 2015), little is understood about non-*Saccharomyces* physiology associated with wine fermentation. The yeast species *T. delbrueckii* has been investigated alongside *S. cerevisiae* for its ecological interactions, flavour modulation and especially decreased volatile acidity in wine (Bely et al. 2008) and, more recently, in beer and mead (Barry et al. 2018, Canonico et al. 2016, Canonico et al. 2017). Its favourable oenological traits have led to its commercialisation and adoption in the wine industry (Benito 2018). Despite this, *T. delbrueckii* is still poorly characterized at a genetic and molecular level (Albertin et al. 2014), with no complete genomes reported from wine isolates (Gordon et al. 2011, Gomez-Angulo et al. 2015). Moreover the physiology of the stress response has been limited to the hyperosmotic challenges (Hernandez-Lopez et al. 2006a, Hernandez-Lopez et al. 2006b), with only a phenotypic characterization in wine-like conditions (van Breda et al. 2013). Therefore, this study sought to investigate an indigenous Australian isolate of *T. delbrueckii* to highlight its physiological behaviour during wine fermentation. The understanding of its stress response *per se* and its relation to its metabolic pathways will allow winemakers to make better use of such microbial tools to achieve a desired wine sensory outcome.

The main aims of this project were therefore:

1. To investigate and compare the physiology of metabolic pathways between two wine-related indigenous yeasts, *T. delbrueckii* and *S. cerevisiae*. To our knowledge this is the first study in which desired oenological properties of the metabolism of the non-conventional yeast, *T. delbrueckii*, were analysed at a transcriptional level during fermentation, and related to the observed growth or metabolic phenotypes. Such information would potentially identify novel regulatory mechanisms for fermentation aroma production under winemaking conditions in a synthetic wine matrix.

2. To provide higher-quality assemblies, which give a clearer view of the genetic differences between yeasts and provide a better resource for new gene discovery and comparative genomics analysis. To our knowledge, the *T. delbrueckii* COFT1 genome will be the first for describing a wine–related isolated of this species. In particular, we sought to develop a novel method that distinguished and aligned with high accuracy and sensitivity Illumina reads between different species, in order to analyse RNA from mixed fermentations. This would potentially identify new or species/strain specific metabolic functions, and provide a better resolution for further comparative genomics and transcriptomic analyses.

3. To elucidate the effect of high sugar synthetic grape juice media on the physiology and signalling pathways in indigenous *T. delbrueckii* and *S. cerevisiae*, providing information on whether simultaneous stress and nutrient exposure impacts growth and viability. To our knowledge, this is the first study to assess and compare the early transcriptional adaptation (0–2 h) of *T. delbrueckii* in response to multiple stimuli (stress, starvation and, potentially, cell–to–cell communication). This will potentially identify gene-environment interactions, which could relate common or specific mechanisms of sensing and response to the environment to the diverse phenotypes that the two species exhibit.

1.6. References

Albertin, W., L. Chasseriaud, G. Comte, A. Panfili, A. Delcamp, F. Salin, at al. (2014). Winemaking and bioprocesses strongly shaped the genetic diversity of the ubiquitous yeast *Torulaspora delbrueckii*. PLoS One, 9(4): e94246.

Azzolini, M., B. Fedrizzi, E. Tosi, F. Finato, P. Vagnoli, C. Scrinzi. At al. (2012). Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. European Food Research and Technology, 235(2): 303-313.

Barry, J. P., M. S. Metz, J. Hughey, A. Quirk, and M. L. Bochman (2018). Two novel strains of *Torulaspora delbrueckii* isolated from the honey bee microbiome and their use in honey fermentation. Fermentation, 4(2), 22.

Bartowsky, E. J. and I. S. Pretorius (2009). Microbial formation and modification of flavor and off-flavor compounds in wine. Springer, 209-231.

Bauer, F. and I. S. Pretorius (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine-a review. South African Journal for Enology and Viticulture, 21: 27-51.

Belda, I., E. Navascués, D. Marquina, A. Santos, F. Calderon and S. Benito (2015). Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. Applied Microbiology and Biotechnology, 99(4): 1911-1922.

Belda, I., Ruiz, J., Beisert, B., Navascués, E., Marquina, D., Calderón, F., at al. (2017). Influence of *Torulaspora delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations. International Journal of Food Microbiology, 257, 183-191

Bely, M., P. Stoeckle, I. Masneuf-Pomarède and D. Dubourdieu (2008). Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevi*siae culture on high-sugar fermentation. International Journal of Food Microbiology, 122(3): 312-320.

Benito, S. (2018). The impact of *Torulaspora delbrueckii* yeast in winemaking. Applied Microbiology and Biotechnology, 102(7): 3081-3094.

Bisson, L. F., J. E. Karpel, V. Ramakrishnan and L. Joseph (2007). Functional genomics of wine yeast *Saccharomyces cerevisiae*. Advances in Food and Nutrition Research, 53: 65-121.

Bisson, L. F. and J. E. Karpel (2010). Genetics of yeast impacting wine quality. Food Science and Technology, 1.

Bokulich, N. A. and D. A. Mills (2012). Next-generation approaches to the microbial ecology of food fermentations. Bmb Reports, 45(7): 377-389.

Bokulich, N. A., J. H. Thorngate, P. M. Richardson and D. A. Mills (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proceedings of the National Academy of Sciences, 111(1): E139-E148.

Brion, C., D. Pflieger, S. Souali-Crespo, A. Friedrich and J. Schacherer (2016). Differences in environmental stress response among yeasts is consistent with species-specific lifestyles. Molecular Biology of the Cell, 27(10): 1694-1705.

Broach, J. R. (2012). Nutritional control of growth and development in yeast. Genetics, 192(1), 73-105.

Buermans, H. and J. Den Dunnen (2014). Next generation sequencing technology: advances and applications. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1842(10): 1932-1941.

Canonico, L., A. Agarbati, F. Comitini and M. Ciani (2016). *Torulaspora delbrueckii* in the brewing process: a new approach to enhance bioflavour and to reduce ethanol content. Food Microbiology, 56: 45-51.

Canonico, L., F. Comitini and M. Ciani (2017). *Torulaspora delbrueckii* contribution in mixed brewing fermentations with different *Saccharomyces cerevisiae* strains. International Journal of Food Microbiology, 259: 7-13.

Chen, R. E. and J. Thorner (2007). Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1773(8): 1311-1340.

Ciani, M. and F. Maccarelli (1997). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World Journal of Microbiology and Biotechnology, 14(2): 199-203.

Ciani, M., P. Morales, F. Comitini, J. Tronchoni, L. Canonico, J. A. Curiel, at al. (2016). Non-conventional yeast species for lowering ethanol content of wines. Frontiers in Microbiology, 7: 642.

Cocolin, L., L. F. Bisson and D. A. Mills (2000). Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology Letters, 189(1): 81-87.

Cordero-Bueso, G., Esteve-Zarzoso, B., Cabellos, J. M., Gil-Díaz, M., and Arroyo, T. (2013). Biotechnological potential of non-*Saccharomyces* yeasts isolated during spontaneous fermentations of Malvar (*Vitis vinifera* cv. L.). European Food Research and Technology, 236(1), 193-207.

Conterno, L., C. L. Joseph, T. J. Arvik, T. Henick-Kling and L. F. Bisson (2006). Genetic and physiological characterization of *Brettanomyces bruxellensis* strains isolated from wines. American Journal of Enology and Viticulture, 57(2): 139-147.

Čuš, F., and Jenko, M. (2013). The influence of yeast strains on the composition and sensory quality of Gewürztraminer wine. Food Technology and Biotechnology, 51(4), 547-553.

Christie, K. R., Weng, S., Balakrishnan, R., Costanzo, M. C., Dolinski, K., Dwight, at al. (2004). *Saccharomyces* Genome Database (SGD) provides tools to identify and analyze sequences from *Saccharomyces cerevisiae* and related sequences from other organisms. Nucleic Acids Research, 32(suppl_1), D311-D314.

Enjalbert, B., A. Nantel and M. Whiteway (2003). Stress-induced gene expression in *Candida albicans*: absence of a general stress response. Molecular Biology of the Cell, 14(4): 1460-1467.

Erasmus, D., G. Vandermerwe and H. Vanvuuren (2003). Genome-wide expression analyses: Metabolic adaptation to high sugar stress. FEMS Yeast Research, 3(4): 375-399.

Faria-Oliveira, F., R. H. Diniz, F. Godoy-Santos, F. B. Piló, H. Mezadri, I. M. Castro, at al. (2015). The role of yeast and lactic acid bacteria in the production of fermented beverages in South America. Food Production and Industry, IntechOpen.

Fleet, G. H. (2003). Yeast interactions and wine flavour. International Journal of Food Microbiology, 86(1-2): 11-22.

Fleet, G. H. (2008). Wine yeasts for the future. FEMS Yeast Research, 8(7): 979-995.

Fraile, P., J. Garrido and C. Ancín (2000). Influence of a *Saccharomyces cerevisiae* selected strain in the volatile composition of rose wines. Evolution during fermentation. Journal of Agricultural and Food Chemistry, 48(5): 1789-1798.

Fuchs, B. B. and E. Mylonakis (2009). Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryotic cell, 8(11): 1616-1625.

Garde-Cerdán, T. and C. Ancín-Azpilicueta (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. LWT-Food Science and Technology, 41(3): 501-510.

Gasch, A. P. (2003). The environmental stress response: a common yeast response to diverse environmental stresses. Yeast stress responses, Springer, 11-70.

Gasch, A. P. (2007). Comparative genomics of the environmental stress response in ascomycete fungi. Yeast, 24(11): 961-976.

Godden, P. and R. Muhlack (2010). Trends in the composition of Australian wine. Australian and New Zealand Grapegrower and Winemaker, 558 (2010): 47.

Goffeau, A. (2000). Four years of post-genomic life with 6000 yeast genes. FEBS Letters, 480(1): 37-41.

Gomez-Angulo, J., L. Vega-Alvarado, Z. Escalante-García, R. Grande, A. Gschaedler-Mathis, L. Amaya-Delgado, at al. (2015). Genome sequence of *Torulaspora delbrueckii* NRRL Y-50541, isolated from mezcal fermentation. Genome Announcements, 3(4): e00438-00415.

Gonzalez, R., P. Morales, J. Tronchoni, G. Cordero-Bueso, E. Vaudano, M. Quirós, at al. (2016). New genes involved in osmotic stress tolerance in *Saccharomyces cerevisiae*. Frontiers in Microbiology, 7: 1545.

Gordon, J. L., D. Armisen, E. Proux-Wera, S. S. Oheigeartaigh, K. P. Byrne and K. H. Wolfe (2011). Evolutionary erosion of yeast sex chromosomes by mating-type switching accidents. Proceedings of the National Academy of Sciences USA, 108.

Grangeteau, C., C. Roullier-Gall, S. Rousseaux, R. D. Gougeon, P. Schmitt-Kopplin, H. Alexandre, at al. (2017). Wine microbiology is driven by vineyard and winery anthropogenic factors. Microbial Biotechnology, 10(2): 354-370.

Hazelwood, L. A., J.-M. Daran, A. J. van Maris, J. T. Pronk and J. R. Dickinson (2008). The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Applied and Environmental Microbiology, 74(8): 2259-2266.

Hittinger, C. T., Rokas, A., Bai, F. Y., Boekhout, T., Goncalves, P., Jeffries, T. W., ... & Sampaio, J. P., (2015). Genomics and the making of yeast biodiversity. Current Opinion in Genetics & Development, 35, 100-109

Hernandez-Lopez, M. J., J. Panadero, J. A. Prieto and F. Randez-Gil (2006). Regulation of salt tolerance by *Torulaspora delbrueckii* calcineurin target Crz1p. Eukaryotic Cell, 5(3): 469-479.

Hernandez-Lopez, M. J., F. Randez-Gil and J. A. Prieto (2006). Hog1 mitogen-activated protein kinase plays conserved and distinct roles in the osmotolerant yeast *Torulaspora delbrueckii*. Eukaryotic Cell, 5(8): 1410-1419.

Hernández-Orte, P., M. Ibarz, J. Cacho and V. Ferreira (2006). Addition of amino acids to grape juice of the Merlot variety: effect on amino acid uptake and aroma generation during alcoholic fermentation. Food Chemistry, 98(2): 300-310.

Hohmann, S. (2009). Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. FEBS Letters, 583(24): 4025-4029.

Huang, C.-W., M. E. Walker, B. Fedrizzi, M. Roncoroni, R. C. Gardner and V. Jiranek (2016). The yeast *TUM1* affects production of hydrogen sulfide from cysteine treatment during fermentation. FEMS Yeast Research, 16(8).

Ishmayana, S., R. P. Learmonth and U. J. Kennedy (2011). Fermentation performance of the yeast *Saccharomyces cerevisiae* in media with high sugar concentration. Proceedings of the 2nd International Seminar on Chemistry: Chemistry for a Better Future, (ISC 2011), Padjadjaran University.

Jimenez-Marti, E., A. Zuzuarregui, M. Gomar-Alba, D. Gutierrez, C. Gil and M. del Olmo (2011). Molecular response of *Saccharomyces cerevisiae* wine and laboratory strains to high sugar stress conditions. International Journal of Food Microbiology, 145(1): 211-220.

Jolly, N. P., C. Varela and I. S. Pretorius (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Research, 14(2): 215-237.

Krantz, M., E. Becit and S. Hohmann (2006). Comparative genomics of the HOG-signalling system in fungi. Current Genetics, 49(3): 137-151.

Kurtzman, C., J. W. Fell and T. Boekhout (2011). The yeasts: a taxonomic study. Elsevier.

Lambrechts, M. and I. Pretorius (2000). Yeast and its importance to wine aroma-a review. South African Journal for Enology and Viticulture, 21: 97-129.

Landolfo, S., H. Politi, D. Angelozzi and I. Mannazzu (2008). ROS accumulation and oxidative damage to cell structures in *Saccharomyces cerevisiae* wine strains during fermentation of high-sugar-containing medium. Biochimica et Biophysica Acta (BBA)-General Subjects, 1780(6): 892-898.

Li, H., G. Du, H.-L. Li, H.-L. Wang, G.-L. Yan, J.-C. Zhan, at al. (2010). Physiological response of different wine yeasts to hyperosmotic stress. American Journal of Enology and Viticulture, 61(4): 529-535.

Liu, S. Q. (2002). Malolactic fermentation in wine–beyond deacidification. Journal of Applied Microbiology, 92(4): 589-601.

Loira, I., R. Vejarano, M. Bañuelos, A. Morata, W. Tesfaye, C. Uthurry, at al. (2014). Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. LWT-Food Science and Technology, 59(2): 915-922.

Marks, V. D., S. J. Ho Sui, D. Erasmus, G. K. Van Der Merwe, J. Brumm, W. W. Wasserman, at al. (2008). Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Research, 8(1): 35-52.

Marsit, S. and S. Dequin (2015). Diversity and adaptive evolution of *Saccharomyces* wine yeast: a review. FEMS Yeast Research, 15(7).

Minnaar, P. P., Ntushelo, N., Ngqumba, Z., Van Breda, V., and Jolly, N. P. (2015). Effect of *Torulaspora delbrueckii* yeast on the anthocyanin and flavanol concentrations of Cabernet franc and Pinotage wines. South African Journal of Enology and Viticulture, 36(1), 50-58.

Nishino, H., S. Miyazaki and K. Tohjo (1985). Effect of osmotic pressure on the growth rate and fermentation activity of wine yeasts. American Journal of Enology and Viticulture, 36(2): 170-174.

Oliver, S. G. (2002). Functional genomics: lessons from yeast. Philosophical Transactions of the Royal Society B: Biological Sciences, 357(1417), 17.

Padilla, B., J. V. Gil and P. Manzanares (2016). Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Frontiers in Microbiology, 7: 411.

Pizarro, F., F. A. Vargas and E. Agosin (2007). A systems biology perspective of wine fermentations. Yeast, 24(11): 977-991.

Pratt, P. L., J. H. Bryce and G. G. Stewart (2003). The effects of osmotic pressure and ethanol on yeast viability and morphology. Journal of the Institute of Brewing, 109(3): 218-228.

Pretorius, I. S. (2003). The genetic analysis and tailoring of wine yeasts. Functional genetics of industrial yeasts. Springer, 99-142.

Puig, S. and J. E. Pérez-Ortín (2000). Expression levels and patterns of glycolytic yeast genes during wine fermentation. Systematic and Applied Microbiology, 23(2): 300-303.

Querol, A. and U. Bond (2009). The complex and dynamic genomes of industrial yeasts. FEMS Microbiology Letters, 293(1): 1-10.

Querol, A., M. T. Fernández-Espinar, M., li del Olmo and E. Barrio (2003). Adaptive evolution of wine yeast. International Journal of Food Microbiology, 86(1-2): 3-10.

Renault, P., Coulon, J., de Revel, G., Barbe, J. C., and Bely, M. (2015). Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. International Journal of Food Microbiology, 207, 40-48.

Renault, P., Coulon, J., Moine, V., Thibon, C., and Bely, M. (2016). Enhanced 3-sulfanylhexan-1-ol production in sequential mixed fermentation with *Torulaspora delbrueckii/Saccharomyces cerevisiae* reveals a situation of synergistic interaction between two industrial strains. Frontiers in Microbiology, 7, 293

Riley, R., Haridas, S., Wolfe, K. H., Lopes, M. R., Hittinger, C. T., Göker, M., at al. (2016). Comparative genomics of biotechnologically important yeasts. Proceedings of the National Academy of Sciences, 113(35), 9882-9887.

Romano, P., C. Fiore, M. Paraggio, M. Caruso and A. Capece (2003). Function of yeast species and strains in wine flavour. International Journal of Food Microbiology, 86(1-2): 169-180.

Rossignol, T., L. Dulau, A. Julien and B. Blondin (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast, 20(16): 1369-1385.

Rossouw, D., N. Jolly, D. Jacobson and F. F. Bauer (2012). The effect of scale on gene expression: commercial versus laboratory wine fermentations. Applied Microbiology and Biotechnology, 93(3): 1207-1219.

Saerens, S. M., F. R. Delvaux, K. J. Verstrepen and J. M. Thevelein (2010). Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microbial Biotechnology, 3(2): 165-177.

Saito, H. and F. Posas (2012). Response to hyperosmotic stress. Genetics 192(2): 289-318.

Saxena, A. and R. Sitaraman (2016). Osmoregulation in *Saccharomyces cerevisiae* via mechanisms other than the high-osmolarity glycerol pathway. Microbiology, 162(9): 1511-1526.

Steensels, J. and K. J. Verstrepen (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. Annual Review of Microbiology, 68: 61-80.

Styger, G., B. Prior and F. F. Bauer (2011). Wine flavor and aroma. Journal of Industrial Microbiology & Biotechnology, 38(9): 1145.

Suárez-Lepe, J. and A. Morata (2012). New trends in yeast selection for winemaking. Trends in Food Science & Technology, 23(1): 39-50.

Swiegers, J. and I. Pretorius (2005). Yeast modulation of wine flavor. Advances in Applied Microbiology, 57: 131-175.

Taillandier, P., Lai, Q. P., Julien-Ortiz, A., and Brandam, C. (2014). Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content. World Journal of Microbiology and Biotechnology, 30(7), 1959-1967.

Tronchoni, J., J. A. Curiel, P. Morales, R. Torres-Pérez and R. Gonzalez (2017). Early transcriptional response to biotic stress in mixed starter fermentations involving *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. International Journal of Food Microbiology, 241: 60-68.

Van Breda, V., Jolly, N., and van Wyk, J. (2013). Characterisation of commercial and natural *Torulaspora delbrueckii* wine yeast strains. International Journal of Food Microbiology, 163(2-3), 80-88.

Varela, C., J. Cárdenas, F. Melo and E. Agosin (2005). Quantitative analysis of wine yeast gene expression profiles under winemaking conditions. Yeast, 22(5): 369-383.

Walker, M. E., T. D. Nguyen, T. Liccioli, F. Schmid, N. Kalatzis, J. F. Sundstrom, at al. (2014). Genome-wide identification of the Fermentome; genes required for successful and timely completion of wine-like fermentation by *Saccharomyces cerevisiae*. BMC Genomics, 15.

Statement of Authorship

Title of Paper	Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains
Publication Status	[X] Published [ ] Accepted for Publication [X] Submitted for Publication [ ] Unpublished and Unsubmitted work written in manuscript style
Publication Details	INTERNATIONAL JOURNAL FOOD MICROBIOLOGY

Principal Author

Name of Principal Author (Candidate)	FEDERICO TONDINI
Contribution to the Paper	CONCEPTION DRAFTING ANALYSIS
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.
Signature	Date: 26/6/18

Co-Author Contributions

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

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

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

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

Name of Co-Author	TOM LANG
Contribution to the Paper	Analysis
Signature	Date: 22/6/18

Name of Co-Author	LIANG CHEN
Contribution to the Paper	Analysis
Signature	Date: 15-June-2018

Name of Co-Author	MARKUS HERDERICH	MARKUS HERDERICH
Contribution to the Paper	- CONCEPTION - DRAFTING	Conception Drafting
Signature		Date 03.07.2018

Name of Co-Author	VLADIMIR JIRANEK	VLADIMIR JIRANEK
Contribution to the Paper	- CONCEPTION - DRAFTING	Conception Analysis
Signature		Date 26.6.18

Please cut and paste additional co-author panels here as required.

Chapter 2: Linking gene expression and oenological traits: comparison between indigenous *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains

Federico Tondini,[1,2] Tom Lang,[1] Liang Chen, [1] Markus Herderich,[2,3] Vladimir Jiranek*[1,2]

[1] Department of Wine & Food Science, University of Adelaide, Glen Osmond, South Australia 5064, Australia.

[2] Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production, Glen Osmond, South Australia 5064, Australia.

[3] The Australian Wine Research Institute (AWRI), Glen Osmond, South Australia 5064, Australia.

*The Corresponding Author: vladimir.jiranek@adelaide.edu.au

Department of Wine & Food Science, University of Adelaide, Glen Osmond, South Australia 5064, Australia.

Declarations of interest: none

International Journal of Food Microbiology 294 (2019) 42–49

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains

Federico Tondini[a,b], Tom Lang[a], Liang Chen[a], Markus Herderich[b,c], Vladimir Jiranek[a,b,*]

[a] Department of Wine & Food Science, University of Adelaide, Glen Osmond, South Australia 5064, Australia
[b] Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production, Glen Osmond, South Australia 5064, Australia
[c] The Australian Wine Research Institute, Glen Osmond, South Australia 5064, Australia

ARTICLE INFO

Keywords:
Indigenous yeast
Wine isolates
Transcripts profile
BAP2

ABSTRACT

Wine fermentations typically involve the yeast *Saccharomyces cerevisiae*. However, many other yeast species participate to the fermentation process, some with interesting oenological traits. In this study the species *Torulaspora delbrueckii*, used occasionally in mixed or sequential fermentation with *S. cerevisiae* to improve wine sensory profile, was investigated to understand the physiological differences between the two. Next generation sequencing was used to characterize the transcriptome of *T. delbrueckii* and highlight the different genomic response of these yeasts during growth under wine-like conditions. Of particular interest were the basic differences in the glucose fermentation pathway and the formation of aromatic and flavour compounds such as glycerol, esters and acetic acid. Paralog genes were missing in glycolysis and glycerol biosynthesis in *T. delbrueckii*. Results indicate the tendency of *T. delbrueckii* to produce less acetic acid relied on a higher expression of alcoholic fermentation related genes, whereas acetate esters were influenced by the absence of esterases, *ATF1–2*. Additionally, in the Δbap2 *S. cerevisiae* strain, the final concentration of short branched chain ethyl esters (SBCEEs) was related to branched chain amino acid (BCAA) uptake. In conclusion, different adaption strategies are apparent for *T. delbrueckii* and *S. cerevisiae* yeasts, an understanding of which will allow winemakers to make better use of such microbial tools to achieve a desired wine sensory outcome.

1. Introduction

Fermented foods have been produced for millennia, via process that was developed as a preservation method. Their traditional preparation relied on a spontaneous fermentation by 'wild' microorganisms. These practises promoted the adaptation and domestication of microorganisms to anthropic habitats. In wine, microorganisms had to adapt to harsh and dynamic environments through beneficial genomic variations that provided a fitness advantage. Yeast, along with lactic acid bacteria, were therefore selected in order to preserve food and beverages and also improve the sensory attributes, and addition of a pure selected yeast starter forms part of current fermentation practices (Mortimer, 2000).

Saccharomyces cerevisiae is undoubtedly the most well-known and broadly used of the yeasts. Its genome has a median length of 12 Mb and is divided into sixteen chromosomes (O'Leary et al., 2015). It encodes > 5400 genes and its selected oenological phenotype comprises several desired traits: tolerance to ethanol (10–14% v/v) and sulfur dioxide (SO_2), complete sugar catabolism (residual of < 5 g/L), low production of volatile acidity, low assimilable nitrogen consumption, high growth rate and fermentation reproducibility (Suárez-Lepe and Morata, 2012). Many of the non-*Saccharomyces* yeasts involved in wine fermentation are considered spoilage, due to their negative sensory traits, lower ethanol tolerance and low fermentation ability (Tataridis et al., 2013). However, more recently, the contribution of these to wine quality has been reconsidered (Jolly et al., 2014) with several investigations reporting many positive effects on wine sensory attributes amongst selected strains (Bely et al., 2008; Ciani and Picciotti, 1995). *Torulaspora delbrueckii* is a non-*Saccharomyces* yeast that can be found metabolically active on grape berries and juice of spontaneous or yeast inoculated fermentations (Albertin et al., 2014; Comitini et al., 2011). Previously, this species was identified as *Saccharomyces rosei*, which suggests a similar taxonomic lineage and function to that of *S. cerevisiae* (Bely et al., 2008). Evolutionarily, *Torulaspora* spp. and *Saccharomyces*

Abbreviations: Chemically Defined Grape Juice Medium, (CDGJM); Short chain ethyl esters, (SCEEs); Short branched chain ethyl esters, (SBCEEs); Branched chain amino acids, (BCAAs); Medium chain fatty acids, (MCFAs)

* Corresponding author at: Department of Wine & Food Science, University of Adelaide, Glen Osmond, South Australia 5064, Australia.
E-mail address: vladimir.jiranek@adelaide.edu.au (V. Jiranek).

https://doi.org/10.1016/j.ijfoodmicro.2019.01.014
Received 14 September 2018; Received in revised form 21 January 2019; Accepted 22 January 2019
Available online 02 February 2019
0168-1605/

spp. separated approximately 100–150 million years ago (Hagman et al., 2014). *Torulaspora delbrueckii* has a 9.7 Mb genome, with nearly 5000 open reading frames (ORFs) divided into 8 chromosomes (Gordon et al., 2011). Under typical winemaking conditions, *Torulaspora* sp. has a fermentative metabolism (Brandam et al., 2013) and can produce lower amounts of acetaldehyde, acetoin, acetate (approximately 0.2 g/L, as reported in the best scenarios) and ethyl acetate compared to *Saccharomyces cerevisiae* (Bely et al., 2008; Martinez et al., 1990; Taillandier et al., 2014), even if, to a certain degree, strain-dependent phenotypic variance have been observed (Velázquez et al., 2015; Van Breda et al., 2013). Its metabolism differs from that of *S. cerevisiae*, especially under hyperosmotic stress, where it produces lower levels of acetic acid (Blomberg and Adler, 1992). Furthermore, *T. delbrueckii* fermentation in combination with *S. cerevisiae* exhibits a different production of fusel alcohols, esters, terpenes and phenolic aldehydes (Azzolini et al., 2012; Azzolini et al., 2015; Renault et al., 2015; Velázquez et al., 2015). Empirically, it added sensory complexity described as “wild” or “natural”, which provides the final wine with desired regional characteristics, usually referred as an expression of terroir (Raynal et al., 2011; Tataridis et al., 2013).

