ORGANIC ACID METABOLISM IN SACCHAROMYCES CEREVISIAE

GENETIC AND METABOLIC REGULATION

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Declaration

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Summary

Organic acids are major contributors to the organoleptic properties of wine. Each acid indeed contributes to the overall acidity of the product, which is an essential feature of wine quality. In addition, and an aspect that has been neglected in many evaluations in the past, each acid also imparts its own sensory characteristic to the wine. Changes in organic acid profiles therefore define relevant sensory features of wine beyond the general perception of acidity.

The main objective of this study was to investigate how different yeast strains and a number of environmental factors (such as aeration, initial pH, temperature and sugar content) influence the organic acid levels in fermenting musts at three critical physiological stages (exponential, early stationary and late stationary phase). Five commercial wine yeast strains (VIN13, EC1118, BM45, 285 and DV10) were selected and these strains were subjected to two widely differing fermentation conditions. The data showed significant variation in organic acid concentrations in the final product depending on the yeast strain, and a more multifactorial experimental design was adopted to investigate the impact of environmental parameters. The impact on both grape-derived (tartaric, citric and malic acid) and fermentation-derived (succinic, acetic and pyruvic acid) acids was evaluated. Condition-dependent shifts in the production of specific organic acids were observed. The multifactorial experimental design evaluated environmental parameters that can be at least partially controlled or managed in the cellar. The influence of individual and /or combinatorial factors such as temperature, pH and sugar content of the must were also shown to affect acid profiles of the synthetic wines.

A further goal of this project was to identify genes that are involved in organic acid metabolism. Transcriptome data of the five yeast strains was analyzed in order to identify genes which showed differential expression between strains and/or time points paralleled by differences in organic acids for the same comparisons. A correlation model was constructed for genes identified in this manner and model predictions were compared/aligned to observed changes in acid levels in response to deletion of the target genes. This approach provided some predictive capacity for modelling the impact of target genes on acid levels. Although some predictions based on gene expression to acid correlations were not validated experimentally, the analysis as a whole provided new insights into organic acid evolution mechanisms of different strains at different stages of fermentation.

Overall, the use of a multifactorial experimental design in the current study confirmed existing knowledge and sheds new light on factors which, either on their own or in combination with other factors, impact on individual organic acids in wine. As a practical outcome, the data can serve for the development of guidelines for winemakers with regard to strain selection and management of fermentation parameters in order to better control wine acidity and wine organic acid profiles.

Opsomming

Organiese sure is vername bydraers tot die organoleptiese kenmerke van wyn. Trouens dra elke suur by tot die algehele suurheid van die produk, wat ’n noodsaaklike kenmerk van wynkwaliteit is. Daarbenewens – en dit is ’n aspek wat in baie analises in die verlede afgeskeep is – verleen elke suur ook sy eie sensoriese kenmerk aan die wyn. Veranderinge in organiese suurprofiele definieer dus die relevante sensoriese kenmerke van wyn verby die algemene waarneming van suurheid. Die vernaamste doelwit van hierdie studie was om te ondersoek hoe verskillende gisrasse en ’n aantal omgewingsfaktore (soos belugting, aanvanklike pH, temperatuur en suikergehalte) die vlakke van organiese suur op drie kritiese stadiums in gistende mos beïnvloed (eksponensieel, vroeë stasionêre en laat stasionêre fase). Vyf kommersiële wyngisrasse (VIN13, EC1118, BM45, 285 en DV10) is geselekteer en aan twee baie verskillende gistingstoestande blootgestel. Die data toon noemenswaardige verskille in die konsentrasies van organiese suur in die finale produk, afhangend van die gisras, en ’n meer multifaktoriale eksperimentele ontwerp is gekies om die impak van omgewingsparameters te ondersoek. Die impak op beide druifafgeleide (wynsteen-, sitroen- en melksuur) en gistingsafgeleide (suksien-, asyn en piruvaatsuur) sure is geëvalueer. Toestand-afhanklike skuiwe in die produksie van spesifieke organiese sure is waargeneem. Die multifaktoriale eksperimentele ontwerp het omgewingsparameters geëvalueer wat ten minste gedeeltelik in die kelder beheer of bestuur kan word. Daar is aangedui dat die invloed van individuele en/of gesamentlike faktore soos die temperatuur, pH en suikergehalte van die mos ’n invloed het op die suurprofiele van die sintetiese wyne. Nóg ’n doelwit van hierdie projek was om die gene te identifiseer wat in metabolisme van organiese suur betrokke is. Transkriptoomdata van die vyf gisrasse is geanaliseer om die gene te identifiseer wat differensiële uitdrukking tussen rasse en/of tydpunte getoon het, parallel aan verskille in organiese sure vir dieselfde vergelykings. ’n Korrelasiemodel is gekonstrueer vir die gene wat op hierdie wyse geïdentifiseer is en modelvoorspellings is vergelyk/belyn met die waargenome veranderinge in suurvlakke in reaksie op die delesie van die teikengene. Hierdie benadering het ’n mate van voorspellende kapasiteit verskaf vir die modellering van die impak van teikengene op suurvlakke. Hoewel sommige voorspellings op die basis van geenuitdrukking op suurkorrelasies nie eksperimenteel bevestig is nie, het die analise in sy geheel insigte verskaf in die meganisme van die evolusie van organiese sure van verskillende rasse tydens verskillende fases van gisting. Oor die algemeen het die gebruik van ’n multifaktoriale eksperimentele ontwerp in die huidige studie die bestaande kennis bevestig en nuwe lig gewerp op faktore wat alleen, of in kombinasie met ander faktore, ’n impak het op die individuele organiese sure in wyn. As ’n praktiese uitkoms kan die data dien vir die ontwikkeling van riglyne vir wynmakers met betrekking tot rasseleksie en die bestuur van gistingsparameters om sodoende beter beheer te verkry oor wynsuurheid asook die organiese suurprofiel van wyn.

This dissertation is dedicated to

My Family

Biographical sketch

Boredi Silas Chidi was born (06 July 1980) in Ga-Mphahlele-Seleteng and matriculated from Sehlaku secondary School in 1997. He enrolled at the University of Limpopo (Former University of the North), where he obtained his BSc, BSc (Hons) and Masters in Biochemistry in 2006 before joining the University of Stellenbosch in 2007 and the Agricultural Research Council in 2012.

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Preface

This dissertation is presented as a compilation of 6 chapters. In Chapter 1 the general aims and motivation for this study are introduced. Chapter 2 is the literature review covering the fundamental reasoning of the research. Chapters 3, 4 and 5 are the research chapters which cover the aims, experimental work and the findings of this research. Chapter 6 focuses on the conclusions and general discussion intending to link the reported outcomes of the research.

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Chapter 1

Introduction and project aims

CHAPTER 1

General Introduction

According to a study, commissioned by the SA Wine Industry Information & Systems (SAWIS) and published in January 2015, South Africa produces 4.2% of the world's wine (2014) and is ranked globally as number seven in overall volume production of wine. It is one of the biggest agro-processing industries in South Africa and an estimated 270 000 people are currently employed both directly and indirectly in the wine industry. The industry faces a competitive global market, and the development of new wine styles and continued improvement of product quality are considered essential to ensure the competitive success of SA wines. For this reason, continuous research and innovation is considered essential to maintain the competitive edge of the SA wine industry. As for most food products, consumer preference in wine is to a large degree linked to the overall sensory character of the product. The sensory properties of wines are influenced by several complex and often interacting factors which together contribute to the flavour, aroma, mouth feel and aftertaste of the wine. The final aroma and taste of a wine is dependent on the chemical composition of the starting must, which is subsequently transformed and conditioned by wine microorganisms such as yeast and bacteria that are responsible for alcoholic and malolactic fermentation. Such fermentation outcomes are thus dependent on microbial factors as well as the physico-chemical factors that prevail during fermentation (Mendoza *et al*., 2009; Styger *et al.* 2011). In addition to such biological factors, the process is also characterised by many spontaneous chemical reactions (Oliveira *et al.* 2008).

Primary fermentation compounds are those derived from, or produced as intermediates of the primary energy –generating pathways of the yeast (glycolysis, TCA cycle. etc.). These compounds (such as ethanol and glycerol) are produced in high concentrations by the yeast, but have low odour activity values (OAVs) and do not themselves present strong aromatic impacts (Lambrechts and Pretorius, 2000). However, these compounds influence the structure and body of the wine, and influence the volatility and perception of the secondary compounds. Likewise, several organic acids (i.e. acetic, succinic and pyruvic acid) may be produced at comparatively high concentrations compared to most esters and higher alcohols. Despite having low OAVs, the organic acids influence the overall acid-balance of the wine, as wines with too low acid contents will taste flat, while too high acid levels will lead to wines with an excessively sharp acidic or sour taste (Mato *et al*., 2005). Secondary metabolites, particularly higher alcohols and esters, are also produced by yeast and bacteria during alcoholic fermentation, and because of their mostly highly volatile nature are of particular relevance to the aroma of wine (Styger *et al.* 2011).

As grapes ripen, their sugar concentrations increase while acidity declines. As a result, cooler wine regions generally have lower sugar levels and higher levels of acidity, which is attributed to slower grape ripening compared to grapes from warmer climate areas (Darias-Martin *et al*., 2000). Grape derived organic acids include primarily tartaric, malic and citric acid, while other acids (e.g. succinic, acetic and pyruvic acid) evolve during alcoholic fermentation (Volschenk *et al*., 2006). All of these acids make an important contribution to the character and quality of the finished wine by impacting the organoleptic characteristics and influencing microbiological stability (Lambrechts and Pretorius, 2000). Some of these acids are also important from a quality control perspective, as acids such as malic acid are often monitored to measure the progress of malolactic fermentation while acetic acid is monitored to assess spoilage. Although grape derived organic acids contribute the highest proportion of titratable acidity in wines (Defilippi *et al*., 2009), it has been shown that fermentation derived acids such as succinic, acetic, pyruvic and lactic acid also contribute to the taste (fresh, tart, sour, sharp), composition and stability of wines (Tita *et al*., 2006). The first three acids are mainly produced by yeast via (i) the tricarboxylic acid cycle which is directly involved in the formation of most intermediate carboxylic acids including succinic acid (Fernie *et al*., 2004), (ii) the glycolytic pathway involving the conversion of glucose to pyruvate and (iii) the glyoxylate pathway that is essential for growth on two-carbon compounds such as ethanol and acetate, and plays an anaplerotic role in the provision of precursors for biosynthesis (Kornberg and Madsen, 1958). In addition, acetic acid production under fermentative conditions is also linked to glycerol formation via redox balancing (Remize *et al*., 1999; Eglinton *et al*., 2002). However, there are several other enzymatic reaction that can lead to acetic acid formation (Jost and Piendl, 1975). Finally, lactic acid is primarily a product of malolactic fermentation which is carried out by lactic acid bacteria, and is therefore not further discussed in this work.

Despite the importance of acid balance to wine quality, the production and consumption of organic acids by yeast has received less attention than secondary metabolism related to aroma compound production. Most studies on acids in wine have focussed on total acidity as opposed to the balance of specific organic acids. Acetic acid has also been singled out in many studies as this acid is the main acid associated with spoilage and acidity problems at high concentrations. Furthermore, several studies have addressed the impact of individual wine–relevant parameters on organic acid concentration. In these mono-factorial studies, the impacts of parameters such as fermentation temperature, initial must nitrogen, initial sugar concentrations, must pH and the level of aeration have been considered.

Such mono-factorial studies have revealed key findings in the past: For example, a direct proportional relationship was established between pH and organic acids such as succinic acid in early studies (Thoukis *et al*., 1965; Shimazu and Watanabe, 1981). Apart from succinic acid, other acids (i.e. pyruvic acid) have also shown pH and strain dependent variations under fermentative

conditions (Rankine, 1967; Agarwal *et al*., 2007). The impact of fermentation temperature on organic acid production has also proven to be a critical factor influencing the production of organic acids under fermentative conditions (Torija *et al*., 2003). In addition, aeration as well as the sugar content of the grape juice has been reported to increase organic acids such as acetic acid during fermentation (Lee *et al*., 1999).

In addition to the impacts of such factors, several studies have also focused on the impact of different yeast strains (i.e. different *S. cerevisiae* genetic backgrounds) on organic acid production in wine (Charoenchai *et al*., 1998; Erasmus *et al*., 2004; Pigeau *et al*., 2007; Magyar *et al*., 2014). However, as stated previously, these studies mostly used single experimental settings, or varied only one or at most two, parameters. To better understand such a complex metabolic system, and to account for the complexity of interactions which arise as different abiotic parameters interplay with one another, and with the differences in genetic backgrounds, a combinatorial approach is required to model acid evolution in wine. Such a holistic approach towards organic acids in wine requires a multifactorial framework comparing different yeast strains. This approach should reveal new features previously overlooked in single factorial experiments. The use of statistically designed multi-factorial experiments has indeed proven valuable in terms of facilitating a better understanding of microbial metabolic processes (Lotfy *et al*., 2007).

In the present study, the impact of several parameters on five different commercial wine strains, EC1118, DV10, VIN13, BM45 and 285, was evaluated using a multifactorial experimental design. These strains were selected as they have previously been studied and exhibited different characteristics in terms of their fermentation profiles, stress tolerance as well as the production of aroma compounds (Rossouw *et al*., 2008, 2009). Fermentations were conducted in different synthetic grape musts of varying composition (a range of pH and sugar values), at different temperatures and under both aerobic and anaerobic conditions. Chemical analyses were conducted at three physiological stages (exponential, early stationery and late stationery growth phases).

The present study also aimed at integrating data from whole transcriptome profiling of the five yeast strains at different time points during fermentation in order to correlate intra- and inter -strain gene expression patterns with experimentally determined organic acid concentrations at the same time points. “Omics” tools such as transcriptomics generate valuable information which expand our understanding of the systems level function of living cells (Brown and Botstein, 1999; Bruggeman and Westerhoff, 2007). Systems biology studies of yeast under wine fermentation conditions are numerous (Erasmus *et al.,* 2003; Marks *et al*., 2008; Mendes-Ferreira *et al.,* 2007; Pizarro *et al*., 2008; Rossignol *et al.,* 2003; Varela *et al.,* 2005). The integration of metabolome and transcriptome datasets in particular have shed light on the regulation of various industrially -relevant aspects of

yeast metabolism, for example the production of important volatile flavour and aroma compounds (Rossouw *et al*., 2008).

The current study is the first to our knowledge which attempts to investigate the transcriptomes of different yeast strains with a focus on organic acid concentrations during fermentation across different time points. This comparative transcriptomic and metabolomic approach was employed to identify genes which may play significant roles in organic acid metabolism during fermentation. It has previously been shown that the use of transcriptomic studies can provide information with regard to the specific function of genes or groups of genes, as well as highlighting their regulation (Hirasawa *et al*., 2010). In this study, several potentially organic acid –relevant genes identified in this manner were targeted for further investigation/validation in deletion studies.

Deletion and overexpression studies focussing on genes involved in organic acid metabolism have been undertaken to understand the role of certain yeast genes in organic acid metabolism (Monschau *et al*., 1997; de Barros *et al*., 2000; Albers *et al*. 2003; Otero *et al*., 2013;). However, the selection of genes in these studies was not based on the relatively unbiased comparative analyses of gene expression and organic acid patterns. In our study, the genes selected for evaluation included *ADH3, AAD6, SER33, ICL1, GLY1, SFC1, SER1, KGD1, AGX1, OSM1 and GPD2*. Fermentations conducted with yeast strains carrying deletions for these genes were characterised with regards to primary fermentation profiles and organic acid concentrations at different time points. The observed changes in the organic acid profiles of the deletion strains were aligned with model predictions based on the correlations of gene expression and acid content.

Collectively, the study represents the most large scale study of its kind on acid evolution during fermentation. The work has been divided into three research chapters that systematically address issues related to the impact of wine yeast strains in two wine-representative conditions (chapter 3), as well as changes in such conditions (chapter 4) on wine acid profiles. Chapter 3 establishes that differences between two relatively extreme conditions (representative of “white” and “red” wine fermentations) with regards to acid profiles produced by different yeast strains were large. This led to a more multifactorial approach to understand the combinatorial impact of fermentation conditions on acid profiles. The parameters investigated included initial pH, temperature and sugar content in both anaerobic and aerobic conditions. The data were generated for three different stages of fermentation. The outcomes of this study are presented and discussed in Chapter 4. Finally, these data were used to query previously generated transcriptome data sets to identify genetic elements that might be linked to or be responsible for the observed differences (Chapter 5). Potential target genes were explored through investigation of deletion mutants to identify whether these genes may play a role in defining the organic acid production patterns observed in chapter 3 and 4.

More specifically, the specific aims of the present study therefore were to:

1. Assess the impact of different yeast strains on the organic acid profiles of two conditions that are broadly representative of “white” and “red” wine fermentations.
2. Assess the impact of environmental parameters, including temperature, nitrogen, pH and sugar concentrations on the acid profile/composition using a multifactorial experimental design.
3. Integrate large-scale multi time-point gene expression data for five yeast strains with organic acid data generated in parallel in order to identify genes with potentially important roles in organic acid production.
4. Investigate the impact of some of the genes identified in point 3 (above) on organic acid production by carrying out fermentations with the relevant deletion strains.

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Chapter 2

Literature review

Overview of organic acid biosynthesis, degradation, analysis, regulation and management in yeast and wine

CHAPTER 2

Overview of organic acid biosynthesis, degradation, analysis, regulation and management in yeast and wine

2.1 Abstract

Grape sugar conversion to ethanol and carbon dioxide is the primary biochemical reaction in alcoholic wine fermentation, but microbial interactions as well as complex secondary metabolic reactions are equally relevant in terms of the composition of the final wine produced. The chemical composition of a wine determines the taste, flavour and aroma of the product, and is determined by many factors such as grape variety, geographical and viticultural conditions, microbial ecology of the grapes and of the fermentation processes as well as winemaking practices. Through the years, major advances have been made in understanding the biochemistry, ecology, physiology and molecular biology of the various yeast strains involved in wine production and how these yeasts impact on wine chemistry and wine sensory properties. However, many important aspects of the impact of yeast on specific wine-relevant sensory parameters remain little understood. One of these areas of limited knowledge is the contribution of individual wine yeast strains to the total organic acid profile of wine. Wine quality is indeed very directly linked to what wine tasters frequently refer to as the sugar - acid balance. Total acidity of a wine is therefore of prime sensory importance, and acidity adjustments are a frequent and legal practice in many wineries. However, the total acidity is the result of the sum of all the individual organic acids that are present in wine. Importantly, each of these acids has its own sensory attributes, with descriptors ranging from fresh to sour to metallic. It is therefore important to not only consider total acidity, but also the contribution of each individual acid to the overall acid profile of the wine. This review will summarise the current knowledge about the origin, synthesis and analysis of organic acids in wine, as well as on the management of wine acidity.

2.2 Introduction

Organic acids and total acidity play a pivotal role in wine sensory perception, and directly influence the overall organoleptic character of wines. It is generally acknowledged that too much acidity will taste excessively sour and sharp while wines with too little acidity will taste flabby and flat, and present a less defined flavour profile (Mato *et al*., 2005). Desirable acidity is also a function of wine sweetness, which is mostly, but not uniquely, derived from residual grape sugars. Sweeter wines usually require higher levels of acidity to be considered of good sensory quality (Schmit *et al*., 2013). Organic acid concentrations in grape musts are primarily a function of grape maturity and variety (Conde *et al*., 2007). Alcoholic fermentation will however change the concentration and content of wine acidity, and may result in higher or lower total acidity of the wines (Volschenk *et al*.,

2006). Importantly, different organic acids have different organoleptic properties, and the impact of organic acids is therefore not only linked to total acidity and pH, but to the specific concentration of each acid in the wine.

In general, malic, citric and tartaric acids are the primary acids in wine grapes and these acids also contribute the highest proportion of acidity (known as titratable acidity) in the final wine (Defilippi *et al*., 2009). However, during alcoholic fermentation several other important organic acids such as succinic, pyruvic, lactic and acetic acid are produced by yeast and bacteria and are mainly associated with the fresh, tart, sour and sometimes metallic taste of wines (Usseglio, 1995; Margalit, 1997; Bely *et al*., 2003;). These acids have also been found to contribute to the stability of wines, especially white wines (Tita *et al*., 2006). Moreover, depending on the requirements for acid balance and maintenance as well as the wine making practices of some wines, acids such as ascorbic, sorbic and sulfurous acids are also used during wine making.