An enhancement of volatile compounds, such as esters, higher alcohols, and an acceptable low production of off-flavours, have been established as criteria for selecting new yeasts for use in winemaking (Pacheco et al., 2012). An increased influence of non-*Saccharomyces* species during fermentation may be a way to enhance the production of desirable compounds, in order to produce more complex and higher quality wines. Nevertheless, there is a need for a better understanding of the molecular basis behind the production of these compounds by non-*Saccharomyces* yeasts under different winemaking conditions. Variations in yeasts activity of aroma-active metabolic pathways will produce organoleptic difference in wine (Bisson and Karpel, 2010). Such pathways include those involved in the production of volatile acidity (as acetate off-flavours), the Ehrlich pathway (fusel alcohols and aldehydes) and the acetate and ethyl ester pathways (Bisson and Karpel, 2010; Chidi et al., 2016; Procopio et al., 2011). Yeast cells use these pathways in response to stress to maintain intracellular redox balance and to adjust the pool of essential cofactors and amino-acids (Hazelwood et al., 2008). To investigate these aspects, next generation RNA sequencing (using an ILLUMINA platform) has emerged as a powerful tool to provide a comprehensive analysis of the simultaneous expression of thousands of genes (Marks et al., 2008). Comparative transcriptomics investigation of *Saccharomyces* vs. non-*Saccharomyces* yeast species could increase our knowledge on genes associated with the production of aroma compounds characteristic of unique wine styles (Masneuf-Pomarede et al., 2015). Findings could aid in the identification of wine yeast strains with promising sensory characteristics and higher fermentation efficiencies (Erasmus et al., 2004).

The aim of this work was to identify the genes responsible for oenological differences between *S. cerevisiae* and *T. delbrueckii*. We also explored if the differences in metabolite production during fermentation between the two species were related to their different genetic backgrounds and/or the manner in which relevant genes and metabolic pathways were regulated.

2. Materials and methods

2.1. Yeast selection and fermentation procedure

Indigenous strains of *S. cerevisiae* YAL1 and *T. delbrueckii* COFT1 were originally isolated from spontaneous fermentations conducted at Yalumba Winery (Angaston, South Australia). Glycerol stocks were streaked onto YEPD agar plates (1% yeast extract, 2% bacto peptone, 2% glucose, 2% agar) and grown for 48 h at 28 °C. For each species, a single colony was isolated and grown overnight in YEPD medium (1% yeast extract, 2% bacto peptone, 2% glucose). A 20 µL cell suspension was then transferred into 150 µL phosphate buffer saline (PBS) solution, stained with 10 µL of 1 mg mL^{-1} propidium iodide (Deere et al., 1998) before 20 µL of Flow-Count fluorospheres (Beckman Coulter, USA) were added to three separate subsamples and a count made by a flow cytometer (FACSCalibur; Becton Dickinson, USA). Accordingly, the appropriate volume of starter culture was added to 200 mL Chemically Defined Grape Juice Medium (CDGJM, modified from McBryde et al., 2006, Supplementary File 2; 250 g/L equimolar glucose and fructose; 400 mg/L nitrogen) in 250 mL Erlenmeyer flasks to provide an initial inoculation rate of 1×10^6 cells/mL. Fermentations were conducted in triplicate with shaking at 120 rpm, to homogenise the samples and ensure common gene expression between all cells (Puig and Pérez-Ortín, 2000) and, at 22 °C, an average temperature between red and white wine fermentations. After initiating the fermentations and consequent establishment of anaerobic condition, 10 mL samples were taken every 24 h, from which 5 mL were divided into 5 aliquots and frozen immediately in liquid nitrogen for subsequent RNA extraction (Piper et al., 2002). The supernatant from the remaining 5 mL subsample was divided into 5 equal aliquots, which were frozen for subsequent metabolite analysis. Yeast growth during fermentation was quantified by measuring cell density at 660 nm with a Tecan Infinite 200 spectrophotometer (Tecan, Switzerland).

2.2. Yeast transformation

Genomic DNA was isolated from BY4741 bap2::KanMX4 yeast strain according to Amberg et al. (2005). PCR amplification of the *BAP2* deletion cassette (bap2::KanMX4) was undertaken using VELOCITY DNA Polymerase (Bioline, UK) according to the manufacturer's instructions and primers BAP2 A (CTTCAACGGTAAATATGTCAGCAG) and BAP2 D (AATATCCTTTCCATTACCCAAAGAG; www-sequence.stanford.edu/group/yeast_deletion_project). PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) following gel electrophoresis. The identified *BAP2* deletion cassette was subsequently transformed into the haploid wine yeast AWRI 1631, according to Gietz and Schiestl (2007). Heat-shocked cells were incubated for 2.5 h in 1 mL YEPD, prior to plating on YEPD agar containing 200 mg/L G418. Plates were incubated at 28 °C for approximately 48 h. Individual colonies were selected and genomic DNA was isolated. Gene deletions were verified by PCR amplification using primers BAP2 A and Kan B (CTGCAGCGAGGAGCCGTAAT) primers.

2.3. Metabolite analysis

Sugars (glucose, fructose) and yeast metabolites (ethanol, malic acid, acetic acid, citric acid, glycerol, succinic acid) were measured by HPLC following the protocol of Liccioli et al. (2011). Profiling of volatile compounds was conducted by Metabolomics Australia (Adelaide, Australia). The quantitation of ethyl esters was carried out using the headspace–solid phase micro extraction – gas chromatography with mass spectrometry (HS-SPME-GC–MS) method. HS-SPME was performed using a Gerstel MPS autosampler fitted with a 1 cm preconditioned divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (50/30 µm). An aliquot of wine (0.5 mL), 4.5 mL of Milli-Q water, 10 µL of internal standard 2-octanol solution, and 2 g of sodium chloride were added into a 20 mL clear, screw-cap GC–MS vial, which was then sealed with PTFE-lined cap. Samples were incubated under agitation (250 rpm) at 35 °C for 30 min followed by extraction at 35 °C for 30 min. Samples were analysed by an Agilent 7890 GC with 5897 mass selective detector (MSD) equipped with a DB-WAX column (60 m × 0.25 mm × 0.25 µm; Agilent Technologies, USA). Ultrapure helium was used as carrier gas with a constant flow rate of 2 mL/min. The oven program was 40 °C for 4 min and then increased to 220 °C at 5 °C/min, and held at 220 °C for 10 min. Temperatures for inlet, transfer line, MS source and MS quadrupole were set at 200, 250, 250 and 150 °C, respectively. Positive ion electron impact energy was 70 eV, and the mass range was 35–350 amu. Samples were analysed in triplicate.

2.4. RNA extraction and sequencing

Three different time points were chosen to investigate the transcriptional profile of each strain: 24 h (exponential growth phase), 140 h (early stationary phase) and 240 h (late stationary phase). RNA extraction was performed using the Trizol protocol described by Chomczynski and Sacchi (2006). To obtain high quality RNA, an RNeasy mini kit (QIAGEN, Melbourne, Australia) was used to purify and concentrate the samples in 2 mL microfuge tubes. The resulting extracts were treated with RNase-free DNase (Life Technologies, United States). Quality check, cDNA preparation, library preparation and Illumina sequencing were performed by the Australian Genomics Research Facility (AGRF, Melbourne, Australia) using a Hiseq 2000 platform. Only high quality RNA was sequenced, with an RNA integrity number (RIN) of > 8. Approximately 130 gigabytes of FASTQ files were obtained, which contained 100 bp paired-end reads.

2.5. Transcriptome assembly and analysis

Approximately 10 million reads/sample were obtained and a total of 30 million reads were used for assembly of the transcriptome for each species. Reads were quality trimmed (phred score > 30) using Trimmomatic (Bolger et al., 2014) to remove both low quality bases and Illumina adapter. The results were checked with FastQC (Andrews, 2010). Quality trimmed reads were mapped to the species reference genomes (*S. cerevisiae* and *T. delbrueckii* NCBI IDs 15 and 12254, respectively; O'Leary et al., 2015) using Tophat (Trapnell et al., 2009). The mapped reads were used to assemble the transcriptome using Trinity with the genome-guided option (Grabherr et al., 2011). Downstream analyses followed the Trinity protocol (Haas et al., 2013), and included the following steps: principal component analysis of the transcriptomes (Yeung and Ruzzo, 2001), abundance estimation of the transcripts using RSEM (Li and Dewey, 2011), normalization of expression values between samples of the same species with the trimmed mean of M-values normalization method (TMM; Robinson and Oshlack, 2010), and differential expression (DE) statistical analysis with the edgeR R package (Robinson et al., 2010). Quality of the assembly was evaluated using Ex50 stats (Haas, 2016), principal component analyses (Yeung and Ruzzo, 2001) and Benchmarking Universal Single Copy Orthologues of fungi (BUSCO) (Simão et al., 2015).

Functional annotation, functional enrichment and gene ontology of the transcriptome genes were made using Trinotate procedures (Finn et al., 2011; Kanehisa et al., 2011; Krogh et al., 2001; Lagesen et al., 2007; Petersen et al., 2011; Powell et al., 2012; Punta et al., 2011). Enzymatic pathways were modelled with Cytoscape (Shannon et al., 2003).

2.6. Statistical analysis

Statistical analysis was made with GraphPad Prism 7.02 (GraphPad Software, USA). Standard deviation and *p*-values were calculated on fermentation replicates. The statistical level of significance was set at p-value of ≤ 0.05 for metabolite concentrations.

3. Results

3.1. Fermentation

Differences were observed in the fermentation performance between *T. delbrueckii* and *S. cerevisiae*. *S. cerevisiae* consumed 25% more sugars and fermented faster than *T. delbrueckii*, consequently producing higher ethanol concentrations (Fig. 1A). After 10 days, neither strain was able to fully complete the fermentation (Fig. 1A).

The production of secondary metabolites such as acetic acid, glycerol, acetates, esters and higher alcohols highlighted differences in the metabolism of the two species. In *S. cerevisiae*, the production of glycerol and acetic acid followed similar trends to those described by Eglinton et al. (2002). Production increased during exponential growth before a modest decrease over the remainder of the experiment (Fig. 1B). Instead, for *T. delbrueckii*, only glycerol production was similar to that of *S. cerevisiae*. In the case of acetic acid, yields were ~10–20 times lower than *Saccharomyces* (Fig. 1B).

More differences between the species were apparent from volatile analyses. With the exception of ethyl propanoate, 2&3-methylbutanol, butanol and ethyl hexanoate, less ester and higher alcohol production was noted in *T. delbrueckii* fermentations (Table 1).

3.2. Transcriptional landscape during fermentation

In order to characterize the different responses of the two yeast species we monitored the evolution of RNA during fermentation using Next Generation sequencing (Linde et al., 2015; Marks et al., 2008). Three different phases of growth (i.e. mid exponential growth, early stationary, late stationary), instead of equivalent fermentation stages, portrayed the transcriptional landscape during fermentation of each of the two species (Puig and Pérez-Ortín, 2000; Duc et al., 2017), to better characterize the metabolic differences. The assembly strategy generated 14,454 transcripts for the *S. cerevisiae* strain and 8853 for the *T. delbrueckii* strain. To assess the quality of the assemblies, the presence of Benchmarking Universal Single Copy Orthologues of fungi (BUSCO) in the transcriptome was checked. Completeness ranged from 74% for *S. cerevisiae* to 83% for *T. delbrueckii* with a moderate extent of fragmentation. Nevertheless, the missing genes were < 5% in each case (Supplementary File 1). The sequences of the unigenes were annotated and approximately 87% had a match in the Uniprot Database (Bairoch and Apweiler, 2000); specifically, a total of 12,846 transcripts for *S. cerevisiae* and 7691 transcripts for the *T. delbrueckii* strain (Supplementary File 1). Ninety percent of *Torulaspora* sp. sequences had significant homology matches in the SWISS-PROT database with an *E*-value cut-off of 10^{-5}. Species specific distribution analysis indicated that 89% of the *Torulaspora* sequences matched genes of *S. cerevisiae*, followed by yeasts belonging to the class *Saccharomycetes* (*Candida glabrata* 2.3%, *Zygosaccharomyces rouxii* 2%, *Kluyveromyces lactis* 1.6%, *Vanderwaltozyma polyspora* 1.3%, *Eremothecium gossypii* 1%), *Schizosaccharomycetes* (*Schizosaccharomyces pombe* 1.3%) and others (1.7%; Supplementary Fig. 2). Similarities between the species could be confirmed by analysis of the predicted proteins: the two species share 82.4% of the annotated proteins, while *Saccharomyces* has 14.6% and *Torulaspora* 3%, respectively, of unique annotated proteins (Supplementary Figs. 2, 3).

Both species indeed had a relatively strong and broad differential expression of genes during the progression of fermentation. In *S. cerevisiae* 11% of the unigenes (1746) were differentially expressed with at least a four-fold increase between the exponential phase and early stationary phase, whereas in *T. delbrueckii* 16% of its total unigenes (2333) were overexpressed with 110 transcripts increased at least by eight-fold. Response to stress, catabolic process, cellular protein modification process were all enriched functions, present in approximately 10% of these genes (data not shown). However no large differences were noted for *Torulaspora* between early and late stationary phases, whereas 177 *S. cerevisiae* genes were differentially expressed by more than an eight-fold change (Supplementary File 1, Supplementary Fig. 1).

3.3. Glucose fermentation pathway

S. cerevisiae and *T. delbrueckii* exhibited different fermentation rates (Fig. 1A). We compared the expression of genes involved in sugar catabolism to highlight differences between sugar consumption and ethanol production of *Saccharomyces* and the less active *Torulaspora*. Patterns of expression of low affinity glucose transporter (*HXT1*) and high affinity glucose transporters (*HXT2* for *Torulaspora* and *HXT6* for *Saccharomyces*) were similar. Both species initially transcribed *HXT1*

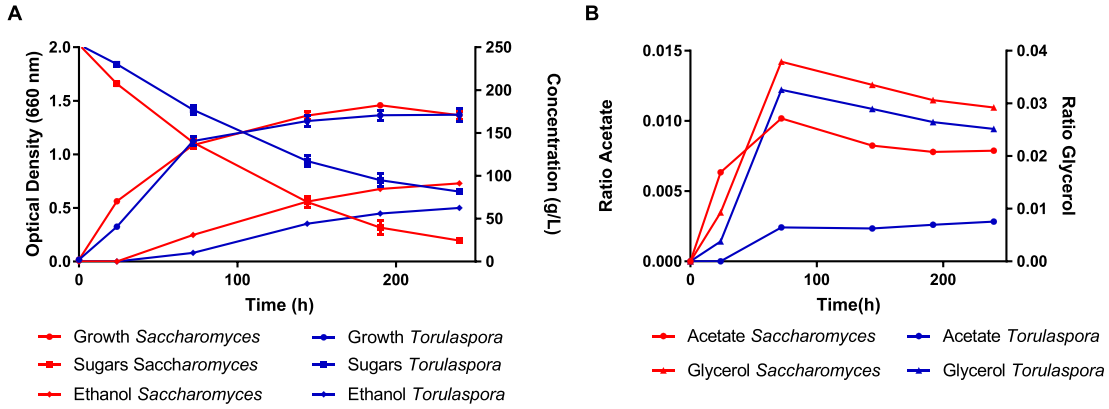


Fig. 1. Fermentation and metabolite production profiles. (A) *S. cerevisiae* and *T. delbrueckii* growth, sugar consumption and relative ethanol production. (B) Production of secondary metabolites during fermentation. Specific acetate and glycerol production (g per gram of sugar consumed) by *S. cerevisiae* and *T. delbrueckii*. (COLOR).

Table 1
Quantification (integrated peak area; relative to internal standards) of ethyl esters, acetate esters and higher alcohol in *S. cerevisiae* and *T. delbrueckii* fermentations.

Compound	Relative quantification		p-Value
	S. cerevisiae	*T. delbrueckii*	
Ethyl acetate	1.29 ± 0.06	1.02 ± 0.05	0.0049
Ethyl propanoate	2.45 ± 0.14	4.95 ± 0.55	0.0016
Ethyl 2-methylpropanoate	9.60 ± 0.24	1.02 ± 0.24	< 0.0001
2-Methylpropyl acetate	4.36 ± 0.50	1.90 ± 0.12	0.0004
Ethyl butanoate	2.20 ± 0.24	0.70 ± 0.87	0.0005
Ethyl 2-methylbutanoate	1.60 ± 0.60	< LOD	N/C
Ethyl 3-methylbutanoate	1.15 ± 0.10	< LOD	N/C
2-Methylpropanol	12.80 ± 0.06	9.40 ± 1.50	0.0234
2-Methylbutyl acetate	1.80 ± 0.24	< LOD	N/C
3-Methylbutyl acetate	1.42 ± 0.26	0.10 ± 0.01	0.0009
Butanol	8.70 ± 0.39	9.80 ± 1.00	0.1532
2&3-Methylbutanol	1.82 ± 0.10	1.88 ± 0.20	0.7266
Ethyl hexanoate	8.80 ± 1.50	8.48 ± 0.60	0.9000
Hexyl acetate	< LOD	< LOD	N/C
Hexanol	< LOD	< LOD	N/C
Ethyl octanoate	5.16 ± 1.40	3.00 ± 0.70	0.0039
Ethyl decanoate	1.00 ± 0.20	< LOD	N/C

LOD, limit of detection; N/C not calculable.

but with the progress of fermentation, switched to expressing high affinity transporters *HXT2/HXT6*. The glycolytic pathway of *Torulaspora* has all steps necessary to convert glucose in pyruvate, but compared to *S. cerevisiae* is missing paralog genes encoding glyceraldehyde-3-phospate dehydrogenase, enolase and pyruvate kinase. Furthermore the transcript of *PGI1*, *TPI1*, *ENO2* in *T. delbrueckii* were more similar to *Candida glabrata*, while *PYK2* was more similar to *Lachancea thermotolerans*. In the fermentation pathway, *T. delbrueckii* expressed only one pyruvate decarboxylase gene (*PDC1*, Fig. 2B), while *S. cerevisiae* expressed all three (*PDC 1*, *5* and *6*; Fig. 2A). Moreover, the sequence of the *T. delbrueckii PDC1* gene was more similar to that of *Kluyveromyces* sp. than *S. cerevisiae* (Supplementary File 1). All seven alcohol dehydrogenases (*ADH1–7*) responsible of converting acetaldehyde to ethanol, were found in *S. cerevisiae* but only four were detected in *Torulaspora* (*ADH1*, *ADH3*, *ADH4* and *ADH6*, Fig. 2). As expected, we observed a broad reduction of expression between exponential and stationary phase in both species, except for *ADH1* and *PDC1* in *T. delbrueckii*, which expression levels increased during fermentation.

3.4. Glycerol and acetic acid pathways

Glycerol is an important secondary metabolite of alcoholic fermentation: it is produced in wine at a concentration of 2–11 g/L and it is known to contribute positively to the perception of body and viscosity in wine (Gawel et al., 2007, Remize et al., 2003,). Both *T. delbrueckii* and *S. cerevisiae* produced similar amounts of glycerol during fermentation, proportional to the amount of sugar consumed, therefore *S. cerevisiae* ended up with 6.67 ± 0.29 g/L compared to 4.32 ± 0.12 for *T. delbrueckii*. Transcriptome expression analysis, however, showed differences between the two species for the genes involved (Fig. 3; Ansell et al., 1997). *T. delbrueckii* lacked *GPD2* and *GPP2*. Transcription of the glycerol pathway genes seemed to be higher in the exponential growth phase (24 h) in both yeast species (Fig. 3). *T. delbrueckii* expressed more *GPP1* compared to *GPD1*, contrary to *S. cerevisiae*.

Acetic acid is responsible for the negative attribute of volatile acidity in wine. In agreement with earlier reports (Bely et al., 2008; Blomberg and Adler, 1992), *T. delbrueckii* produces less acetic acid than *S. cerevisiae* (Fig. 1B). Overall production of acetic acid was 0.486 ± 0.07 g/L (2 mg/g of sugar consumed) for *T. delbrueckii* compared to 1.80 ± 0.12 g/L (8 mg/g of sugar consumed) for *S. cerevisiae*. Transcripts for aldehyde dehydrogenases *ALD2–6*, enzymes involved in acetic acid production, were all expressed in *S. cerevisiae* while *T. delbrueckii* lacked *ALD3* expression. Nevertheless, acetyl-CoA synthetases (*ACS1* and *ACS2*) and acetyl-CoA hydrolase (*ACH1*) transcripts were present in both transcriptomes. Expression analysis of these genes during fermentation, showed two different patterns in metabolic flow. Low acetate producer, *T. delbrueckii* consistently expressed *ADHs* transcripts higher than *ALDs* transcripts during fermentation. In contrast, *Saccharomyces ALD* transcript expression was dominant, with *Saccharomyces* producing 10-times more acetic acid that *Torulaspora* (Figs. 1B, 3). Also *T. delbrueckii* tended to express *ACSs* more than *ACH1*, while in *Saccharomyces* expression was similar.

3.5. Higher alcohols, ethyl and acetate esters

T. delbrueckii and *S. cerevisiae* differed in their production of acetate esters, ethyl esters and higher alcohols at the end of fermentation (Table 1). *Saccharomyces* produced a wider range of acetate and ethyl esters, while *Torulaspora* instead produced higher amounts only of ethyl propanoate, as previously reported (Ramírez et al., 2016; Renault et al., 2015; Velázquez et al., 2015). Transcriptome analysis revealed *T. delbrueckii* lacked the main genes responsible for acetate ester production, *ATF1–2* (Fig. 4B; Lilly et al., 2000). A minimal production of these esters was seen and might be explained by the expression of *YPL272C* (uncharacterized protein) and *SLI1* (*n*-acetyl-transferase; Supplementary File 1). The amino acid sequence of both of these genes includes an alcohol acetyltransferase domain.

Regarding ethyl esters, genes *EEB1* and *EHT1* were expressed in

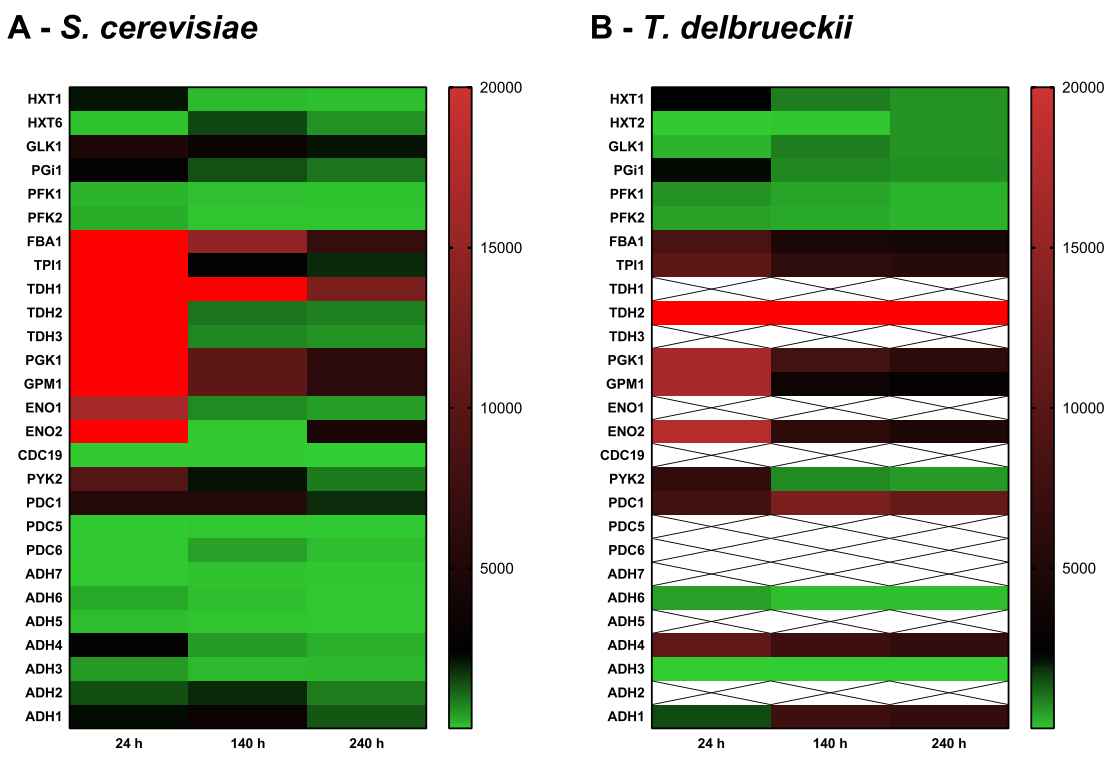


Fig. 2. Expression of genes of the glucose fermentation pathway. Expression values (FPKM) of the genes involved in the glucose fermentative pathway of (A) *S. cerevisiae* and (B) *T. delbrueckii*. Expression is showed from low (green) to high (red). Crossed squares indicate no expression. (COLOR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

both transcriptomes (Fig. 4; Saerens et al., 2010, Tehlivets et al., 2007). *T. delbrueckii* mainly expressed *ETH1*, while *S. cerevisiae* expressed *EEB1* to a greater extent. However ethyl ester production has also been related to the availability of their precursors, medium chain fatty acids (MCFAs; Saerens et al., 2006) and therefore to enzymes acetyl–CoA carboxylase (*ACC1*) and the fatty acid synthase (FAS) complex (*FAS1/FAS2*; Bardi et al., 1998, Dufour et al., 2008). Interestingly *S. cerevisiae* expressed *ACC1* highly and the *FAS* complex during all stages of fermentation, which might result in a higher accumulation of MCFAs. Contrarily *T. delbrueckii*, in which *FAS2* transcript is more closely related to *L. thermotolerans* (Supplementary File 1), mainly expressed the pathway at the beginning of fermentation.

T. delbrueckii produced a lower concentration of higher alcohols. Formation of higher alcohols can be directly related to the catabolism of branched chain amino-acids (BCAAs; leucine, valine, and isoleucine) through the Ehrlich pathway (Hazelwood et al., 2008). *BAT1*, *BAT2* and *BAP2* have already been identified as being involved in this pathway (Colón et al., 2011; Procopio et al., 2011). We found all three of these genes expressed in *Saccharomyces*, but no transcripts for the BCAAs permease *BAP2* and for the BCCAs transaminase *BAT2* in *T. delbrueckii* (Fig. 4B). This could indicate an impaired amino acid uptake and Ehrlich pathway in *Torulaspora*.

3.6. Effect of BAP2 deletion on short chain ethyl esters production in S. cerevisiae

Contrary to acetate esters and higher alcohols, ethyl esters production is related to branched chain amino acid uptake (Saerens et al., 2008). Branched chain amino acid uptake is mainly achieved in *S. cerevisiae* by the amino acid permease *BAP2*. Deletion and overexpression, respectively, show decreases or increases in BCCA uptake (Grauslund et al., 1995). To test the effect of an impaired uptake of BCAA in producing the patter of esters seen in *T. delbrueckii*, CDGJM was fermented with a wild type haploid *S. cerevisiae* AWRI 1631 and the corresponding AWRI 1631 Δ*bap2* deletant. The effect of *BAP2* deletion on ethyl esters was determined via five compounds: ethyl propanoate, ethyl 2–methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate and ethyl 3-methyl butanoate. No differences between the strains were seen in extent of sugar consumption after 10 days of fermentation (approx. 14 g/L residual sugar, *p*-value 0.33). Three SBCEEs were significantly lower, whereas the not branched short ethyl esters, ethyl propanoate and ethyl butanoate, weren't significantly reduced (Table 2). AWRI 1631 Δ*bap2* strain related SBCEEs final concentration with BCAAs uptake.

4. Discussion

Torulaspora delbrueckii shows great potential as an alternative to an *S. cerevisiae* inoculum during wine fermentation due to its different production of secondary metabolites. Its reduced production of volatile acidity and its sensory characteristics have not been previously investigated at the transcriptome and metabolome level. In this study, we have shown how transcriptional differences reflect the different metabolic characteristics of those yeasts and help highlight the genetic differences between a well-annotated species (*S. cerevisiae*) and a non-reference species with a sparsely annotated genome (*T. delbrueckii*).

The transcript analysis showed *T. delbrueckii* is missing multiple genes, results of the complex program of whole genome duplication in *S. cerevisiae* (Wolfe, 2015). Paralog genes are missing in the glycolic and fermentation pathways, in amino acid uptake and in production of secondary metabolites such as acetate and glycerol biosynthesis. Glycerol production is similar in both yeast species, in contrast to acetic acid; the results confirm *T. delbrueckii* produces less volatile acidity, under wine-like fermentation conditions, instead redirecting carbon flux through alcoholic fermentation.

Comparative analysis between metabolite profiles and transcriptomes also highlighted the link between the limited production of higher alcohols and esters to the lack or lower expression of related

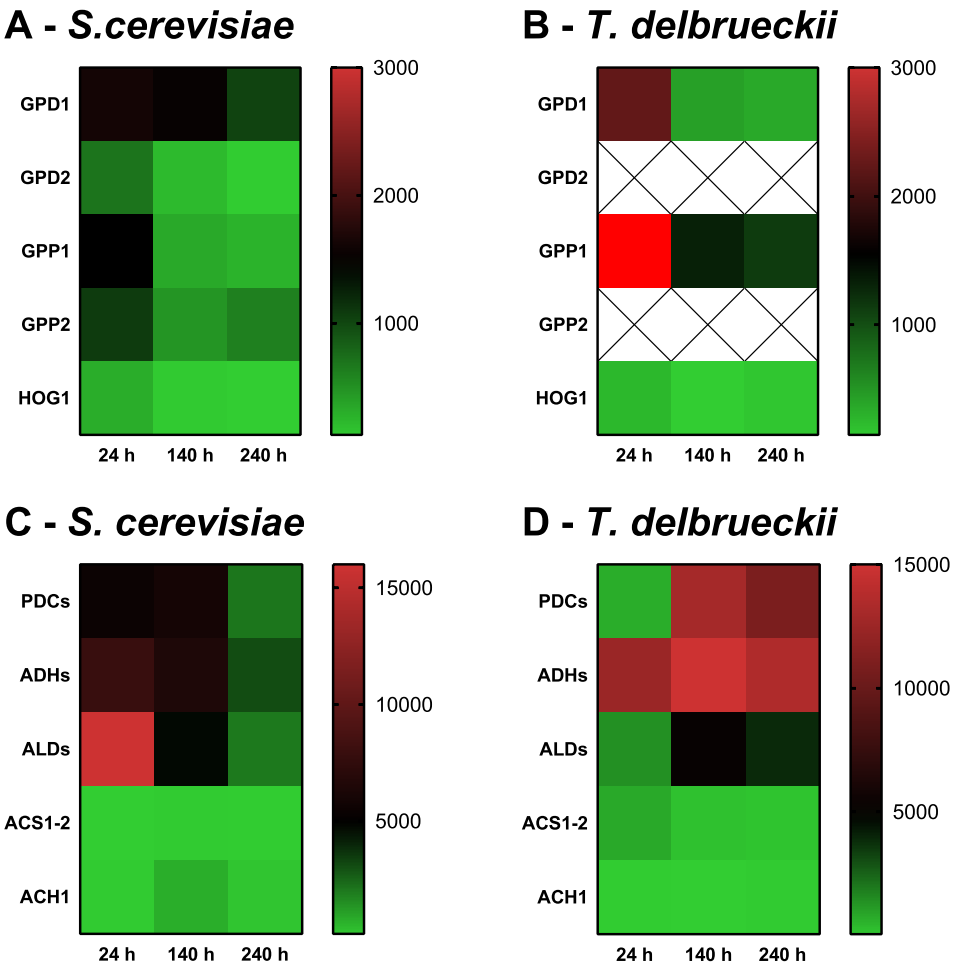


Fig. 3. Expression of genes of the glycerol and acetate pathways. Expression values (FPKM) of the genes involved in the glycerol pathway for (A) *S. cerevisiae* and (B) *T. delbrueckii*. (D) Normalised expression (see Material and methods) of the enzymes involved in the Acetate/Acetyl-CoA pathway for (C) *S. cerevisiae* and (D) *T. delbrueckii*. Expression is showed from low (green) to high (red). Crossed squares indicate no expression. (COLOR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

genes. In the case of *T. delbrueckii*, which showed reduced production of higher alcohols and acetate esters, an absence of transcripts of key enzymes in these pathways was revealed. Instead, the lower production of ethyl esters by *Torulaspora* seemed to be related to the down-regulation of fatty acids biosynthesis. Based on (i) differences in expression of amino acid permeases between the two species and their influence on aromas (Supplementary File 1; Stahl and James, 2014), (ii) lower SBCEEs production by *T. delbrueckii* (Table 1) and *S. cerevisiae* Δ*bap2* (Table 2) and (iii) analysis of the structure and biosynthesis pathway of SBCEEs (KEGG compound, KEGG pathway; Kanehisa et al., 2011) we suggest the formation of SBCEEs depends on metabolism of BCAAs through a modified Ehrlich pathway, under these oenological conditions. Intracellular accumulation of α-ketoacids is a consequence of amino acid metabolism (Procopio et al., 2011). In the cytosol excess

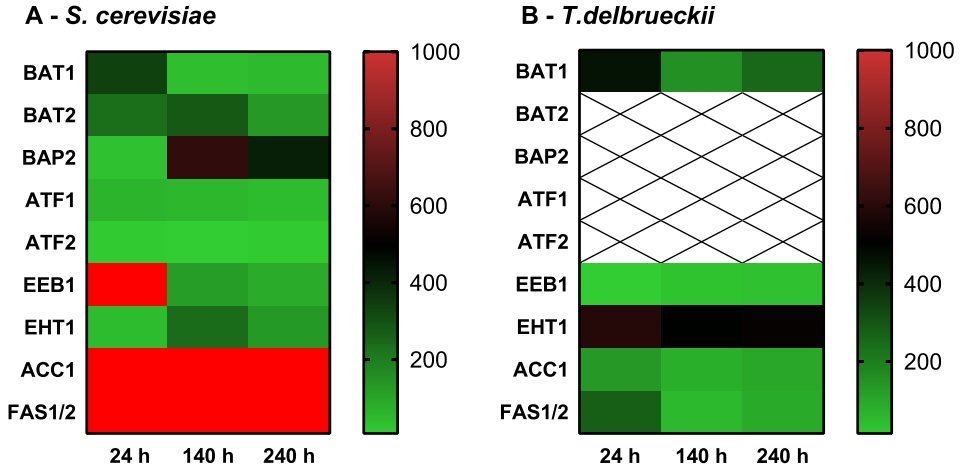


Fig. 4. Expression of genes involved in formation of higher alcohols, acetates and ethyl esters. Expression values (FPKM) of the genes involved in higher alcohols, acetates and ethyl esters pathways of (A) *S. cerevisiae* and (B) *T. delbrueckii*. Expression is showed from low (green) to high (red). Crossed squares indicate no expression. (COLOR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Quantification (integrated peak area; relative to internal standards) of ethyl esters, in *S. cerevisiae* AWRI 1631 and *S. cerevisiae* AWRI 1631 Δ*bap2*.

Compounds	Relative quantification		p-Value
	AWRI 1631	AWRI 1631 Δbap2	
Ethyl propanoate	5.7 ± 0.6	5.1 ± 0.3	0.2694
Ethyl 2 – methylpropanoate	9.1 ± 0.3	6.7 ± 0.4	0.0038
Ethyl butanoate	1.2 ± 0.9	1.1 ± 0.35	0.1237
Ethyl 2 – methylbutanoate	9.3 ± 2.5	3.1 ± 0.2	0.0259
Ethyl 3 – methylbutanoate	8.4 ± 0.7	6.6 ± 0.3	0.0255

of α-ketoacid is processed through the Ehrlich pathway (Avalos et al., 2013; Hazelwood et al., 2008). However, in the mitochondria, an excess of these substrates may be processed by other enzymes. For example, an enzyme complex with branched-chain α-ketoacid dehydrogenase activity (similar to 2-oxoacid dehydrogenase; EC 1.2.4.4) has already been reported in *Saccharomyces*. This metabolic route was previously detected by ^{13}C-labeled intermediate products (Dickinson et al., 1997; Dickinson, 1999) but its physiological role is still unknown (Dickinson and Dawes, 1992; Sinclair et al., 1993). The catalytic subunit E3 of this complex, the protein encoded by *LPD1* gene, has been found to be expressed in both transcriptomes (Supplementary File 1). The reaction would produce the related acyl–CoA, and finally esterases would form an SBCEE. Further characterisation of *T. delbrueckii* amino acid metabolism is however necessary to understand the genetic basis for its distinct profile to provide winemakers a useful tool to predict spontaneous and co-inoculated fermentations.

In conclusion the comparative transcriptomic approach described here can not only be used to search for specific oenological traits in non-conventional yeast (Rossignol et al., 2003), but further used to explore the natural biodiversity of yeast communities and their interaction. New sequencing technology based on long DNA fragments may help differentiate transcriptomes and therefore further analyse the effects of mixed population and their effects on the wine sensory output, closely relating laboratory outputs to the wine industry conditions.

Declarations of interest

None.

Acknowledgement

This work was supported by the Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production (Project No. IC130100005) and Australian Wine Research Institute (AWRI). University of Adelaide and AWRI are Wine Innovation Cluster (WIC) members. Metabolic Australia laboratory at AWRI is founded by Bioplatform Australia as part of the Commonwealth Government National Collaborative Research Infrastructure Strategy (NCRIS). The authors wish to thank the Yalumba Wine Company for providing the wine samples. Yeast AWRI 1631 was provided by AWRI Wine Microorganism Culture Collection (AWMCC).

Appendix A. Supplementary data

Supplementary 1, Supplementary Figs. 1–3 can be found online: http://dx.doi.org/10.17632/v8g3y2f35f.1

Supplementary File 2 (CDGJM composition) can be found online: http://dx.doi.org/10.17632/j9g3mxsh23.2

Appendix B. Raw sequences

Raw sequences can be found on SRA database, accession: **SRP136972**.

References

Albertin, W., Chasseriaud, L., Comte, G., Panfili, A., Delcamp, A., Salin, F., Marullo, P., Bely, M., 2014. Winemaking and bioprocesses strongly shaped the genetic diversity of the ubiquitous yeast *Torulaspora delbrueckii*. PLoS One 9 (4), e94246.
Amberg, D.C., Burke, D.J., Strathern, J.N., 2005. Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual.
Andrews, S., 2010. FastQC: a Quality Control Tool for High Throughput Sequence Data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M., Adler, L., 1997. The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. 16 (9), 2179–2187.
Avalos, J.L., Fink, G.R., Stephanopoulos, G., 2013. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat. Biotechnol. 31 (4), 335–341.
Azzolini, M., Fedrizzi, B., Tosi, E., Finato, F., Vagnoli, P., Scrinzi, C., Zapparoli, G., 2012. Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. Eur. Food Res. Technol. 235 (2), 303–313.
Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., Zapparoli, G., 2015. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 31 (2), 277–293.
Bairoch, A., Apweiler, R., 2000. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 28 (1), 45–48.
Bardi, L., Crivelli, C., Marzona, M., 1998. Esterase activity and release of ethyl esters of medium-chain fatty acids by *Saccharomyces cerevisiae* during anaerobic growth. Can. J. Microbiol. 44 (12), 1171–1176.
Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122 (3), 312–320.
Bisson, L.F., Karpel, J.E., 2010. Genetics of yeast impacting wine quality. Annu. Rev. Food Sci. Technol. 1, 139–162.
Blomberg, A., Adler, L., 1992. Physiology of osmotolerance in fungi. Adv. Microb. Physiol. 33, 145–212.
Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120.
Brandam, C., Lai, Q.P., Julien-Ortiz, A., Taillandier, P., 2013. Influence of oxygen on alcoholic fermentation by a wine strain of *Torulaspora delbrueckii*: kinetics and carbon mass balance. Biosci. Biotechnol. Biochem. 77 (9), 1848–1853.
Chidi, B.S., Rossouw, D., Bauer, F.F., 2016. Identifying and assessing the impact of wine acid-related genes in yeast. Curr. Genet. 62 (1), 149–164.
Chomczynski, P., Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. Nat. Protoc. 1 (2), 581–585.
Ciani, M., Picciotti, G., 1995. The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. Biotechnol. Lett. 17 (11), 1247–1250.
Colón, M., Hernández, F., López, K., Quezada, H., González, J., López, G., Aranda, C., González, A., 2011. *Saccharomyces cerevisiae Bat1* and *Bat2* aminotransferases have functionally diverged from the ancestral-like *Kluyveromyces lactis* orthologous enzyme. PLoS One 6 (1), e16099.
Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol. 28 (5), 873–882.
Deere, D., Shen, J., Vesey, G., Bell, P., Bissinger, P., Veal, D., 1998. Flow cytometry and cell sorting for yeast viability assessment and cell selection. Yeast 14 (2), 147–160.
Dickinson, J.R., 1999. Branched-chain keto acid dehydrogenase of yeast. Methods Enzymol. 324, 389–398.
Dickinson, J.R., Dawes, I.W., 1992. The catabolism of branched-chain amino acids occurs via 2-oxoacid dehydrogenase in *Saccharomyces cerevisiae*. Microbiology 138 (10), 2029–2033.
Dickinson, J.R., Lanterman, M.M., Danner, D.J., Pearson, B.M., Sanz, P., Harrison, S.J., Hewlins, M.J., 1997. A ^{13}C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 272 (43), 26871–26878.
Duc, C., Pradal, M., Sanchez, I., Noble, J., Tesnière, C., Blondin, B., 2017. A set of nutrient limitations trigger yeast cell death in a nitrogen-dependent manner during wine alcoholic fermentation. PLoS One 12 (9), e0184838.
Dufour, J., Malcorps, P., Silcock, P., 2008. Control of ester synthesis during brewery fermentation. In: Brewing Yeast Fermentation Performance.
Eglinton, J.M., Heinrich, A.J., Pollnitz, A.P., Langridge, P., Henschke, P.A., de Barros Lopes, M., 2002. Decreasing acetic acid accumulation by a glycerol overproducing strain of *Saccharomyces cerevisiae* by deleting the *ALD6* aldehyde dehydrogenase gene. Yeast 19 (4), 295–301.
Erasmus, D.J., Cliff, M., van Vuuren, H.J., 2004. Impact of yeast strain on the production of acetic acid, glycerol, and the sensory attributes of icewine. Am. J. Enol. Vitic. 55 (4), 371–378.
Finn, R.D., Clements, J., Eddy, S.R., 2011. HMMER web server: interactive sequence similarity searching. Nucleic Acids Res. 39, W29–W37.
Gawel, R., Sluyter, S.V., Waters, E.J., 2007. The effects of ethanol and glycerol on the body and other sensory characteristics of Riesling wines. Aust. J. Grape Wine Res. 13 (1), 38–45.
Gietz, R.D., Schiestl, R.H., 2007. Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2 (1), 38.
Gordon, J.L., Armisén, D., Proux-Wéra, E., ÓhÉigeartaigh, S.S., Byrne, K.P., Wolfe, K.H.,

2011. Evolutionary erosion of yeast sex chromosomes by mating-type switching accidents. Proc. Natl. Acad. Sci. U. S. A. 108 (50), 20024–20029.
Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29 (7), 644–652.
Grauslund, M., Didion, T., Kielland-Brandt, M.C., Andersen, H.A., 1995. *BAP2*, a gene encoding a permease for branched-chain amino acids in *Saccharomyces cerevisiae*. BBA-Mol. Cell. Res. 1269 (3), 275–280.
Haas, B., 2016. Trinity Transcriptome Contig Nx and ExNy Statistics. https://github.com/trinityrnaseq/trinityrnaseq/wiki/Transcriptome-Contig-Nx-and-ExN50-stats.
Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., 2013. De novo transcript sequence reconstruction from RNA-seq using the trinity platform for reference generation and analysis. Nat. Protoc. 8 (8), 1494–1512.
Hagman, A., Säll, T., Piškur, J., 2014. Analysis of the yeast short-term Crabtree effect and its origin. FEBS J. 281 (21), 4805–4814.
Hazelwood, L.A., Daran, J.-M., van Maris, A.J., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl. Environ. Microbiol. 74 (8), 2259–2266.
Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14 (2), 215–237.
Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., Tanabe, M., 2011. KEGG for integration and interpretation of large-scale molecular data sets. Nucleic Acids Res. 40, D109–D114.
Krogh, A., Larsson, B., Von Heijne, G., Sonnhammer, E.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305 (3), 567–580.
Lagesen, K., Hallin, P., Rødland, E.A., Stærfeldt, H.-H., Rognes, T., Ussery, D.W., 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35 (9), 3100–3108.
Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinf. 12 (1), 1.
Liccioli, T., Tran, T.M., Cozzolino, D., Jiranek, V., Chambers, P.J., Schmidt, S.A., 2011. Microvinification—how small can we go? Appl. Microbiol. Biotechnol. 89 (5), 1621–1628.
Lilly, M., Lambrechts, M., Pretorius, I., 2000. Effect of increased yeast alcohol acetyltransferase activity on flavour profiles of wine and distillates. Appl. Environ. Microbiol. 66 (2), 744–753.
Linde, J., Duggan, S., Weber, M., Horn, F., Sieber, P., Hellwig, D., Riege, K., Marz, M., Martin, R., Guthke, R., 2015. Defining the transcriptomic landscape of *Candida glabrata* by RNA-Seq. Nucleic Acids Res. 43 (3), 1392–1406.
Marks, V.D., Sui, S.J.H., Erasmus, D., Van Der Merwe, G.K., Brumm, J., Wasserman, W.W., Bryan, J., Van Vuuren, H.J., 2008. Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res. 8 (1), 35–52.
Martinez, J., Toledano, F., Milla, C., Ortega, J., 1990. Development of alcoholic fermentation in non-sterile musts from 'Pedro Ximenez' grapes inoculated with pure cultures of selected yeasts. Food Microbiol. 7 (3), 217–225.
Masneuf-Pomarede, I., Bely, M., Marullo, P., Albertin, W., 2015. The genetics of non-conventional wine yeasts: current knowledge and future challenges. Front. Microbiol. 6, 1563.
McBryde, C., Gardner, J.M., de Barros Lopes, M., Jiranek, V., 2006. Generation of novel wine yeast strains by adaptive evolution. Am. J. Enol. Vitic. 57 (4), 423–430.
Mortimer, R.K., 2000. Evolution and variation of the yeast (*Saccharomyces*) genome. Genome Res. 10 (4), 403–409.
O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., 2015. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 44 (D1), D733–D745.
Pacheco, A., Leão, C., Almeida, J., Santos, J., Sousa, M.J., Chaves, S., 2012. The emerging role of the yeast *Torulaspora delbrueckii* in bread and wine production: using genetic manipulation to study molecular basis of physiological responses. In: Structure and Function of Food Engineering. InTech.
Petersen, T., Brunak, N.S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8 (10), 785–786.
Piper, M.D., Daran-Lapujade, P., Bro, C., Regenberg, B.S., Knudsen, B., Nielsen, J., Pronk, J.T., 2002. Reproducibility of oligonucleotide microarray transcriptome analyses an interlaboratory comparison using chemostat cultures of *Saccharomyces cerevisiae*. J. Biol. Chem. 277 (40), 37001–37008.
Powell, S., Szklarczyk, D., Trachana, K., Roth, A., Kuhn, M., Muller, J., Arnold, R., Rattei, T., Letunic, I., Doerks, T., 2012. eggNOG v3. 0: orthologous groups covering 1133 organisms at 41 different taxonomic ranges. Nucleic Acids Res. 40 (D1), D284–D289.
Procopio, S., Qian, F., Becker, T., 2011. Function and regulation of yeast genes involved in higher alcohol and ester metabolism during beverage fermentation. Eur. Food Res. Technol. 233 (5), 721–729.
Puig, S., Pérez-Ortín, J.E., 2000. Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. Yeast 16 (2), 139–148.
Punta, M., Coggill, P.C., Eberhardt, R.Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., 2011. The Pfam protein families database. Nucleic Acids Res. 40, D290–D301.
Ramírez, M., Velázquez, R., Maqueda, M., Zamora, E., López-Piñeiro, A., Hernández, L.M., 2016. Influence of the dominance of must fermentation by Torulaspora delbrueckii on the malolactic fermentation and organoleptic quality of red table wine. Int. J. Food Microbiol. 238, 311–319.
Raynal, C., Wardrop, F., Pillet, O., Amos, J., Languet, P., Dumont, A., Ortiz-Julien, A., 2011. An innovative tool for winemakers' sequential inoculation with a non-*Saccharomyces* yeast and a *Saccharomyces cerevisiae* yeast. Wine Vitic J. 1, 22–31.
Remize, F., Cambon, B., Barnavon, L., Dequin, S., 2003. Glycerol formation during wine fermentation is mainly linked to Gpd1p and is only partially controlled by the HOG pathway. Yeast 20 (15), 1243–1253.
Renault, P., Coulon, J., de Revel, G., Barbe, J.C., Bely, M., 2015. Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. Int. J. Food Microbiol. 207, 40–48.
Robinson, M.D., Oshlack, A., 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 11 (3), 1.
Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26 (1), 139–140.
Rossignol, T., Dulau, L., Julien, A., Blondin, B., 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 20 (16), 1369–1385.
Saerens, S.M., Verstrepen, K.J., Van Laere, S.D., Voet, A.R., Van Dijck, P., Delvaux, F.R., Thevelein, J.M., 2006. The *Saccharomyces cerevisiae EHT1* and *EEB1* genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. J Biol. Chem. 281 (7), 4446–4456.
Saerens, S.M.G., Delvaux, F., Verstrepen, K.J., Van Dijck, P., Thevelein, J.M., Delvaux, F.R., 2008. Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. Appl. Environ. Microbiol. 74 (2), 454–461.
Saerens, S.M., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3 (2), 165–177.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13 (11), 2498–2504.
Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., Zdobnov, E.M., 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinfo. 31 (19), 3210–3212.
Sinclair, D.A., Dawes, I.W., Dickinson, J.R., 1993. Purification and characterization of the branched chain alpha-ketoacid dehydrogenase complex from *Saccharomyces cerevisiae*. Int. J. Bioorganic. Chem. Mol. Biol. 31 (5), 911–922.
Stahl, N., James, U., 2014. Amino acid permeases and their influence on flavour compounds in beer. Brew Sci. 67, 120–127.
Suárez-Lepe, J., Morata, A., 2012. New trends in yeast selection for winemaking. Trends Food Sci. Technol. 23 (1), 39–50.
Taillandier, P., Lai, Q.P., Julien-Ortiz, A., Brandam, C., 2014. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: influence of inoculation and nitrogen content. World J. Microbiol. Biotechnol. 30 (7), 1959–1967.
Tataridis, P., Kanellis, A., Logothetis, S., Nerantzis, E., 2013. Use of non-*Saccharomyces Torulaspora delbrueckii* yeast strains in winemaking and brewing. Zbornik Matice srpske za prirodne nauke. 124, 415–426.
Tehlivets, O., Scheuringer, K., Kohlwein, S.D., 2007. Fatty acid synthesis and elongation in yeast. Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1771 (3), 255–270.
Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinfomatics 25 (9), 1105–1111.
Van Breda, V., Jolly, N., van Wyk, J., 2013. Characterisation of commercial and natural Torulaspora delbrueckii wine yeast strains. Int. J. Food Microbiol. 163 (2–3), 80–88.
Velázquez, R., Zamora, E., Álvarez, M.L., Hernández, L.M., Ramírez, M., 2015. Effects of new *Torulaspora delbrueckii* killer yeasts on the must fermentation kinetics and aroma compounds of white table wine. Front. Microbiol. 6, 1222.
Wolfe, K.H., 2015. Origin of the yeast whole-genome duplication. PLoS Biol. 13 (8), e1002221.
Yeung, K.Y., Ruzzo, W.L., 2001. Principal component analysis for clustering gene expression data. Bioinfomatics 17 (9), 763–774.

Chapter 3: Third generation genomes for indigenous yeast isolates

3.1. Introduction

As the wine market demands new and improved wine yeast strains, the potential of non-*Saccharomyces* yeast has been re-evaluated for positive quality contributions (greater complexity of flavour and stylistic distinction) in commercial wine production (Viana et al. 2008, Barbosa et al. 2015). These yeast species have particularly received heightened attention because of their glucophilic character, fermentative metabolism and improved organoleptic characteristics (Fleet 2003), influencing fermentations by both direct and indirect mechanisms (Padilla et al. 2016). Their impact has been shown to be highly dependent on many factors other than the species, such as nutrient composition (Bely et al. 2008, Ciani et al. 2010, Jolly et al. 2014).