In general, and as grapes ripen, their sugar concentrations increase while acidity declines. It has been shown that grapes from cooler wine regions generally have higher levels of acidity, which is attributed to slower grape ripening compared to grapes from warmer climate areas (Schmit *et al*., 2013). It has also been reported that lower acidity levels in white wine is often the cause of polymerization of phenolic compounds resulting in brown deposits, therefore causing darkening of white wine (Darias-Martin *et al*., 2000). On the other side of the acidity spectrum, general concerns about undesirably high levels of acidity are common in oenology and winemakers in some cases can resort to malolactic fermentation as a way of reduce wine acidity (Lopez *et al*., 2008). Although malolactic fermentation is considered the most natural method for wine acidity adjustment, microbial stability and organoleptic complexity, there are a number of concerns such as spoilage (especially in warm viticultural regions with grapes containing less malic acid) and undesirable changes in wine flavour associated with the metabolic activity of lactic acid bacteria, making this technique inappropriate for certain types of wine (Bauer and Dicks, 2005).

Acidity is a primary driver for important management decisions related to contamination risks and sensorial properties (Akin *et al*., 2008). In terms of contamination risks, it is well established that lower acidity and higher pH generally support the growth of microorganisms, including several unwanted or spoilage species (Bisson and Walker, 2015). High pH wines therefore usually require more careful microbiological management, including the use of higher amounts of SO_2. Acidity and pH are also central features of the sensorial properties of wine, although pH and acid taste are not always directly correlated. For this reason, the adjustment of acid in grape must is a critical part of winemaking. Under normal alcoholic fermentation conditions, titrable acidity (TA) of wine increases by 1 to 2 g/L from the start to finish of alcoholic fermentation as a result of the evolution of acids such as succinic, acetic, lactic, malic and pyruvic acids (Volschenk *et al*., 2006). While it is

essential to monitor pH and acidity throughout fermentation, acid management includes the addition of acids, mostly tartaric acid to low-acid, high pH grape must (Petrie and Sadras, 2007). This practice is of particular importance in warm viticultural regions, where tartaric acid is most commonly added at the start of alcoholic fermentation in order to prevent the proliferation of spoilage LAB and other bacteria during alcoholic fermentation (Volschenk *et al*., 2006).

Acid control and regulation in wine is therefore regarded as a key process for wine makers to control wine character and quality, and combining controlled pH adjustments and informed yeast selection and management. However, the impact of many other environmental and nutritional management practices which may modulate yeast organic acid metabolism, and thus final wine acidity, during the wine making process has not yet been fully elucidated.

2.3 Organic acids in wine

Organic acids in wine derive either directly from the grape, or are the result of microbiological activities that take place before, during or after alcoholic and malolactic fermentation. While the most commonly measured feature of wine acidity is the total acidity (TA) and pH, some of organic acids are important markers for fermentation management and wine flavour and aroma. Malic acid is monitored to measure the progress of malolactic fermentation, acetic acid is monitored as an indicator of fermentation problems or of spoilage, and citric acid may be added to adjust acidity and chelate metal ions to prevent nutrients from precipitation resulting from the interaction of nutrients with metal ions, such as iron precipitating with phosphorus (Fowles, 1992).

2.4 Wine organic acids derived from grapes

2.4.1 Tartaric acid

Unlike most other fruits, grapes contain significant amounts of tartaric acid. It is regarded as the main contributor to wine acidity, and presents a tart taste in wine (Volschenk *et al*., 2006). Tartaric acid is not metabolized by grape berry cells via respiration in the same manner as malic acid, and the level of tartaric acid in the grapes remains relatively consistent throughout the ripening process. The concentration of tartaric acid in grapes depends largely on the grape variety and soil composition of the vineyard. Levels usually range from 4.5 -10 g/L at the end of the grape vegetative growth phase (Ribereau *et al*., 2006). In cold climates, concentrations of above 6 g/L are commonly reached, while low levels of 2 - 4 g/L are more commonly observed in warm climates (Apichai *et al*., 2007). Because of its stability, and the fact that yeast and other microorganisms are unable to metabolise tartaric acid, it is the most commonly employed acid for pH adjustment in the wine industry (Volschenk *et al*., 2006).

2.4.2 Malic acid

L-malic acid is commonly found in many fruits such as green apples and grapes (Krueger, 2012). Mature grapes contain between 2 and 6.5 g/L of L-malic acid (Ribéreau-Gayon *et al*., 2000). Excessive amounts of malic acid (15 to 16 g/L) may be present in grapes harvested from exceptionally cool-climatic regions (Gallander, 1977). The highest concentration of malic acid attained varies depending on the grape variety with some, such as Barbera, Carignan and Sylvaner, being naturally prone to higher malic acid levels. Before the colour change of grapes at veraison, the malic acid content can reach up to 25 g/L before declining to 2 - 6.5 g/L by maturation (Rebereau-Gayon *et al*., 2000). When malic acid levels are too high, wines may taste sour and may require the use of lactic acid bacteria to convert malic acid to the less harsh and softer lactic acid. The induction of malolactic fermentation is beneficial to some wines but in white wines such as Chenin Blanc, it may result in the production of off-flavours such as diacetyl (Bartowsky and Henschke, 2004).

2.4.3 Citric acid

Citric acid is an intermediate of the TCA cycle and is widespread in nature (e.g. lemons). It plays a critical role in the biochemical processes of grape berry cells, bacteria and yeast. High citric acid levels during fermentation could lead to a slower yeast growth rate (Nielsen and Arneborg, 2007). However, concentrations of citric acid in must and wine prior to malolactic fermentation are usually relatively low, between 0.5 and 1 g/L. (Kalathenos *et al*., 1995). Citric acid addition during fermentation influences the acidity and flavour of wines by promoting the perception of “freshness“, while on the other hand, promoting microbial instability and the growth of unwanted microorganisms.

2.5 Organic acids derived from fermentation

2.5.1 Succinic acid

Succinic acid occurs widely in nature in both plants and animals. Succinic acid levels vary between grape varieties as concentrations are usually very low in white cultivars but slightly higher in red grapes. Succinic acid is one of the most important acids which develop during fermentation due to yeast metabolism, with concentrations averaging approximately 0.5 - 1.5 g/L in wine. It is a dicarboxylic acid produced mainly as an intermediate of the tricarboxylic acid (TCA) cycle during aerobic respiration, but is also one of the fermentation end-products of anaerobic metabolism. Song *et al*. (2006) reported that the organic acid responsible for the largest part of the increase in titrable acidity during fermentation was succinic acid. The same observations were previously reported by Bertolini *et al*. (1996) where succinic acid accounted for 50% (1.23 g/L) of the observed increase in wine acidity. In general, it is expected that during fermentation the formation

of non-volatile organic acids ranges between 1 to 4 g/L but such ranges vary significantly with different fermentation conditions (Lamikanra, 1997). The organoleptic character of succinic acid has been described as sour with a salty, bitter taste and its threshold concentration is approximately 35 mg/L (Benito *et al*., 1999). Because of its bitter-salty flavour, winemakers pay particular attention to succinic acid levels in wine. Although succinic acid is relatively resistant to microbial utilisation under fermentative conditions, it cannot be used as an acidulating agent due to this bitter-salty taste attribute (Ribéreau-Gayon *et al*., 2006).

2.5.2 Lactic acid

Lactic acid is an organic acid which also contributes to the overall acidity of wine. The reason why it is attractive to winemakers is because it is much softer on the palate than malic acid (Robinson, 2006). Lactic acid concentrations normally average between 1 - 3 g/L in wines (Boulton *et al*., 1996) but can be higher in wines that have undergone malolactic fermentation whereby malic acid is decarboxylated to lactic acid (Volschenk *et al*., 2006). Unlike malic and tartaric acid, lactic acid is a softer and milder acid which contributes to a creamier mouthfeel of the wine. During winemaking, lactic acid production is usually controlled by sulfur dioxide addition which suppresses the metabolic activities of lactic acid bacteria such as those belonging to the *Oenococcus* and *Lactobacillus* genera (Osborne *et al*., 2000). Small amounts of lactic acid can also be synthesized through cellar practises such as maceration and cold stabilisation (Jackson and Schuster, 1997). While high lactic acid levels presents no major problems in wine, lactic acid bacteria are capable of changing the sensorial characteristics of certain wines through degradation of terpenes and other flavour molecules produced during alcoholic fermentation, as well as producing potentially undesirable aromatic compounds such as diacetyl (Lonvaud-Funel, 1999).

2.5.3 Acetic acid

Acetic acid is a two-carbon volatile organic acid produced during wine fermentation and is mostly responsible for sour and vinegary smell and taste in wines. Alcoholic fermentation of grapes usually results in the production of acetic acid. This process occurs mainly at the beginning of alcoholic fermentation and again towards the end (Bartowsky *et al*., 2003). Apart from yeast metabolic activity, the involvement of aerobic acetic acid bacteria during fermentation can also produce acetic acid by oxidizing ethanol (Pronk *et al*., 1996).

In *S. cerevisiae*, a direct relationship has been established between glycerol and acetic acid production during fermentation (Remize *et al*., 1999, Erasmus *et al*., 2004). S*. cerevisiae* continuously has to equilibrate redox imbalances, which are a feature of alcoholic fermentation. Indeed, anabolic reactions related to biomass formation divert glycolytic intermediates away from

ethanol production, requiring other pathways for the regeneration of NAD^+ which is required to maintain flux through glycolysis. NAD^+ is therefore regenerated through glycerol biosynthesis. However, excess production of NAD^+ may occur, which is balanced through production of acetic acid from acetaldehyde, a reaction that works as a redox sink to convert NAD^+ to NADH (Michnick *et al*., 1997; Remize *et al*., 1999). Wine yeasts therefore also produce acetic acid in response to hyperosmotic stress conditions. The primary response to such conditions is indeed the production of glycerol to act as a compatible compound (Hohmann, 2002). As a consequence, the redox balance is disturbed since NADH is oxidised to NAD, leading to acetic acid production to regenerate NADH. Such hyperosmotic conditions tend to prevail at yeast inoculation at the start of alcoholic fermentation due to the high initial sugar concentrations (Erasmus *et al*., 2004).

The critical acetic acid detection threshold in wine is estimated at approximately 600 mg/L. However, the normal desirable acetic acid level in wines is about 100 - 300 mg/L (Ribéreau-Gayon *et al*., 2006). High volatile acidity in wine presents a major problem with most wineries recommending the use of lower initial sugar -containing must to reduce acetic acid formation during fermentation. However, acetic acid concentrations can reach above 1 g/L, depending on environmental factors and the nutritional composition of the must as well as the influence of spoilage yeasts and bacteria (Bely *et al*., 2003). Since the aroma threshold for acetic acid varies depending on the wine variety and style, its maximum acceptable limit for most wines is 1.2 g/L (OIV, 2010). The volatile acidity of ice wines and botrytized wines can however reach maximum acetic acid concentrations of 2.1 g/L (OIV, 2010).

2.5.4 Pyruvic acid

Pyruvic acid is generally present in wine as a secondary product of alcoholic fermentation and the amount of pyruvic acid in wine varies considerably. Concentrations of pyruvic acid average anywhere between 10 – 500 mg/L in dry wines (Usseglio, 1995). In terms of its sensory attributes, this acid imparts a slightly sour taste and it is formed at the onset of fermentation and decreases towards the end of fermentation (Usseglio, 1995). It also plays an indirect role in wine quality due to its ability to bind sulphur dioxide. SO_2 is widely used in winemaking and its germicidal effect is hugely dependent on the levels of free sulphur dioxide. Free SO_2 is indeed the most antimicrobial form of SO_2, and bound SO_2 has much weaker antimicrobial properties (Fugelsang and Edwards, 2007). Binding of SO_2 by pyruvic acid thus enables the growth of bacteria such as those involved in malolactic fermentation (Wells and Osborne, 2012). Any compound which binds sulphur dioxide reduces its effectiveness, and pyruvic acid is second only to acetaldehyde in this regard.

2.6 Yeast metabolism

2.6.1 Yeast central carbon metabolism

Most yeast species have similar central carbon metabolic pathways but differences in nutrient uptake and utilization as well as the regulation of fermentation and respiration have been noted (Flores *et al*., 2000). Few yeast species are capable of growing under close-to-anaerobic conditions as successfully as *S. cerevisiae* (Visser *et al*., 1990; Moller *et al*., 2001). Therefore, the physiology of this organism during fermentative, respiratory and respiro-fermentative conditions has attracted a considerable research interest. This interest is mainly driven by the industrial significance of this species, and linked to its ability to produce ethanol, proteins, cell biomass and other commercially relevant products (Khan and Dwivedi, 2013). The metabolism of yeast, as for all living cells, is interconnected by means of coupling anabolic and catabolic pathways. As summarised in figure 1, ATP is provided by the oxidation of organic carbon sources yielding energy, ethanol, carbon dioxide and various intermediate metabolites such as organic acids (Rodrigues *et al*., 2006).

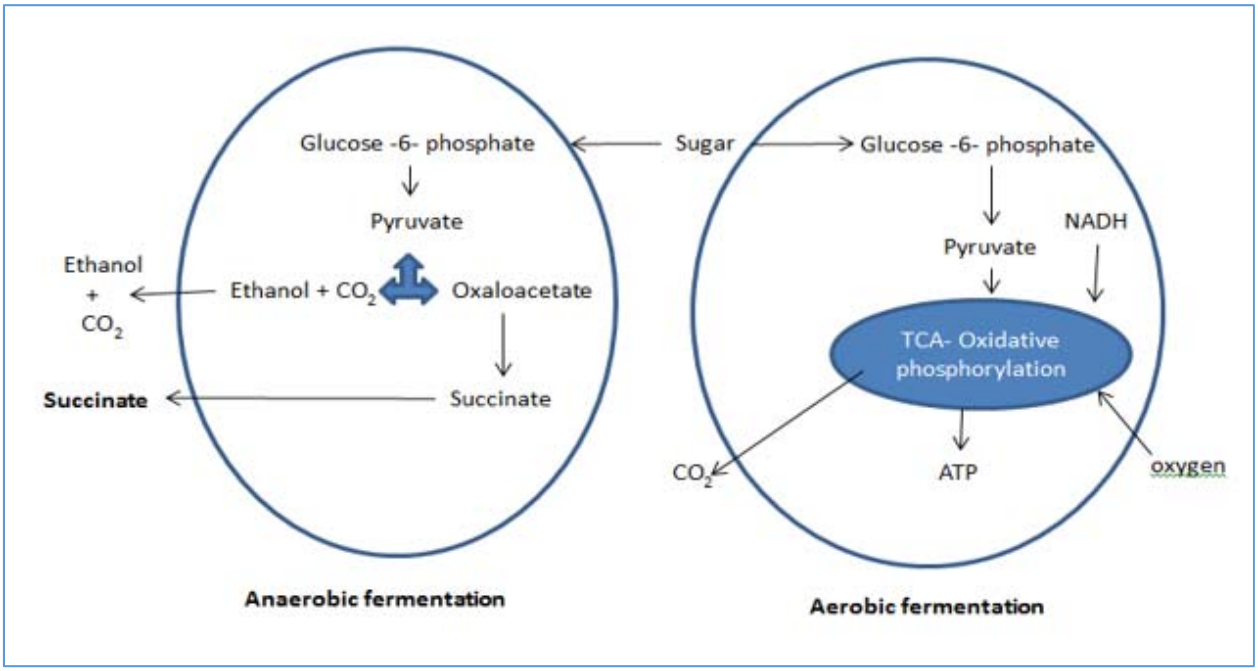

Figure 1: Summary of major sugar catabolic pathways in S. cerevisiae under aerobic versus anaerobic conditions.

2.6.2 Glycolysis

The principal source for energy production in *S. cerevisiae* are hexoses, primarily glucose, and the conversion of such hexoses to pyruvate is achieved via the glycolytic pathway (Fernie *et al*., 2004). Glycolysis provides the yeast with energy, together with essential glycolytic intermediates under both aerobic and anaerobic conditions. Under aerobic conditions the pyruvate formed by glycolysis

enters the TCA cycle and energy is subsequently generated by substrate level phosphorylation in the presence of oxygen.

However sugar dissimilation during anaerobic growth of yeast occurs via alcoholic fermentation which enables the re-oxidation of NADH formed during glycolysis. Moreover, the reduction of the glycolytic dihydroxyacetone phosphate to glycerol-3-phosphate during glycolysis (in the production of glycerol) is also essential to re-oxidise the NADH formed by sugar catabolism under anaerobic conditions. Re-oxidation of NADH provides NAD^+ which enables continuation of glycolysis in the absence of oxygen (and thus without a final electron acceptor). Redox balance is thus maintained by both ethanol and glycerol formation (Rigoulet *et al*., 2004). The glycolytic pathway is also responsible for pyruvate production. Pyruvate is a key metabolite not only in energy generation but also as an intermediate in many other yeast metabolic pathways, including anabolic pathways involved in biomass formation (Zhu *et al.,* 2008). Besides its role in cellular metabolism, it is also an important organic acid which contributes to the overall acid balance and organoleptic properties of wine.

2.6.3 Glyoxylate pathway

Another pathway responsible for the replenishment of TCA intermediates such as oxaloacetate and α-ketoglutarate is referred to as the glyoxylate cycle (fig 2), which is most active when yeast oxidises acetate (Lee *et al*., 2011). This pathway is essential for the continuous flow of carbon through the TCA cycle (Servi, 1990) since when intermediates of the TCA cycle are withdrawn for anabolic reactions, the cycle is replenished by the glyoxylate cycle (Wendisch *et al*., 2006). The enzymes of the TCA cycle and the glyoxylate cycle are physically segregated, with the glyoxylate cycle enzymes of yeast and fungi localized in a specialized organelle called the glyoxysome/peroxisome (Donnelly *et al*., 1998). Glyoxysomes import fatty acids and aspartate, which presents acetyl-CoA to the shunt. During this process, aspartate transaminase converts aspartate into oxaloacetate, permitting incorporation of acetyl CoA into citrate via citrate synthase (Pronk *et al*., 1996). However, the maintenance of the glyoxylate pathway is mostly controlled by the oxidation of succinate to oxaloacetate, which can be converted back to aspartate by aspartate transminase (Popov *et al*., 2005). When the glyoxylate pathway is active, it by-passes some reactions of the TCA cycle in which CO_2 is released, thus conserving 4-carbon compounds responsible for further biosynthesis of other metabolites such as organic acids. (Songa *et al*., 2006). While this pathways is fully active primarily under respiratory conditions, parts of it play important roles during fermentation and act as a source of organic acids such as succinic acid (Heerde and Radler, 1978).

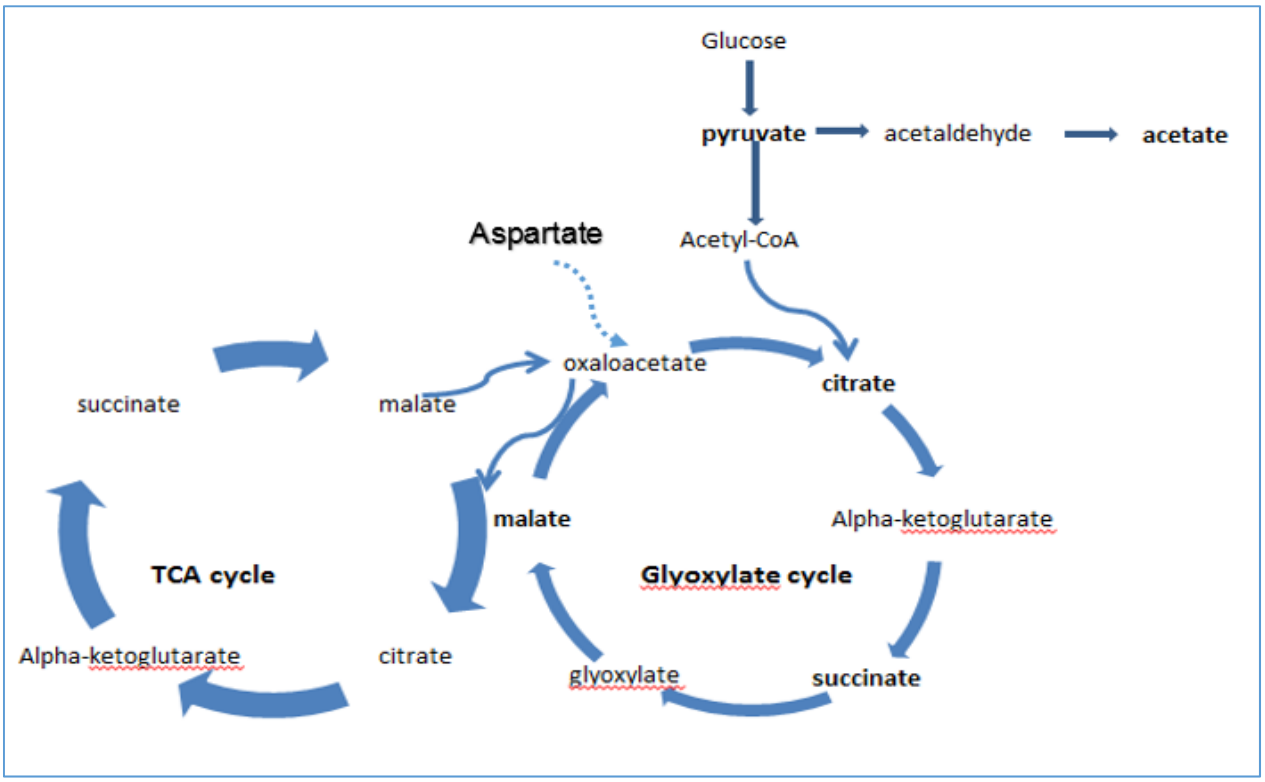

Figure 2: A Simplified pathway diagram showing yeast- derived acids and their connection to the TCA and glyoxylate cycles.