Wine research has benefited enormously from the well characterized *S. cerevisiae* metabolic network, and its metabolic physiology has been the subject of intensive study. (Herrgård et al. 2008, Chambers and Pretorius 2010, Zhao et al. 2016). However, limited genomic information is available for the vast majority of the other species in wine, thus making it difficult to specifically target their physiologic characteristics to winemakers' needs (Masneuf-Pomarede et al. 2016). In the particular case of *Torulaspora delbrueckii,* characteristics such as fermentation kinetics, increased thiol production and lowered production of volatile acidity have been identified as being of potential interest (Renault et al. 2015, Renault et al. 2016). Also, mixed fermentation with *T. delbrueckii* improves the aroma intensity and complexity of wine due to increased levels of several higher alcohols and esters (Ehsani et al. 2012, Belda et al. 2015, Renault et al. 2015, Arslan et al. 2018). Three sequenced genomes of this species are available, but contain gaps, unassembled contigs, but mainly they are poorly annotated. At any rate, none of the sequenced isolates can be directly linked to wine, but only other fermentation

processes (Gordon et al. 2011, Gomez-Angulo et al. 2015, Wu et al. 2015). The challenge of understanding the imprint of non-conventional yeasts on winemaking is the reconstruction of metabolic networks from environmental sequence information (Konwar et al. 2013). System-wide "Omics" approaches have proven to be useful to boost our understanding of biotechnologically relevant phenotypes of *S. cerevisiae* strains (Rossouw and Bauer 2009). Transcriptomics highlighted broad genetic expression in continuously changing environmental conditions such as wine fermentation, allowing the identification of stress response and metabolic mechanisms that are active under these conditions (Alexandre et al. 2001, Erasmus et al. 2003, Marks et al. 2008, Hazelwood et al. 2009). In particular, transcriptomic analysis has provided valuable insights into the molecular basis by which the nutritional composition of the growth medium, particularly the initial concentration of nitrogen, impacts growth and performance of fermentations undertaken by *S. cerevisiae* wine yeasts (Backhus et al. 2001, Erasmus et al. 2003, Varela et al. 2005, Marks et al. 2008). These findings have helped to develop strategies to optimize yeast performance, manage and prevent slow and sluggish fermentations, and improve organoleptic outcome (Orellana et al. 2014, Barbosa et al. 2015). With the successful achievement of a consensus reconstruction based on RNA sequencing, similar strategies should benefit systems biology for other organisms (Zhao et al. 2016). In Chapter 2, a transcriptomic approach was used to investigate the genome-wide remodelling of two indigenous yeasts, a *T. delbrueckii* and a *S. cerevisiae*. Two main strategies for transcriptome assembly were available: genome-guided and *de novo* (genome-independent; Grabherr et al. 2011, Liu et al. 2016). The *de novo* protocol performed by the Trinity algorithm (Haas et al. 2013), which first aligns reads to the genome, partitioning them according to locus, and then effects a *de novo* transcriptome assembly at each locus, was chosen to investigate non-conventional yeasts, and therefore possible non-conventional features. This method was seen as the best option to investigate oenologically interesting isolates with sparse genome

information, whilst not missing complex and atypical genes or transcripts. The primary aim was to provide an in-depth comparative analysis of the temporal expression profile of genes and any abundance differences between the two species during fermentation. Secondly, the aim was to elucidate the differences in metabolic pathways that may have led to their physiological properties. Hence, particular attention was given to metabolite production. The results were useful in elucidating *T. delbrueckii* metabolic features and thereby, for example, linking branched chain amino acid permease *BAP2* and some ethyl esters for the first time and elucidating a different regulation of genes involved in acetic acid production.

This approach has proven itself to be fit for the purpose of first analysis of indigenous single isolates, contextualizing high-throughput data and directing hypothesis-driven discovery. However, some limitations were apparent: (i) fragmented genes, (ii) the presence of chimeric gene isoforms and (iii) a high rate of multi-mapping reads, all of which reduced the accuracy of the analysis. Therefore, we wanted to validate previous experiments and further characterize the two indigenous isolates, in order to be able to generate high-resolution transcriptome maps for non-conventional yeast or for interrogation of multispecies relationships (Zhao et al. 2016). The genomes were sequenced using third generation sequencing (Oxford Nanopore minion; Deamer et al. 2016). The aim was to develop a method to employ in mixed population RNA-seq experiments, which would not be compromised by low species-specificity read alignments. The superior capability of very long sequence reads for *de novo* genome assembly (Jenjaroenpun et al. 2018) showed improved downstream gene expression quantification, compared to the *de novo* transcriptome assembly.

3.2. Materials & Methods

3.2.1. Genome sequencing, annotation and comparison

T. delbrueckii strain COFT1 and *S. cerevisiae* strain YAL1 were isolated as described in Chapter 2 and stored in glycerol. Yeast glycerol stocks were streaked onto YEPD agar plates (1% yeast extract, 2% bacto peptone, 2% glucose, 2% agar) and then a single colony was isolated and grown overnight in YEPD medium (1% yeast extract, 2% bacto peptone, 2% glucose). High molecular weight genomic DNA was extracted following the phenol/chloroform protocol (Sambrook and Russell 2006), including a 2 h pre-incubation with zymolase. DNA was prepared for sequencing with the MinION device using the SQK-LSK108 library prep kit (protocol GDE_9002_v108_revT_18Oct2016) and R9.4 chemistry. Fast5 files were base called using Albacore v.2.0.2. Pass reads were trimmed for adapters using PoreChop v.0.2.3 and then assembled using SMARTdenovo v 1.0 (https://github.com/ruanjue/smartdenovo). Contigs obtained from the assembly were polished using Racon v. 0.5.0 (Vaser et al. 2017) and Nanopolish v. 0.8.5 (Loman et al. 2015). Final polish of the assembly was performed with Pilon V. 1.22 using Illumina Hiseq RNA reads extracted from pure culture laboratory ferments with strain COFT01. The mitochondrial chromosome was assembled using Canu (v.1.7; Koren et al. 2017) and polished as above. The genome was firstly annotated with YGAP (Proux-Wéra et al. 2012), and the annotation was improved with MAKER2 (Holt and Yandell 2011), providing STAR RNA alignment information (Dobin and Gingeras 2015) for the prediction of protein-coding genes. Pathway reconstruction started with functional annotation based on sequence homology using BLAST (Madden 2013, Kanehisa et al. 2016) against the SWISS-PROT protein sequence database (e-value = 1e-5; Bairoch and Apweiler 2000), followed by KAAS (Moriya et al. 2007) and KEGG pathways mapper (Kanehisa et al. 2011) to reconstruct pathways and project them into symbolic representations of metabolism. Genome completeness was evaluated with

Benchmarking Universal Single-Copy Orthologs (BUSCO; Simão et al. 2015). Genome sequences were aligned with MUMmer (Delcher et al. 2003). Protein alignment used CLUSTAL multiple sequence alignment by MUSCLE (3.8; Edgar 2004).

3.2.2. RNA alignments and differential gene expression (DE) analysis

Quality trimmed RNA reads from Chapter 2 were aligned to genomes and the reference genome using STAR (Dobin and Gingeras 2015) or to transcriptomes using Salmon (Patro et al. 2015). The transcriptome for the newly assembled genome was generated using Gffread utility (Trapnell et al. 2012). Gene expression analysis was performed using Bioconductor package DESeq2 (Love et al. 2014) and then normalised to the average base expression using z-score transformation (Cheadle et al. 2003).

3.3. Results

3.3.1. *Torulaspora delbrueckii* COFT1 genome

The final genome of this isolate has been deposited in the NCBI Genbank database under accession numbers CP027647 to CP027655. The final assembly had no gaps with a total length of 9,356,826 bp arranged in 9 chromosomes (incl. 1 mitochondrial chromosome) and an average GC content of 42%. A total of 4,831 protein coding genes were identified, compared to 4,714 and 4,972 in previously reported genomes (Gordon et al. 2011, Gomez-Angulo et al. 2015). BUSCO assessment revealed a genome completeness of 98%. Alignment with reference genome *Torulaspora delbrueckii* ASM24337v1, individuated 36,821 single nucleotide polymorphisms (SNPs).

3.3.2. *Saccharomyces cerevisiae* YAL1 genome

This *Saccharomyces* genome has been deposited in the NCBI Genbank database under accession numbers QQBK01000001 to QQBK01000025. After correcting for misassembles the genome size was 12.1 Mb, fragmented into 25 contigs: 22 belonging to the nuclear genome, 1 contig belonging to the 2 µm plasmid and 2 contigs corresponding to the mitochondrial genome. Most chromosomes of the assembly were formed by a single contig: 7 contigs were flanked by telomere repeats while 9 had telomere repeats on one side. BUSCO analysis revealed a genome completeness of 93%. We successfully annotated 6,155 protein coding genes.

3.3.3. Alignment to genome and chimeric transcriptome

S. cerevisiae YAL1 and *T. delbrueckii* COFT1 Illumina reads, obtained from previous single culture fermentations (Chapter 2), were respectively aligned to their closest reference genome obtained from NCBI (*S. cerevisiae* S288C ID 15, *T. delbrueckii* ASM24337v1; O'Leary et al. 2015) and then to the newly assembled genome. Strict alignment, which excludes multi mapping and mismatched reads, was improved for both species, whereas unique mapping reads were increased by roughly 5% (Table1). The improvement was mostly dependent on the greater accuracy of the alignment, detecting mismatch between the query (reads) and the genome. The increase in accuracy, arising from a more accurate genome sequence, was tested against a chimeric genome. Two chimeric transcriptomes of the two species were generated, starting from the two reference genomes and the newly assembled ones.

	Read length	Mapped unique (%)	Mapped Length	Unmapped multi+ (%)	Unmapped short (%)
CBS	199	88.6	189.8	2.0	9.4
COFT1	199	93.4	196.3	1.9	4.6
S228C	199	85.5	188.2	2.6	11.9
YAL1	199	91.8	194.8	2.4	5.8

Table 1: STAR alignment statistics. *T. delbrueckii* COFT1 reads aligned versus *T. delbrueckii* ASM24337v1 and COFT1 genomes. *S. cerevisiae* YAL1 reads aligned versus S288C and YAL1 genomes.

Illumina reads were aligned to chimeric transcriptomes with and without restrictions (i.e. no mismatch and no multi mapping, Table 2). For COFT1 reads, loose alignments against the newly assembled chimeric transcriptome had the same unique mapped reads but a reduced number of ambiguous reads in the order of 6% to less than 1%.

		Reference Chimeric Transcriptome		**New Chimeric Transcriptome**	
		loose	**strict**	**loose**	**strict**
COFT1	**Unique**	7.4×10^6	4.4×10^6	7.4×10^6	5.8×10^6
	Ambiguous	4.8×10^5	0	9.6×10^3	0
YAL1	**Unique**	5.6×10^6	3.1×10^6	5.5×10^6	4.2×10^6
	Ambiguous	3.4×10^5	2×10^2	3×10^5	0

Table 2: SALMON alignment statistics. *T. delbrueckii* COFT 1 and *S. cerevisiae* YAL1 reads aligned versus the Chimeric Transcriptome generated from CBS and S288C genomes and versus the Chimeric Transcriptome formed by COFT1 and YAL1 genomes.

In the case of strict alignment for the same reads, no ambiguous reads were detected but we reported 1.4 million more reads uniquely aligned. YAL1 reads aligned loosely to a similar extent to both transcriptomes, whereas in the case of a strict alignment, we reported 1.1 million more reads uniquely aligned.

3.4.4. Validation of previous experiments

We used the newly assembled genome for the species under investigation in order to evaluate the *de-novo* transcriptome method and validate the findings shown in Chapter 2. The reported absence of paralog genes in the glycolytic (*TDH1*, *TDH3*, *ENO1*, *CDC19*), fermentation (*PDC5*, *PDC6*, *ADH2*, *ADH5*, *ADH7*), glycerol (*GDP2*, *GPP2*), acetic acid (*ALD3*), higher alcohol (*BAT2*, *BAP2*) and acetate ester (*ATF1*, *ATF2*) pathways was confirmed by annotation of *T. delbrueckii* genome. Moreover, we reported the absence of gene *EEB1*, involved in ethyl ester formation pathways.

To confirm the observation of a different regulation of the fluxes between the acetic acid and fermentation pathways between the strains, we compared the mean basal expression of the alcohol and aldehyde dehydrogenases. However, instead of using the Trinity protocol to create a *de novo* transcriptome we generated the transcriptome directly from the Nanopore genomes and quantified the gene expression with a genome-guided approach (Patro et al. 2015). Results confirmed that *T. delbrueckii* expressed more alcohol than aldehyde dehydrogenase, with a total *ADHs* expression 2.5 more times higher than the *ALDs*, during the course of fermentation. *S. cerevisiae* instead showed a higher expression of aldehyde dehydrogenases, compared to alcohol dehydrogenases. Contrarily, the analyses did not support the observation relating to the FAS1-FAS2 complex, involved in ethyl ester formation, which may therefore require a more focused investigation.

3.4.5. Functional genomics for oenological traits: *IRC7*

A higher release, compared to *S.* cerevisiae, of varietal thiols has been identified as a desirable oenological trait of certain *T. delbrueckii* strains (Renault et al. 2016, Belda et al. 2017). *IRC7* has been identified in both species under investigation to be related to the genetic basis for their thiol-releasing capability. However, in most wild strains of *S. cerevisiae* a truncated form of the enzyme is present, translating into a less active protein (Roncoroni et al. 2011). *S. cerevisiae* S288c carries the truncated form of the gene, *T. delbrueckii* CBS1146 the full-length gene copy (Belda et al. 2017). The functional copy in *T. delbrueckii* COFT1 has just one amino acid difference from CBS1146 (Figure 1). *S. cerevisiae* YAL1 carries a gene copy with two truncated regions (Figure 1). Looking at the transcriptome, another difference can be found between the strains: *T. delbrueckii* expressed *IRC7* more during the course of the fermentation with a z- score of 0.77, compared to *S. cereviasiae* with a z-score of -1.18.

```
COFT1
MHASKDLSEFNAGTVLSLLGRNPSKQEGFLNPSLYKGSTVIHPNLESLENLGGRFWYGTA
CBS1146
MHASKDLSEFNAGTVLSLLGRNPSKQEGFLNPSLYKGSTVIHPNLESLESLGGRFWYGTA
S288C
MIDRTELSKFGITTQLSVIGRNPDEQSGFVNPPLYKGSTIILKKLSDLEQRKGRF-YGTA
YAL1
MIDRTELSKFGITTQLSVIGRNPDEQSGF----------------------------GTA
*   .:**:*.  * **::****.:*.**                                ***

COFT1
GSPTIANLEDSWTELTGAAGTVLSPTGLGSISLAILSVVKHGDHILLPISVYGPTANFCN
CBS1146
GSPTIANLEDSWTELTGAAGTVLSPTGLGSISLAILSVVKHGDHILLPISVYGPTANFCN
S288C
GSPTIDNLENAWTHLTGGAGTVLSASGLGSISLALLALSKAGDHILMTDSVYVPTRMLCD
YAL1
GSPTIDNLENAWTHLTGGAGTVLSASGLGSISLALLALSKAGDHILMTDSVYVPTRMLCD
***** ***::** ***.******.:********:*:: * *****:. *** **  :*:
```

```
COFT1
NVLRKFGVNSEFYDPLIGKDIEELIKTNTSLIFLESPCSQTMEIQDVPAIVKVAKKHNVK
CBS1146
NVLRKFGVNSEFYDPLIGKDIEELIKTNTSLIFLESPCSQTMEIQDVPAIVKVAKKHNVK
S288C
GLLAKFGVETDYYDPSIGKDIEKLVKPNTTVIFLESPGSGTMEVQDIPALVSVAKKHGIK
YAL1
GLLAKFGVETDYYDPSIGKDIEKLVKPNTTVIFLESPGSGTMEVQDIPALVSVAKKHGIK
.:* ****:::::*** ******:*:*.**::****** * ***:**:**:*.*****.:*


COFT1
TVLDNTWATPLFFKAHDYGIDISVEAGTKYVGGHSDLLLGLTSANSRCWPALRSTYDAMG
CBS1146
TVLDNTWATPLFFKAHDYGIDISVEAGTKYVGGHSDLLLGLTSANSRCWPALRSTYDAMG
S288C
TILDNTWATPLFFDAHAHGIDISVEAGTKYLGGHSDLLIGLASANEECWPLLRSTYDAMA
YAL1
TILDNTWATPLFFDAHAHGIDISVEAGTKYLGGHSDLLIGLASANEECWPLLRSTYDAMA
*:***********.** :************:*******:**:***. *** ********.


COFT1
MLPGADDCHLALRGLRTLQLRVKEAERKALILAEWLEARNEVERILHPAFEDCPGHSLWV
CBS1146
MLPGADDCHLALRGLRTLQLRVKEAERKALILAEWLEARNEVERILHPAFEDCPGHSLWV
S288C
MLPGAEDCQLALRGMRTLHLRLKEVERKALDLAAWLGNRDEVEKVLHPAFEDCPGHEYWV
YAL1
MLPGAEDCQLALRGMRTLHLRLKEVERKALDLAAWLGNRDEVEKVLHPAFEDCPGHEYWV
*****:**:*****:***:**:**.***** ** **  *:***.:***********. **

COFT1
RDYSGSTGVFTFILKDQFSRAGLKDMLEKMQIFKLGYSWGGYESLLIPVNSTSRDRIRTW
CBS1146
RDYSGSTGVFTFILKDQFSRAGLKDMLEKMQIFKLGYSWGGYESLLIPVNSTSRDRIRTW
S288C
RDYKGSSGLFSIVLKNGFTRA---------------------------------------
YAL1
RDYKGSSGLFSIVLKNGFTRAGLEKMVEGMKVLQLGFSWGG------------------
***.**:*:*:::**: *:**

COFT1
SHFGYALRIQVGLEDIDDQLRDLEMGFELLKSHVSDVLQARL
CBS1146
SHFGYALRIQVGLEDIDDQLRDLEMGFERLKSHVSDVLQARL
S288C
-------------------------------------------
YAL1
-------------------------------------------
```

Figure 1*: IRC7* amino acid sequences alignments by MUSCLE.

3.5. Discussion and conclusion

With the application of genomic techniques, the understanding of wine strains of *Saccharomyces cerevisiae* has been greatly enhanced for both native and laboratory strains S288C. The application of these approaches to non-conventional yeasts poses considerable difficulties: (i) differences to the genome structure of *S. cerevisiae* and (ii) the complexity of the interaction with the fermentation environment (Bisson et al. 2007). The lack of available genomic information constitutes a bottleneck in the study of the genetic determinants of the impact of non-*Saccharomyces* species on wine characteristics, including *T. delbrueckii* (Belda et al. 2017). The *de novo* transcriptome approach was proven to be quite a useful and accurate tool to investigate the genomic information of novel yeast species, but it was not optimal due to chimeric gene isoforms, which led, for example, to the incorrect identification of *EEB1* in *T. delbrueckii*. This was the primary reason for sequencing the genome of *T. delbrueckii* COFT1 with the latest technology, and thereby making the first wine-related annotated genome of the species available. In fact, the complete sets of encoded genes and the complete sequences could be used to compare genomes, obtaining (i) insight into the genetic basis of differences in phenotypical traits, and (ii) enabling common or specific pathway identification. Therefore, improved genetic resources for *T. delbrueckii* could drive genetic screens to highlight physiological properties and unveil the contribution of this yeast to fermentation. This is especially important because research has indicated that the majority of the desired oenological properties identified in *T. delbrueckii* are strain dependent, thus demanding proper selection processes to identify appropriate strains (Azzolini et al. 2012, Benito 2018).

One of these oenological traits, first studied in *S. cerevisiae*, is the release of varietal thiols during fermentation (Howell et al. 2005, Roncoroni et al. 2011, Belda et al. 2016). Key aroma volatile thiols are 4-mercapto-4-methylpentane-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3MHA; Swiegers et al. 2005, Fedrizzi et al. 2009). The first

two are sulfur compounds, which are released in fermenting grape must from a form conjugated with cysteine or glutathione by several gene products with β-lyase activity (Dubourdieu et al. 2006). The latter is produced through the esterification of 3MH by yeast (Swiegers and Pretorius 2007). *IRC7* was found to have a major influence on the release of thiols from the precursors (Roncoroni et al. 2011, Pretorius et al. 2012), whereas *ATF1* has been identified as being responsible for 3MHA production (Swiegers and Pretorius 2007). In *T. delbrueckii* several studies analysed different strain phenotypes, characterized by undetectable 3MHA production or partially contradictory results regarding 4MMP and 3MH formations (Renault et al. 2016, Belda et al. 2017). The genome of *T. delbrueckii* COFT1 indicates a lack of *ATF1*, and therefore the inability of this strain to produce 3MHA. On the other hand, *IRC7* was found in both species under investigation, but in *S. cerevisiae* YAL1 the protein sequenced matched the truncated and less functional copy. *T. delbrueckii* had a higher expression of *IRC7* too throughout fermentation, which indicates the ability to produce 3MH and 4MMP and which could be related to the notably higher thiol release reported of the species compared to *S. cerevisiae* (Renault et al. 2016, Belda et al. 2017, Benito 2018).

Production of extracellular enzymes (e.g. esterases, proteases, pectinases), amino acid uptake or any other oenological properties considered necessary for the selection process could be further investigated with comparative functional genomics. Other than wine, the genomic screening for genes involved in metabolic pathways of interest can be extended to other industrial applications, such as beer production. In brewery wort, maltose is the most abundant sugar (He et al. 2014). In order to be able to metabolise maltose, *S. cerevisiae* needs at least a copy of a multigene complex (e.g. *MAL6*), which includes an activator of transcription (*MAL63*), maltose permease (*MAL61*) and maltase (*MAL62*; Novak et al. 2004). Some *T. delbrueckii* have been reported to be able to ferment beer wort (Canonico et al. 2016, Michel et al. 2016) but genomic analysis of *T. delbrueckii* COFT1 suggested an inability of the

indigenous strain to ferment maltose due to a lack of a maltase gene, which was confirmed experimentally (data not shown).

Comparative functional genomics approaches covered some of the differences that underlie phenotypic diversity, but yeast contribution to fermentation and volatile aroma profile is not limited to the presence of metabolic pathways, but also how the cell uses these during fermentation. This mainly depends on the physiology of the cell, which is influenced by external stimuli, such as stresses and nutrient availability (Bauer and Pretorius 2000, Fairbairn 2012). Moreover, the inability to sense and modulate intracellular changes needed to adapt to stress has been linked, in *S. cerevisiae*, to slow or sluggish fermentations (Fairbairn 2012). Comparative transcriptomics is another of the tools used to correlate oenologically relevant phenotypes to specific gene expression patterns. Our results indicate the use of newly sequenced genomes reduced ambiguous alignment, making downstream gene expression quantification more accurate, and consequently applicable in comparative transcriptome analysis of mixed strain fermentations. This opens new opportunities to investigate the physiology of yeast in wine-like conditions, where multiple species are present at the same time. Of particular interest is the response to high sugar conditions of the two species that usually occurs at the beginning of particularly harsh fermentations. Changes in viability and aroma profile, after hyperosmotic stress, have been determined for multiple *S. cerevisiae* strains (Fairbairn 2012, Heit 2014). These changes were characterized in wine strains belonging to *T. delbrueckii* species, whereby some of them exhibited a desirable higher tolerance to high sugar conditions and lower acetic acid production than *S. cerevisiae* (Hernandez-Lopez et al. 2003, Bely et al. 2008). However, the adaptation to such fermentation media has not been investigated physiologically for *T. delbrueckii*. Therefore, further investigation aimed at analysing the hyperosmotic stress response of the two yeast strains COFT1 and YAL1 needs to highlight the physiological basis of the phenotypic difference between the two species, and to validate a

method for a more complex transcriptome analysis which involves multiple species simultaneously. This will potentially identify complex gene-environment interactions, where different stimuli are present (i.e. nutrient availability, osmotic pressure, intra and inter species cell to cell contact). Different or common mechanisms of sensing and responding to the environment could be related to the diverse phenotypes that the two species exhibit and be of enormous interest in the understanding of *T. delbrueckii* selection processes in the laboratory and its uses in winemaking.

3.6. Acknowledgement

The authors wish to thank the Yalumba Wine Company for providing the wine samples and the Genomics Facility at SAHMRI (Adelaide, South Australia).

3.7. Funding

This work was supported by the Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production (Project IC130100005) and Wine Australia (Project AGT1502).

3.8. Appendix

Genome announcement and sequences

The final genome of *T. delbrueckii* COFT1 has been deposited in NCBI Genbank database under accession number CP027647 to CP027655.

Genome sequence of *S. cerevisiae* YAL1 has been deposited in NCBI, under accession number from QQBK01000001 to QQBK01000025.

Genomes annotation can be found online: https://data.mendeley.com/datasets/c8854nww3p/3

Statement of Authorship

Title of Paper	Genome Sequence of Australian Indigenous Wine Yeast *Torulaspora delbrueckii COFT1 Using Nanopore Sequencing*
Publication Status	[X] Published [ ] Accepted for Publication [ ] Submitted for Publication [ ] Unpublished and Unsubmitted work written in manuscript style
Publication Details	GENOME ANNOUNCEMENTS

Principal Author

Name of Principal Author (Candidate)	FEDERICO TONDINI
Contribution to the Paper	Conception Writing Analysis
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.
Signature	Date 27/6/18

Co-Author Contributions

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

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

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

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

Name of Co-Author	VLADIMIR JIRANEK
Contribution to the Paper	Conception Drafting
Signature	Date 26.6.18

Name of Co-Author	PAUL GRBIN
Contribution to the Paper	Conception Drafting
Signature	Date 25/06/2018

Name of Co-Author	Cristobal A. Onetto
Contribution to the Paper	Conception Drafting Analysis
Signature	Date 26/6/18

Please cut and paste additional co-author panels here as required.

AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements™

EUKARYOTES

Genome Sequence of Australian Indigenous Wine Yeast *Torulaspora delbrueckii* COFT1 Using Nanopore Sequencing

Federico Tondini,[a,b] Vladimir Jiranek,[a,b] Paul R. Grbin,[a,b] Cristobal A. Onetto[a]

[a]Department of Wine & Food Science, University of Adelaide, Adelaide, South Australia, Australia
[b]Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production, Adelaide, South Australia, Australia

ABSTRACT Here, we report the first sequenced genome of an indigenous Australian wine isolate of *Torulaspora delbrueckii* using the Oxford Nanopore MinION and Illumina HiSeq sequencing platforms. The genome size is 9.4 Mb and contains 4,831 genes.

Torulaspora delbrueckii occurs saprophytically on wine grape surfaces worldwide (1). Under winemaking conditions, it displays a less vigorous fermentation phenotype than *Saccharomyces cerevisiae*, differing in flavor and aroma compound production (2). Its favorable oenological traits, such as low acetic acid production and osmotic tolerance, have led to its commercialization and adoption for use in the wine industry (3). Despite this, *T. delbrueckii* is still not well characterized at a molecular level, with no other genomes reported from wine isolates.

For a better understanding of *T. delbrueckii* oenological traits, RNA sequencing (RNA-seq) studies under wine-like conditions are required. However, in the absence of a closely related reference sequence, significant challenges remain when it comes to assembling short reads into full-length gene and transcript models. In order to facilitate future wine-related studies, the genome of a wine isolate of *T. delbrueckii* was sequenced and characterized.