2.6.4 TCA cycle

The tricaboxylic acid (TCA) cycle is directly involved in the formation of most intermediate carboxylic acids including succinic acid. Under aerobic conditions, the TCA cycle's main function is the reduction the coenzymes that are necessary for the full operation of the respiratory electron transport chain (Fernie *et al*., 2004). Its role in anaerobic conditions had been understated in the past, but proof of the TCA cycle's importance in anaerobic fermentation was provided by showing that all of its enzymes were present within anaerobically grown yeast cells (Kuyper *et al*., 2004). Under anaerobic conditions, the TCA pathway however more frequently operates in a branched manner, with a reductive arm working in the reverse direction of the normal cycle and leading to the formation of succinate, and an oxidative arm leading to the formation of α-ketoglutarate (Tu *et al*., 2005).

The TCA cycle is in large part responsible for citrate, malate and succinate production (Heerde and Radler 1978; Albers *et al*., 1996). While citric acid and malic acid depend mostly on TCA cycle reactions, succinic acid can be formed in yeast via four main pathways including amino acid catabolism, depending on the growth conditions and the availability of nitrogen sources in the culture media (Cartledge, 1987; Finley *et al.,* 2012). Under fermentative conditions the TCA cycle operates in a branched manner with a reductive branch leading to succinate formation and the oxidative branch leading to α-ketoglutarate. However, the flux through these pathways depends on

nitrogen availability since α-ketoglutarate is the primary ammonium fixing compound. The reductive branch of the TCA cycle is the principle metabolic pathway for succinate formation under anaerobic conditions, particularly in the absence of glutamate. In contrast, glutamate is responsible for the production of succinate via the oxidative arm of the TCA cycle. Pyruvate and aspartate also play an important role in the formation of succinic acid via the reductive branch of the cycle, or from pyruvate via the oxidative branch.

2.6.5 Nitrogen metabolism as another source of organic acids

Nitrogen availability has a noteworthy (though indirect) impact on organic acids formed via the TCA cycle. For instance, glutamate (a preferred amino acid) can lead to the production of succinic acid via the deamination of glutamate to α-ketoglutarate (Sanborn *et al*., 1979) (fig 3). In a study by Alberts *et al*. (1994) the assimilation of 3-^{13} C glutamate led to significant succinate concentrations which were labelled at the second and third carbon positions. The results proved that 3-^{13} C glutamate was deaminated to 3-^{13} C α-ketoglutarate, which was then oxidatively decarboxylated to succinyl CoA and succinate. In their observations, 17% of the carbon from glutamate was converted to succinate, which further support succinate synthesis from glutamate. When glutamate is used as a nitrogen source the reduced synthesis of 2-oxoglutarate from glucose causes fewer reducing equivalents to be formed, which often reduces the glycerol and ethanol production (Camarasa *et al*., 2003). As a consequence, a metabolic flux (shift) towards organic acid synthesis such as succinic acid might occur.

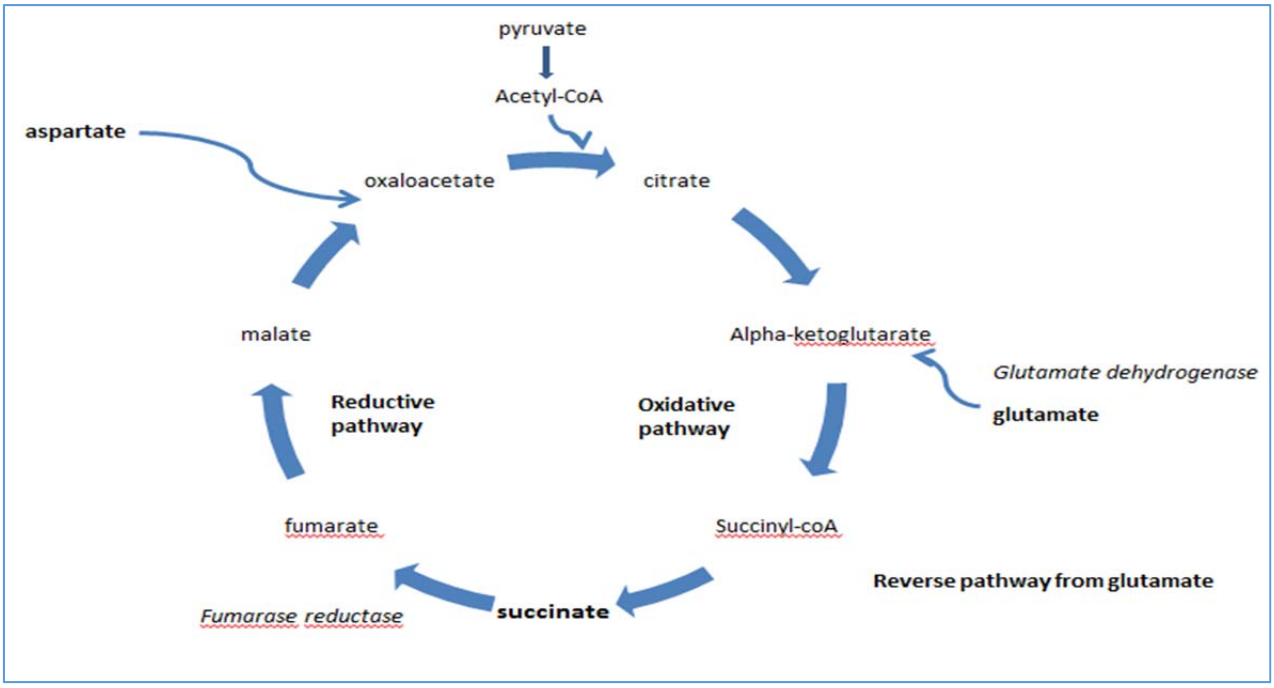

Figure 3: Summary of the main pathways involved in succinic acid production/utilisation during anaerobic fermentation.

2.6.6 Acetic acid metabolism

The yeast biochemical pathways as well as the individual enzymatic reactions involved in acetic acid formation during fermentation are reasonably well characterised (Jost and Piendl, 1975). They include (1) the reversible formation from acetyl Co-A and acetyl adenylate through acetyl Co-A synthetase; (2) the cleavage of citrate by citrate lyase; (3) the production from pyruvate by pyruvate dehydrogenase (PDH) yielding acetyl Co-A that can be hydrolysed into acetate through acetyl Co-A hydrolase; (4) the reversible formation from acetyl-phosphate by acetyl kinase; (5) and the oxidation of acetaldehyde by aldehyde dehydrogenase (Boulton *et al*., 1996; Ribéreau-Gayon *et al*., 2006). In wine, acetic acid production is mainly due to the latter enzymatic oxidation of acetaldehyde to acetate by acetaldehyde dehydrogenases (Remize *et al*., 2000).

Acetic acid production under fermentative conditions is also linked to glycerol formation via redox balancing (Remize *et al*., 1999; Eglinton *et al*., 2002). Glycerol is formed in order to reoxidize the NADH formed during glycolysis (Jackson, 2008), and *S. cerevisiae* may convert NAD^+ back to NADH through acetic acid production. Alterations in glycerol metabolism, such as increased glycerol production, are generally accompanied by an increase in acetic acid production to compensate for changes to the cellular redox balance (de Barros Lopes *et al*., 2000; Prior *et al*., 2000).

2.7 Factors affecting organic acid production in wine

The sensor systems of the yeast cell act to identify variations in environmental conditions (osmolarity, temperature, pH, nitrogen and carbon starvation, chemical and physical agents etc.). The responses to the sensing of changes in conditions are set in motion by a series of signal transduction pathways, which result in changes to gene expression networks, synthesis of protective molecules and /or modulation of protein activity by post-translational modifications or sub-cellular localization (Estruch, 2000). The downstream effects of the regulatory responses to these physiochemical factors will in most cases have an impact on the primary and secondary metabolism of the yeast, thus affecting the quality and organoleptic characters of the wines produced. These environmental and compositional factors are thus important focus areas in wine research due to their impact on the attributes and perceived quality of the final product. Several influential and wine-relevant environmental and nutritional factors which require extensive investigation as indicated in the current review include temperature, aeration, pH, nitrogen levels, osmotic stress and vitamins. Furthermore, the effect of individual strains with different genetic backgrounds on organic acid production is also an important area to consider.

2.7.1 Effect of pH on organic acid production in *S. cerevisiae* and other species

The large majority of wines show pH values between 3 and 4. As with sugars, the pH level will vary according to ripeness of the grapes, with increasing ripeness leading to lower acidity and increased pH. For white wines, winemakers often recommend pH levels between 3.1 and 3.2, and if the pH is too high (>pH 3.4), it may be a sign that the grapes are overripe. It is generally known that the activity of enzymes involved in central carbon metabolism is pH sensitive, which could account for the pH related changes in the production levels of several organic acids in wine (Agarwal *et al*., 2007). For this reason, the mono-factorial impacts of pH on organic acid production have been studied and several authors have presented significant variation effects of pH on yeast and bacterial strains in terms of organic acid profiles. The impact of pH on organic acid productivity is often related to the specific strain employed which may account for the different trends observed in different studies. For example, high succinic acid levels have been linked to an increased initial fermentation pH (Thoukis *et al*., 1965). The authors observed a slight increase in succinic acid by *S. cerevisiae* when the grape must was adjusted to pH values ranging from 3.0 to 3.8, but more when it was raised from 3.8 to 4.8. The study also indicated a strong direct relationship between glycerol and succinic acid formation at a higher initial pH of the must. Recently, Liu *et al*. (2015) reported that lower initial pH (2.50; pH 2.75 and 2.50) showed the properties of prolonged yeast lag phase, increased acetic acid levels, and decreased final content of succinic acid when 3 *S. cerevisiae* strains were tested under wine making conditions. Several authors have also noted a significant increase in pyruvic acid levels when fermentation pH was increased (Graham *et al*., 1979; Samuelov *et al*., 1998). Rankine (1967) also showed that yeast strain variability and pH appeared to be the two most influential factors affecting the pyruvic acid content in wines. In this study however pyruvic acid production only showed pH dependent trends for some of the strains evaluated.

2.7.2 Effect of aeration on organic acid production in *S. cerevisiae* and other species

The fermenting must is easily saturated in oxygen (6 - 8 mg/L) at cellar temperature (Du Toit *et al*., 2006). Several techniques are available to provide at least limited amounts of oxygen during the fermentation process, including pumping-over and micro-oxygenation. Pumping-over is not recommended in white winemaking because of oxidation concerns, however oxygen can be introduced during pressing of whole clusters, transfer from tank to tank, filtration, racking, centrifugation, bottling and barrel aging (Cheynier, 1993). According to Saa *et al*. (2012), for oxygen solubility in fermentation media at 25 °C (i.e. about 7 mg/L), 15% oxygen saturation (with air) would be equivalent to around 1 mg/L of molecular oxygen. Aeration has been shown to play a fundamental role in the production of many secondary metabolites. For example, the level of citric acid production by yeast as well as other fungi was previously shown to be strongly dependent on the oxygen availability in the fermentation vessel (Sakurai *et al*., 1996). Sufficient oxygen supply is

extremely important during citric acid metabolism since even short interruptions in oxygen supply can result in a complete loss of citric acid formation (Yigitoglu, 1992). Previously, Rehm *et al*. (1980) indicated that the formation rate of citric acid strongly coincides with the ratio between ATP and ADP at various pH values. The authors also indicated that the energy generated by substrate-level phosphorylation is used to promote the excretion of organic acids (in particular citric acid) by the pH -dependent active transport system.

In many cases, aeration during fermentation might increase the production of several secondary metabolites such as succinic, pyruvic and acetic acid. This behaviour has been correlated to the increase in the levels or activity of the TCA enzymes involved in the biosynthesis of these secondary metabolites during aeration, of which succinic acid is an intermediate (Boulton *et al*., 1996). The synthesis of succinic acid has also been previously tested in apple and grape juices. For instance, when *Saccharomyces cerevisiae EC1118* was assessed in white wine fermentations, acetic acid was produced only under conditions of strict anaerobiosis (0.3 C-mmol g /DW/ h). On the other hand, a striking and significant increase in the level of succinic acid production occurred between 1.2 and 2.7 µM dissolved oxygen conditions (from 0.02 to 0.27 C-mmol g/ DW/ h) (Aceituno *et al*., 2012). In another study (Estela *et al*., 2012), compounds of sensory importance produced by *Saccharomyces cerevisiae RIVE V 15-1-416* cultivated in apple juice at 28 ◦C in static and agitated cultivation (200 rpm) were analysed. At the end of fermentation, the authors reported an increase (0.77 g/L under static and 1.32 g/L under agitate cultivation) in succinic acid levels under agitated cultivation whereas acetic acid levels decreased (51.0 g/L under agitated and 266 g/L under static cultivation). Recently, oxygenation levels in wine were assayed with *M. pulcherrima CECT12841* and *S. cerevisiae EC1118* containing 10 or 25% air (maximum dissolved oxygen levels around 0.7 and 1.7 mg/L respectively). A negative correlation between air concentration and ethanol yield was found while a positive correlation was confirmed between acetic acid yield and oxygenation level (Morales *et al*., 2015).

2.7.3 The impact of temperature on organic acid production

Wine fermentations are generally conducted across a wide range of temperatures, with red wine fermentation being carried out at higher (18 – 25 ^{o}C) and white wine fermentation at lower temperatures (10 – 15 ^{o}C). The higher fermentation temperature in red wines is essential for the extraction of anthocyanins and other non-volatile compounds from grape skins during fermentation while lower temperatures are recommended for the retention of fruity volatile compounds and the prevention of undesirable volatile flavour compounds in white wines (Lambrecht and Pretorius, 2000; Styger *et al*., 2011).

Torija *et al*. (2003) observed temperature effects on the growth of strains of *S. cerevisiae* in grape must and noted a significant increase in succinic and acetic acid as fermentation temperature was increased. Aragon *et al*. (1998) investigated the influence of yeast type and fermentation temperature on organic acid contents and observed that wines fermented at 18 ^{o}C showed lower succinic acid contents compared to those fermented at 21 ^{o}C. Significant differences were also observed for acetic acid and citric acid concentrations, Taing and Taing. (2007) also found that temperature (optimum at 25 ^{o}C) increased succinic and malic acid levels in high sugar fermented food.

Data also suggest that the intrinsic ability of yeast strains to grow at different temperatures appears to be another influential element in terms of temperature-dependent succinic acid production during fermentation. Castellari *et al*. (1994) showed that mesophilic strains *AWIR 796* and *Endoferm M2* were average producers of succinic acid but cryo-tolerant strains of *S. bayanus* produced an additional 0.8 g/L succinic acid. The study concluded that succinic acid production was strain dependent. The same study also showed variations in acetic acid production among the wine yeast strains tested.

2.7.4 Effect of vitamins on organic acid production in *Saccharomyces cerevisiae*

According to United States Department of Agriculture (National Nutrient Database for Standard Reference Release 28), 100 grams of grapes comprise of about 5.4 mg of vitamin C, 0.09 mg of vitamin B1, 0.2 mg of vitamin B2, 0.08 mg of vitamin B6, 0.70 mg of vitamin E and 0.2 mg of niacin. Vitamins commonly required by yeast include the following: Biotin (which serves as a cofactor in carboxylase-catalysed reactions), pantothenic acid (the functional group of coenzyme A which is involved in acetylation reactions), nicotinic acid (in the form of nicotinamide which is involved in redox reactions) and thiamine (in the form of thiamine pyrophosphate which is involved in decarboxylation reactions, Walker, 1998). Organic co-factors such as vitamins are required for the enzymatic complexes of several intermediates such as organic acids (Tu *et al*., 2005). The study of Ribereau *et al*. (1956) demonstrated that the absence of individual vitamins such as thiamine, biotin and pantothenate in synthetic medium significantly reduced succinic acid concentrations in wine, whilst increasing acetic acid and ethanol yields. In addition, a vitamin, nicotinic acid, was the limiting factor for lactic acid production during fermentation with the K1-LDH strain in batch conditions (Colombie and Sablayrolles, 2004).

2.7.5 Effect of nitrogen on organic acid production in *Saccharomyces cerevisiae* and other yeast species

The Yeast Available Nitrogen (YAN, mainly ammonium and amino acids, with the exception of proline) range recommended by oenologists varies from 150 mg/l YAN (Weeks and Henschke,

2013) to 400 mg of nitrogen per litre (Ugliano et al., 2007). However, Bruce and Zoecklein. (1998), showed that maximum fermentation rates can be achieved with YAN in the 400 to 500 mg N/L range although most winemakers do not prefer higher nitrogen concentrations due to the impact that it can have on wine aroma. According to Bisson and Butzke. (2000), in order to successfully complete fermentations, optimal nitrogen levels at harvest should be part of viticulture considerations. Sugar-nitrogen balancing is also an important aspect of fermentation. For this reason, UC Davis Department of Viticulture and Enology made some recommendations to yeast and nutrient manufacturers which were made based on harvest brix level (a measure of must sugar). They have recommended that, at the brix level of 21 - 27°Brix, about 200 - 350 mg N/L should be made available in order to successfully complete fermentations.

The nitrogen content of yeast cells is approximately 10% of cellular dry weight (Walker, 1998). Although yeast is unable to fix molecular nitrogen, simple inorganic nitrogen sources such as ammonium salts (e.g. ammonium sulfate) can be effectively utilised (Matthews and Web, 1991). Yeast can also utilize nitrate and a variety of organic nitrogen compounds such as amino acids, peptides, purines, pyrimidines and amine as nitrogen sources (Webb and Lee, 1990). Insufficient nitrogen during fermentation is one of the biggest challenges faced by yeast under fermentative conditions. Although grape must contains a broad variety of nitrogen sources such as amino acids and ammonia, sluggish or stuck fermentation are often observed when these nitrogen sources are limited or depleted before the end of fermentation (Bisson, 1999).

Must nitrogen content influences organic acid production in wine in many ways via mechanisms that have only been partially elucidated. According to Remize *et al*. (2000) the relationship between the initial nitrogen content (ammonium sulphate) and organic acids such as acetic acid in *Saccharomyces cerevisiae* during fermentation is very complex. The effect of yeast assimilable nitrogen addition indeed showed an inverse relationship with acetic acid production for the initial stages of fermentation but not at the later stages of fermentation.

The impact of different nitrogen sources on *S. cerevisiae* general metabolism has also been evaluated in relative depth. Camarassa *et al*. (2003) showed that glutamic acid has a large influence on the formation and production of α-ketoglutaric acid, succinic acid, and acetic acid. Increased concentrations of these compounds were produced during fermentation when glutamate was used as a nitrogen source as compared to ammonium and other amino acids. In a different study, increased *S. cerevisiae* growth and succinic acid production has also been observed with increased nitrogen levels, (up to 500 mg/L) by *S. cerevisiae*. However further nitrogen increases above the 500 mg/L level had little additional impact on the production of succinic acid and acetic acid (Heerde and Radler, 1978). Vilanova *et al*. (2007) also revealed that ammonium supplementation increased the final concentration of L-malic acid, acetic acid and glycerol in wine.

The availability of nitrogen sources is central to the utilization of aspartate and glutamate via the reductive and oxidative arm of the TCA cycle respectively (Camarassa *et al*., 2003). Several authors have reported an increase in succinic acid production when growth media contained glutamate and aspartate as the major nitrogen sources for yeast (Roustan and Sablayrolles, 2002). Agarwal *et al*. (2007) also investigated the effect of nitrogen sources (among others peptone, urea, tryptone and ammonium sulfate) on enzymes involved in succinic acid production. Supplying tryptone as a nitrogen source resulted in elevated formation of succinic acid (3.8 g/L) as activities of enzymes involved in succinic acid production were found to be increased. Among the inorganic nitrogen sources tested ammonium hydrogen phosphate yielded a maximum of 2.43 g/L of succinic acid.

2.7.6 Effect of sugar on organic acid production in *Saccharomyces cerevisiae*

Under normal circumstances, viticulturists prefer to pick the grapes when the sugars are in specific ranges, depending on the varietal and the style of wine that is targeted. Most wines are harvested at sugar levels of between 190 and 250 g/L, composed of similar amounts of glucose and fructose (Boulton *et al*., 1995). *Saccharomyces* usually converts 95% of the sugar to ethanol and carbon dioxide, 1% is converted to cellular material, and the remaining 4% is converted to other end products such as organic acids, higher alcohols and esters and, to a lesser extent, aldehydes (Rapp, 1991). However, there are other end products such as acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl which may be regarded as undesirable when present in excess concentrations.