T. delbrueckii strain COFT1 was isolated from a spontaneous wine fermentation at the Yalumba Wine Company (Angaston, South Australia, Australia). High-molecular-weight genomic DNA was extracted according to the phenol-chloroform protocol (4), including a 2-h preincubation with Zymolase. DNA was prepared for sequencing with the MinION device using the SQK-LSK108 library prep kit (protocol GDE_9002_v108_revT_18Oct2016) and R9.4 chemistry. A total of 138,992 reads were obtained, for a total of 1,214 Mbp (130× coverage) and an average length of 8,737 bp. Fast5 files were base called using Albacore version 2.0.2. Passed reads were trimmed for adapters using PoreChop version 0.2.3 and then assembled using SMART*denovo* version 1.0 (https://github.com/ruanjue/smartdenovo). Contigs obtained from the assembly were polished using Racon version 0.5.0 (5) and Nanopolish version 0.8.5 (6). A final polish of the assembly was performed with Pilon version 1.22 using Illumina HiSeq RNA reads extracted from pure culture laboratory ferments with strain COFT1. The final assembly had no gaps, with a total length of 9,356,826 bp arranged in 9 chromosomes (1 mitochondrial chromosome) and an average GC content of 42%. The genome was first annotated with YGAP (7), and 5,231 genes were predicted. An improved annotation was performed with MAKER2 (8), providing STAR RNA alignment information (9) for the prediction of protein-coding genes. A total of 4,831 protein-coding genes were identified, compared to 4,714 and 4,972 protein-coding genes reported in previous genomes (10, 11). Functional annotation of the predicted protein sequences was performed using BLASTP (12) against the Swiss-Prot protein sequence database (E value = $1e^{-5}$) (13). BUSCO assessment (14) revealed a genome completeness of 98%.

Received 23 March 2018 **Accepted** 24 March 2018 **Published** 26 April 2018

Citation Tondini F, Jiranek V, Grbin PR, Onetto CA. 2018. Genome sequence of Australian indigenous wine yeast *Torulaspora delbrueckii* COFT1 using nanopore sequencing. Genome Announc 6:e00321-18. https://doi.org/10.1128/genomeA.00321-18.

Address correspondence to Vladimir Jiranek, vladimir.jiranek@adelaide.edu.au.

The functional annotation of the genome, as well as its completeness, allows comparison with the species *S. cerevisiae* and a better understanding of the oenological traits of this yeast. The genome sequence reported here will assist in delivering clearer transcriptional results in complex wine-like fermentations (e.g., mixed fermentations), providing useful insight into these processes for the wine industry.

Accession number(s). The final genome sequence has been deposited to NCBI GenBank database under accession numbers CP027647 to CP027655.

ACKNOWLEDGMENTS

We thank the Yalumba Wine Company for providing the wine samples and the Genomics Facility at SAHMRI (Adelaide, South Australia) for the use of the TapeStation 2200.

This work was supported by the Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production (project IC130100005) and Wine Australia (project AGT1502).

REFERENCES

1. Renouf V, Claisse O, Lonvaud-Funel A. 2005. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. Aust J Grape Wine Res 11:316–327. https://doi.org/10.1111/j.1755-0238.2005.tb00031.x.
2. Jolly NP, Varela C, Pretorius IS. 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14:215–237. https://doi.org/10.1111/1567-1364.12111.
3. Ciani M, Morales P, Comitini F, Tronchoni J, Canonico L, Curiel JA, Oro L, Rodrigues AJ, Gonzalez R. 2016. Non-conventional yeast species for lowering ethanol content of wines. Front Microbiol 7:642. https://doi.org/10.3389/fmicb.2016.00642.
4. Sambrook J, Russell DW. 2006. Purification of nucleic acids by extraction with phenol: chloroform. Cold Spring Harb Protoc 2006:pdb.prot4045. https://doi.org/10.1101/pdb.prot4045.
5. Vaser R, Sović I, Nagarajan N, Šikić M. 2017. Fast and accurate *de novo* genome assembly from long uncorrected reads. Genome Res 27:737–746. https://doi.org/10.1101/gr.214270.116.
6. Loman NJ, Quick J, Simpson JT. 2015. A complete bacterial genome assembled *de novo* using only nanopore sequencing data. Nat Methods 12:733. https://doi.org/10.1038/nmeth.3444.
7. Proux-Wéra E, Armisén D, Byrne KP, Wolfe KH. 2012. A pipeline for automated annotation of yeast genome sequences by a conserved-synteny approach. BMC Bioinformatics 13:237. https://doi.org/10.1186/1471-2105-13-237.
8. Holt C, Yandell M. 2011. MAKER2: an annotation pipeline and genome-database management tool for second-generation genome projects. BMC Bioinformatics 12:491. https://doi.org/10.1186/1471-2105-12-491.
9. Dobin A, Gingeras TR. 2015. Mapping RNA-seq reads with STAR. Curr Protoc Bioinformatics 51:11–19. https://doi.org/10.1002/0471250953.bi1114s51.
10. Gomez-Angulo J, Vega-Alvarado L, Escalante-García Z, Grande R, Gschaedler-Mathis A, Amaya-Delgado L, Arrizon J, Sanchez-Flores A. 2015. Genome sequence of *Torulaspora delbrueckii* NRRL Y-50541, isolated from Mezcal fermentation. Genome Announc 3:e00438-15. https://doi.org/10.1128/genomeA.00438-15.
11. Gordon JL, Armisén D, Proux-Wéra E, ÓhÉigeartaigh SS, Byrne KP, Wolfe KH. 2011. Evolutionary erosion of yeast sex chromosomes by mating-type switching accidents. Proc Natl Acad Sci U S A 108:20024. https://doi.org/10.1073/pnas.1112808108.
12. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403. https://doi.org/10.1016/S0022-2836(05)80360-2.
13. Bairoch A, Apweiler R. 2000. The Swiss-Prot protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res 28:45–48. https://doi.org/10.1093/nar/28.1.45.
14. Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31:3210–3212. https://doi.org/10.1093/bioinformatics/btv351.

3.9. References

Alexandre, H., V. Ansanay-Galeote, S. Dequin and B. Blondin (2001). Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Letters 498(1): 98-103.

Arslan, E., Z. Çelik and T. Cabaroglu (2018). Effects of pure and mixed autochthonous *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on fermentation and volatile compounds of Narince wines. Foods 7(9): 147.

Azzolini, M., B. Fedrizzi, E. Tosi, F. Finato, P. Vagnoli, C. Scrinzi et al. (2012). Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. European Food Research and Technology 235(2): 303-313.

Backhus, L. E., J. DeRisi, P. O. Brown and L. F. Bisson (2001). Functional genomic analysis of a commercial wine strain of *Saccharomyces cerevisiae* under differing nitrogen conditions. FEMS Yeast Research 1(2): 111-125.

Bairoch, A. and R. Apweiler (2000). The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Research 28(1): 45-48.

Barbosa, C., A. Mendes-Faia, P. Lage, N. P. Mira and A. Mendes-Ferreira (2015). Genomic expression program of *Saccharomyces cerevisiae* along a mixed-culture wine fermentation with *Hanseniaspora guilliermondii*. Microbial Cell Factories 14(1): 124.

Bauer, F. and I. S. Pretorius (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine-a review. South African Journal for Enology and Viticulture 21: 27-51.

Belda, I., E. Navascués, D. Marquina, A. Santos, F. Calderon and S. Benito (2015). Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality. Applied Microbiology and Biotechnology 99(4): 1911-1922.

Belda, I., J. Ruiz, E. Navascués, D. Marquina and A. Santos (2016). Improvement of aromatic thiol release through the selection of yeasts with increased β-lyase activity. International Journal of Food Microbiology 225: 1-8.

Belda, I., J. Ruiz, B. Beisert, E. Navascués, D. Marquina, F. Calderón et al. (2017). Influence of *Torulaspora delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations. International Journal of Food Microbiology 257: 183-191.

Bely, M., P. Stoeckle, I. Masneuf-Pomarède and D. Dubourdieu (2008). Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. International Journal of Food Microbiology 122(3): 312-320.

Benito, S. (2018). The impact of *Torulaspora delbrueckii* yeast in winemaking. Applied Microbiology and Biotechnology 102(7): 3081-3094.

Bisson, L. F., Karpel, J. E., Ramakrishnan, V., and Joseph, L. (2007). Functional genomics of wine yeast *Saccharomyces cerevisiae*. Advances in Food and Nutrition Research, 65–121.

Canonico, L., A. Agarbati, F. Comitini and M. Ciani (2016). *Torulaspora delbrueckii* in the brewing process: a new approach to enhance bioflavour and to reduce ethanol content. Food Microbiology 56: 45-51.

Chambers, P. J. and I. S. Pretorius (2010). Fermenting knowledge: the history of winemaking, science and yeast research. EMBO Reports 11(12): 914-920.

Cheadle, C., M. P. Vawter, W. J. Freed and K. G. Becker (2003). Analysis of microarray data using Z score transformation. The Journal of Molecular Diagnostics 5(2): 73-81.

Ciani, M., F. Comitini, I. Mannazzu and P. Domizio (2010). Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. FEMS Yeast Research 10(2): 123-133.

Deamer, D., M. Akeson and D. Branton (2016). Three decades of nanopore sequencing. Nature Biotechnology 34(5): 518-524.

Delcher, A. L., S. L. Salzberg and A. M. Phillippy (2003). Using MUMmer to identify similar regions in large sequence sets. Current Protocols in Bioinformatics (1): 10.13. 11-10.13. 18.

Dobin, A. and T. R. Gingeras (2015). Mapping RNA-seq Reads with STAR. Current Protocols in Bioinformatics: 11.14. 11-11.14. 19.

Dubourdieu, D., T. Tominaga, I. Masneuf, C. P. des Gachons and M. L. Murat (2006). The role of yeasts in grape flavor development during fermentation: the example of Sauvignon blanc. American Journal of Enology and Viticulture 57(1): 81-88.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32(5), 1792-1797.

Ehsani, M., P. Renault and V. Puente-López (2012). L'impact organoleptique de *Torulaspora delbrueckii* en conditions oenologiques lors d'une inoculation séquentielle avec *Saccharomyces cerevisiae*. Revue des Oenologues et des Techniques Vitivinicoles et Oenologiques 39: 23-26.

Erasmus, D., G. Vandermerwe and H. Vanvuuren (2003). Genome-wide expression analyses: metabolic adaptation to high sugar stress. FEMS Yeast Research 3(4): 375-399.

Fairbairn, S. (2012). Stress, fermentation performance and aroma production by yeast. Stellenbosch: Stellenbosch University.

Fedrizzi, B., K. H. Pardon, M. A. Sefton, G. M. Elsey and D. W. Jeffery (2009). First identification of 4-S-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon blanc juice. Journal of Agricultural and Food Chemistry 57(3): 991-995.

Fleet, G. H. (2003). Yeast interactions and wine flavour. International Journal of Food Microbiology 86(1-2): 11-22.

Gomez-Angulo, J., L. Vega-Alvarado, Z. Escalante-García, R. Grande, A. Gschaedler-Mathis, L. Amaya-Delgado et al. (2015). Genome sequence of *Torulaspora delbrueckii* NRRL Y-50541, isolated from mezcal fermentation. Genome Announcements 3(4): e00438-00415.

Gordon, J. L., D. Armisen, E. Proux-Wera, S. S. Oheigeartaigh, K. P. Byrne and K. H. Wolfe (2011). Evolutionary erosion of yeast sex chromosomes by mating-type switching accidents. Proceedings of the National Academy of Sciences USA 108.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit et al. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29(7): 644.

Haas, B. J., A. Papanicolaou, M. Yassour, M. Grabherr, P. D. Blood, J. Bowden et al. (2013). *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols 8(8): 1494-1512.

Hazelwood, L. A., M. C. Walsh, M. A. Luttik, P. Daran-Lapujade, J. T. Pronk and J. M. Daran (2009). Identity of the growth-limiting nutrient strongly affects storage carbohydrate accumulation in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. Applied Environmental Microbiology 75(21): 6876-6885.

He, Y., J. Dong, H. Yin, Y. Zhao, R. Chen, X. Wan et al. (2014). Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer–a review. Journal of the Institute of Brewing 120(3): 157-163.

Heit, C. (2014). Hyperosmotic stress and the impact on metabolite formation and redox balance in *Saccharomyces cerevisiae* and *Saccharomyces bayanus* strains. Faculty of Mathematics and Science, Brock University.

Hernandez-Lopez, M., J. Prieto and F. Randez-Gil (2003). Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. Antonie van Leeuwenhoek 84(2): 125-134.

Herrgård, M. J., N. Swainston, P. Dobson, W. B. Dunn, K. Y. Arga, M. Arvas et al. (2008). A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. Nature Biotechnology 26(10): 1155.

Holt, C. and M. Yandell (2011). MAKER2: an annotation pipeline and genome-database management tool for second-generation genome projects. BMC Bioinformatics 12(1): 491.

Howell, K. S., M. Klein, J. H. Swiegers, Y. Hayasaka, G. M. Elsey, G. H. Fleet et al. (2005). Genetic determinants of volatile-thiol release by *Saccharomyces cerevisiae* during wine fermentation. Applied and Environmental Microbiology 71(9): 5420-5426.

Jenjaroenpun, P., T. Wongsurawat, R. Pereira, P. Patumcharoenpol, D. W. Ussery, J. Nielsen et al. (2018). Complete genomic and transcriptional landscape analysis using third-generation sequencing: a case study of *Saccharomyces cerevisiae* CEN. PK113-7D. Nucleic Acids Research 46(7): e38-e38.

Jolly, N. P., C. Varela and I. S. Pretorius (2014). Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. FEMS Yeast Res 14(2): 215-237.

Kanehisa, M., S. Goto, Y. Sato, M. Furumichi and M. Tanabe (2011). KEGG for integration and interpretation of large-scale molecular data sets. Nucleic acids research 40(D1): D109-D114.

Kanehisa, M., Y. Sato and K. Morishima (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. Journal of Molecular Biology 428(4): 726-731.

Konwar, K. M., N. W. Hanson, A. P. Pagé and S. J. Hallam (2013). MetaPathways: a modular pipeline for constructing pathway/genome databases from environmental sequence information. BMC Bioinformatics 14(1): 202.

Koren, S., B. P. Walenz, K. Berlin, J. R. Miller, N. H. Bergman and A. M. Phillippy (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Research 27(5): 722-736.

Liu, J., T. Yu, T. Jiang and G. Li (2016). TransComb: genome-guided transcriptome assembly via combing junctions in splicing graphs. Genome Biology 17(1): 213.

Loman, N. J., J. Quick and J. T. Simpson (2015). A complete bacterial genome assembled *de novo* using only nanopore sequencing data. Nature Methods 12(8): 733.

Love, M. I., W. Huber and S. Anders (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15(12): 550.

Madden, T. (2013). The BLAST sequence analysis tool. Madden, T. (2013). The BLAST sequence analysis tool. The NCBI Handbook [Internet]. 2nd edition. National Center for Biotechnology Information (US).

Marks, V. D., S. J. Ho Sui, D. Erasmus, G. K. Van Der Merwe, J. Brumm, W. W. Wasserman et al. (2008). Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Research 8(1): 35-52.

Masneuf-Pomarede, I., M. Bely, P. Marullo and W. Albertin (2016). The genetics of non-conventional wine yeasts: current knowledge and future challenges. Frontiers in Microbiology 6: 1563.

Michel, M., J. Kopecká, T. Meier-Dörnberg, M. Zarnkow, F. Jacob and M. Hutzler (2016). Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. Yeast 33(4): 129-144.

Moriya, Y., M. Itoh, S. Okuda, A. C. Yoshizawa and M. Kanehisa (2007). KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Research 35(suppl_2): W182-W185.

Novak, S., V. Zechner-Krpan and V. Maric (2004). Regulation of maltose transport and metabolism in *Saccharomyces cerevisiae*. Food Technology and Biotechnology 42(3): 213-218.

O'Leary, N. A., M. W. Wright, J. R. Brister, S. Ciufo, D. Haddad, R. McVeigh et al. (2015). Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Research 44. D1 (2015): D733-D745.

Orellana, M., F. F. Aceituno, A. W. Slater, L. I. Almonacid, F. Melo and E. Agosin (2014). Metabolic and transcriptomic response of the wine yeast *Saccharomyces cerevisiae* strain EC1118 after an oxygen impulse under carbon-sufficient, nitrogen-limited fermentative conditions. FEMS Yeast Research 14(3): 412-424.

Padilla, B., J. V. Gil and P. Manzanares (2016). Past and future of non-*Saccharomyces* yeasts: from spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Frontiers in Microbiology 7: 411.

Patro, R., G. Duggal and C. Kingsford (2015). Salmon: accurate, versatile and ultrafast quantification from RNA-seq data using lightweight-alignment. BioRxiv 021592.

Pretorius, I. S., C. D. Curtin and P. J. Chambers (2012). The winemaker's bug: from ancient wisdom to opening new vistas with frontier yeast science. Bioengineered 3(3): 149-158.

Proux-Wéra, E., D. Armisén, K. P. Byrne and K. H. Wolfe (2012). A pipeline for automated annotation of yeast genome sequences by a conserved-synteny approach. BMC Bioinformatics 13(1): 237.

Renault, P., J. Coulon, G. de Revel, J.-C. Barbe and M. Bely (2015). Increase of fruity aroma during mixed *T. delbrueckii/S. cerevisiae* wine fermentation is linked to specific esters enhancement. International Journal of Food Microbiology 207: 40-48.

Renault, P., J. Coulon, V. Moine, C. Thibon and M. Bely (2016). Enhanced 3-sulfanylhexan-1-ol production in sequential mixed fermentation with *Torulaspora delbrueckii/Saccharomyces cerevisiae* reveals a situation of synergistic interaction between two industrial strains. Frontiers in Microbiology 7: 293.

Roncoroni, M., M. Santiago, D. O. Hooks, S. Moroney, M. J. Harsch, S. A. Lee et al. (2011). The yeast *IRC7* gene encodes a β-lyase responsible for production of the varietal thiol 4-mercapto-4-methylpentan-2-one in wine. Food Microbiology 28(5): 926-935.

Rossouw, D. and F. F. Bauer (2009). Comparing the transcriptomes of wine yeast strains: toward understanding the interaction between environment and transcriptome during fermentation. Applied Microbiology and Biotechnology 84(5): 937.

Sambrook, J. and D. W. Russell (2006). Purification of nucleic acids by extraction with phenol: chloroform. Cold Spring Harbor Protocols 2006(1): pdb. prot4455.

Simão, F. A., R. M. Waterhouse, P. Ioannidis, E. V. Kriventseva and E. M. Zdobnov (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31(19): 3210-3212.

Swiegers, J., E. Bartowsky, P. Henschke and I. Pretorius (2005). Yeast and bacterial modulation of wine aroma and flavour. Australian Journal of grape and wine research 11(2): 139-173.

Swiegers, J. and I. Pretorius (2007). Modulation of volatile sulfur compounds by wine yeast. Applied Microbiology and Biotechnology 74(5): 954-960.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley et al. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nature Protocols 7(3): 562-578.

Varela, C., J. Cárdenas, F. Melo and E. Agosin (2005). Quantitative analysis of wine yeast gene expression profiles under winemaking conditions. Yeast 22(5): 369-383.

Vaser, R., I. Sovic, N. Nagarajan and M. Šikic (2017). Fast and accurate *de novo* genome assembly from long uncorrected reads. Genome Research.

Viana, F., J. V. Gil, S. Genovés, S. Vallés and P. Manzanares (2008). Rational selection of non-*Saccharomyces* wine yeasts for mixed starters based on ester formation and enological traits. Food Microbiology 25(6): 778-785.

Wu, B., A. Buljic and W. Hao (2015). Extensive horizontal transfer and homologous recombination generate highly chimeric mitochondrial genomes in yeast. Molecular Biology and Evolution 32(10): 2559-2570.

Zhao, Y., Y. Wang, L. Zou and J. Huang (2016). Reconstruction and applications of consensus yeast metabolic network based on RNA sequencing. FEBS Open Bio 6(4): 264-275.

Statement of Authorship

Title of Paper	**Early adaptation strategies of *Saccharomyces cerevisia* and *Torulaspora delbrueckii* to co-inoculation in high sugar grape-like media.**
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	FOOD MICROBIOLOGY

Principal Author

Name of Principal Author (Candidate)	FEDERICO TONDINI
Contribution to the Paper	CONCEPTION DRAFTING ANALYSIS
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 27/6/18

Co-Author Contributions

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

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

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

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

Name of Co-Author	CRISTOBAL A. ONETTO
Contribution to the Paper	ANALYSIS DRAFTING
Signature	Date 26/6/18

Name of Co-Author	VLADIMIR JIRANEK
Contribution to the Paper	CONCEPTION DRAFTING
Signature	Date 26·6·18

Please cut and paste additional co-author panels here as required.

Chapter 4. Early adaptation strategies of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* to co-inoculation in high sugar grape-like media.

Federico Tondini[1*], Cristobal A. Onetto[1†], Vladimir Jiranek[1]

[1] Department of Wine & Food Science, University of Adelaide and Australian Research Council Training Centre for Innovative Wine Production, Glen Osmond, South Australia 5064, Australia.

[*]Corresponding author: federico@aebusa.com

[†]Current address: The Australian Wine Research Institute, Glen Osmond, South Australia 5064, Australia.

4.1. Abstract

Torulaspora delbrueckii and *Saccharomyces cerevisiae* are yeast species found concurrently in wine. In order to commence fermentation, they adapt to the initial harsh environment, maintaining cellular homeostasis and promoting metabolism. These actions involve an intricate regulation of stress tolerance, growth and metabolic genes. Their phenotypes are influenced by the fermentation environment and physiological state of the cell, but such gene-environment interactions are poorly understood. This study aimed to compare the cell physiology of the two species, through genome-wide analysis of gene expression, coupling Oxford Nanopore MinION and Illumina Hiseq sequencing platforms. The early transcriptional responses to stress, nutrients and cell-to-cell communication were analysed. Particular attention was given to the fundamental gene modulations, leading to an understanding of the physiological changes needed to maintain cellular homeostasis, exit the quiescent state and establish dominance in the fermentation. Our findings suggest the existence of species-specific adaptation strategies in response to growth in a high sugar synthetic grape juice medium.

4.2. Introduction

Yeasts are unicellular organisms observed on fruit, flowers and bark worldwide (Renouf at al., 2005, Morrison-Whittle and Goddard, 2018). The naturally occurring yeasts associated with grapes and their processing are occasionally used to produce wines in a so-called 'spontaneous' fermentation process (Pretorius, 2017). This fermentation technique does not use an inoculum of a selected yeast but sees a complex and sequential succession of indigenous yeast species (Barata at al., 2012, Masneuf-Pomarede at al., 2016). During the fermentation process, changes in environmental conditions and nutrient availability pose simultaneous or sequential stresses that yeast must overcome in order to survive (Gasch, 2007, Landolfo at al., 2008, Fuchs and Mylonakis, 2009). Accordingly, these organisms have evolved elaborate strategies to rapidly respond to and counteract their environment so as to maintain intracellular homeostasis and normal physiology (Zuzuarregui and del Olmo, 2004, Gasch, 2007). The ability of yeast to promptly and successfully react to these stress factors is correlated to the start of wine fermentation, the yeast's lag phase (Ferreira at al., 2017). Knowledge of these stress-induced mechanisms is fundamental to an understanding of how these microbes hold out to drastic environmental changes and, in *S. cerevisiae*, represent a vividly studied field.

The yeast stress response (SR) to external changes relies on a large set of chaperones, heat shock proteins, protective molecules, detoxifying enzymes and transporters (Enjalbert at al., 2003, Jungwirth and Kuchler, 2006). These SRs work in a coordinated fashion, mainly through protein sensors and downstream protein kinases, which help remodel transcription (Matallana and Aranda, 2017). Such complex genome-wide transcriptional remodelling has been defined in *Saccharomyces cerevisiae* as the environmental SR (ESR), where a set of ~900 genes among the ~6,000 genes of the genome are differentially expressed in a similar manner in response to each of 13 different conditional changes (Gasch at al., 2000). In addition, wine-related studies of the *S. cerevisiae* transcriptome identified 223 genes dramatically induced at various time-

points during fermentation, characterising them as a fermentation SR (FSR) (Marks at al., 2008). An in-depth investigation of the laboratory strain S288c plotted the complex interacting pathways of SR signalling (five mitogen-activated protein kinases (MAPK; Chen and Thorner, 2007) and nutrient sensing systems (NS; Jiménez at al., 2015, Duc at al., 2017). Through their transcription factors (TFs), these pathways orchestrate the harmonious operation of the cell in response to inter- and intracellular changes to coordinate growth and development of the cell (Chen and Thorner, 2007, Stephan at al., 2009). Regulation of stress tolerance and growth rate needs to be tuned minutely to gain maximum fitness (Zakrzewska at al., 2011, Broach, 2012).

Other than *S. cerevisiae*, the SR and NS have been investigated in the pathogen *Candida albicans* (Enjalbert at al., 2003, Gasch, 2007), the fission yeast *Schizosaccharomyces pombe* (Gasch, 2007), *Lachancea kluyveri* (Brion at al., 2016) and *Torulaspora delbrueckii* (Tronchoni at al., 2017), but not to the extent seen in *S. cerevisiae*. In terms of wine fermentation, having an accurate picture of how yeast gene expression changes in the adaptation to multiple stresses and nutrient availabilities is critical to understanding the risk of stuck and sluggish fermentations (Bauer and Pretorius, 2000, Fuchs and Mylonakis, 2009, Bendjilali at al., 2017).

This study aimed to further investigate the different SR phenotypes of *T. delbrueckii* compared to *S. cerevisiae* as observed at the beginning of a high sugar fermentation (Hernandez-Lopez at al., 2006). Although generally less efficient than *S. cerevisiae* during fermentation, *T. delbrueckii* owns unique and appealing oenological characteristics such as production of specific aromas and lower acetic acid production (Tondini at al., 2019). Previous observations of *S. cerevisiae* occurred under high hyperosmotic stress, wherein cells died by apoptosis (Silva at al., 2005). Such conditions might not be relevant to wine. Thus, despite great achievements in the last decades, the process of yeast adjustment to winemaking conditions is far from fully understood and could be furthered. As such, we sought to investigate less extreme conditions

and compare cellular responses by identifying differentially expressed genes in the two species. Our findings highlight a species-specific adaptation where conserved signalling pathways are differently regulated in response to the same environment.

4.3. Materials and Methods

4.3.1. Yeast strains and synthetic grape juice fermentations

S. cerevisiae yeast strain YAL1 (accession number QQBK01000001-QQBK01000025) and *T. delbrueckii* COFT1 (Tondini at al., 2018, accession number CP027647 to CP027655) were selected from an in-house collection of isolates obtained from spontaneous grape juice fermentations conducted at the Yalumba Wine Company (Angaston, South Australia). In order to mimic high sugar grape must, we utilised a chemically defined grape juice medium (CDGJM, Supplementary File 1) with 300 g/L equimolar glucose and fructose and 200 mg/L of yeast assimilable nitrogen (as amino acids). Yeasts were grown individually in YEPD (1% yeast extract, 2% bacto peptone, 2% glucose) for 24 h prior to co-inoculation into 100 ml of CDGJM. Cells were inoculated at 10^6 viable cells/ml for monoculture propagations and 10^7 viable cells/ml for each species at the beginning of the mixed culture fermentations. Estimation of cell number and viability was achieved with a Guava® easyCyte Flow Cytometer (Beckman Coulter, USA) using propidium iodide (1% v/v). The initial stress response of the two species was investigated at five timepoints: 0 (T0), 10 (T1), 30 (T2), 60 (T3) and 120 min (T4) after inoculation. Fermentations were conducted in quadruplicate.