Factors that affect the production of some organic acids, and in particular of acetic acid, have been extensively studied (Usseglio, 1995; Bisson, 1999; Bely *et al*., 2005; Ferreira *et al*., 2006). In very high sugar Riesling Icewine juices (over 400 g/L), alcoholic fermentation tends to result in very low ethanol yields and high acetate production (Caridi, 2003). Pigeau et al. (2007) also noted that, increasing Icewine juice sugar concentration from 40 to 46°Brix decreased yeast growth, sugar consumption rate, the total amount of sugar consumed and the total amount of ethanol produced. However, acetic acid levels increased from 0.11 g/L (at 40°Brix) to 0.21 g/L (at 46°Brix). A reasonable hypothesis for these findings could be the increased osmotic stress imposed by higher initial sugar contents, leading to higher initial glycerol formation and thus indirectly acetic acid formation due to redox balancing (Erasmus *et al*., 2004). Based on these observations, the influence of sugar levels on organic acid production appears to be significant, though no conclusive trends have been established. The impact of specific strains was also not taken into consideration in these studies.

2.7.7 Effect of yeast strain on organic acid production in wine

Numerous studies have provided insights on the response of individual strains to grape must of specific cultivars and their impact on the sensory and chemical characteristics of the wines produced.

In the case of *S. cerevisiae* many papers have reported on the influence of yeast strain on wine composition in general (Delfini *et al*., 1994, Galletti *et al*., 1996; Kunicka-Styczyńska and Pogorzelski, 2009). The influence of yeast strain on the organic acid composition of wine was specifically addressed by Aragon *et al*. (1998). The results showed significant differences in acetic, citric and succinic acid production for different yeast strains. Patel *et al*. (2003) also investigated a total of 18 *S. cerevisiae* yeast strains for the production of volatile acidity, primarily acetic acid. Of all the strains, the composition of the volatile acidity produced by *A350/VL1/Fermiblanc* and *T73* yeast strains were significantly different from the other strains and significantly contributed to the final characteristic flavours in the corresponding wines. Previously, Kunicka-Styczyńska and Pogorzelski. (2009) also observed significant variations in pyruvic and acetic acid levels when different *Saccharomyces species* were assessed in apple wines. Although there are many other factors that directly/indirectly affect acid degradation/evolution in wine, strain identity seems to be one of the major drivers.

A diversity of yeast species within the genera *Hanseniaspora*, *Pichia*, *Kluyveromyces*, *Candida* and *Saccharomyces* have long been known to be present in freshly crushed grape juices and the early stages of fermentation (Bisson, 1993). However, very little information exists regarding how these yeast species affect organic acid production in wine.

2.7.8 Impact of osmotic stress on major fermentation products in yeast

The ability of wine yeast to carry out alcoholic fermentation under winemaking conditions is largely influenced by their response to the stress conditions such as osmotic stress (Carrasco *et al*., 2001). Different mechanisms have been developed by the fermenting strains to triumph over these adverse situations. A clear understanding of these mechanisms is essential to improve the overall fermentation process and thus improve the quality of wines (Ivorra *et al*., 2000).

During osmotic stress, glycerol is produced in response to high sugar levels of the must resulting in excess NAD^+. To counterbalance the high levels of NAD^+ production, NADH is regenerated by converting acetaldehyde to acetic acid (Caridi *et al*., 2003). Glycerol metabolism thus plays an essential role in fermentation, not only as an osmo-protectant, but also by aiding equilibration of the intracellular redox balance (Romano, 1993; Jain, 2010). Beney *et al*. (2001) also found that the

resistance of *S. cerevisiae* to high osmotic stress improved at lower temperatures compared to warmer conditions but a link to organic acid levels was not established.

Although the current review focuses much attention on several factors which do or may affect organic acid degradation or evolution in wine, very little literature is available on how multiple changes in two or more parameters may impact on wine acidity. It is not surprising since multifactorial experiments are not easy to interpret. Notwithstanding these obstacles, more work is required to investigate the influences of individual and/or multiple changes in wine yeast strains, fermentation pH, sugar and temperature on acid profiles of different wines.

2.8 Analytical methods for organic acid determination

Several methods have been established for identifying and quantifying organic acids in grape juices and wines. Such methods include non-enzymatic and enzymatic spectrophotometric, chromatographic and electrophoretic methods (Mato *et al*., 2005). Spectrophotometric methods are based on the reaction of the organic acid with a specific substance, resulting in the formation of a compound or coloured complex that can be determined at an appropriate wavelength. In most cases interference can be avoided by isolation of organic acids by precipitation, ionic exchange resins, etc. (Cunha *et al*., 2002). Prior to recently developed analytical techniques, spectrophotometric methods have been used to evaluate organic acids in grape juices and wines. For the organic acids found in wine, Rebelein *et al*. (1961) used spectrophotometric methods and managed to measure organic acids at several wavelengths (490 nm for tartaric acid, 420 nm for malic acid and 530 or 570 nm in the case of lactic acid). The prediction of organic acids and other quality parameters of wine vinegar by near-infrared spectroscopy has also been investigated (Saiz *et al*., 2006).

Enzymatic methods are widely used for the analysis of organic acids such as tartaric, acetic, L-ascorbic, formic, D gluconic/ D glucono-d-lactone, D-isocitric, oxalic and succinic acids (Boehringer Mannheim GmbH, 1995). These methods are based on the measurement of the increase or decrease in absorbance of the coenzymes NADH (nicotinamide adenine dinucleotide, reduced form) or NADPH (nicotinamide–adenine dinucleotide Phosphate, reduced form) which absorb in the distant wavelength region (Boehringer Mannheim GmbH, 1995).Although this technique is not more difficult than other instrumental techniques, it arguably provides greater accuracy since the enzyme only acts on the specific organic acid involved. In addition, the enzymes have the ability to distinguish between the various isomeric forms (Mato *et al*., 2005). Several other methods have also been reported to evaluate organic acids, such as high performance liquid chromatography (Castellari *et al*., 2000; Pereira *et al*., 2010), ion chromatography (Kupina *et al*., 1991), gas chromatography (West and Mauer, 2011) and capillary zone electrophoresis (Kandl and Kupina, 1999; Cortacero *et al*., 2005; Mato *et al*., 2006).

2.9 The importance of "omics" tools in wine research

2.9.1 Transcriptomic and Proteomic approaches in yeast research

Transcriptome and proteome profiles for several wine yeast strains have been established previously under winemaking conditions (Rossouw *et al*., 2008, Gomez *et al*., 2010), and many studies have paid special attention to the intrinsic genetic and regulatory pathways that are actively involved in wine fermentation (Erasmus *et al.,* 2003; Rossignol *et al.,* 2003; Varela *et al.,* 2005; Mendes-Ferreira *et al.,* 2007; Marks *et al*., 2008; Pizarro *et al*., 2008). A global analysis of gene expression and protein profiles plays an important role in increasing our understanding of how yeast cells adapt to environmental changes, and how their response to environmental conditions impact cellular metabolism and the production of primary and secondary compounds important to wine quality.

Wine fermentation is a process during which yeast must adapt to the significant changes which occur during vinification (Zuzuarregui *et al*., 2006). At the end of the biological information transfer system (from genome, to transcriptome, to proteome), the post-translational modifications at the protein level and/or protein activity plays an essential role in further adjustments of the cell to these changing external conditions. Efforts have been made in recent years to investigate gene expression profiles during vinification. The detailed analysis of subsets of genes, transcriptional regulation and gene expression profiles in yeast under winemaking conditions has been the focus of several studies (Puig *et al*., 2000; Rossignol *et al*., 2006; Rossouw *et al*., 2008).

Previously, commercial wine strains have been screened and selected on the basis of desired physiological traits for optimised fermentation performance but this has not been accompanied by knowledge with regard to the molecular basis for the fermentation phenotypes of these selected strains (Aranda *et al*., 2004). The study of Zuzuarregui *et al*. (2006) offered the first proteomic and transcriptomic comparisons involving two commercial strains *(ICV 16* and *ICV 27*) with different fermentative performances. Since then, several other combinatorial transcriptomic-proteomic studies of wine yeast have been carried out (Rossignol *et al.,* 2003; Gomez *et al*., 2010). The use of these global analysis strategies has made it possible to analyse variations in gene expression and protein levels at different time points during vinification and have greatly enhanced our understanding of yeast metabolism during fermentation (Marks *et al*., 2003). However, and to our knowledge, such approaches have not yet been applied with regards to the genetic regulation of organic acid production. The potential thus exists to utilise these tools with a specific focus on organic acid metabolism in different yeast strains to gain new insights into the regulation of acid metabolism on a molecular level.

2.9.2 Metabolomics of yeast

The major goal of system biology is to acquire an overall quantitative description of systems which occur inside of the cell. It is a challenging task as the components and interactions involved in these cellular systems are both numerous and complex (Cakir *et al*., 2006). Although transcriptome data supplies an overview of the broad expression patterns and regulation of genes involved in metabolism, understanding functional cellular physiology requires metabolomic data to complete the systems picture (Nielsen *et al*., 2003).

The metabolomics of wine have been studied extensively over the past few years (Van Dorsten *et al*., 2009; Son *et al*., 2009; Cuadros *et al*., 2010). Cuadros *et al*. (2010) provided data demonstrating that unbiased and objective analytical chemistry in combination with multivariate statistical methods allow reproducible classification of wine attributes such as variety, origin, vintage, and quality through metabolomics studies. Son *et al*. (2009) investigated the changes in metabolites such as pyruvate, succinate, citrate, malate and tartrate in musts during alcoholic fermentation and during aging by coupling ^{1}H NMR spectroscopy with multivariate statistical analysis. Elsewhere, the contribution of individual volatile aroma compounds to the overall volatile composition was also accomplished through the development of automated metabolomics data analysis of GC-MS profiles of wines (Schmidtke *et al*., 2013). In addition, the analysis of wine micro-oxygenation has also been attempted by untargeted LC-MS (Arapitsas *et al*., 2012).

Functional analysis of cellular metabolism and integration of metabolome data with other omics-data (e.g. transcriptome data) necessitates the large-scale detection and quantification of metabolites of interest. However a noteworthy challenge is the shortage of targeted quantitative analyses for metabolomics approaches (Nielsen *et al*., 2003). A high-throughput GC-MS method for quantifying metabolites that permits semi-quantitative analysis of several metabolites in *Saccharomyces cerevisiae* was generated by Devantier *et al*. (2005). Similar metabolomics techniques could also act as valuable tools that will expand our knowledge regarding organic acid metabolism of yeast in wine.

2.10 Conclusion

Wine acidity contributes significantly towards consumer perception of wine quality. Winemakers have always been challenged in terms of acid management and the balance between sugar and acidity in their wines (both in warm and cool climate viticultural regions). While researchers are currently investigating acid evolution in wine, the key issue that is often overlooked by winemakers is how individual organic acids contribute towards the flavour profile and organoleptic characteristics of wines. In recent years the development of several analytical techniques (i.e. electrophoretic, chromatographic and enzymatic) for metabolite quantification has received

considerable attention in wine science. Rapid, sensitive and accurate analytical techniques for quantification of organic acids that are present in wine provide the platform for detailed analysis of organic acids in wine across a range of experimental conditions. Intelligent experimental design combined with suitable analytical techniques form the foundation of meaningful studies of yeast acid metabolism. This will ultimately provide wine makers with a better understanding of acid development in their wines, and adjustment or management practices in the cellar to favour desirable acid profiles. Factors which impact significantly on the production of organic acids in wine must be thoroughly investigated (with the help of complementary systems biology approaches) in order understand the fundamental metabolic regulation underlying the evolution of acidity during fermentation.

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Chapter 3

Research results

Determining the impact of industrial wine yeast strains on organic acid production

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CHAPTER 3

Determining the impact of industrial wine yeast strains on organic acid production

3.1 Abstract

Organic acids are major contributors to wine flavour and aroma. In the past, the scientific focus has mostly been on organic acids derived from grapes or on the transformation of malic acid to lactic acid by lactic acid bacteria, since these acids contribute significantly to the final total acidity of wine. However, the organic acid concentration and composition also changes significantly during alcoholic fermentation, yet only limited information regarding the impact of different yeast strains on these changes has been published. Here we report on changes in organic acid composition during fermentation by five widely used industrial wine yeast strains in a synthetic grape must (MS300) in two very different, but both wine-like, fermentation conditions. Samples were obtained from three physiological stages during fermentation, namely the exponential growth phase (day 2), early stationary phase (day 5) and late stationary phase (day 14). These different stages were selected to provide more information about acid evolution throughout fermentation as well as the impact of nutritional and environmental conditions during aerobic and anaerobic fermentation. The data clearly demonstrate that different strains have different acid consumption and production patterns, and presents a first step towards enabling winemakers to appropriately select strains for acid management during fermentation.

3.2 Introduction

Winemakers are faced with many challenges to adjust products according to consumer demands. The most prominent and easily perceived sensory balance in wine is between acidity and sweetness. Elevated initial sugar concentrations and relatively low acidity of grapes at harvest, as is frequently experienced in particular in warmer vintages or regions, present a particular challenge in this regard and various wine making strategies have been proposed over the years (Coulter *et al*., 2008; Santos *et al*., 2008). Many studies have been dedicated to understanding how the metabolism of wine yeast strains contributes and may help to improve the sensory properties of the finished product (Borneman *et al*., 2008; (Gonzalee *et al*., 2014). However, much remains to be learned in this regard.

One area of relatively limited knowledge in wine yeast metabolism is the profile of the organic acids that are produced or metabolised by yeast strains during fermentation. Organic acids play a significant role in the perception of wine quality since their presence directly impacts on the perceived and total acidity of wines (Cole and Noble, 1997). However, several different organic acids are found in wines, and each of these acids contributes differently to total wine acidity, and

each also has specific and unique organoleptic properties (table 1). While the overall impact of fermentation on total acidity is a relatively well-established parameter, and is commonly monitored, very few data have been published relating to the impact of individual wine yeast strains on the specific concentrations of the different organic acids present in wine. This information will be important to improve the control and management of wine acidity.

In general, tartaric, citric and malic acids are the most notable primary organic acids present in grapes, and also contribute the largest proportion of titratable acidity in wine (Shiraishi *et al*., 2010). They are also known to contribute, directly or indirectly, to wine colour and stability (Boulton *et al*., 1996). In the case of wines with below desirable levels of acidity, acidification is often accomplished by tartaric acid addition, a practice that is commonly applied in warmer climates where grapes are frequently harvested with relatively low acidity levels and high sugar contents.

Grape acids are in most cases unaffected by alcoholic fermentation, although some reports have indicated that malic acid in particular can be impacted by the process. However, yeast releases a number of additional organic acids, resulting in changes to the final taste and an overall acidification of the wine (Volschenk *et al*., 2006). The predominant organic acids produced by the yeast include succinic, pyruvic and acetic acid. These acids are often associated with the fresh, tart and sour taste of wines (table 1).

Table 1: Summary of the organic acids and their characteristics in wine.

Organic acid	Level ranges (g/L)	Characteristic	Reference
Tartaric acid	4.5 - 15	Tart taste	Ribéreau *et al*., 2006
Malic acid	2 - 6.5	Sour taste	Ribéreau *et al*., 2000
Citric acid	0.5 -1.0	Freshness	Kalathenos *et al*., 1995
Acetic acid	0.2 - 0.6	Vinegar sour aroma	Bely *et al*.,2005
Pyruvic acid	0.01 - 0.5	Slightly sour	Usseglio, 1995
Succinic acid	0.5 - 1.5	Salty bitter taste	Margalit, 1997

Organic acid consumption and production by yeast during wine fermentation has been primarily linked to central carbon metabolism. Indeed, organic acids are intermediates or by-products of glycolysis or of the TCA cycle, but may also be derived from the glyoxylate pathway and from nitrogen metabolism (Kornberg and Madsen, 1958; Popov *et al*., 2005). The three most prominent acids produced by yeast during fermentation are succinic, acetic and pyruvic acid, while the release of small amounts of fumaric and malic acid has also been reported. Under anaerobic conditions, the TCA cycle operates in a branched manner, reductive or oxidative (Buckel *et al*. 1974; Tu *et al*., 2005). In these conditions succinic acid is primarily produced as an end product of the reductive branch of the TCA cycle. Acetic acid, which at higher concentrations is highly undesirable because of its volatile nature and vinegary character, is primarily derived from the oxidation of acetaldehyde by acetaldehyde dehydrogenases (Remize *et al*. 2000). Pyruvic acid, on

the other hand, is produced as a product of glycolysis, in particular when downstream metabolic pathways, such as the production of ethanol from pyruvate which would otherwise utilise pyruvic acid, are overwhelmed.

Factors that affect the production of some of these organic acids, and in particular of acetic acid, have been extensively studied (Usseglio, 1995; Bisson, 1999; Bely *et al*., 2005; Ferreira *et al*., 2006). Bely *et al*. (2005) noted a sharp increase in acetic acid levels of high sugar containing botrytized wines. Similar findings were also reported for high sugar medium by Ferreira *et al*. (2006). Other acids such as succinic and pyruvic acid have also been found to be influenced by temperature and pH variations in wine (Usseglio, 1995; Bisson, 1999). Torija *et al*. (2003) observed temperature effects on the growth of strains of *S. cerevisiae* in grape must and noted significant increase in succinic and acetic acid as fermentation temperature was increased.

In addition, the influence of different yeast species and strains on organic acid production has also been considered in a few earlier studies: Several authors have reported on the influence of *Saccharomyces*, as well as of some non-*Saccharomyces* yeast, on some organic acids present in wine (Charoenchai *et al*., 1998; Erasmus *et al*., 2004; Pigeau *et al*., 2007; Magyar *et al*., 2014). However, these studies were focusing on individual factors such as yeast strain, pH, sugar or temperature, and did not consider the complexity of the combination of different wine yeast genetic backgrounds combined with differences and changes in environmental parameters as well as the composition of the must. The current study therefore investigates yeast strain dependent differences within the context of different fermentation conditions and environmental parameters.

The primary objective of this study was to investigate the impact of different commercial yeast strains in wine-like conditions under both anaerobic and aerobic conditions. For this purpose, the investigation compared the organic acid profiles produced by five widely used wine yeast strains, EC1118, DV10, VIN13, BM45 and 285 in two different synthetic musts and under different fermentation temperatures. These yeast strains have previously been shown to exhibit different characteristics in terms of fermentation kinetics, stress tolerance as well as the production of volatile aromatic compounds (Rossouw *et al*., 2008, 2009). The two fermentation conditions were selected to reflect relatively extreme wine-like conditions, the first condition corresponding to what may be encountered in a cool climate white wine fermentation (150 g/L of sugar, a low pH of 3, and a cold fermentation temperature of 15 ^{o}C), while the second may closely reflect a warmer climate red wine fermentation (250 g/L of sugar, a pH of 4 and a fermentation temperature of 30 ^{o}C). The widely divergent and relatively extreme nature of these two conditions should allow describing the broader organic acid production space of yeast and of strain-related acid variations in the broader fermentation context.

3.3 Materials and Methods

3.3.1 Strains and culture conditions

The commercial yeast strains as well as some of their relevant characteristics as described by the manufacturers are listed in table 2. Yeast cells were cultivated at 30 ^{o}C in YPD synthetic media [1% yeast extract; (Biolab, South Africa), 2% peptone (Fluka, Germany), 2% dextrose (Sigma, Germany). Solid medium was supplemented with 2% agar (Biolab, South Africa)]. Pre-culture medium was sterilized at 121 ^{o}C for 15 min, maintained at 4 ^{o}C and renewed monthly. Yeast cultures were grown in 50 ml shake-flasks on YPD at 30 ^{o}C and at 250 rpm. These cultures were harvested, washed with sterile distilled water and the cells were inoculated at an OD_{600} of 0.1 (i.e. an initial cell density of approximately 10^6 cfu/ml).

Table 2: Industrial yeast strains information and their fermentative characteristics.

Strain	Commercial Supplier	Characteristics
VIN13	Anchor Yeast	Short lag phase; ferments sugar fully even at low temperature. Low nitrogen requirements
EC1118	Lallemand	It ferments well at low temperatures and flocculates well with very compact lees. Fast fermenter and a killer yeast. Low nitrogen requirements.
BM45	Lallemand	BM45 is a relatively slow starter and is well suited for long maceration programs. It has high nitrogen requirements
DV10	Lallemand	DV10 has strong fermentation kinetics over a wide temperature range and relatively low nitrogen demands. DV10 is famous for its ability to ferment under stressful conditions of low pH, high total SO_2 and low temperature.
285	Lallemand	Ideal for aromatic white and Rosé wines with high alcohol potential, low fermentation temperatures and low nitrogen level requirements.

3.3.2 Fermentation medium

Fermentation experiments were conducted in the defined synthetic must MS300, which resembles a natural must as previously described (Bely *et al.,* 1990). The medium contained equimolar amounts of glucose and fructose at a total of either 150 or 250 g/L (for the 'cool' and 'warm' climate setting respectively), 6 g/L of citric acid, 6 g/L of tartaric acid, 6 g/L of malic acid, 13.09 ml/L of amino acid stock, 1 ml/L of oligoelements, 10 ml/L of vitamin stock solution, 1 ml/L of anaerobic factors, 0.46 g/L of ammonium chloride (120 mg/Lg/L N ammoniacal), 0.75 g/L of potassium dihydrogen phosphate, 0.5 g/L of di-potassium sulphate, 0.25 of magnesium sulphate, 0.2 g/L of calcium chloride and 0.2 g/L of sodium chloride. Amino acids, oligoelements, vitamins and anaerobic factors are listed in the supplementary section (table A1). Temperature and pH were set at 15 and 30 ^{o}C and 3.0 and 4.0, respectively. The initial pH was adjusted with sodium hydroxide. Two sets of fermentations were carried out under aerobic and anaerobic conditions in 250 ml Erlernmeyer flasks (containing 100 ml of the medium). Anaerobic fermentations were sealed with rubber stoppers with a CO_2 opening while under aerobic conditions constant stirring at 200 rpm

was performed during the course of the fermentation. The fermentations were monitored for a period of 14 days. All batch fermentations were carried out in triplicate. The fermentation progress was monitored by daily CO_2 weight loss measurements and samples from the fermentation media were taken at days 2 (exponential phase), 5 (early stationary phase) and 14 (late stationary phase).

3.3.3 Growth measurement

Cell propagation (i.e. growth) was determined spectrophotometrically by measuring the optical density at 600 nm of samples taken from re-suspended cell cultures during the fermentation period.

3.3.4 Experimental design

The environmental conditions were set as indicated in table 3. The five yeast strains (table 2) were all inoculated separately to ferment in the two different synthetic musts (150 g/L of sugar_ pH 3_15 ^{o}C and 250 g/L of sugar_pH 4_30 ^{o}C) shown below, both under aerobic and anaerobic conditions. All fermentations were carried out in triplicate.

Table 3: Experimental design for five wine yeast under anaerobic and aerobic conditions using varying temperature, pH, sugar and yeast physiological stages.

EXPERIMENTAL DESIGN	Sugar	pH	Temperature	Sampling time (Days)
Aerobic/Anaerobic_150_3_15	150	3	15	2/5/14
Aerobic/Anaerobic _250_4_30	250	4	30	2/5/14

3.3.5 Chemical analysis

An improved capillary electrophoresis (CE) method adapted from Soga and Ross (1997) was used to analyse and quantify organic acid contents in synthetic must (MS300). High performance capillary electrophoresis (HPCE) was used with Hewlett-Packard's G1600A HP3DCE system (Agilent Technologies, Waldbronn, Germany), fitted with a built-in photodiode array detector. HP3D Chemstation software was used for system control, data control and data handling. Samples were diluted with a 5 mM morpholino ethanesulphonic acid (MES) buffer (pH = 6.20), which also contained 0.5 mM sodium formate as an internal standard and 10 mg/L sodium azide. The separation electrolyte (pH = 5.60) was filtered through 0.45 micron Nylon membrane filters consisting of 20 mM pyridine-2,6-dicarboxylic acid (PDC), 0.5 mM cetyltrimethylammonium bromide (CTAB) and 20% acetonitrile. Electrophoresis was carried out at 10°C in a fused-silica capillary column with an internal diameter of 50 microns and total length of 91.5 cm (60.0 cm effective length; Agilent Technologies, Germany). Separated occurred at -10 kV. Between each

separation series, the capillary was automatically rinsed with 0.1 mol/L NaOH for 5 min, with water for 2 min and with running buffer for 5 min. Approximately 16 nL of sample was injected into the column by applying pressure (50 mbar) at the inlet side of the capillary. A small amount of separation electrolyte (~2 nL) was injected into the column directly afterwards. The electric potential was ramped from zero to −25 kV within half a minute and then kept constant at −25 kV for the remainder of each run. Absorbance detection at 210 nm was used throughout the experiments. The sample injection was set to a constant value of ~2 nL (hydrodynamic injection at 0.5 psi for 5 s). The residual sugars and ethanol were analysed by using FTIR spectrometry (FOSS wine scan) as described by Nieuwoudt *et al*. (2006).

3.3.6 Data analysis

3.3.6.1 Multivariate data analyses

The trends within various sets of data were investigated by principal-component analysis (PCA; Latentix 2.0, BRANDON GRAY INTERNET SERVICES, INC. DBA). By plotting the principal components it is possible to analyse statistical relationships linking different variables in complex datasets, identify and deduce sample groupings, similarities or differences, as well as the associations among the different variables (Mardia *et al*., 1979). The PCA data was transformed using the auto scale function and the PCA models were calculated. Based on the experimental design, the samples represent the different fermentations (three independent replicates for each of the five strains) at different time points. The trends and variables considered are as the result of changes in organic acid contents in the MS00 by wine yeast strains subjected to different environmental conditions as well as the inter-strain and intra-strain dependent differences between different yeast strains and different time point (2, 5 and 14 days).

3.4 Results and Discussion

3.4.1 Fermentation profile of strains subjected to warm climate “red” wine and cool climate “white” wine fermentation conditions

In all conditions, yeast growth and fermentation profile of all of the strains followed similar trends, with some minor differences in the absolute values of certain parameters between strains. Changes in environmental conditions impacted in similar ways on the growth and fermentation rates of the different strains. Aerobic fermentations of all strains reached the stationary growth phase earlier and completed fermentation faster than the anaerobic fermentations (fig 1). This is not surprising; although *S. cerevisiae* is one of the few yeast species which grows well under anaerobic conditions, oxygen availability (particularly in the early stages of fermentation) supports mixed respiro-fermentative metabolism leading to increased fermentation rate and higher production of other secondary metabolites (Rigoulet *et al*., 2004).

Ethanol production as well as glucose and fructose utilisation of BM45, DV10, EC1118, 285 and VIN13 at different initial sugars (Supplementary fig A1), pH (Supplementary fig A2) and fermentation temperature (Supplementary fig A3) was further analysed. In all likelihood the high initial sugar content (250 g/L) of the “red wine” setting and the low temperature and pH of the “white wine” setting were responsible for fermentations with slightly higher residual sugars. Nevertheless, most fermentations reached the official dryness threshold of below 5 g/L of residual sugar. Ethanol production, as expected, correlated to the initial sugar concentration (Supplementary fig A1).

Overall, VIN13 fermentations presented the lowest levels of residual sugars (glucose and/or fructose) while inoculation of BM45 generally resulted in the highest amounts (see supplementary fig A1 –A5). As is usually observed with *S. cerevisiae*, fructose was the major contributing hexose to residual sugar levels, but conditions clearly impacted on the levels of glucose and fructose. However, no general trends could be observed regarding strains and/or environmental conditions with regard to this parameter.

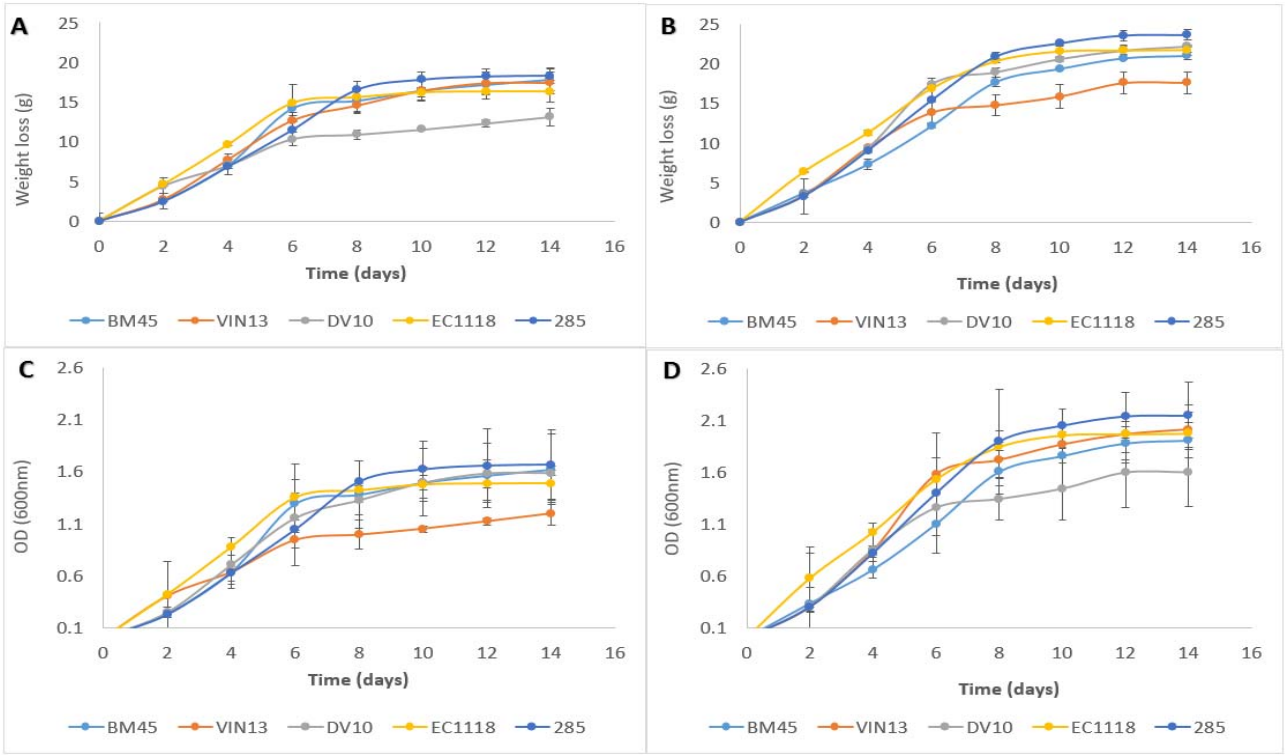

Figure 1: Fermentation profile of wine yeast under “red wine” settings. Anaerobic fermentation rates (frame A), aerobic fermentation rates (frame B), anaerobic growth rates (frame C) and aerobic growth rates (frame D). Results are the average of three biological repeats ± standard deviations.

3.4.2 The influence of yeast strains on grape-derived acid production

Organic acid analysis was carried out at three different time points for the “red” and “white” wine fermentations inoculated with the five different wine yeast strains. Concentrations of acids and changes to the major grape derived acids such as tartaric, malic and citric acid were evaluated. In general, a slight decrease in the overall concentration of the grape acids was observed (fig. 2A and B). The concentration of all three grape acids diminished by approximately 10 - 20% of the initial amounts under both aerobic and anaerobic conditions. Under aerobic conditions, differences between strains (fig 2B) were less significant than under anaerobic conditions. While there were no significant differences in grape derived acids between most of the strains under anaerobic conditions, DV10 fermentations resulted in significantly reduced levels of all three acids at the end of fermentation (fig 2A). Although precipitation is a main cause of tartaric acid reduction in some young bottled wines, we did not observe any precipitate in any of the wines based on visual inspection. There is also no evidence that yeast strains of *Saccharomyces* can effectively transport or degrade any of these acids. But for DV10, the observed differences between the strains are statistically insignificant, and there were no major changes between these acids.

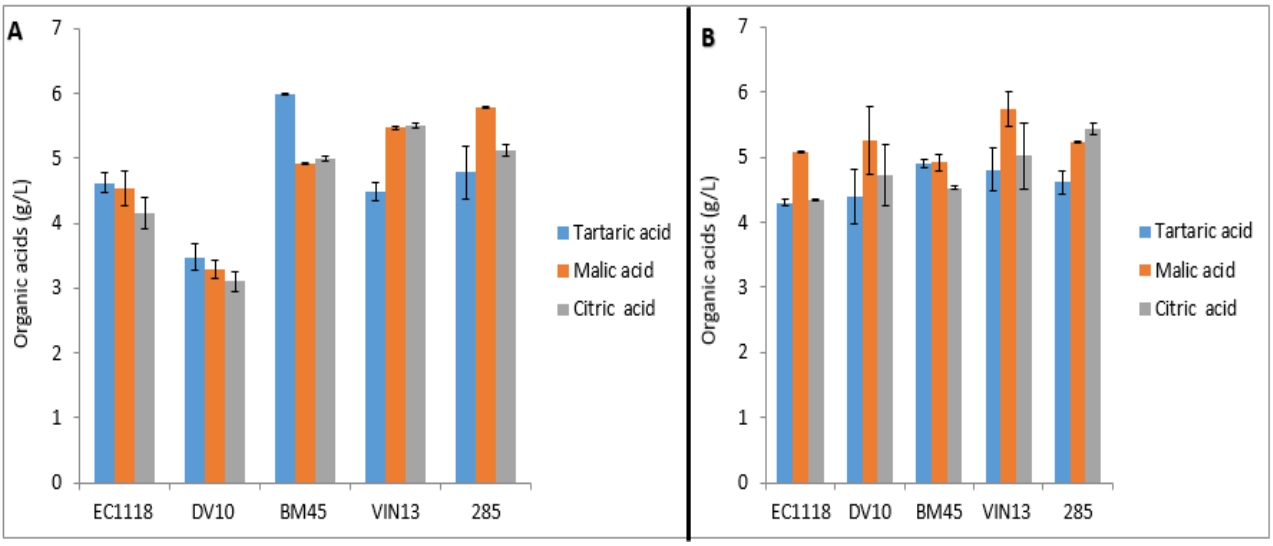

Figure 2: Grape derived acid production by different yeast strains under the “red wine” setting at the end of fermentation under anaerobic (frame A) and aerobic (frame B) conditions. Results are the average of three biological repeats ± standard deviation.

3.4.3 The impact of yeast strain on organic acid production under different winemaking conditions

3.4.3.1 Succinic acid

Succinic acid increased throughout fermentation for all strains and all environmental conditions, but significant differences were observed between strains in terms of the actual concentrations of

succinic acid produced. As an example, figure 3 shows succinic acid accumulation at days 2, 5 and 14 for strain EC1118 in both white (fig 3A) and red (fig 3B) wine settings. Similar succinic acid accumulation trends were also observed for other strains under similar conditions (data not shown). The increase in succinic acid levels during fermentation has been reported previously (Peynaud and Blouin, 1996; Ribéreau-Gayon *et al*., 2006), however little information exists regarding how different strains of yeast influence succinic acid accumulation in wine.

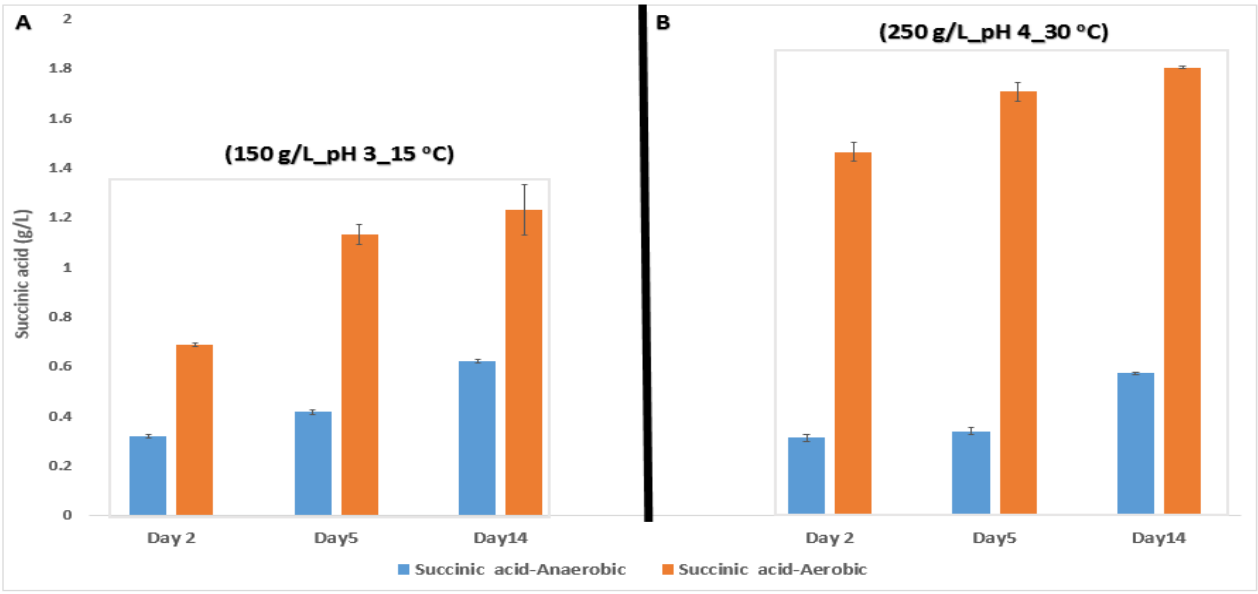

Figure 3: Succinic acid production by EC1118 at different fermentation stages (days 2, 5 and 14) under anaerobic (blue bars) and aerobic (orange bars) conditions. The graph shows succinic acid concentrations at three time points under white wine (A) and red wine (B) fermentation settings. Results are the average of three biological repeats ± standard deviations.

Strain genetic background showed a strong impact on succinic acid production. In anaerobic red wine conditions, VIN13 produced higher succinic acid levels while other strains such as DV10 produced relatively lower succinic acid levels than the rest by the end of fermentation (fig 4A). Similar differences were also observed between these two strains in the white wine anaerobic settings (fig 4A) at this and the other time-points of fermentation (days 2 and 5; Supplementary fig A5). The three other strains, BM45, 285 and EC1118 showed intermediate levels of this acid (fig 4A).

Compared to anaerobic conditions, succinic acid levels were significantly higher under aerobic conditions at all time-points in both red and white fermentation conditions (fig 3). This observation is in line with expectations since it has been reported that organic acid levels in wine will generally be significantly augmented under aerobic conditions compared to anaerobic conditions (Wiebel *et al*., 2008). The increased succinic acid concentration has previously been linked to the shift in

central carbon metabolism of yeast from fermentative to respiratory (Larsson *et al.,* 1993; Coulter *et al*. 2004).

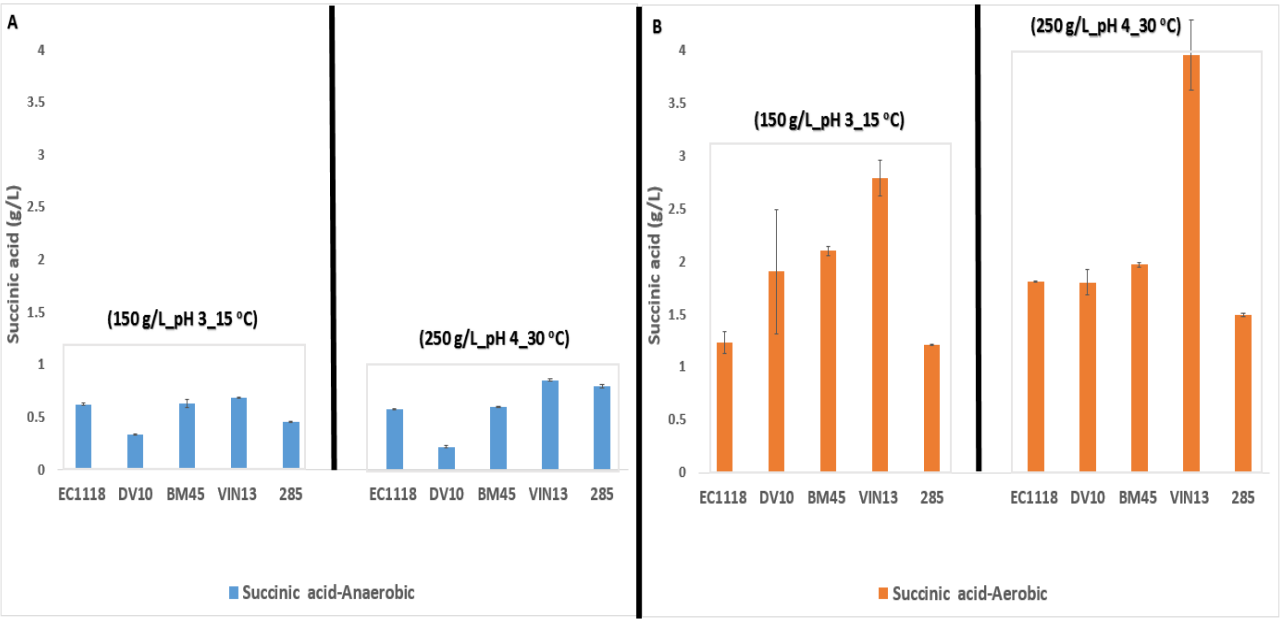

Figure 4: Succinic acid concentrations at the end of fermentaion for five yeast strains in white wine (150 g/L of sugar_pH 3_15 ^{o}C) and red wine (250 g/L of sugar_pH 4_30 ^{o}C) fermentation settings under aerobic (A) and anaerobic fermentation conditions (B). Results are the average of three biological repeats ± standard deviations.