4.3.2. RNA extraction, transcriptome assembly and gene expression data analysis

Cells were divided into 5 aliquots of 25 ml for each fermentation and were snap-frozen immediately (Piper at al., 2002). High quality RNA extraction was performed using the Trizol protocol described by Chomczynski and Sacchi (2006). A RNeasy mini kit (QIAGEN, Melbourne, Australia) was used to purify and concentrate the samples in 2 ml microfuge tubes.

The resulting extracts were treated with RNase-free DNase (Life Technologies, United States). Quality check, cDNA preparation, library preparation and Illumina sequencing were performed by the South Australian Health & Medical Research Institute (SAHMRI, Australia), using a Hiseq 2500 Illumina platform and the Rapid Run v2 chemistry. Only high-quality RNA with an integrity number between 9.5 and 10 (Tapestation 2200; Agilent Technologies, USA) was sequenced. Approximately 300 million, 100-bp paired-end reads were obtained in total, resulting in 15 million reads per sample (SRA submission number SUB4270844). Raw reads were quality trimmed to remove <33 phread score sequences with Trimmomatic-0.36 (Bolger at al., 2014). High quality reads were then aligned to a chimeric genome derived from *S. cerevisiae* YAL1 and *T. delbrueckii* COFT1, using STAR (Dobin and Gingeras, 2015). A chimeric transcriptome was generated using Gffread (Trapnell at al., 2012). Only uniquely mapped reads with no mismatched bases of each species (Borneman at al., 2008, Tronchoni at al., 2017) were fed to SALMON 0.8.2 (Patro at al., 2015) for transcript quantification (transcript per million: TPM). For analysis of differentially expressed genes between time points we separated the genes based on name suffix, which enabled differentiation of the two species. TPM values were fed to DESeq2 to normalize gene expression and quantify the differential expression (p-value < 0.001; Storey, 2002, Love at al., 2014). Z-score transformation was performed on normalised TPM values (Cheadle at al., 2003). Principal component analysis of the normalised RNA data and gene clustering was done using R (Team, 2013, Gu at al., 2014).

4.4. Results

4.4.1. Overview of the transcriptional response to high sugar CDGJM

For each time point, the use of third-generation sequencing genomes improved the unique aligned reads by 6% (from 80% to 86%) compared to the alignment rate to the chimeric genome

obtained by merging reference CBS1146 and S288c (NCBI IDs 12254 and 15, respectively). Single species reads, obtained from a previous experiment (Tondini at al., 2018), under the same alignment condition, had on average only a 0.01-0.02% unique match to the opposite genome. Therefore, third-generation sequencing genomes reduced mismatch and multi-mapped read errors in gene quantifications and, moreover, enabled the analysis of the proportion of the genome that was unique to these particular indigenous wine yeast isolates.

To identify the processes required for stress survival, various physiological reactions were investigated in response to the same harsh environment, with a monitoring changes in genome-wide expression patterns between T0 and 10 (T1), 30 (T2), 60 (T3) and 120 min (T4). The global change in gene expression was analysed in *S. cerevisiae* and *T. delbrueckii* (Supplementary File 2). Between 0 and 10 min, no pronounced changes were observed (Supplementary Figure 1A and 2A. However, large differences were observed at 2 h for *S. cerevisiae* (Supplementary Figure 1B) and at 30-60 min for *T. delbrueckii* (Supplementary Figure 2B). In particular, *S. cerevisiae* had a slower but vast remodelling of its transcriptome (up to 40% of differentially expressed genes after 2 h), while *T. delbrueckii* had a limited response (261 genes out of 4,796), displaying transcription peaks at 30-60 min (Supplementary Figure 2B). Only 1% of differentially expressed (DE) genes were observed after 2 h in *T. delbrueckii*.

Transcription of *CWP1* (Table 1) and *HXT1* (Table 2), a cell wall protein involved in stress response (Boorsma at al., 2004) and a sugar transporter respectively, were among the most expressed genes in both species.

4.4.2. Environmental stress response (ESR) pathway

Diverse studies have investigated the genomic expression of the ESR pathway in ‘model’ ascomycete fungi, identifying its role in maintaining homeostasis in the face of variable

conditions (Gasch, 2007). Under suboptimal conditions, initiation of the stress response is at the cost of optimal growth, and its transcriptional response is modulated by paralogs, the Msn2p/Msn4p transcription factors (Gasch at al., 2000). Only one orthologue transcript for Msn4p was observed in *T. delbrueckii*, compared to indigenous *S. cerevisiae*, which expresses both.

Transcript profiling of approximately 400 genes, whose expression has been correlated to ESR activation (Gasch at al., 2000), showed unexpected and contrasting behaviours between the two species. The majority of *S. cerevisiae* genes analysed changed expression after the first 30 minutes, but not as expected. Genes that were supposed to be overexpressed (such as carbohydrate metabolism, detoxification of reactive oxygen species, protein folding and degradation) showed downregulation compared to T0, and vice versa (Figure 1). Instead, *T. delbrueckii* expressed the ESR pathway differently, in a pattern similar to previously reported experiment by Gasch at al. (2000). ESR activation occurred between 10 min and 1 h, to disappear at 2 h (Figure 1). ESR expression in the last time point, was similar between the two species.

4.4.3. High osmolarity glycerol (HOG) and C5-C6 polyols pathways

One of the reported physiological roles of the HOG pathway is to guide the adaptation of yeast cells to high osmolarity conditions, which otherwise cause water loss and shrinking of the cells (Novo at al., 2007, Hohmann, 2009, Saito and Posas, 2012). In *S. cerevisiae*, osmotic stress is sensed by receptors and then signalled intracellularly through the MAPK cascade pathway to activate Hog1p (Hohmann, 2002, O'Rourke at al., 2002). Following hyperosmotic shock, activated Hog1p works to control water and glycerol homeostasis (Lee and Levin, 2015) and expressing genes throughout different TFs, Hot1p, Sko1p and Msn2/Msn4 (Hohmann at al., 2007).

Conservation of HOG pathway genes was observed between *S. cerevisiae* and *T. delbrueckii*, (Supplementary File 2; Wu at al., 2010, Feng at al., 2017). Paralog genes in *S. cerevisiae*, which arose from a whole genome duplication event, were missing in *T. delbrueckii* (Liu at al., 2009). The expression of multiple HOG pathway genes was similar for *S. cerevisiae* and *T. delbrueckii* (Table 1). In *S. cerevisiae* we observed an overexpression of the glycerol pathway (glycerol importer *SLT1*, glycerol metabolic genes *GPD1/2* and *GPP1/2*). *CTT1* and *HSP12*, whose activation depends on the TF Msn2/4p, were already highly expressed and then downregulated (Table 1; Fuchs and Mylonakis, 2009, Wu at al., 2010). On the contrary, upon exposure to high sugar CDGJM, *T. delbrueckii* already expressed *CTT1* and *HSP12*, before raising their transcript levels from 10 to 60 min (Table 1). Of the HOG pathway genes, only *GPD1* was highly expressed but not *GPP1*. The two species showed similar expression of *GRE2*, involved in ergosterol synthesis related to the osmotic stress response (Warringer and Blomberg, 2006).

Interestingly, transcript levels of genes involved in multiple glycerol catabolic pathways (*GUT1*, *GUT2*, *DAK1*, *DAK2*; Klein at al., 2017), which are highly expressed by *S. cerevisiae* and then downregulated after exposure to CDGJM, were poorly expressed in *T. delbrueckii*, except for *GUT1* (Figure 2) and *DAK1*, which are overexpressed in the mid-part of the stress response (10-30 min). This suggests a different use of glycerol-3-phospate by *T. delbrueckii*, one where it does not serve a pivotal role in osmoprotection.

Aside from glycerol, yeast cells accumulate polyols to facilitate osmotic adjustment and support redox control (Shen at al., 1999). Polyols are the product of the reduction of a variety of sugars (Song and Vieille, 2009, Toivari at al., 2010). *T. delbrueckii* up-regulated the general aldo-keto reductases *GRE3* (Figure 2), *YPR1* and *YJR096W* (Table 1). These genes were previously identified in *S. cerevisiae* as responsible for the first step of sugar reduction (Träff at al., 2002), and could be responsible for the higher production of polyols previously reported in *T. delbrueckii* (Mbuyane at al., 2018).

4.4.4. Oxidative stress response

Oxidative stress is suffered by yeast in the presence of reactive oxygen species (ROS). ROS can damage cellular constituents such as DNA, lipids and proteins (Matallana and Aranda, 2017). Yeast cells possess enzymatic (catalase and peroxidase) and small molecule (glutathione) defences to maintain the cellular redox state (Jamieson 1998) and avoid cell damage (Morano at al., 2012). Expression of genes *CPP1*, *SOD2* (peroxidases) and *CTA1* (catalase) were downregulated in *S. cerevisiae*, while they were upregulated in the 10 to 60 min response in *T. delbrueckii* (Table 1). Metacaspase *MCA1* and nuclear serine protease *NMA111* (Figure 2) were overexpressed in *S. cerevisiae*, genes that are involved in the ROS and hyperosmotic programmed cell death pathways (PCD; Madeo at al., 2004, Silva at al., 2005, Mazzoni and Falcone, 2008). No significant changes in the expression levels of *NMA111* and *MCA1* were observed in *T. delbrueckii*.

4.4.5. Other stresses: Cell Wall Integrity (CWI) pathway and weak acid response

The cell wall is essential for protection against environmental changes (Sanz at al., 2017). β1, 3-glucan, β1,6-glucan, chitin and mannoproteins form the molecular membrane architecture, which is highly dynamic in order to maintain yeast osmotic integrity (Rodicio and Heinisch, 2010, Orlean, 2012). This dynamism is provided by enzymes (glucanosyl transferase and chitinase), plus a multitude of cell wall and secretory glycoproteins (Ene at al., 2015). The two species expressed different cell wall related proteins at the beginning of the experiment (Table 1). In *S. cerevisiae*, *FKS1* (1,3-β-glucan synthase, *CHS1* (chitin synthase) and *UTR2* (chitin transglycosylase) were highly expressed from a low basal level following exposure to high sugar CDGJM. These changes indicated assembly of new cell wall and preparation of the budding site for *S. cerevisiae*. Almost no changes in transcription of these genes were seen for *T. delbrueckii*, which routinely showed a strong expression of *FKS1*. Both species

overexpressed *CWP1* (cell wall mannoprotein; Figure 2), suggesting a specific function in the high sugar grape-like stress response for this protein in both species.

As well as osmotic pressure, weak acid and low pH can also impact the cell wall and viability at the beginning of fermentation (Kapteyn at al., 2001). In response to high sugar CDGJM, *S. cerevisiae* cells upregulated the proton pump, *PMA1*, and plasma membrane transporter, *PDR12*, followed by downregulation of weak acid importers, *JEN1* (Figure 2) and *ADY2* (Table 1). In contrast, *T. delbrueckii* downregulated *PMA1* and increased the expression of *JEN1*. The weak acid exporter *ADY2* was not present in the genome of *T. delbrueckii.*

4.4.6. Cell cycle and glycolysis regulation by stress and the Protein A Kinase response

Most organisms have evolved to confront diverse environmental conditions by ensuring the optimal combination of proliferation and survival (Zakrzewska at al., 2011). Duplication/cell cycle progression rely on cyclin-dependent kinases (Cdc28p) and their interaction with proteins that are varyingly expressed during the cell cycle, namely the cyclins (*CNL 1-3* for G1/S phase, *CLB 5-6* for entering S phase and *CLB 1-4* to enter M phase (Bloom and Cross, 2007, Jiménez at al., 2015). In growing cells, upon exposure to fermentation stress, the ESR, HOG and CWI pathways can cause a delay in the cell cycle (Gray at al., 2004, Clotet and Posas, 2007). However, on a fermentable carbon source, glucose counter-acts this by positively regulating growth through nutrient sensors and the downstream PKA pathway (Rubio-Texeira at al., 2010), promoting growth and repressing stress-related gene expression (Swinnen at al., 2006). Cell growth is therefore modulated by this complex network of intracellular signals. The progression of the cell cycle through expression of different cyclins was monitored (Figure 3). Cyclin expression patterns indicated both species were in a G0/ G1 physiological state once inoculated, with higher expression of *CLN* cyclins, compared to *CLB* cyclins (Table 2, Figure 3).

Transcript profiles of cyclin expression showed different responses in CDGJM; *S. cerevisiae* stopped cyclin expression in the short stress response (5-10 min), before increasing their expression after 30 min of stress exposure. Instead, *T. delbrueckii* downregulated the expression of cyclins during the initial 30 min, only increasing after 1 hr of incubation (Figure 3).

Glycolytic gene expression followed the trend of the cell cycle genes in *T. delbrueckii* (Table 2). Most of glycolytic genes were expressed at the beginning of the experiment, followed by a decrease and a final restoration to initial levels of expression. Contrarily, in *S. cerevisiae*, expression of the glycolytic genes did not relate to cyclin expression but to the PKA energy pathway. Activity of PKA was monitored by the glycolytic gene hexoglucokinase 2 (*HXK2*) (Table 2, Figure 4), a key player in glucose repression/PKA activation (Vega at al., 2016). Hxk2p was not expressed in *S. cerevisiae* initially but showed an exponential increase throughout the experiment. Glycolytic genes, as for *HXK2*, were poorly expressed but showed a dramatic increase during exposure to CDGJM. In *T. delbrueckii*, *HXK2* was highly expressed and its transcriptional profile followed the same trend of other glycolytic genes, suggesting a different mechanism of action of the carbon catabolite repression between yeast species. To further investigate the observed differences in regulation, we checked the expression of genes highly affected by PKA activity (induced and repressed), as reported by Livas at al. (2011) (Table 2). The two species showed opposite behaviours, in accordance with previous observations, further supporting that PKA activation in *S. cerevisiae* as a response to CDGJM is not observed in *T. delbrueckii.*

4.5. Discussion and Conclusion

The first objective of this study was to comparatively analyse the physiological response, at a gene expression level, of two different wine yeast strains. Cellular responses to environmental stress are fundamental to cellular physiology, an understanding of which will provide

significant insight into the metabolic features activated under sub-optimal growth conditions (Molin at al., 2003) such as winemaking. The use of third-generation sequencing technologies (Oxford nanopore, MinION) helped deliver an increased quality of genome assemblies (Bleidorn, 2016), expanding the opportunities in mixed population transcriptomic studies, and providing new in-depth tools to investigate cellular physiology. Strict alignment of RNA Illumina reads to their higher quality genomes gave more precise results in our experimental setting than previously utilised reference genomes. Therefore, this approach was used to compare the genomic and transcriptomic differences between *T. delbrueckii* strain COFT1 and *S. cerevisiae* YAL1 in high sugar CDGJM.

This complex medium imposed multiple stresses and nutrient signals on the cell, which resulted in two different adaptation mechanisms across the species. The global view of the results leads to the conclusion that *S. cerevisiae* and *T. delbrueckii* reacted differently to CDGJM. Contrarily to previously reported stress responses of *S. cerevisiae* (Gasch at al., 2006), exposure to CDGJM did not activate the ESR pathway in this yeast species. *T. delbrueckii* instead had a broad activation of the ESR pathway, showing a species-specific stress pathway activation.

Important differences in utilisation of glycerol as an osmoprotectant were also observed. Unlike *S. cerevisiae*, in which glycerol (C4 polyol) has a well-documented function after exposure to osmotic stress, *T. delbrueckii* seemed to favour accumulation of other polyols (C5-C6). The role and possible accumulation during fermentation of these sugar alcohols requires further investigation, since they could impact the desirable characteristics of the resulting wine.

Two different modes of cell wall and plasma membrane protein regulation appeared to be in use between the two species. In *S. cerevisiae*, activation of chitin related genes strongly suggests cell wall remodelling, initiation of assembly of the yeast cell wall and therefore preparation to cell division by budding. Instead, *T. delbrueckii* expressed these genes to a

higher basal level without significantly altering their expression under the experimental conditions, suggesting a less dynamic cell wall structure. On the same note, the transcriptional data showed a different relationship between growth-related and stress-related genes in the two species. As previously describe by Spor at al. (2008), cell division cyclins and glycolytic genes were expressed sooner in *S. cerevisiae*, while ESR genes were poorly activated. This metabolic activity, in fact, was not followed by an increase in oxidative stress related genes, but by PCD genes. This observation is in accordance with a previous report (Costa and Moradas-Ferreira, 2001), which indicates how the general stress response and specific stress response (oxidative) could be inhibited by the glucose catabolite repression system.

Two species-specific lifestyles appear to exist to exit the quiescence–like state that cells were exhibiting at inoculation. The *T. delbrueckii* response relied on the broader activation of the ESR, while *S. cerevisiae* activated specific branches of the HOG and CWI pathway. *S. cerevisiae* promoted growth and sugar consumption exhibiting strong glucose catabolite repression activity, while *T. delbrueckii* downregulated glycolysis and overexpressed catalases. Increased viability for higher resistance to H_2O_2 has already been related to *T. delbrueckii* freeze-tolerance (Alves-Araújo at al., 2004). Apoptosis-related genes, both cytosolic and nuclear, were upregulated, possibly as a result of accumulation of high levels of ROS in *S. cerevisiae*, while *T. delbrueckii* seemed to better control intracellular ROS balance. Coupling physiological observations with previous phenotypic characterisation, the higher osmotolerance of *T. delbrueckii* could be a consequence of slower growth combined with increased stress tolerance (Vilaprinyo at al., 2006, Vilaprinyo at al., 2010). On the other side, *S. cerevisiae* adaptation strategies, called adaptive re-growth, have already been observed in the later stages of fermentation, precisely upon ethanol stress (Zambrano at al., 1993, Carmona-Gutierrez at al., 2012). A positive correlation between superoxide and mutation frequency has indicated that with shortened life span cells may adapt better to a new environment (Fabrizio

at al., 2003, Fabrizio at al., 2004, Herker at al., 2004). We suggest human domestication of yeast (Albertin at al., 2014, Gallone at al., 2018) shaped and exploited the adaptation strategies of the two species in opposite directions. Nutrient driven adaptation provided *S. cerevisiae* with benefits via the make-accumulate-consume strategy whereas a stress-driven adaptation may enable *T. delbrueckii* to survive in a high osmolarity environment. Further studies are needed in order to understand these processes of adaptation, which could help in creating yeast strains more suited to future winemaking challenges.

In the same direction, the regulation of two genes, *JEN1* and *CWP1*, could be of oenological interest. The first could be disassociated from glucose repression in order to obtain a *S. cerevisiae* strain producing lower acetic acid. The latter could be constitutively overexpressed to provide a higher protection of the cell wall.

In conclusion, this study, in the footsteps of only a few previous ones, enlightens a research field that has countless opportunities to expand with new technologies. The physiological response can now be broadened to investigate more yeast species in-depth, implementing knowledge on (i) stress, (ii) potentially on cell communication and interaction, (iii) nutrient competition and (iv) metabolic pathway regulation, which will have a profound impact on future winemaking techniques.

4.6. Acknowledgements

The authors wish to thank the Yalumba Wine Company for providing the wine samples and the Genomics Facility at SAHMRI (Adelaide, South Australia).

4.7. Funding

This work was supported by the Australian Research Council Training Centre for Innovative Wine Production (Project IC130100005 and IC170100008) and Wine Australia (Project AGT1502).

4.8. Authors contributions

F.T. conceived and designed the experiment, collected and performed the data analysis. C.A.O. contributed to data and analysis tools, V.J. supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

4.9. Nucleotide sequences

The genome sequence of *T. delbrueckii* COFT1 has been deposited at NCBI, under accession numbers CP027647 to CP027655.

The genome sequence of *S. cerevisiae* YAL1 has been deposited at NCBI, under accession numbers QQBK01000001 to QQBK01000025.

The transcriptome reads have been deposited at NCBI, under SRA submission number SUB4270844.

4.10. Supplementary Data

Supplementary data to this article can be found online:

Supplementary File 1:

https://data.mendeley.com/datasets/j9g3mxsh23/1

Supplementary File 2, Supplementary Figure 1-2:

https://data.mendeley.com/datasets/c8854nww3p/3

4.11. Figure Legends

Figure 1: ESR changes in *S. cerevisiae* and *T. delbrueckii* gene expression in response to high sugar CDGJM. Upregulated and downregulated genes reported by Gasch (2000) are highlighted in red and green, respectively. Differential expression is measured by LogFC between each timepoint (T4, T3, T2, T1) and T0.

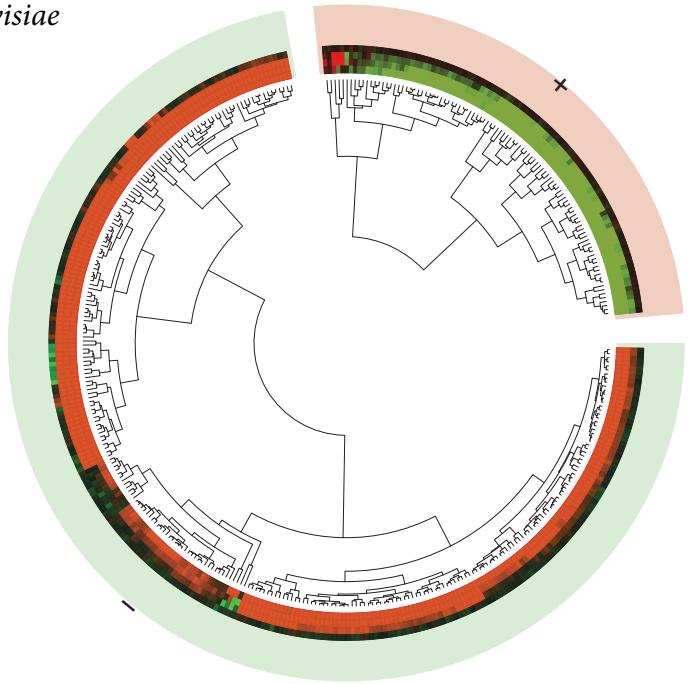
Figure 2: Dot-plot of the expression of genes of interest (LogFC vs Z-score) of *S. cerevisiae* and *T. delbrueckii.*

Figure 3: Stacked histogram of the cyclin expression values for each time point for *S. cerevisiae* and *T. delbrueckii.*

Figure 4: (a) Z-scores and Log_FC values (b) of *T. delbrueckii* and *S. cerevisiae HXK2* expression.