Under aerobic conditions VIN remained the highest producer of succinic acid under both the white and red wine conditions (fig 4B). The levels of succinic acid produced by the other four strains for both fermentation settings under aerobic conditions were more or less similar at the end of fermentation (fig 4B).

A significant increase in the level of succinic acid was previously reported when EC1118 was grown under aeration, carbon-sufficient and nitrogen-limited oenological conditions (Aceituno *et al*., 2012). Although higher succinic acid levels of all strains were observed under aerobic compared to anaerobic conditions in the current study, strain EC1118 produced relatively lower succinic acid when compared to some of the other strains such as VIN13 at the end of fermentation (fig 4B).

DV10 was the lowest producer of succinic acid under anaerobic conditions in both the white and red wine settings (fig 4A) while 285 was the lowest producer of succinic acid under aerobic conditions in both these settings (fig 4B) compared to the other strains. Strain DV10 and 285 also behaved similarly at days 2 and 5 of fermentation under both white and red wine settings (Supplementary fig A5 and A6). It is known that DV10 is more tolerant to low pH wines, and it is a preferred yeast for Champagne base wine. The ability of DV10 to reduce overall acidity might therefore be a specific adaptation for such conditions.

VIN13 and 285 were the most prolific in terms of succinic acid production, particularly in the red wine setting (under anaerobic conditions for 285 and under both aerobic and anaerobic conditions in the case of VIN13).

3.4.3.2 Acetic acid

All strains behaved similarly with regard to acetic acid production at the different time points throughout fermentation. Figure 5 shows a representative dataset for one of the strains, namely EC1118. Acetic acid levels in all fermentations increased rapidly at the beginning of fermentation. At the later time points, aerobic and anaerobic fermentations showed divergent patterns: Under aerobic conditions, a continuous decrease throughout fermentation was observed in the case of the white wine setting (150 g/L_pH 3_15 ^{o}C), whereas a continuous increase in acetic acid was noted in the red wine setting (250 g/L_pH 4_30 ^{o}C) (fig 5B). In contrast, the levels of this acid remained more or less the same across all time points in both white and red anaerobic conditions (fig 5).

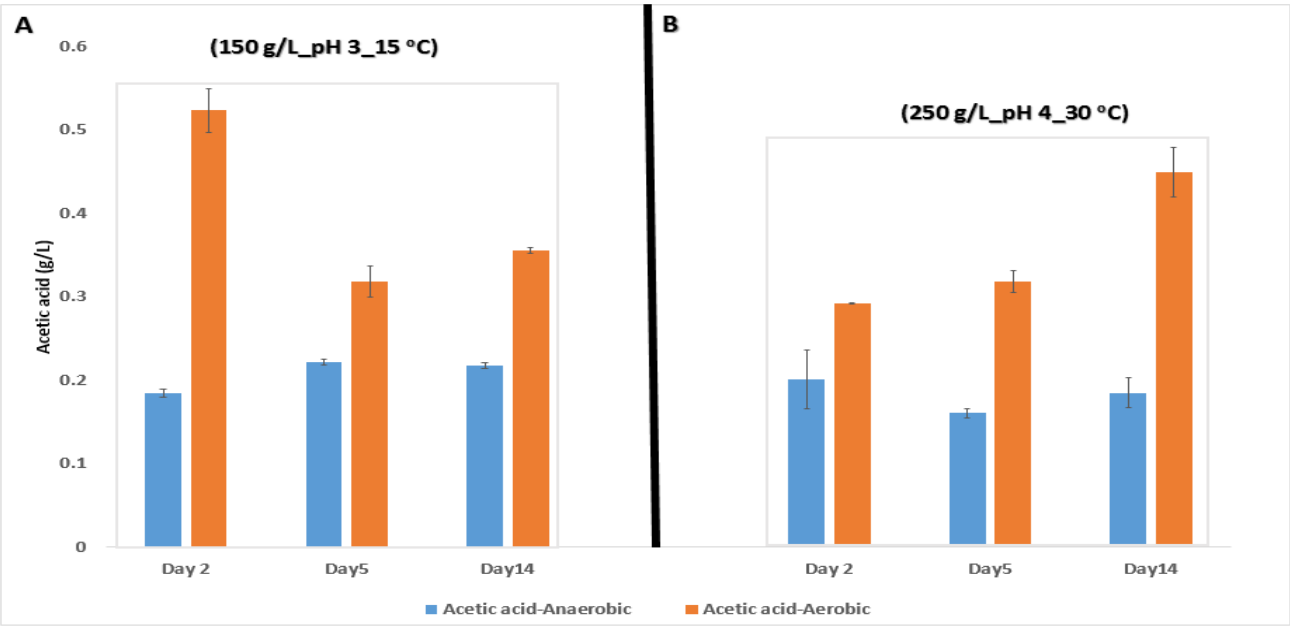

Figure 5: Acetic acid production by EC1118 at different physiological and fermentation stages (day 2, 5 and 14) under anaerobic (blue bars) and aerobic (orange bars) conditions. The graph shows succinic acid variations over-time in white wine (A) and red wine (B) fermentation conditions. Results are the average of three biological repeats ± standard deviations.

Strain genetic background strongly impacted on acetic acid production: Under anaerobic red wine conditions, strain 285 produced relatively higher acetic acid levels (up to 0.4 g/L) compared to other strains such as DV10 (as low as 0.136 g/L; fig 6B). Similar strain behaviour was also observed at the exponential phase (day 2) and early stationery phase (day 5), highlighting the

strong impact of the selection of yeast strains on acetic acid production, even at different fermentative stages (Supplementary fig A4). On the other hand, fermentations carried out in the white wine setting (under anaerobic conditions) showed no significant variations in acetic acid levels among strains, with the exception of strain DV10 which produced extremely low levels of acetic acid (fig 6A).

Under aerobic conditions, acetic acid concentrations were the highest (up to 0.49 g/L) in fermentations carried out with EC1118 and 285 in the red wine setting. For this treatment the other three strains produced similar levels of acetic acid (fig 6B). The same trends were observed at days 2 and 5 (supplementary fig A4). Compared to all other strains, DV10 fermentations resulted in the lowest acetic acid levels in both white (fig 6A) and red (fig 6B) wine settings under anaerobic conditions, however under aerobic conditions, acetic acid levels were similar for DV10, EC1118, VIN13 and 285 in the white wine setting. In this case, BM45 produced the lowest levels of acetic acid. Although, the influence of strain variability on acetic acid production under wine making conditions has not been fully elucidated, the current study supports the findings of previous work which has shown remarkable variations in acetic acid levels among 20 *Saccharomyces cerevisiae* strains tested in wine (Romano *et al*., 2003). Our data also highlights the important point that, while certain strains could generally be described as lower acetic acid producers, this phenotype can be modulated by the prevailing fermentation conditions.

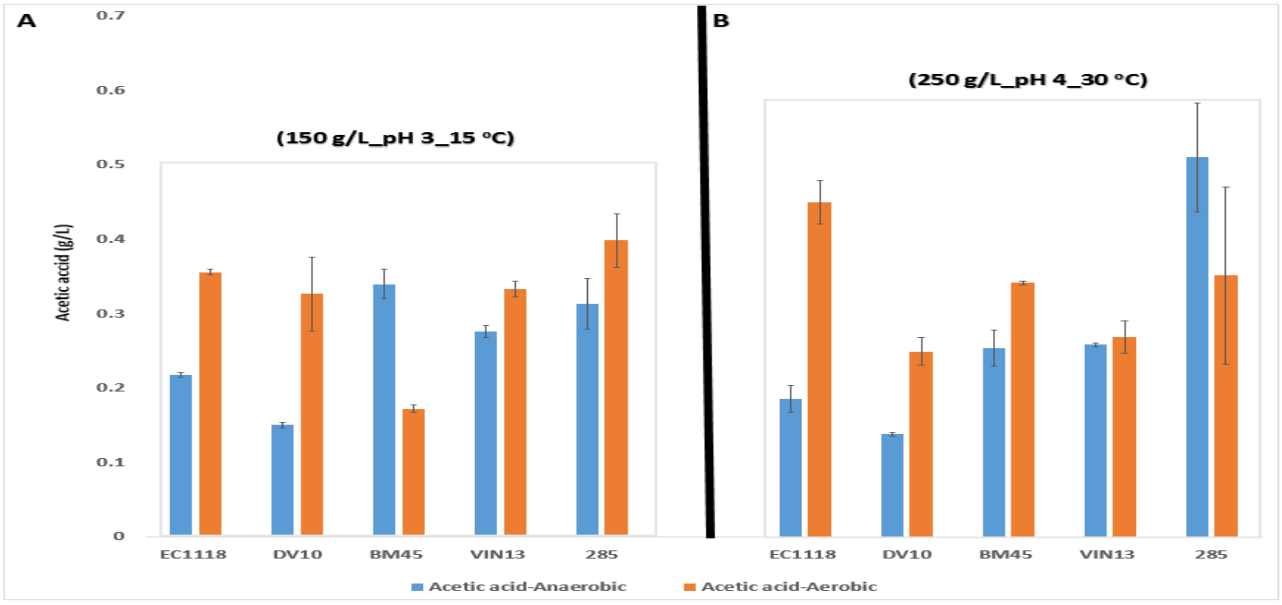

Figure 6: Acetic acid levels at the end of fermentation for five different yeast strains in white wine (A) and red wine (B) fermentation settings. Both aerobic (orange bars) and anaerobic (blue bars) conditions were analysed. Results are the average of three biological repeats ± standard deviations.

3.4.3.3 Pyruvic acid

With regards to pyruvic acid production across time points (days 2, 5 and 14) most strains behaved similarly with regards to overall production trends for this acid. Figure 7 shows a representative dataset for strain EC1118. Pyruvic acid was produced early during fermentation, and slowly re-absorbed in the white wine anaerobic setting (fig 7A). Similar observations have been reported elsewhere under wine making conditions (Ribéreau *et al*., 2006). However the opposite trend was observed in the case of the red wine fermentation setting: Here, pyruvic acid levels were initially lower in the exponential phase but increased throughout fermentation (fig 7A). Under aerobic conditions the trends were very different: In the white wine setting pyruvic acid levels were initially low for the exponential phase measurements, then increased towards the early stationary phase, before decreasing again (possibly due to reabsorption) by the late stationary phase (fig 7B). However, in the red wine aerobic setting, pyruvic acid was produced early during fermentation and slowly reabsorbed as fermentation proceeded (similar to the white wine setting under anaerobic conditions).

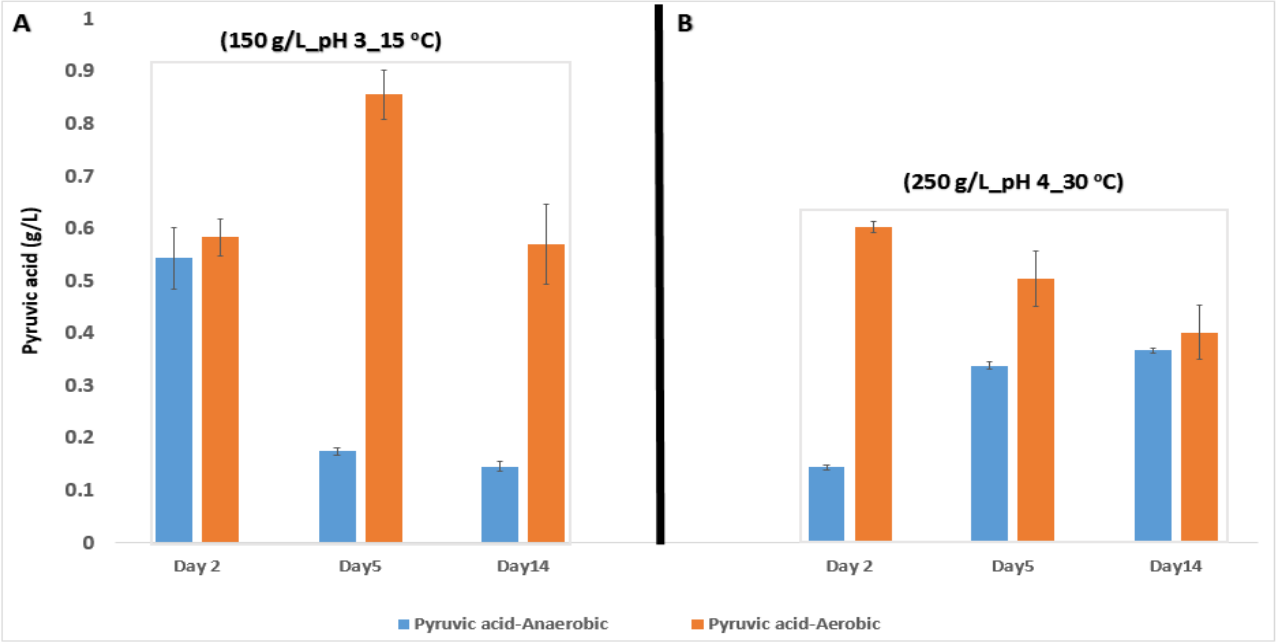

Figure 7: Pyruvic acid concentrations of EC1118 inoculated fermentations at different fermentation stages (day 2, 5 and 14) under anaerobic (blue bars) and aerobic (orange bars) conditions. The graph shows pyruvic acid variations over time in simulated white wine (150 g/L, pH 3, 15 ^{o}C) and red wine (250 g/L, pH 4, 30 ^{o}C) fermentation conditions. Results are the average of three biological repeats ± standard deviations.

The impact of yeast strain identity on pyruvic acid production was evident: There were no major significant differences among strains at day 5 (the early stationary phase) with regard to pyruvic acid concentrations when red wine aerobic settings were evaluated. However, VIN13 and 285 strain produced higher concentrations of pyruvic acid in white wine settings under similar

conditions (Supplementary fig A7). A different profile was however noted at day2 (the exponential phase) since significant strain differences were evident under all conditions tested. Here, strains BM45 and VIN13 produced consistently low pyruvic acid levels in red wine settings compared to the other three strains under aerobic conditions (Supplementary fig A7). Similar strain behaviour was also seen in white wine aerobic settings, where pyruvic acid levels at exponential phase were highest for strains EC1118, 285 and DV10 both the red and white wine aerobic fermentations (Supplementary fig A7). BM45 and VIN13 produced lower pyruvic acid levels under these conditions.

A completely different pattern was observed when the yeast strains were subjected to anaerobic fermentation conditions, as significant strain dependent differences in pyruvic acid production were observed (Supplementary fig A7). Fermentations at exponential phase in the white wine conditions showed an increase in pyruvic acid production/release by EC1118 while for strain 285 the levels of pyruvic acid were below the detection threshold. At this stage there were no significant strain dependent differences in the red wine setting. However, by the early stationary phase there were significant differences in the red wine conditions as fermentations conducted by EC1118, VIN13 and 285 showed significantly higher pyruvic acid concentrations compared to others. In contrast, pyruvic acid levels in the white wine fermentation were extremely low for all five strains (Supplementary fig A7).

Under aerobic conditions, strain VIN13 produced higher pyruvic acid levels (up to 0.7 g/L) by the end of fermentation while strain DV10, BM45 and 285 produced the lowest levels in white wine simulated conditions (fig 8A). These trends are very different from those of the red wine conditions, where VIN13 was one of the lower pyruvic acid producers compared to all other strains (fig 8B).

Anaerobic fermentations conducted by DV10 resulted in a relatively low pyruvic acid levels in both white and red wine settings while strain 285 resulted in undetectable levels of pyruvic acid in the white wine setting (fig 8A). EC1118, VIN13 and 285 were the highest producers of pyruvic acid in the red wine setting (fig 8B). This highlights the impact of fermentation conditions in terms of changing (exacerbating, eliminating or completely reversing) inter-strain trends in organic acid production.

Large variations in pyruvic acid production in wine (especially under anaerobic conditions) have been reported previously and the predicted range of this acid falls within a wide range of 10 - 500 mg/L (Usseglio, 1995). The factors responsible for these large variations in pyruvic acid production have however not received much scientific attention. Our data suggests a strong influence of (i) strain variability, (ii) stage of fermentation and (iii) environmental and chemical conditions during fermentation.

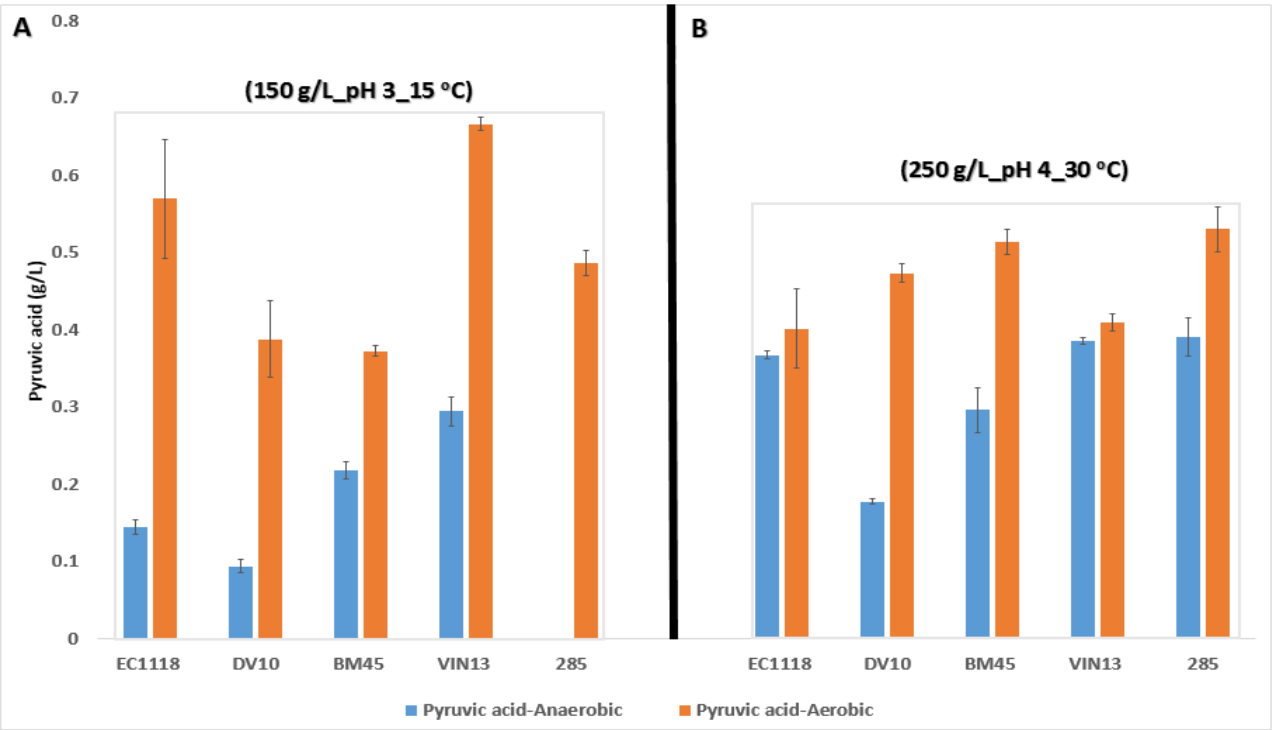

Figure 8: Pyruvic acid levels at the end of fermentaion for five yeast strains in white wine (A) and red wine (B) fermentation setting conditions. Both aerobic (orange bars) and anaerobic (blue bars) fermentation conditions were analysed. Results are the average of three biological repeats ± standard deviation.

3.4.4 Trends in organic acid production by different yeast strains

For the purpose of this study, principal component analysis (PCA) was conducted to generate and overview of treatment effects on sample groupings. Only data generated under anaerobic conditions are shown here as this condition is more relevant from a winemaking perspective and the aerobic PCAs did not contribute additional insights on strain-specific behaviour.

The PCA clearly demonstrates the impact of yeast strain on the overall organic acid profile, which applies to all three stages of fermentation considered in our study. The PCA was performed using organic acid data (succinic, pyruvic and acetic acid) for the three different fermentation timepoints (Supplementary figs A8-10).

Fig 9 illustrates the influence of individual yeast strains on organic acid profiles at the exponential phase (day 2) in the white wine –like fermentation conditions. The first two principle components together accounted for 95.4% of the total explained variance. Replicates clustered well, indicative of good sample reproducibilty. Samples were clearly separated in strain-specific clusters, with some strains such as BM45 and VIN13 exhibiting similar organic acid profiles at this timepoint (fig 9). Separation of samples along the first principal component axis was primarily driven by

differences in acetic and succinic acid production. Similar groupings were also evident in the PCA analysis of data generate in the red wine setting (Supplementary fig A8).

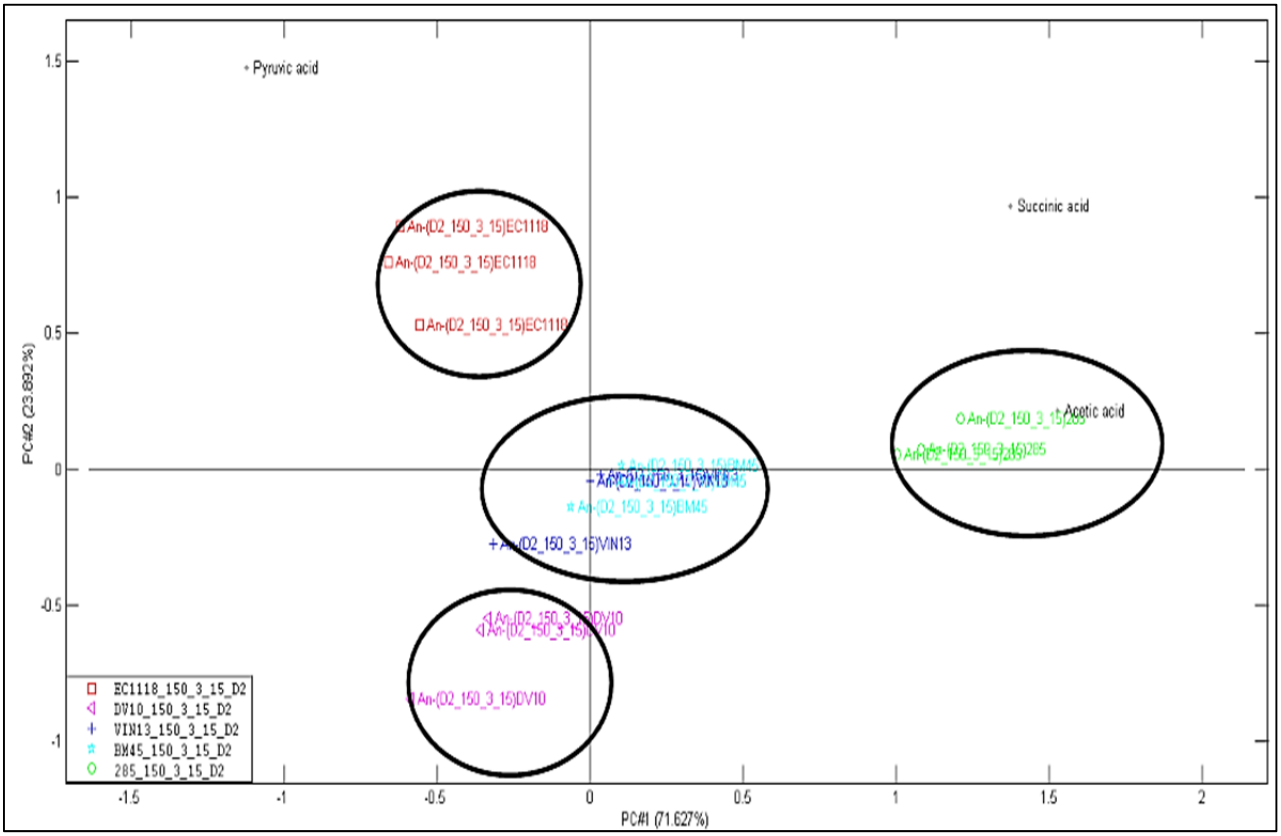

Figure 9: PCA bi-plot showing sample groupings for triplicate fermentations of strains DV10 (pink-dataset), BM45 (sky blue), VIN13 (blue), 285 (green) and EC1118 (red) at day 2 (D2) of fermentation. Fermentation were carried out under anaerobic white wine -like conditions (150 g/L, pH 3 and 15 ^{o}C).

The PCA in figure 10 accounts for 96.1% of total explained variance in the dataset. This analysis highlights the differences in interstrain organic acid profiles at the early stationary phase. Once again, replicates clustered well along both principal components, indicative of good sample reproducibilty. The main drivers of sample separation along the first principal component axis was succinic acid, while pyruvic acid, and to a lesser extent acetic acid, were associated with variation along the second principal component axis. At this stage of fermentation, strains BM45 and 285 were most similar in terms of their total organic acid profiles, while DV10 was the most dissimilar to the rest of the strains in the white wine setting fermentations. Similar groupings were observed for the red wine –like conditions (Supplementary fig A9).

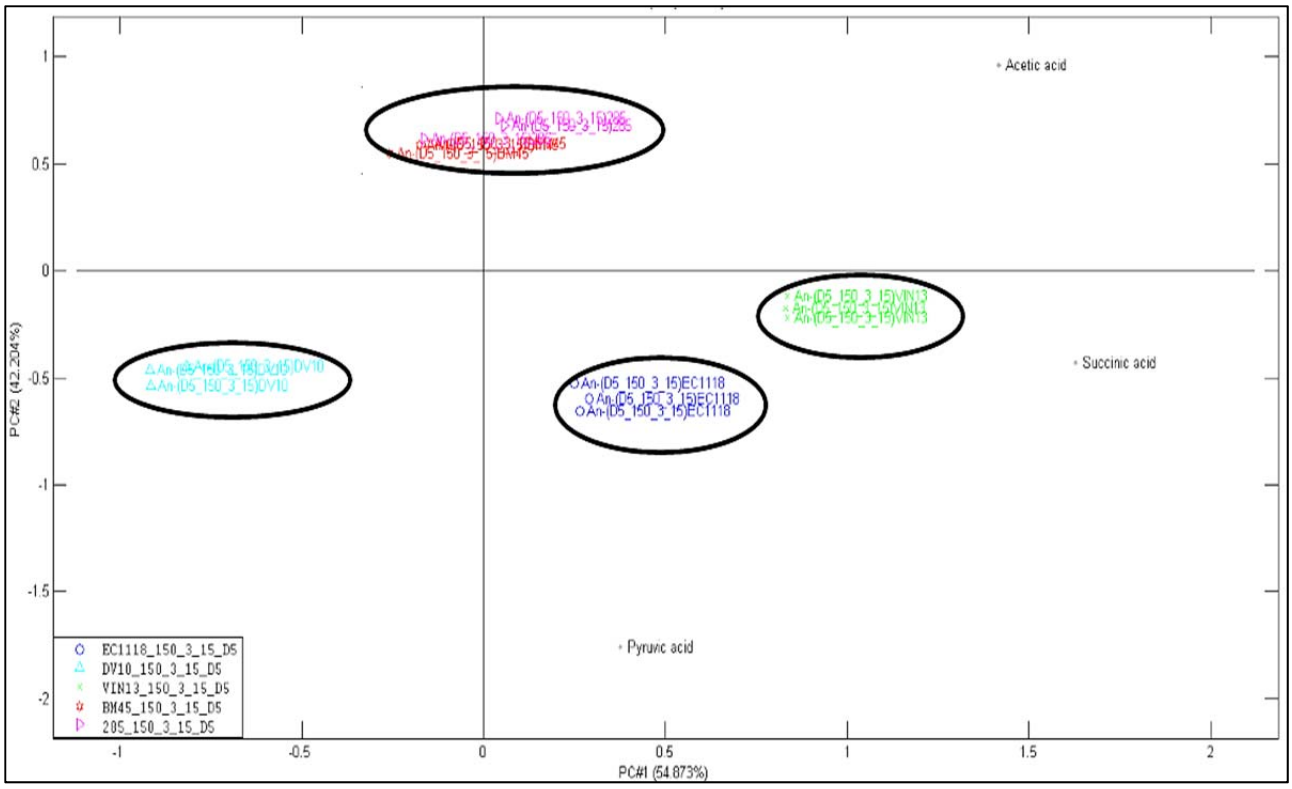

Figure 10: PCA bi-plot showing sample groupings for triplicate fermentations of strains DV10 (sky blue-dataset), BM45 (red), VIN13 (green), 285 (pink) *and* EC1118 (blue) at day 5 (D5) of fermentation. Fermentations were carried out in anaerobic white wine –like conditions (150 g/L, pH 3 and 15 ^{o}C).

Though changes in organic acid levels do occur throughout fermentation, winemakers are more concerned with the impact of a yeast strain on the final concentration of acids in the wine. In light of the importance of end point organic acid profiles, a PCA describing the influence of different strains on the late stationary phase organic acid composition is also shown (fig 11). Once again clear strain-specific groupings are evident. At the end of fermentation (in the simulated white wine under anaerobic conditions) the overal acid profiles of VIN13 and EC1118 are most similar, with strain BM45 showing the most dissimilar organic acid profile compared to the other strains. A different strain cluster arrangement was, however observed in the red wine setting (Supplementary fig A10). Here the overall acid profiles of strains 285 and VIN13 were most similar with BM45 again showing the most dissimilar acid profile. The main driver for separation of samples along the first principal component was acetic and pyruvic acid, and succinic acid for the second component.

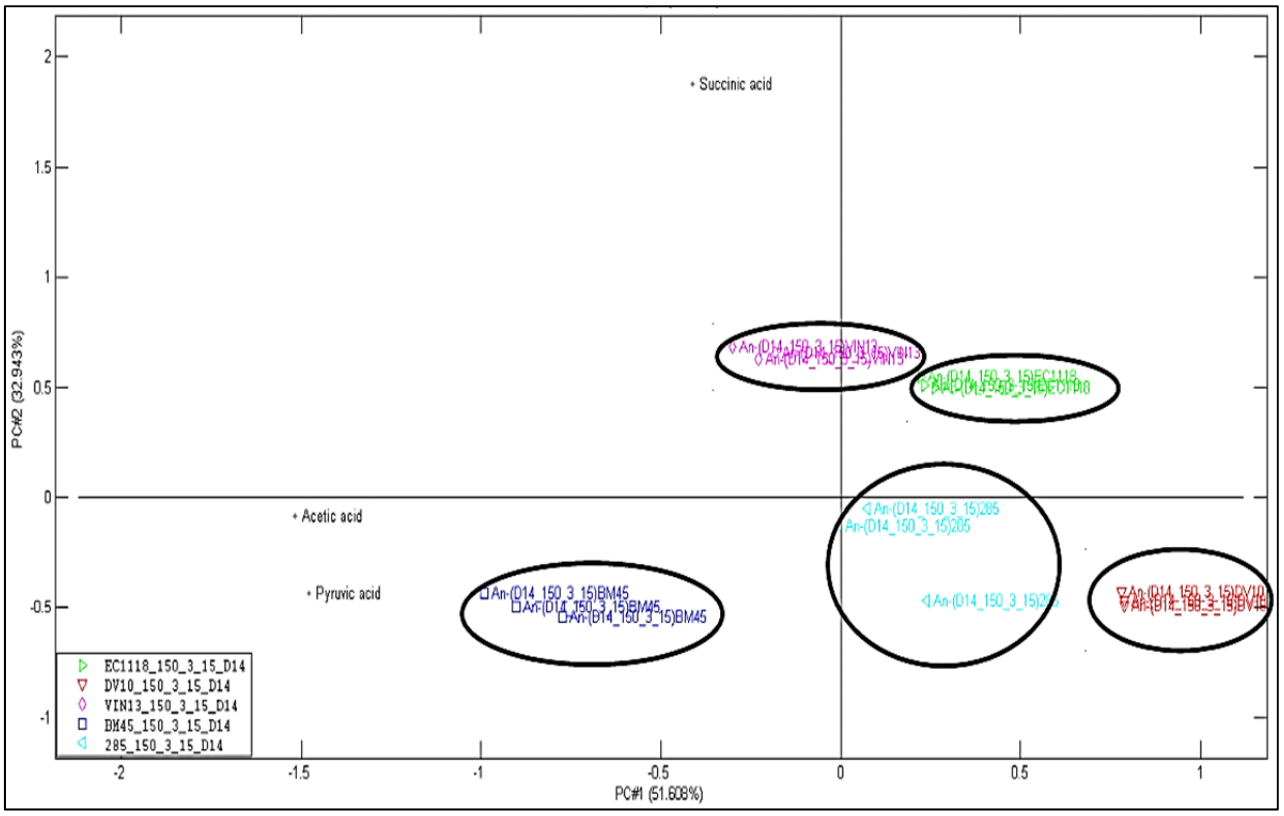

Figure 11: PCA bi-plot showing sample groupings for triplicate fermentations of strains DV10 (red-dataset), BM45 (blue), VIN13 (pink), 285 (sky blue) *and* EC1118 (green) at day 14 (D14) of fermentation. Fermentations were carried out under anaerobic white wine –like conditions (150 g/L, pH 3 and 15 ^{o}C).

3.5 Conclusion

The current study evaluated five commercially available wine yeast strains (subjected to two very different fermentation conditions) with regards to their strain-specific influence on both grape- and fermentation- derived organic acids at different time points throughout fermentation. The study explored inter-strain differences in acid production/release at different physiological stages of growth (different time-points throughout fermentation) and under very different, yet possible wine making conditions which could be encountered in industry. The strains displayed minor differences in their fermentation profiles but in many cases significant differences in organic acid production were observed. Only extracellular organic acids were measured (obtained from the fermentation medium). It should be noted that these acids may not necessarily represent the *in vivo* metabolic production of these acids. Indeed, these organic acids are primarily intermediates of central metabolic pathways, and most are involved in several different metabolic reactions and may be produced from different precursors. This may explain the somewhat divergent results obtained here when comparing different strains.

While many other known and unknown factors (apart from yeast and environmental factors) may significantly impact on the organic acid levels of wines, the particular focus of the current study was

the influence of strain identity on organic acid production. Our data highlight the fact that acid production characteristics of strains are not embedded phenotypes (i.e. consistent irrespective of conditions). Clearly the acid evolution of a given strain, while displaying certain trends, is conditioned by the interplay of various physico-chemical parameters. Differences between strains in our study were moderated, or strongly influenced by changes to the prevailing fermentation conditions, including changes to the pH, fermentation temperature, initial sugar concentration and aeration.

Furthermore this study provides novel information which could be incorporated into practical guidelines for winemakers seeking to manage wine organic acid profiles through appropriate strain selection. Our results identified higher, moderate and lower producers of specific organic acids in both white and red wine fermentation settings. The current study will therefore assist wine makers to make informed decisions relating to strains that will improve the management of acidity and the organoleptic properties of their wines.

Although there are many other factors which may impact on acid production during fermentation, the current study highlights strain-dependent impacts on organic acid production/release. The data presented here is the first to focus on the influence of yeast strain identity on organic acid production within a multi-condition framework.

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Appendix A

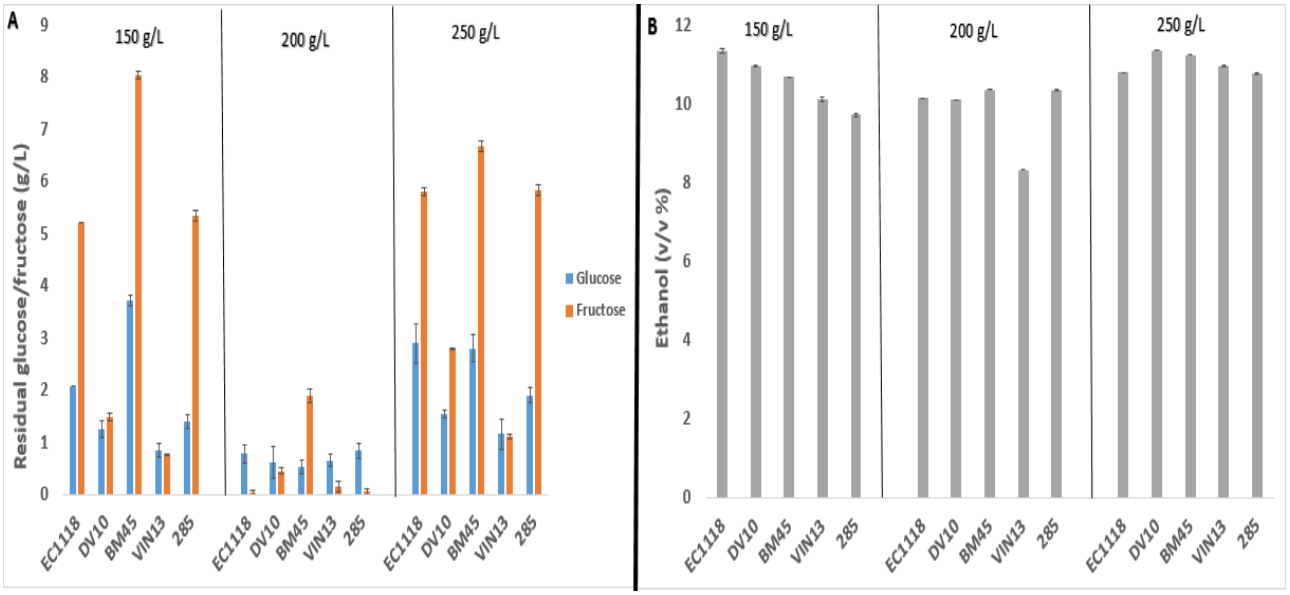

Figure A1. The influence of different initial must sugar contents on glucose/fructose utilisation (A) and ethanol production (B) of BM45, EC1118, VIN13, DV10 and 285 at the end of fermentation. (The initial fermentation temperature and pH of the must were kept constant at 30 ^{o}C and pH 4.0, respectively).

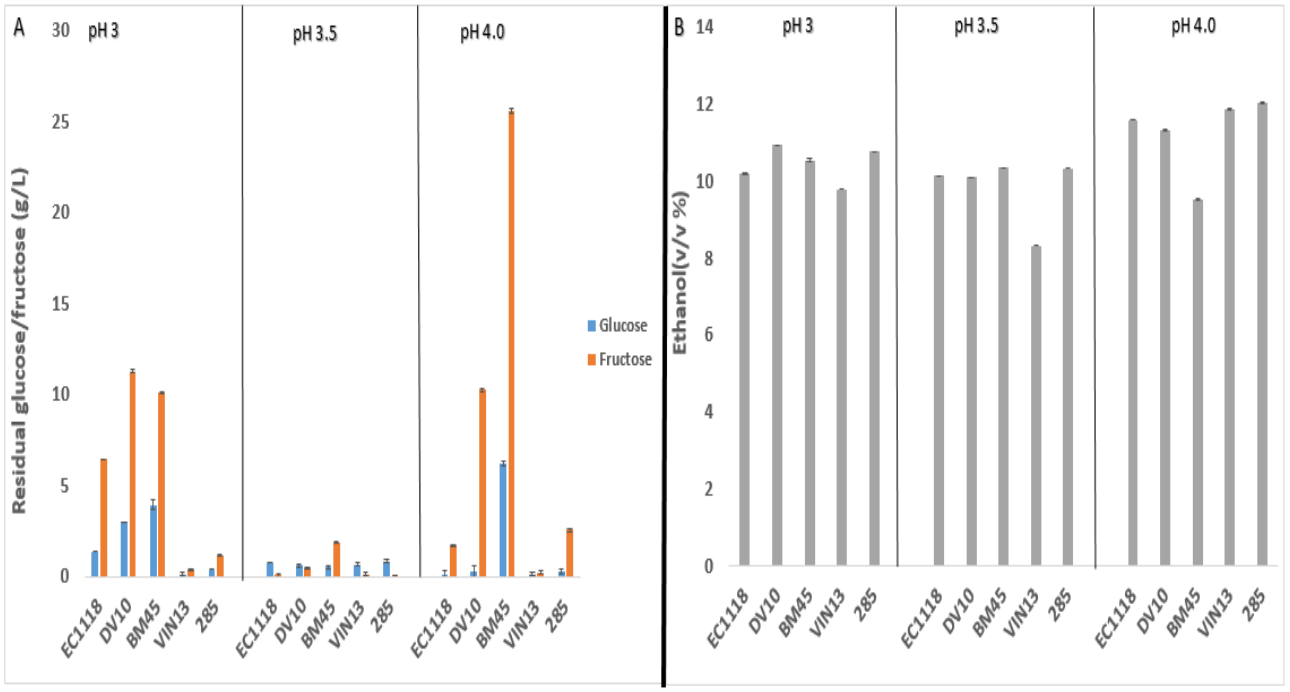

Figure A2. The influence of different initial must pH on glucose/fructose utilisation (A) and ethanol production (B) of BM45, EC1118, VIN13, DV10 and 285 at the end of fermentation. (The initial fermentation temperature and sugar content of the must were kept constant at 30 ^{o}C and 250 g/L).