Figure 1

S. cerevisiae



T. delbrueckii

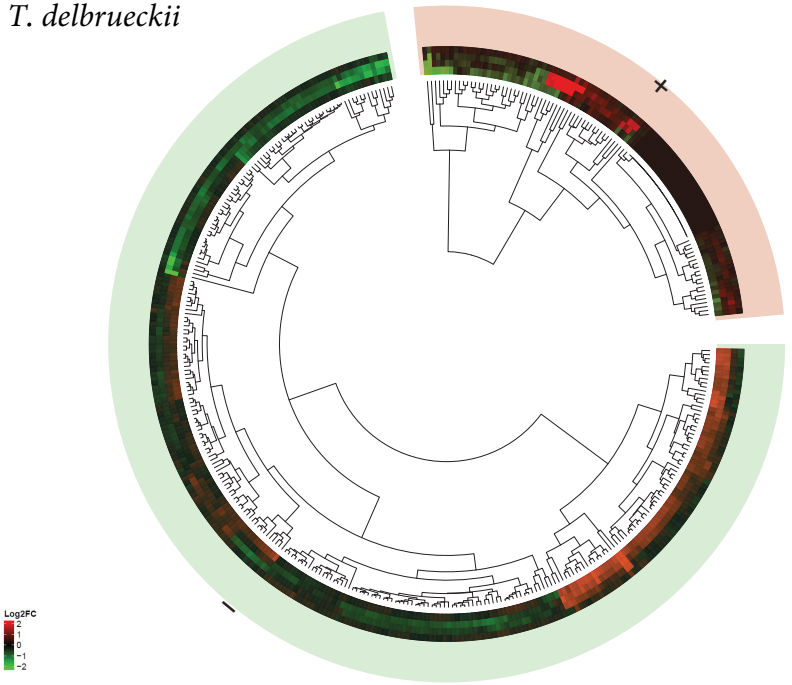


Figure 2

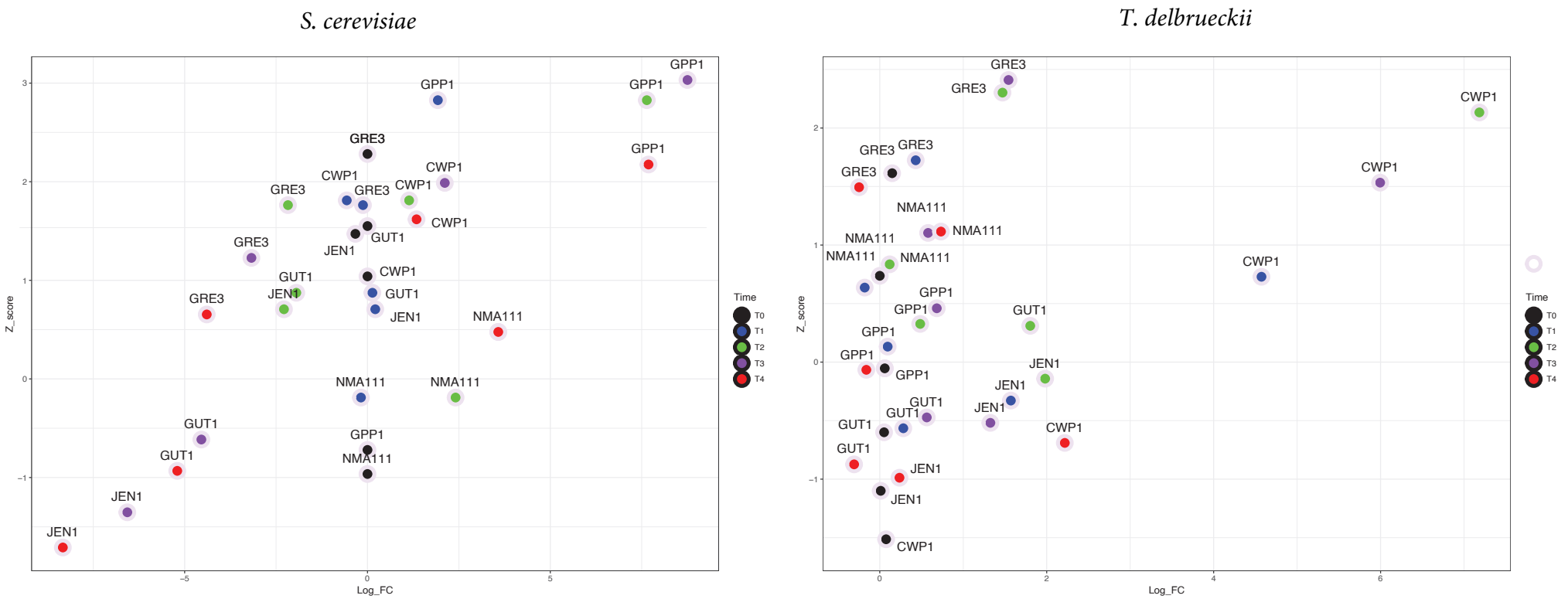

S. cerevisiae
T. delbrueckii
Z_score
Log_FC
Time
T0
T1
T2
T3
T4
GPP1
GRE3
CWP1
GUT1
JEN1
NMA111

Figure 3

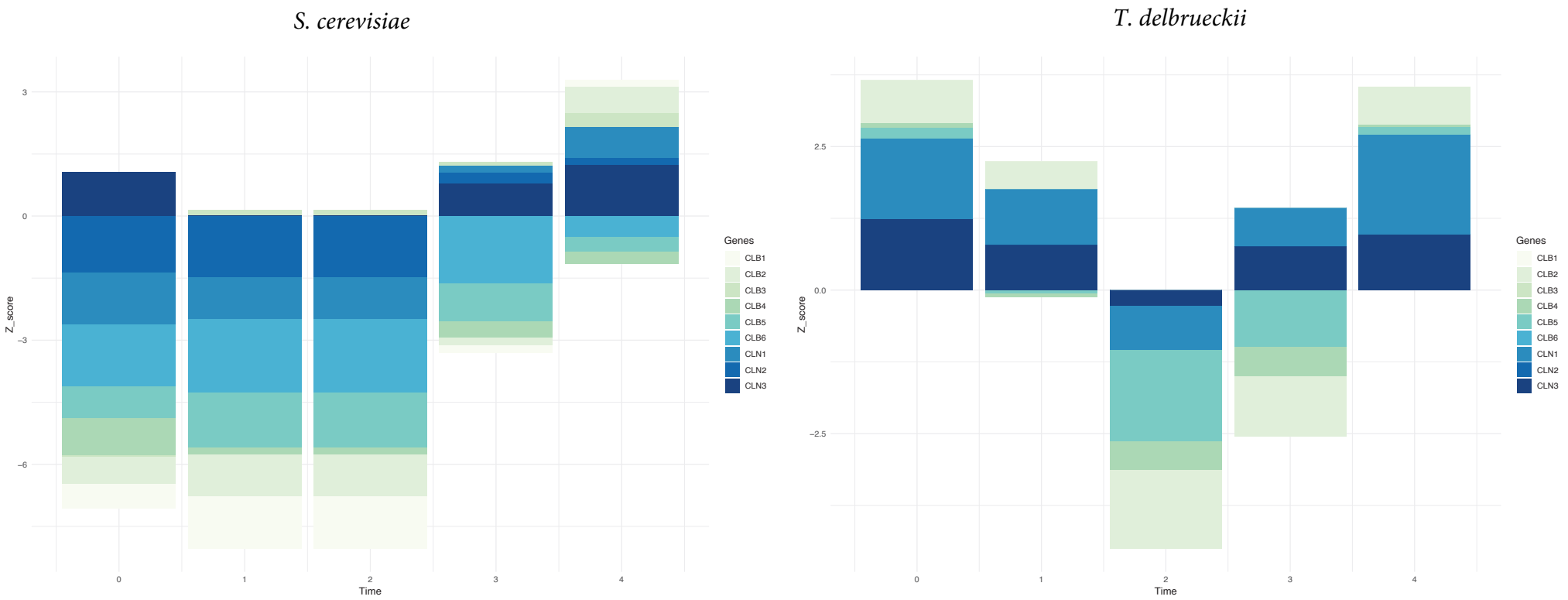
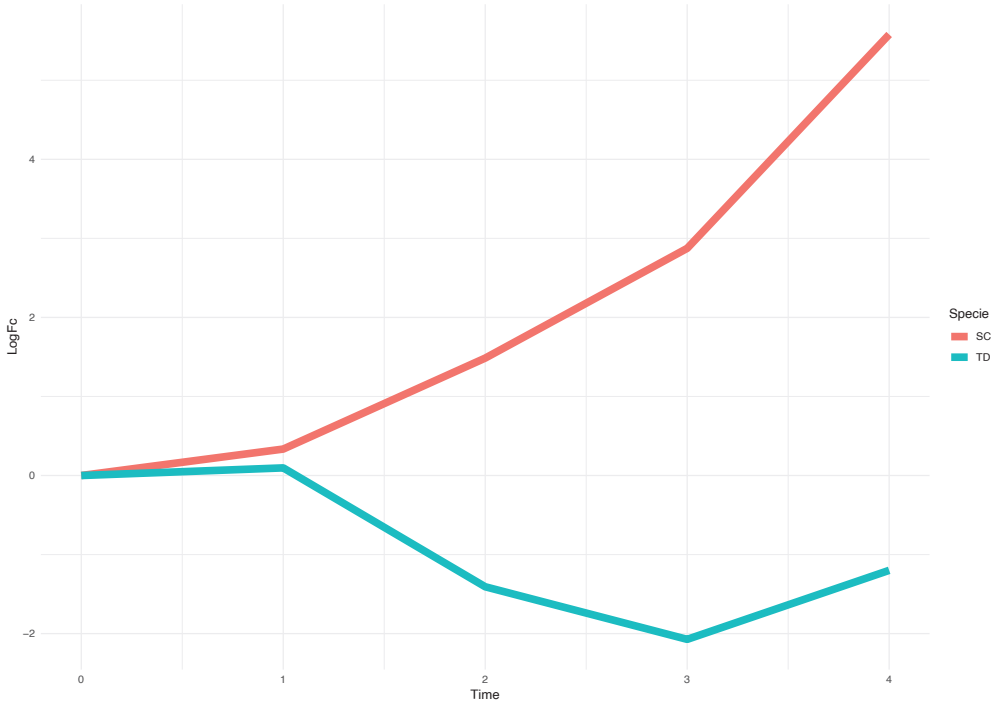
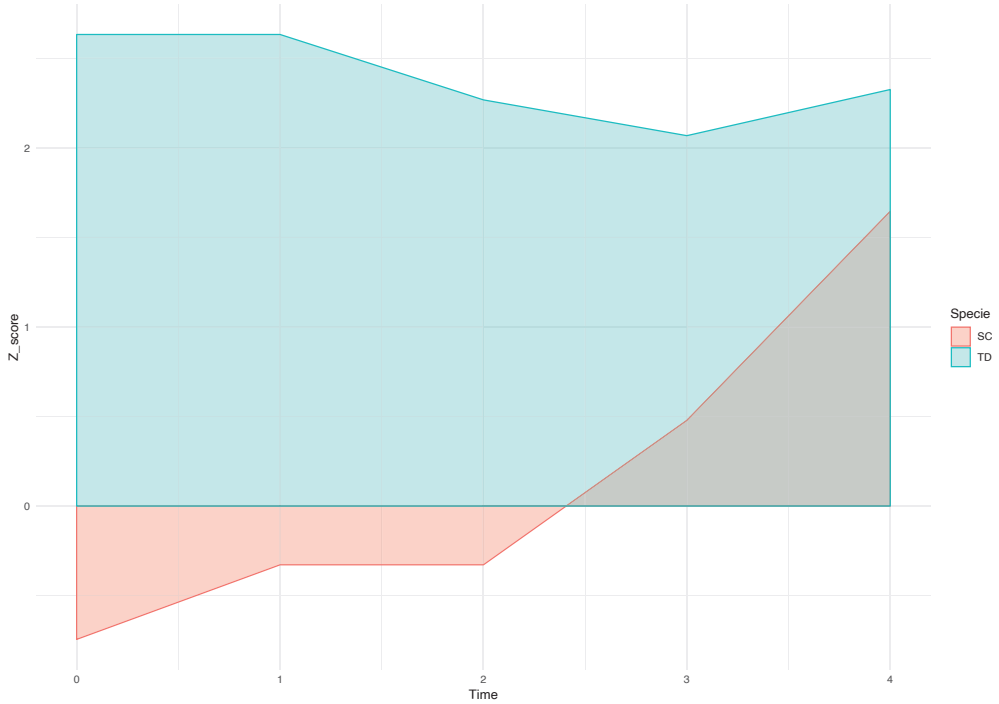

S. cerevisiae
T. delbrueckii
Z_score
Time
Genes
CLB1
CLB2
CLB3
CLB4
CLB5
CLB6
CLN1
CLN2
CLN3

Figure 4

A


LogFc
4
2
0
−2
0
1
2
3
4
Time
Specie
SC
TD

B


Z_score
2
1
0
0
1
2
3
4
Time
Specie
SC
TD

		S. cerevisiae									*T. delbrueckii*								
		DE				Z-score					DE				Z-score				
	Genes	0vs1	0vs2	0vs3	0vs4	0	1	2	3	4	0vs1	0vs2	0vs3	0vs4	0	1	2	3	4
OSMOTIC	STL1	0.10	6.43	7.53	0.52	-0.44	-0.42	2.62	2.99	-0.34	-0.41	-0.62	-0.40	0.34	-0.80	-1.01	-1.14	-1.04	-0.65
	GPD1	0.10	1.74	2.65	-1.16	2.01	2.06	3.07	3.25	1.52	0.25	0.81	0.48	0.33	2.35	2.40	2.73	2.80	2.63
	GPD2	0.32	1.24	0.15	-0.30	0.64	0.78	1.46	0.75	0.53	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	GPP1	1.92	7.64	8.75	7.69	-0.84	-0.24	2.59	2.96	2.23	0.09	0.48	0.68	-0.16	0.05	0.13	0.33	0.46	-0.07
	GPP2	2.45	6.77	7.46	4.14	-0.51	0.42	2.68	2.85	1.23	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CTT1	-0.06	-1.77	-3.40	-7.26	3.03	3.03	2.99	2.10	0.18	0.30	2.62	2.57	-1.02	1.91	2.01	2.96	3.00	1.49
	HSP12	0.05	-0.85	-1.14	-4.56	2.68	2.73	2.90	2.64	0.96	0.39	1.74	2.51	-0.73	0.63	0.84	1.65	2.09	0.18
	GUT1	0.13	-1.95	-4.54	-5.20	2.05	2.08	1.45	-0.05	-0.29	0.28	1.80	0.56	-0.31	-0.70	-0.57	0.31	-0.47	-0.87
	GUT2	0.02	-2.77	-4.50	-6.08	1.99	2.00	0.88	-0.08	-0.68	0.01	0.16	0.56	-0.49	1.33	1.33	1.52	1.69	1.09
	DAK1	0.11	-0.30	0.37	-0.51	1.79	1.83	2.02	2.14	1.55	0.21	1.36	1.71	-0.20	1.35	1.42	2.11	2.31	1.28
	DAK2	0.10	-3.24	-4.19	-3.98	-1.52	-1.53	-2.06	-2.08	-2.05	-0.13	-0.88	-1.08	-0.95	0.20	0.13	-0.41	-0.51	-0.40
	GRE2	-0.30	2.11	3.91	0.35	-0.19	-0.29	0.82	1.69	-0.08	0.33	2.45	2.30	0.30	1.26	1.40	2.48	2.44	1.45
	GRE3	-0.12	-2.17	-3.17	-4.40	2.25	2.20	1.79	1.07	0.46	0.43	1.47	1.54	-0.25	1.58	1.72	2.30	2.41	1.49
	YPR1	0.20	0.62	1.46	0.58	0.84	0.93	1.34	1.66	1.14	0.32	0.68	0.59	0.60	-0.47	-0.27	-0.13	-0.15	-0.13
	YJR096W	0.15	-1.20	-3.71	-6.05	2.45	2.51	2.50	1.11	-0.03	0.59	1.09	1.56	-0.43	-0.10	0.28	0.56	0.85	-0.38
OXYDATIVE	CCP1	0.16	-0.85	-1.23	-1.55	1.54	1.60	1.40	1.05	0.88	1.28	2.85	2.34	0.78	-1.60	-1.16	-0.44	-0.69	-1.35
	SOD2	0.18	-1.10	-3.39	-3.66	1.93	2.00	1.81	0.43	0.33	0.37	1.37	1.51	-0.36	-0.22	0.02	0.60	0.68	-0.45
	CTA1	0.09	-1.64	-2.35	-2.04	0.61	0.65	-0.22	-0.52	-0.32	0.86	0.86	1.20	0.90	-2.09	-1.99	-2.02	-1.94	-1.95
	MCA1	0.35	0.24	0.83	1.08	0.89	1.04	1.17	1.40	1.39	0.13	-0.34	-0.71	-0.22	0.58	0.66	0.33	0.13	0.42
	NMA111	-0.18	2.41	3.58	3.58	-0.32	-0.39	0.76	1.33	1.23	-0.18	0.04	0.64	0.68	0.74	0.64	0.78	1.11	1.11
OTHER	FKS1	-0.02	1.06	2.68	3.45	1.28	1.25	2.14	2.67	2.70	0.00	-0.85	-0.02	0.26	2.22	2.20	2.04	2.43	2.47
	CHS1	-0.03	-0.05	0.17	1.47	0.01	0.00	-0.08	0.02	0.70	0.15	0.59	0.11	0.14	0.42	0.52	0.79	0.47	0.48
	UTR2	-0.04	4.20	5.34	5.52	-0.72	-0.73	1.06	1.57	1.50	-0.29	-1.37	-0.84	-0.35	1.36	1.21	0.64	0.95	1.20
	CWP1	-0.40	1.14	2.11	1.34	0.68	0.48	1.46	1.81	1.31	4.57	7.18	5.99	2.21	-1.58	0.72	2.13	1.53	-0.69
	PMA1	-0.01	1.71	4.40	5.02	1.02	1.01	2.19	3.12	2.93	0.10	-0.90	-1.32	-0.35	3.00	3.00	2.92	2.96	3.05
	PDR12	-0.15	0.42	1.34	3.62	0.58	0.49	0.88	1.31	2.15	0.16	-0.96	-2.93	-2.19	2.54	2.59	2.36	1.46	1.79
	JEN1	0.21	-2.28	-6.57	-8.33	1.89	1.97	1.02	-0.97	-1.41	1.57	1.98	1.33	0.24	-1.14	-0.33	-0.14	-0.52	-1.01
	ADY2	-0.04	-0.61	-1.61	-1.82	-0.69	-0.71	-1.17	-1.35	-1.37	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 1: Expression of stress related genes for *S. cerevisiae* and *T. delbrueckii*. Differential expression (Log_2 Fold change) and Z-score are measured from transcripts per million values.

		S. cerevisiae									*T. delbrueckii*								
		LogFC				Z-score					LogFC				Z-score				
	Genes	0vs1	0vs2	0vs3	0vs4	0	1	2	3	4	0vs1	0vs2	0vs3	0vs4	0	1	2	3	4
GLYCOLYSIS	HXT1	0.06	6.54	9.00	8.40	-1.33	-1.34	1.11	2.25	1.76	3.41	6.58	6.22	5.05	-0.83	1.08	2.63	2.52	1.93
	HXK2	0.33	1.49	2.87	5.58	-0.48	-0.37	-0.01	0.71	1.83	0.10	-1.41	-2.07	-1.20	2.63	2.63	2.27	2.07	2.32
	PGI1	-0.24	-0.61	0.79	2.71	1.74	1.62	1.80	2.29	2.77	0.09	-1.36	-1.83	-0.60	2.77	2.77	2.53	2.37	2.75
	PFK1	-0.06	0.10	0.57	2.32	1.47	1.44	1.88	1.89	2.46	0.10	-0.86	-1.83	-0.59	2.70	2.73	2.57	2.25	2.69
	PFK2	-0.02	0.27	0.72	2.17	1.47	1.46	1.96	2.00	2.37	0.14	-1.18	-1.78	-0.70	2.67	2.70	2.37	2.23	2.57
	FBA1	-0.35	-1.22	-1.10	1.52	2.79	2.65	2.85	2.75	3.18	0.34	-0.64	-1.26	-0.34	3.28	3.28	3.18	3.11	3.18
	TPI1	-0.19	-1.51	-1.04	1.22	1.87	1.79	1.48	1.56	2.35	0.11	-1.14	-1.81	-0.73	2.29	2.31	2.01	1.67	2.11
	TDH2	-0.24	-0.92	1.42	5.07	1.48	1.36	1.26	2.33	3.35	0.30	-0.29	-1.35	-0.61	3.71	3.71	3.71	3.42	3.71
	TDH3	-0.05	-1.27	-2.56	-0.20	3.25	3.25	3.77	2.77	3.12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	ENO1	-0.08	-1.70	-4.19	-0.52	2.63	2.62	2.48	1.10	2.59	0.31	-0.57	-1.93	-0.67	3.05	3.05	3.05	2.75	3.00
	ENO2	0.00	-1.06	1.29	5.18	1.24	1.23	0.85	2.01	3.07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	PYK2	0.04	-1.77	-4.45	-4.93	1.19	1.20	0.40	-0.82	-0.95	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CDC19	-0.28	-2.20	-1.78	2.07	2.65	2.56	2.33	2.35	3.49	0.10	-0.84	-1.90	-0.23	3.11	3.11	3.00	2.85	3.11
	CDC28	-0.01	0.87	1.72	0.73	0.11	0.09	0.54	0.94	0.44	0.03	-1.65	-0.89	-0.32	-0.06	-0.03	-1.02	-0.63	-0.29
CELL CYCLE	CLN1	0.30	1.97	4.03	5.12	-0.93	-0.86	-0.52	0.52	1.06	-0.81	-3.72	-1.39	0.57	1.40	0.97	-0.77	0.67	1.74
	CLN2	0.05	1.66	5.00	4.91	-1.02	-1.02	-0.80	0.85	0.80	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CLN3	-0.18	-2.20	-0.80	0.24	1.43	1.35	0.47	1.16	1.53	-0.85	-2.52	-0.90	-0.54	1.25	0.79	-0.28	0.76	0.97
	CLB2	-0.31	0.03	1.42	2.89	-0.39	-0.51	-0.61	0.08	0.84	-0.44	-3.89	-3.03	-0.12	0.74	0.48	-1.37	-1.04	0.66
	CLB3	-0.10	0.43	0.43	0.76	0.07	0.02	0.25	0.22	0.42	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	CLB4	-0.16	2.25	1.85	1.85	-0.74	-0.80	-0.06	-0.26	-0.17	-0.26	-0.84	-0.90	-0.06	0.08	-0.06	-0.49	-0.51	0.04
	CLB5	0.06	-0.45	0.30	1.30	-0.61	-0.61	-1.03	-0.71	-0.23	-0.43	-3.62	-2.01	-0.05	0.18	-0.07	-1.59	-0.99	0.14
	CLB6	0.82	0.12	0.43	3.50	-1.32	-1.12	-1.57	-1.42	-0.43	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PKA INDUCED	YMC2	0.88	4.25	7.05	6.76	-1.61	-1.46	-0.80	0.58	0.44	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	STR3	0.18	3.31	4.01	-2.44	-0.94	-0.91	0.14	0.49	-1.65	1.94	2.95	1.45	-0.53	-0.86	0.24	0.86	-0.11	-1.13
	ARO3	-0.06	0.29	0.56	0.61	0.40	0.36	0.57	0.68	0.71	0.20	-0.65	-0.34	0.17	0.94	1.02	0.56	0.74	1.04
	MET5	-0.07	1.41	4.19	-1.06	0.87	0.84	1.85	2.90	0.40	-0.18	-2.00	-1.56	-0.74	1.79	1.69	0.83	1.08	1.51
	AQR1	1.30	3.49	2.60	1.73	-0.39	0.07	1.28	0.70	0.29	-0.25	-4.54	-1.91	-3.17	2.01	1.90	-0.47	1.18	0.42
	ATR1	0.35	0.90	2.07	1.44	-0.43	-0.31	-0.24	0.36	0.09	0.27	0.59	0.26	-0.10	-0.13	0.05	0.18	0.00	-0.23
	SDT1	-0.24	1.43	1.03	0.46	-0.27	-0.34	0.32	0.08	-0.12	-0.30	0.35	-1.38	-2.05	0.67	0.50	0.91	-0.20	-0.58
	UTR2	-0.04	4.20	5.34	5.52	-0.72	-0.73	1.06	1.57	1.50	-0.29	-1.37	-0.84	-0.35	1.36	1.21	0.64	0.95	1.20
PKA REPRESSED	AYR1	-0.22	-2.54	-4.54	-3.17	1.18	1.07	-0.04	-0.85	-0.27	0.07	-0.99	-0.73	-0.86	0.02	0.08	-0.63	-0.48	-0.51
	GPD1	0.10	1.74	2.65	-1.16	2.01	2.06	3.07	3.25	1.52	0.25	0.81	0.48	0.33	2.35	2.40	2.73	2.80	2.63
	PEP4	-0.13	-1.31	-2.47	-2.65	2.15	2.11	2.04	1.24	1.10	0.26	0.19	0.67	-0.73	1.67	1.75	1.89	2.15	1.35
	PRC1	0.18	-1.03	-2.59	-3.65	2.58	2.71	2.75	1.90	1.28	0.14	-0.62	-0.15	-0.68	1.86	1.89	1.70	1.93	1.61
	HSP12	0.05	-0.85	-1.14	-4.56	2.68	2.73	2.90	2.64	0.96	0.39	1.74	2.51	-0.73	0.63	0.84	1.65	2.09	0.18
	GRX2	0.04	-1.21	-2.25	-4.48	0.62	0.64	0.02	-0.47	-1.19	0.04	1.20	1.79	0.18	-0.54	-0.50	0.12	0.53	-0.45
	SDH4	0.08	-1.47	-4.59	-5.42	1.36	1.39	0.79	-0.73	-0.98	0.19	0.35	0.14	-1.43	0.24	0.35	0.41	0.30	-0.64
	LSP1	0.10	-1.00	-2.65	-4.48	2.52	2.60	2.65	1.77	0.83	0.27	0.13	-0.26	-2.19	1.63	1.71	1.82	1.56	0.45

Table 2: Expression of growth-related genes for *S. cerevisiae* and *T. delbrueckii*. Differential expression (Log_2 Fold change) and Z-score are measured from transcripts per million.

References

Albertin, W., L. Chasseriaud, G. Comte, A. Panfili, A. Delcamp, F. Salin, P., at al. (2014). Winemaking and bioprocesses strongly shaped the genetic diversity of the ubiquitous yeast *Torulaspora delbrueckii*. PLoS One 9(4): e94246.

Alves-Araújo, C., M. J. Almeida, M. J. Sousa and C. Leão (2004). Freeze tolerance of the yeast *Torulaspora delbrueckii*: cellular and biochemical basis. FEMS Microbiol. Lett. 240(1): 7-14.

Avbelj, M., J. Zupan, L. Kranjc and P. Raspor (2015). Quorum-sensing kinetics in *Saccharomyces cerevisiae*: a symphony of ARO genes and aromatic alcohols. J. Agric. Food Chem. 63(38): 8544-8550.

Avbelj, M., J. Zupan and P. Raspor (2016). Quorum-sensing in yeast and its potential in wine making. Appl. Microbiol. Biotechnol. 100(18): 7841-7852.

Barata, A., M. Malfeito-Ferreira and V. Loureiro (2012). The microbial ecology of wine grape berries. Int. J. Food Microbiol. 153(3): 243-259.

Bauer, F. and I. S. Pretorius (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine-a review. S. Afr. J. Enol. Vitic. 21: 27-51.

Bendjilali, N., S. MacLeon, G. Kalra, S. D. Willis, A. N. Hossian, E. Avery, at al. (2017). Time-course analysis of gene expression during the *Saccharomyces cerevisiae* hypoxic response. G3 7(1): 221-231.

Bleidorn, C. (2016). Third generation sequencing: technology and its potential impact on evolutionary biodiversity research. Syst. Biodivers. 14(1): 1-8.

Bloom, J. and F. R. Cross (2007). Multiple levels of cyclin specificity in cell-cycle control. Nat. Rev. Mol. Cell Biol. 8(2): 149.

Bolger, A. M., M. Lohse and B. Usadel (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. J. Bioinform. btu170.

Boorsma, A., H. D. Nobel, B. T. Riet, B. Bargmann, S. Brul, K. J. Hellingwerf, at al. (2004). Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. Yeast 21(5): 413-427.

Borneman, A. R., A. H. Forgan, I. S. Pretorius and P. J. Chambers (2008). Comparative genome analysis of a *Saccharomyces cerevisiae* wine strain. FEMS Yeast Res. 8(7): 1185-1195.

Brion, C., D. Pflieger, S. Souali-Crespo, A. Friedrich and J. Schacherer (2016). Differences in environmental stress response among yeasts is consistent with species-specific lifestyles. Mol. Biol. Cell 27(10): 1694-1705.

Broach, J. R. (2012). Nutritional control of growth and development in yeast. Genetics 192(1): 73-105.

Carmona-Gutierrez, D., C. Sommer, A. Andryushkova, G. Kroemer and F. Madeo (2012). A higher spirit: avoiding yeast suicide during alcoholic fermentation. Cell Death Differ. 19(6): 913–914.

Cheadle, C., M. P. Vawter, W. J. Freed and K. G. Becker (2003). Analysis of microarray data using Z score transformation. J. Mol. Diagn. 5(2): 73-81.

Chen, H. and G. R. Fink (2006). Feedback control of morphogenesis in fungi by aromatic alcohols. Genes Dev. 20(9): 1150-1161.

Chen, H., M. Fujita, Q. Feng, J. Clardy and G. R. Fink (2004). Tyrosol is a quorum-sensing molecule in *Candida albicans.* Proc. Natl. Acad. Sci. 101(14): 5048-5052.

Chen, R. E. and J. Thorner (2007). Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. (BBA)- Mol. Cell Res. 1773(8): 1311-1340.

Chomczynski, P. and N. Sacchi (2006). The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. Nat. Protoc. 1(2): 581-585.

Claro, F., K. Rijsbrack and E. V. Soares (2007). Flocculation onset in *Saccharomyces cerevisiae*: effect of ethanol, heat and osmotic stress. J. Appl. Microbiol. 102(3): 693-700.

Clotet, J. and F. Posas (2007). Control of Cell Cycle in Response to Osmostress: Lessons from Yeast. Methods Enzymol. Vol. 428. Academic Press, 63-76.

Costa, V. and P. Moradas-Ferreira (2001). Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. Mol. Aspects Med. 22(4-5): 217-246.

Cullen, P. J. and G. F. Sprague (2000). Glucose depletion causes haploid invasive growth in yeast. Proc. Natl. Acad. Sci. 97(25): 13619-13624.

Dobin, A. and T. R. Gingeras (2015). Mapping RNA-seq Reads with STAR. Curr. Protoc. Bioinformatics 11.14. 11-11.14. 19.

Duc, C., M. Pradal, I. Sanchez, J. Noble, C. Tesnière and B. Blondin (2017). A set of nutrient limitations trigger yeast cell death in a nitrogen-dependent manner during wine alcoholic fermentation. PloS One 12(9): e0184838.

Ene, I. V., L. A. Walker, M. Schiavone, K. K. Lee, H. Martin-Yken, E. Dague, at al. (2015). Cell wall remodeling enzymes modulate fungal cell wall elasticity and osmotic stress resistance. MBio 6(4): e00986-00915.

Enjalbert, B., A. Nantel and M. Whiteway (2003). Stress-induced gene expression in *Candida albicans*: absence of a general stress response. Mol. Biol. Cell 14(4): 1460-1467.

Erasmus, D., G. Vandermerwe and H. Vanvuuren (2003). Genome-wide expression analyses: metabolic adaptation of to high sugar stress. FEMS Yeast Res. 3(4): 375-399.

Fabrizio, P., L. Battistella, R. Vardavas, C. Gattazzo, L.-L. Liou, A. Diaspro, at al. (2004). Superoxide is a mediator of an altruistic aging program in *Saccharomyces cerevisiae*. J. Cell Biol. 166(7): 1055-1067.

Fabrizio, P., L.-L. Liou, V. N. Moy, A. Diaspro, J. S. Valentine, E. B. Gralla, at al. (2003). *SOD2* functions downstream of Sch9 to extend longevity in yeast. Genetics 163(1): 35-46.

Feng, B., Y. Lin, L. Zhou, Y. Guo, R. Friedman, R. Xia, at al. (2017). Reconstructing yeasts phylogenies and ancestors from whole genome data. Sci. Rep. 7(1): 15209.

Ferreira, D. M., Galeote, V., Ortiz, A. J., and S. Dequin (2017). Yeast multi-stress resistance and lag-phase duration in wine fermentation. FEMS Yeast Res. 17(6): fox051.

Fuchs, B. B. and E. Mylonakis (2009). Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot. Cell 8(11): 1616-1625.

Gallone, B., S. Mertens, J. L. Gordon, S. Maere, K. J. Verstrepen and J. Steensels (2018). Origins, evolution, domestication and diversity of *Saccharomyces* beer yeasts. Curr. Opin. Biotechnol. 49: 148-155.

Garde-Cerdán, T. and C. Ancín-Azpilicueta (2008). Effect of the addition of different quantities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. LWT-Food Sci. Technol. 41(3): 501-510.

Gasch, A. P. (2007). Comparative genomics of the environmental stress response in ascomycete fungi. Yeast 24(11): 961-976.

Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein and P. O. Brown (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11(12): 4241-4257.

González-Marco, A., N. Jiménez-Moreno and C. Ancín-Azpilicueta (2010). Influence of nutrients addition to nonlimited in nitrogen must on wine volatile composition. J. Food Sci. 75(4): S206-S211.

Gray, J. V., G. A. Petsko, G. C. Johnston, D. Ringe, R. A. Singer and M. Werner-Washburne (2004). Sleeping beauty: quiescence in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 68(2): 187-206.

Gu, Z., L. Gu, R. Eils, M. Schlesner and B. Brors (2014). Circlize implements and enhances circular visualization in R. Bioinformatics 30(19): 2811-2812.

Herker, E., H. Jungwirth, K. A. Lehmann, C. Maldener, K.-U. Fröhlich, S. Wissing, at al. (2004). Chronological aging leads to apoptosis in yeast. J. Cell Biol. 164(4): 501-507.

Hernandez-Lopez, M., J. Prieto and F. Randez-Gil (2003). Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. Antonie Leeuwenhoek 84(2): 125-134.

Hernandez-Lopez, M. J., F. Randez-Gil and J. A. Prieto (2006). Hog1 mitogen-activated protein kinase plays conserved and distinct roles in the osmotolerant yeast *Torulaspora delbrueckii*. Eukaryot. Cell 5(8): 1410-1419.

Hogan, D. A. (2006). Quorum sensing: alcohols in a social situation. Curr. Biol. 16(12): R457-R458.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. Microbiol. Mol. Biol. Rev. 66(2): 300-372.

Hohmann, S. (2009). Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. FEBS Lett. 583(24): 4025-4029.

Hohmann, S., M. Krantz and B. Nordlander (2007). Yeast osmoregulation. Methods in Enzymol. 428: 29-45.

Jamieson, D. J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. Yeast 14(16): 1511-1527.

Jiménez, J., S. Bru, M. Ribeiro and J. Clotet (2015). Live fast, die soon: cell cycle progression and lifespan in yeast cells. Microb. Cell 2(3): 62.

Jungwirth, H. and K. Kuchler (2006). Yeast ABC transporters–a tale of sex, stress, drugs and aging. FEBS Lett. 580(4): 1131-1138.

Kapteyn, J., B. Ter Riet, E. Vink, S. Blad, H. De Nobel, H. Van Den Ende, at al. (2001). Low external pH induces HOG1-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. Mol. Microbiol. 39(2): 469-480.

Klein, M., S. Swinnen, J. M. Thevelein and E. Nevoigt (2017). Glycerol metabolism and transport in yeast and fungi: established knowledge and ambiguities. Environ. Microbiol. 19(3): 878-893.

Landolfo, S., H. Politi, D. Angelozzi and I. Mannazzu (2008). ROS accumulation and oxidative damage to cell structures in *Saccharomyces cerevisiae* wine strains during fermentation of high-sugar-containing medium. (BBA)-Gen. Subjects 1780(6): 892-898.

Liu, Y., S. Ye and A. M. Erkine (2009). Analysis of *Saccharomyces cerevisiae* genome for the distributions of stress-response elements potentially affecting gene expression by transcriptional interference. In Silico Biol. 9(5, 6): 379-389.

Livas, D., Almering, M. J., Daran, J. M., Pronk, J. T. and J. M. Gancedo (2011). Transcriptional responses to glucose in *Saccharomyces cerevisiae* strains lacking a functional protein kinase A. BMC Genom. 12(1), 405.

Love, M. I., W. Huber and S. Anders (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15(12): 550.

Madeo, F., E. Herker, S. Wissing, H. Jungwirth, T. Eisenberg and K.-U. Fröhlich (2004). Apoptosis in yeast. Curr. Opin. Microbiol 7(6): 655-660.

Marks, V. D., S. J. Ho Sui, D. Erasmus, G. K. Van Der Merwe, J. Brumm, W. W. Wasserman, at al. (2008). Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. FEMS Yeast Res. 8(1): 35-52.

Masneuf-Pomarede, I., M. Bely, P. Marullo and W. Albertin (2016). The genetics of non-conventional wine yeasts: current knowledge and future challenges. Front. Microbiol. 6: 1563.

Matallana, E. and A. Aranda (2017). Biotechnological impact of stress response on wine yeast. Lett. Appl. Microbiol. 64(2): 103-110.

Mazzoni, C. and C. Falcone (2008). Caspase-dependent apoptosis in yeast. (BBA)-Mol. Cell Res. 1783(7): 1320-1327.

Mbuyane, L. L., M. de Kock, F. F. Bauer and B. Divol (2018). *Torulaspora delbrueckii* produces high levels of C5 and C6 polyols during wine fermentations. FEMS Yeast Res. 18(7): foy084.

Molin, M., J. Norbeck and A. Blomberg (2003). Dihydroxyacetone kinases in *Saccharomyces cerevisiae* are involved in detoxification of dihydroxyacetone. J. Biol. Chem. 278(3): 1415-1423.

Morano, K. A., C. M. Grant and W. S. Moye-Rowley (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. Genetics 190(4): 1157-1195.

Morrison-Whittle, P. and M. R. Goddard (2018). From vineyard to winery: a source map of microbial diversity driving wine fermentation. Environ. Microbiol. 20(1): 75-84.

Novo, M., G. Beltran, N. Rozes, J.-M. Guillamon, S. Sokol, V. Leberre, at al. (2007). Early transcriptional response of wine yeast after rehydration: osmotic shock and metabolic activation. FEMS Yeast Res. 7(2): 304-316.

O'Rourke, S. M., I. Herskowitz and E. K. O'Shea (2002). Yeast go the whole HOG for the hyperosmotic response. Trends in Genet. 18(8): 405-412.

Orlean, P. (2012). Architecture and biosynthesis of the *Saccharomyces cerevisiae* cell wall. Genetics 192(3): 775-818.

Patro, R., G. Duggal and C. Kingsford (2015). Salmon: accurate, versatile and ultrafast quantification from RNA-seq data using lightweight-alignment. Biorxiv: 021592.

Piper, M. D., P. Daran-Lapujade, C. Bro, B. Regenberg, S. Knudsen, J. Nielsen, at al. (2002). Reproducibility of oligonucleotide microarray transcriptome analyses an interlaboratory comparison using chemostat cultures of S*accharomyces cerevisiae*. J. Biol. Chem. 277(40): 37001-37008.

Pretorius, I. S. (2017). Solving yeast jigsaw puzzles over a glass of wine: Synthetic genome engineering pioneers new possibilities for wine yeast research. EMBO Rep. 18(11): 1875-1884.

Renouf, V., O. Claisse and A. Lonvaud-Funel (2005). Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. Aust. J. Grape Wine R. 11(3): 316-327.

Rodicio, R. and J. J. Heinisch (2010). Together we are strong—cell wall integrity sensors in yeasts. Yeast 27(8): 531-540.

Rubio-Texeira, M., G. Van Zeebroeck, K. Voordeckers and J. M. Thevelein (2010). *Saccharomyces cerevisiae* plasma membrane nutrient sensors and their role in PKA signaling. FEMS Yeast Res. 10(2): 134-149.

Saito, H. and F. Posas (2012). Response to hyperosmotic stress. Genetics 192(2): 289-318.

Sanz, A. B., R. García, J. M. Rodríguez-Peña and J. Arroyo (2017). The CWI Pathway: regulation of the transcriptional adaptive response to cell wall stress in yeast. J. Fungi 4(1): 1.

Shen, B., S. Hohmann, R. G. Jensen and H. J. Bohnert (1999). Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. 121(1): 45-52.

Silva, R. D., R. Sotoca, B. Johansson, P. Ludovico, F. Sansonetty, M. T. Silva, at al. (2005). Hyperosmotic stress induces metacaspase and mitochondria dependent apoptosis in *Saccharomyces cerevisiae*. Mol. Microbiol. 58(3): 824-834.

Soares, E. V. (2011). Flocculation in *Saccharomyces cerevisiae*: a review. J. Appl. Microbiol. 110(1): 1-18.

Song, S. H. and C. Vieille (2009). Recent advances in the biological production of mannitol. Appl. Microbiol. Biotechnol. 84(1): 55-62.

Spor, A., S. Wang, C. Dillmann, D. De Vienne and D. Sicard (2008). Ant and grasshopper life-history strategies in *Saccharomyces cerevisiae*. PLoS One 3(2): e1579.

Sprague, G. F. and S. C. Winans (2006). Eukaryotes learn how to count: quorum sensing by yeast. Genes Dev. 20(9): 1045-1049.

Stephan, J. S., Y.-Y. Yeh, V. Ramachandran, S. J. Deminoff and P. K. Herman (2009). The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. Proc. Natl. Acad. Sci. 106(40): 17049-17054.

Storey, J. D. (2002). A direct approach to false discovery rates. J. R. Stat. Soc. Ser. B Methodol. 64(3): 479-498.

Swinnen, E., V. Wanke, J. Roosen, B. Smets, F. Dubouloz, I. Pedruzzi, at al. (2006). Rim15 and the crossroads of nutrient signalling pathways in *Saccharomyces cerevisiae*. Cell Div. 1(1): 3.

Team, R. C. (2013). R: A language and environment for statistical computing. http://www.R-project.org/.

Toivari, M. H., H. Maaheimo, M. Penttilä and L. Ruohonen (2010). Enhancing the flux of D-glucose to the pentose phosphate pathway in *Saccharomyces cerevisiae* for the production of D-ribose and ribitol. Appl. Microbiol. Biotechnol. 85(3): 731-739.

Tondini, F., Jiranek, V., Grbin, P. R., and C. A. Onetto (2018). Genome sequence of australian indigenous wine yeast *Torulaspora delbrueckii* COFT1 Using Nanopore Sequencing. Genome Announc. 6(17), e00321-18.

Tondini, F., Lang, T., Chen, L., Herderich, M. and V. Jiranek (2019). Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains. Int. J. Food Microbiol. 294, 42-49.

Träff, K., L. J. Jönsson and B. Hahn-Hägerdal (2002). Putative xylose and arabinose reductases in *Saccharomyces cerevisiae*. Yeast 19(14): 1233-1241.

Tronchoni, J., J. A. Curiel, P. Morales, R. Torres-Pérez and R. Gonzalez (2017). Early transcriptional response to biotic stress in mixed starter fermentations involving *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. Int. J. Food Microbiol. 241: 60-68.

Vilaprinyo, E., R. Alves and A. Sorribas (2006). Use of physiological constraints to identify quantitative design principles for gene expression in yeast adaptation to heat shock. BMC Bioinform. 7(1): 184.

Vilaprinyo, E., R. Alves and A. Sorribas (2010). Minimization of biosynthetic costs in adaptive gene expression responses of yeast to environmental changes. PLoS Comput. Biol. 6(2): e1000674.

Warringer, J. and A. Blomberg (2006). Involvement of yeast *YOL151W/GRE2* in ergosterol metabolism. Yeast 23(5): 389-398.

Wu, X., X. Chi, P. Wang, D. Zheng, R. Ding and Y. Li (2010). The evolutionary rate variation among genes of HOG-signaling pathway in yeast genomes. Biol. Direct 5(1): 46.

Wuster, A. and M. M. Babu (2009). Transcriptional control of the quorum sensing response in yeast. Mol. Biosyst. 6(1): 134-141.

Zakrzewska, A., G. van Eikenhorst, J. E. Burggraaff, D. J. Vis, H. Hoefsloot, D. Delneri, at al. (2011). Genome-wide analysis of yeast stress survival and tolerance acquisition to analyze the central trade-off between growth rate and cellular robustness. Mol. Biol. Cell 22(22): 4435-4446.

Zambrano, M. M., D. A. Siegele, M. Almirón, A. Tormo and R. Kolter (1993). Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science 259(5102): 1757-1760.

Zuzuarregui, A. and M. l. del Olmo (2004). Expression of stress response genes in wine strains with different fermentative behavior. FEMS Yeast Res. 4(7): 699-710.

Discussion and future directions

Non-*Saccharomyces* spp. were considered undesirable or as spoilage issues in most industrial food and beverage bioprocesses, including wine fermentation. Indeed, only recently have winemakers begun viewing indigenous yeasts as integral to the authenticity of their wine, imparting desired and district superior regional characteristics to it. These qualities gave microbiology researchers a purpose to explore these yeasts from the view that their utilization could solve some of the challenges of contemporary winemaking. This study addressed the problem of sluggish and stuck indigenous fermentations, especially arising from high sugar accumulation in grapes. Many factors have been reported to influence the fermentation progress and outcome, thereby making reliability and predictability very complex to understand. Current techniques based on massive sequencing are gaining importance to study the metagenomics, phylogenetic (genomic evolution) and population dynamics at every stage of the wine making process. In order to untangle the matter, 'Omics' high-throughput analyses have been adopted to provide a different point of view from the inside of the yeast cell. Cell physiology has been largely defined in the *S. cerevisiae* laboratory strain S288c but remains unclear for other, indigenous species in wine, where only what (their phenotype) and not why (their physiology) has been investigated. Therefore, we selected two yeast species from spontaneous fermentation samples to explore the application of new generation sequencing technologies to wine-related research. Different new generation sequencing technologies and bioinformatics protocols have been employed throughout this study. Firstly, Illumina technology was used to sequence the RNA of the two species in a single culture. A mixed approach was used to assemble transcriptomes: reads were first aligned to the species reference genome obtained from NCBI, partitioned according to locus, followed by *de novo* transcriptome assembly at each locus. The reason was that the sample genomes were not an exact match to the reference genome. Genome-guided *de novo* assembly should then have

captured the sequence variations, improving the assembly of paralogs or other genes with shared sequences.

Transcriptomes showed good completeness (only 5% missing), but high fragmentation. The technique proved to be useful with sparsely annotated-genomes on one hand and then useful to investigate non-conventional yeasts. But some concerns arose around the techniques used in mixed population samples, due to the presence of chimeric artefacts that needed to be manually curated. Therefore, we decided to further improve the analysis. Using Oxford Nanopore technology with a *de-novo* genome assembly pipeline, we delivered the first genomes for Australian indigenous wine-related *T. delbrueckii* and *S. cerevisiae* yeasts. Especially long reads provided higher and superior contiguity compared to those produced by second generation technologies. In our circumstance, it was beneficial to polish the assembled contigs, using Illumina short reads from the previous RNA experiment to correct smaller indels and substitutions, hence improving the overall assembly quality. In particular the *T. delbrueckii* genome was the first complete genome of the species reported on NCBI. The genomes were annotated in order to allow comparison between the species. Mixed population transcriptomes were then assembled with a genome-guided approach, achieving the goals of (i) reduced fragmentation and (ii) absence of chimeric transcript. Discrimination of the reads between the species, followed by quantification of gene expression for each of them, were made possible by a new strict alignment bioinformatics procedure, which considered only the unique alignments.

High quality, high-throughput data made it possible for comparative genomics and transcriptomic to be able to relate the oenological properties of *T. delbrueckii* and *S. cerevisiae* to their different physiologies. Indeed, pursuing phenotypic evidence for the *T. delbrueckii* property of lower acetic acid production, we could propose two molecular mechanisms. First, the acetic acid importer encoded by *JEN1* was expressed in high sugar must in *T. delbrueckii*,

free from the glucose repression feedback present in *S. cerevisiae*, thereby enabling the consumption of acetic acid from an early stage of fermentation. This characteristic is in concordance with the lower ALDs expression measured during fermentation, as acetic acid could be sourced externally rather than internally. Coupling these properties could lead to the future development of lower acetic acid producing strains of *S. cerevisiae.*

The production of other aroma compounds (esters and higher alcohols), on the other hand, was more closely related to genetic differences, in particular the absence of certain genes in the Ehrlich pathway. The uptake of branched chain amino acids has already been addressed as a major basis for acetate esters and higher alcohols in *S. cerevisiae*. But the molecular evidence in other yeast species was lacking. This study highlighted the differences between the two species and, for the first time, related ethyl ester production to BCCAs uptake through targeted gene deletion in *S. cerevisiae*.

This investigation explored another *T. delbrueckii* characteristic: enhanced osmotolerance under winemaking conditions. Knowledge of stress-induced mechanisms is fundamental to understanding changes in cellular physiology, especially for yeast that encounter dramatic external changes. Such information provides not only an understanding of these stress mechanisms and metabolic features activated under suboptimal growth conditions, but also reveals fundamental mechanisms in basal physiology. In this project, an elucidation of the signalling and associated transcriptional network involved after exposure to CDGJM was sought. The nutrient and stress sensing in the two yeast species shared many of the same genes and signalling pathways but did not show the same regulation. Two different adaptation strategies were identified. *T. delbrueckii* expressed general cell defences, slowed down the cell cycle and metabolism, and protected itself from reactive oxygen species. *S. cerevisiae* instead, followed the motto “live fast, die young”. It expressed only specific osmotic stress responses, promoted glycolysis and growth rather than protecting from metabolisc oxidation, resulting in

the increased expression of apoptosis marker genes and significantly decreased viability. Whether cell suicide could constitute an evolutionary advantage for unicellular organisms is still an open debate. Programmed cell death has already been observed when pheromone mating is not successful, when nutrients are dwindling and in the presence of other yeast killer toxins. More recently it has been associated with environmental adaptation and ROS. Increased ROS enhances the probability of somatic mutation and genomic variance, resulting in shortened lifespan and in better adaptation. Several studies described the phenomena as altruistic aging/adaptive regrowth. These different species-specific lifestyles could explain why the two yeasts are differently adapted to different stresses: *T. delbrueckii* ensures its niche in low temperature and high osmolarity media (freeze-dough) while *S. cerevisiae* dominates alcoholic fermentation. In the light of this suggestion, a new approach has to be developed to direct evolution of *T. delbrueckii* for enhanced ethanol resistance or *S. cerevisiae* for osmotolerance in wine. In the former, fluctuating concentrations of stressor are coupled with a mutagenesing agent to increase mutation rates and to avoid a reduction in population growth. In the latter, *S. cerevisiae* glucose repression needs to be tuned down in order to activate the general stress response. To hijack glucose sensing we could learn from the prion [GAR+] yeast phenotype. The [GAR+] prion results in an impaired glucose-signalling pathway. Its induction depends on the protein Slt1p while its propagation relies upon Pma1p. Positive enhancement of osmotolerance might be achieved by targeted gene editing of these two genes.

This thesis discussed only a part of the considerable possibilities in wine research, and employment of non-conventional yeast. Non-*Saccharomyces* species are described more and more as beneficial in winemaking, but still many unknown factors can influence their effect on wine composition. Future directions further involve (i) expanding the genomic knowledge of the other wine-related yeast species, (ii) investigating other oenological characteristics and their physiologic or genetic explanations and (iii) studying the interaction of these species

during fermentation. Such work could help reveal phenotypes and enlightenment on gene function in a different model than *S. cerevisiae*. The wealth of information which will be generated will help guide construction of commercial stains with tailored traits and more suitable phenotypes according to winemaker needs. Moreover, the discovery of new metabolic pathways could help develop novel industrial processes, based on yeasts.

In conclusion, “Omics’ approaches are now being used frequently in an increasing number of research fields, giving a clearer understanding and better prediction of the interactive mechanisms: this is the future of wine research. Future wine omics will finally shift wine research from descriptive to predictive, and thus will be a milestone in the study of indigenous fermentations. Piece by piece, the prediction of microbial population dynamics and biochemical activities, and consequently the resulting aromatic profile of wine, may overcome the challenges of the enormous variability of grape must compositions. I like to think that this thesis is one of these pieces.

6. Appendix: Poster for presentation at 27th International Conference on Yeast Genetics and Molecular Biology (ICYGMB)

Dealing with Spontaneous Fermentation: Wild Yeast Behaviour

Federico Tondini[1,2], **Ana Hranilovic**[1,2], **Paul Grbin**[1,2], **Markus Herderich**[1,2,3], **Tertius van der Westhuizen**[1,4] **and Vladimir Jiranek**[1,2]

[1] The Australian Research Council Training Centre for Innovative Wine Production

[2]University of Adelaide *Department of Wine & Food Science, PMB 1, Glen Osmond, South Australia 5064, Australia*

[3]The Australian Wine Research Institute *Glen Osmond, South Australia 5064, Australia*

[4]Laffort Australia Pty Ltd *Woodville North SA 5012*

Introduction

'Spontaneous' fermentations, in which the micro-flora present on the grapes/in the winery is responsible for alcoholic fermentation, are favoured for their added complexity in the final wine. However they show higher risk of incomplete sugar consumption and the yeast behaviour is poorly characterized. This project seeks to define the physiological and transcriptional response of indigenous yeast to high sugar fermentation.

Methods

The isolates from spontaneous fermentation at Yalumba Winery (Angaston, South Australia) were genetically identified by sequencing of the 5.8R-ITS region. Stress resistance was tested in a chemically defined grape juice medium (CDGJM) under two conditions: different concentrations of sugar (200,250 or 300 g/L) and ethanol (3,6 or 9% (v/v)). Cell growth was measured by optical density (OD) at 600 nm, sugar consumption was estimated by an enzymatic method and fitness advantage obtained mathematically.

Results & Discussion

Twenty different single colonies were randomly selected and genetically identified to reveal five different yeast genera (Fig.1). In micro-fermentation growth experiments, osmotic stress influenced growth (reduction in relative OD and increase in lag phase). Sugar consumption increased for all the wild strains (Fig. 2D), and interestingly *Hanseniaspora* sp. was the fittest (Fig. 2E).This was not the case for ethanol tolerance. With higher ethanol concentration growth and sugar consumption decreased significantly (Fig 2C-D). *Saccharomyces* sp. was the fittest with increasing ethanol concentration (Fig. 2E).

Transcriptome and metabolome analyses will be performed in order to understand the correlation between stress response, fermentation ability and metabolite production (Fig. 3).

www.agwine.adelaide.edu.au/winemicro

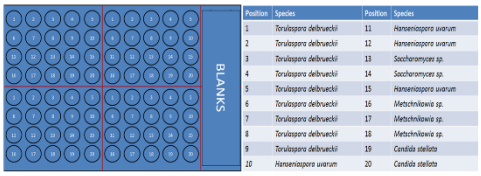


Figure 1: Identified yeast species were spotted in quadruplicate on a 200µl 96-well plate to test stress resistance in micro-fermentation condition.

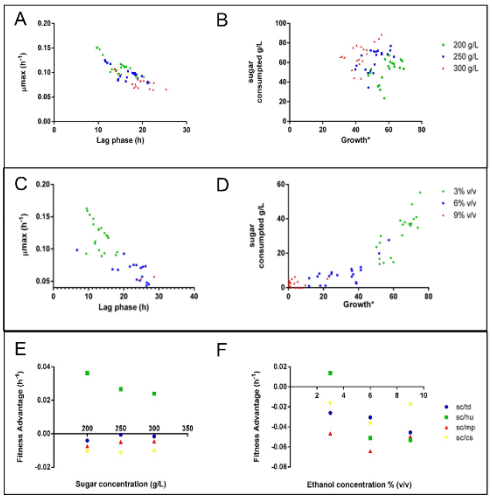


Figure 2: Samples were taken after 30 hours of micro-fermentation in an anaerobic environment. (A) yeast growth parameters (i.e. µmax and lag phase) and (B) yeast overall growth and sugar consumption under different sugar concentrations; (C) yeast growth parameters (i.e. µmax and lag phase) and (D) yeast overall growth and sugar consumption under different ethanol concentrations. (E) Fitness advantage of *Saccaromyces* sp. over non-*Saccharomyces* spp. in sugar conditions and (F) in ethanol conditions.

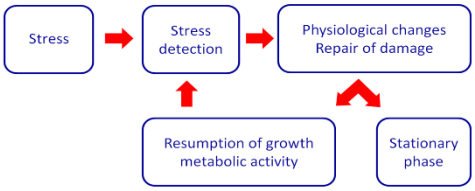


Figure 3: Stress detection and response in yeast cells

Contact: Federico Tondini
Phone: +61 8 8313 0402
Email: federico.tondini@adelaide.edu.au

Acknowledgements: This research was conducted by the Australian Research Council Industrial Transformation Training Centre for Innovative Wine Production (www.adelaide.edu.au/tc-iwp/) (Project No IC130100005) with support from Wine Australia and industry partners: AWRI, Bio SA, Charles Sturt University, CSIRO, DPI NSW, Laffort Oenologie Australia, Lowe Wines, Memstar, National Wine and Grape Industry Centre, Sainsbury's Supermarkets, SARDI, Treasury Wine Estates and Tarac Technologies.

THE UNIVERSITY of ADELAIDE

7. Acknowledgements

I'd be lying if I said I did it alone.

I wouldn't be here if it weren't for very important people in my life, starting from my family, but also and my other family. I've been encouraged, sustained, inspired and tolerated by the greatest groups of friends and colleagues.

Thank you