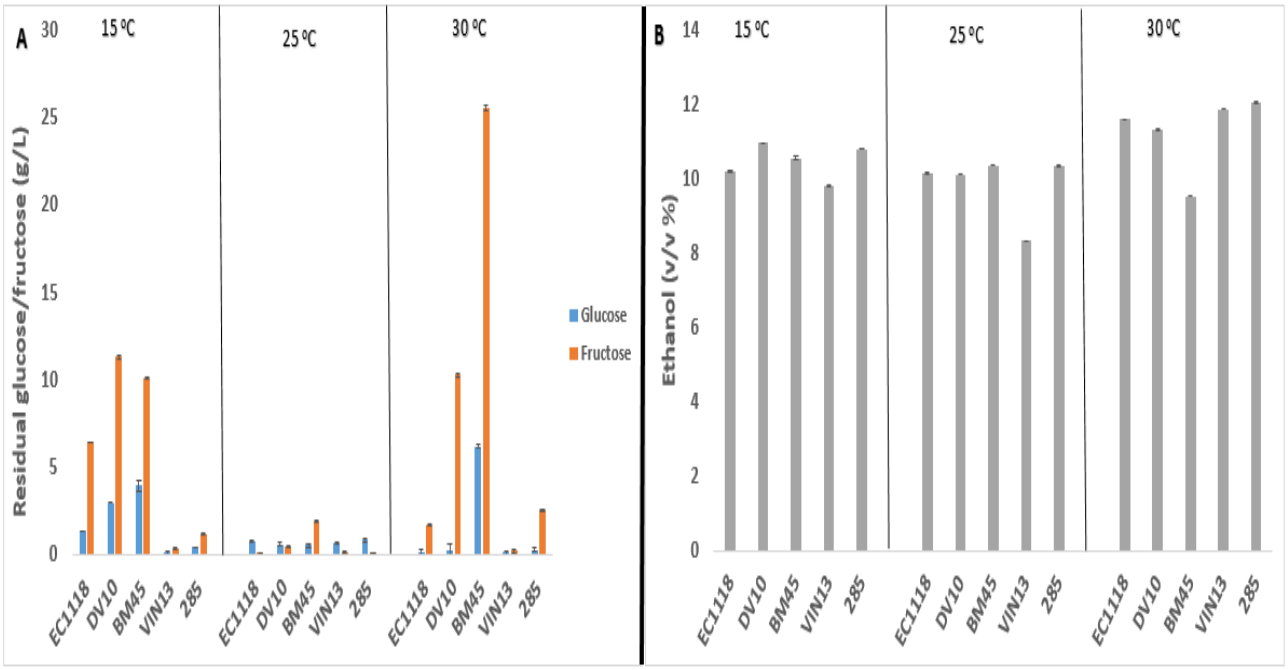

Figure A3. The influence of different fermentation temperatures on glucose/fructose utilisation (A) and ethanol production (B) of BM45, EC1118, VIN13, DV10 and 285 at the end of fermentation. (The initial pH and sugar content were kept constant at pH 4.0 and 250 g/L, respectively).

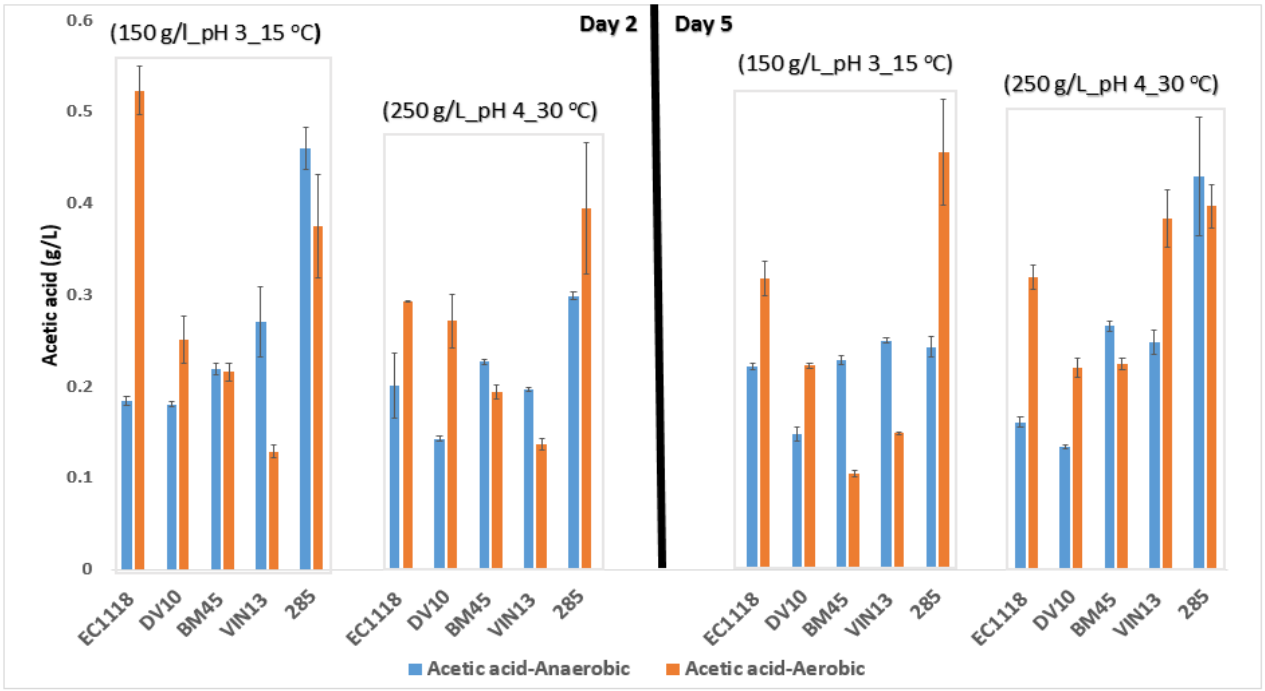

Figure A4. Acetic acid profiles for five yeast strains at different time points (day 2 and 5) under white wine (150 g/L, pH 3 and 15 °C) and red wine (250 g/L, pH 4 and 30 °C) fermentation conditions, both aerobic and anaerobic. Results are the average of three biological repeats ± standard deviations

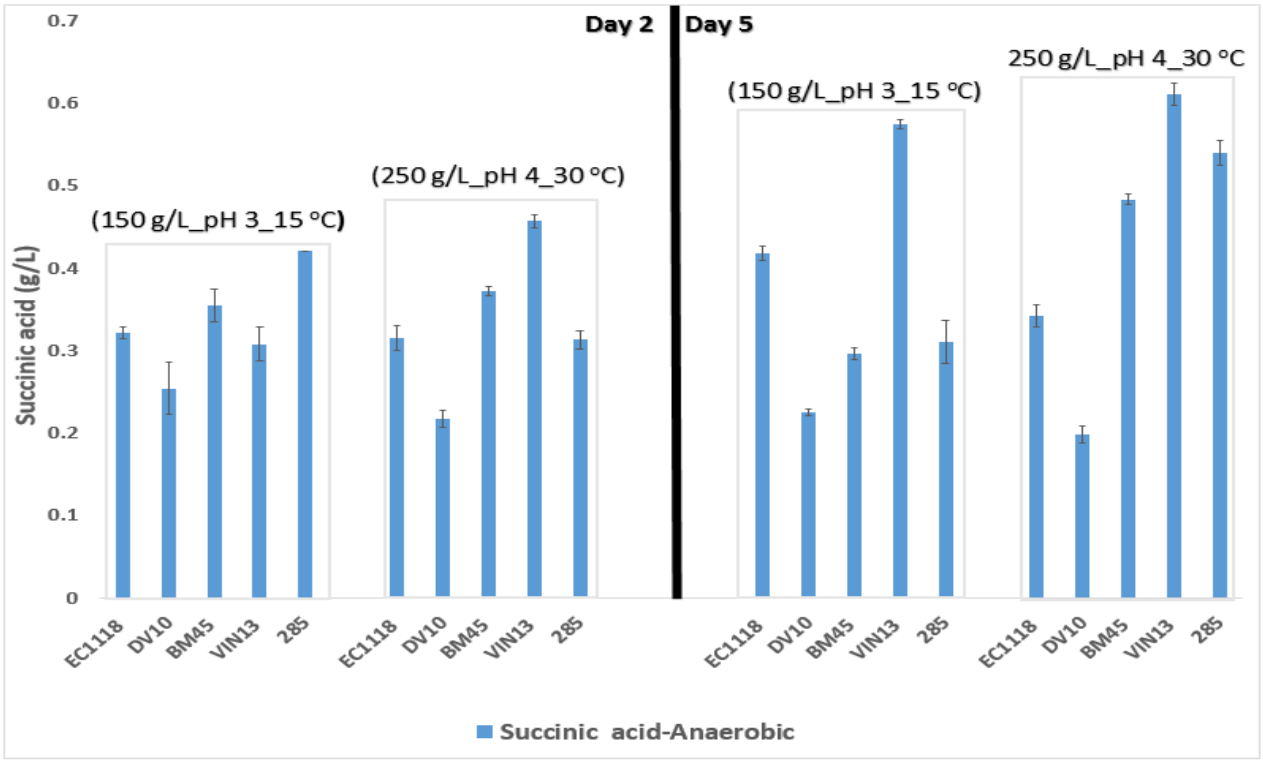

Figure A5. Succinic acid profiles of five yeast strains at different time points (day 2 and 5) under white wine (150 g/L, pH 3 and 15 °C) and red wine (250 g/L, pH 4 and 30 °C) anaerobic fermentation conditions. Results are the average of three biological repeats ± standard deviations.

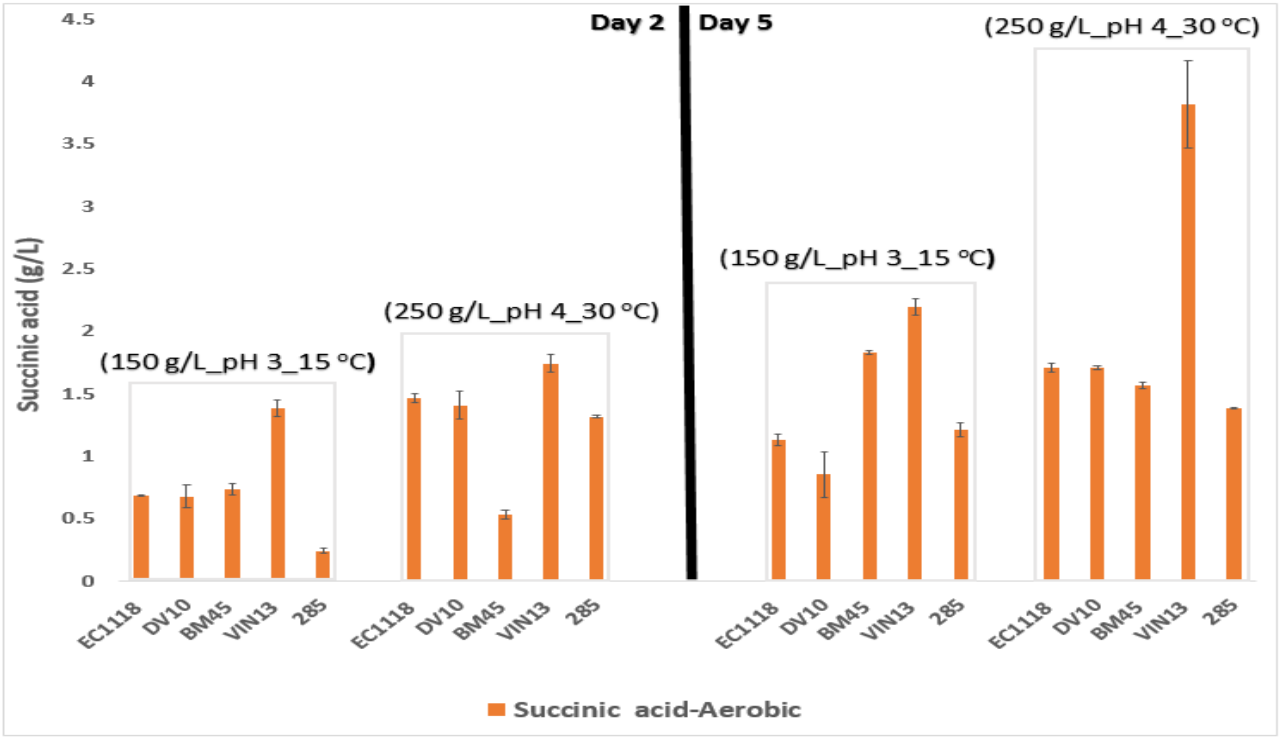

Figure A6. Succinic acid profiles of five yeast strains at different time points (day 2 and 5) under white wine (150 g/L, pH 3 and 15 °C) and red wine (250 g/L, pH 4 and 30 °C) aerobic fermentation conditions. Results are the average of three biological repeats ± standard deviations.

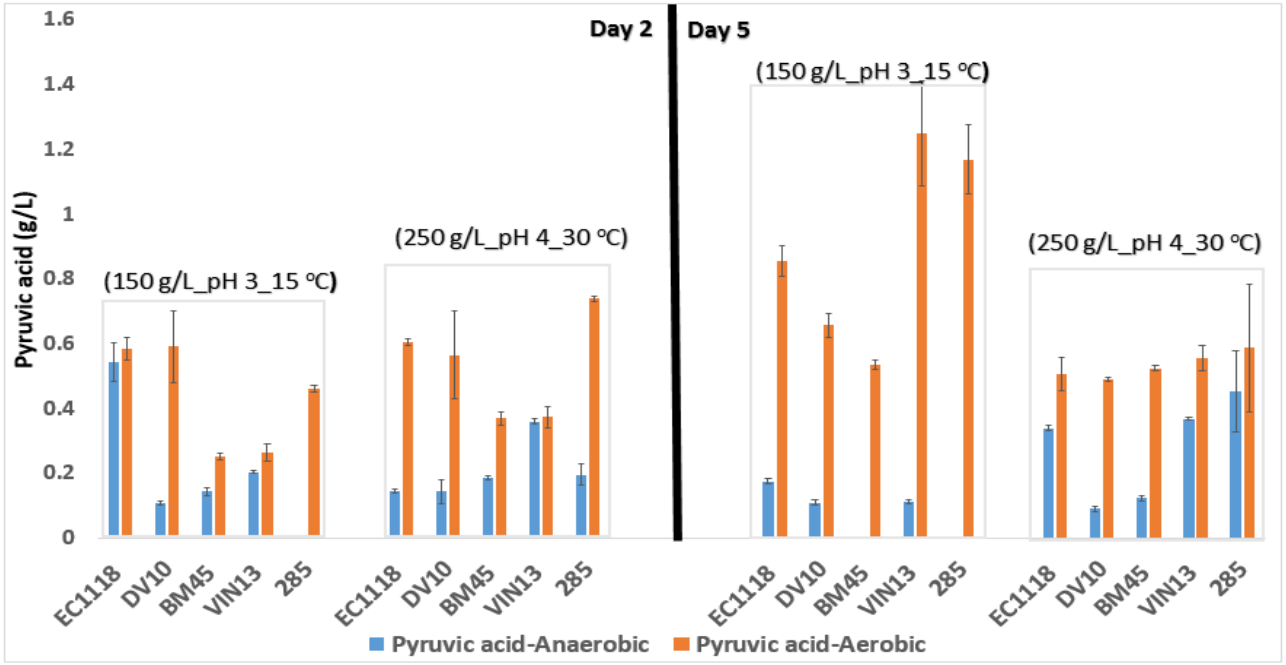

Figure A7. Pyruvic acid profiles of five yeast strains at different time points (day 2 and 5) in white wine (150 g/L, pH 3 and 15 ^{o}C) and red wine (250 g/L, pH 4 and 30 ^{o}C) fermentation conditions, both aerobic and anaerobic. Results are the average of three biological repeats ± standard deviations

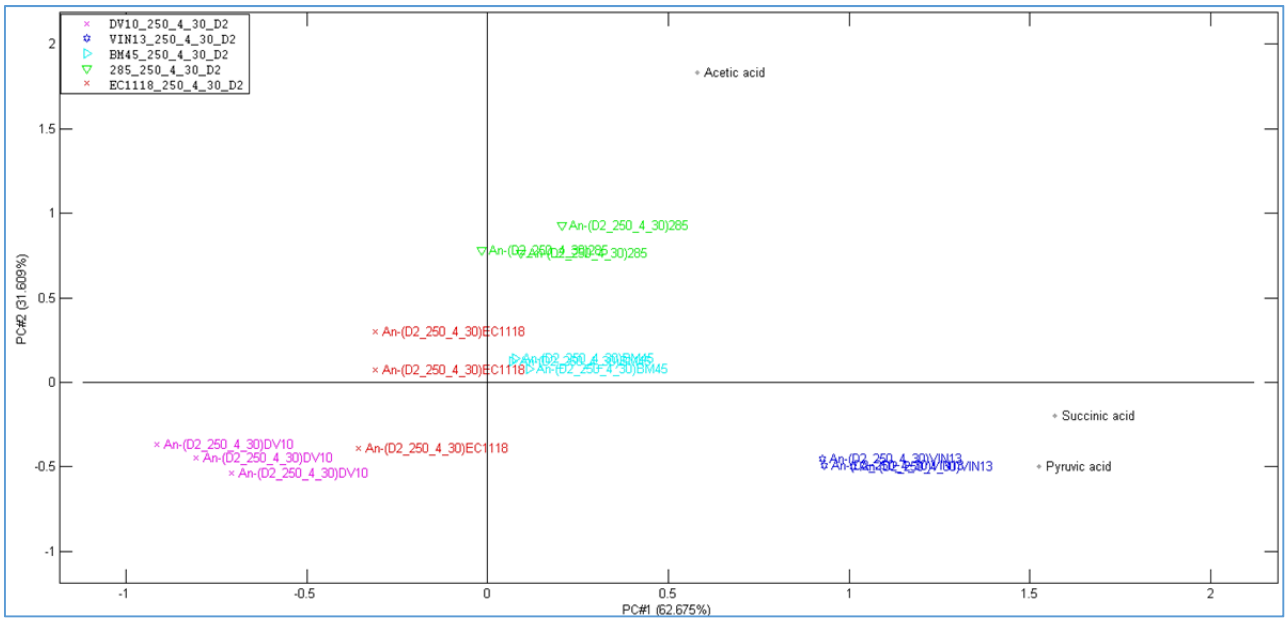

Figure A8. PCA bi-plot showing sample groupings for triplicate fermentations of strains DV10 (pink-dataset), BM45 (sky blue), VIN13 (blue), *285* (green) *and* EC1118 (red) at day 2 (D2) of fermentation. Fermentations were carried out in red wine –like anaerobic conditions (250 g/L, pH 4 and 30 ^{o}C).

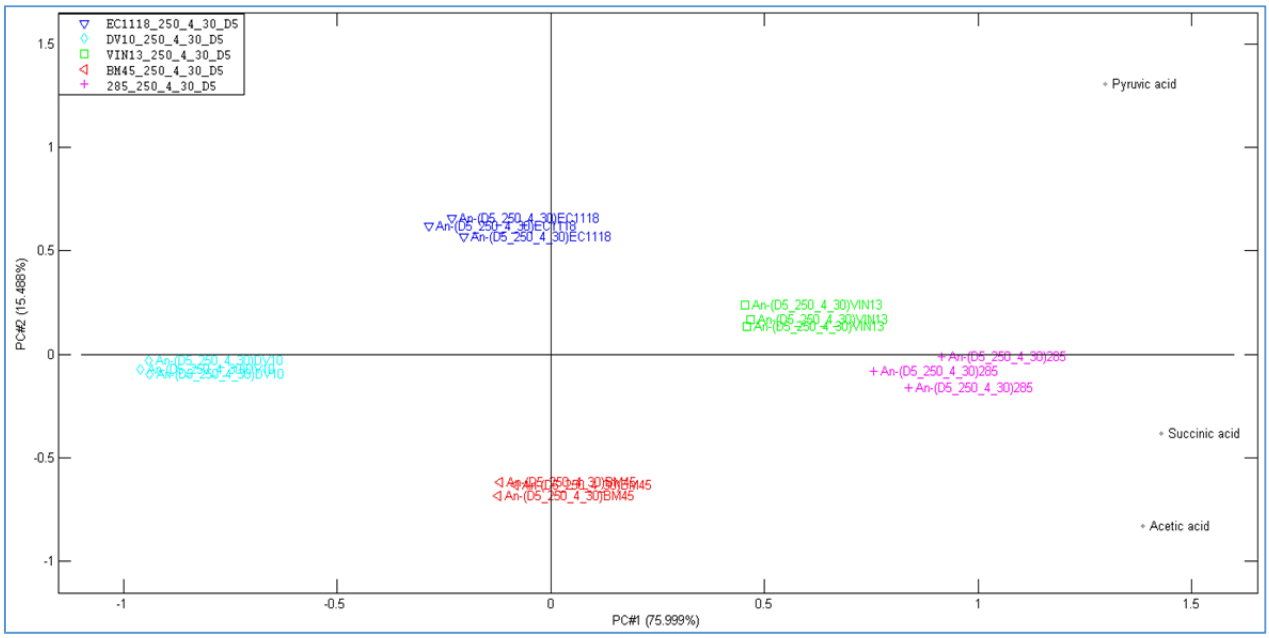

Figure A9. PCA bi-plot showing sample groupings for triplicate fermentations of strains DV10 (sky blue-dataset), BM45 (red), VIN13 (green), 285 (pink) *and* EC1118 (blue) at day 5 (D5) of fermentation. Fermentations were carried out in red wine –like anaerobic conditions (250 g/L, pH 4 and 30 ^{o}C).

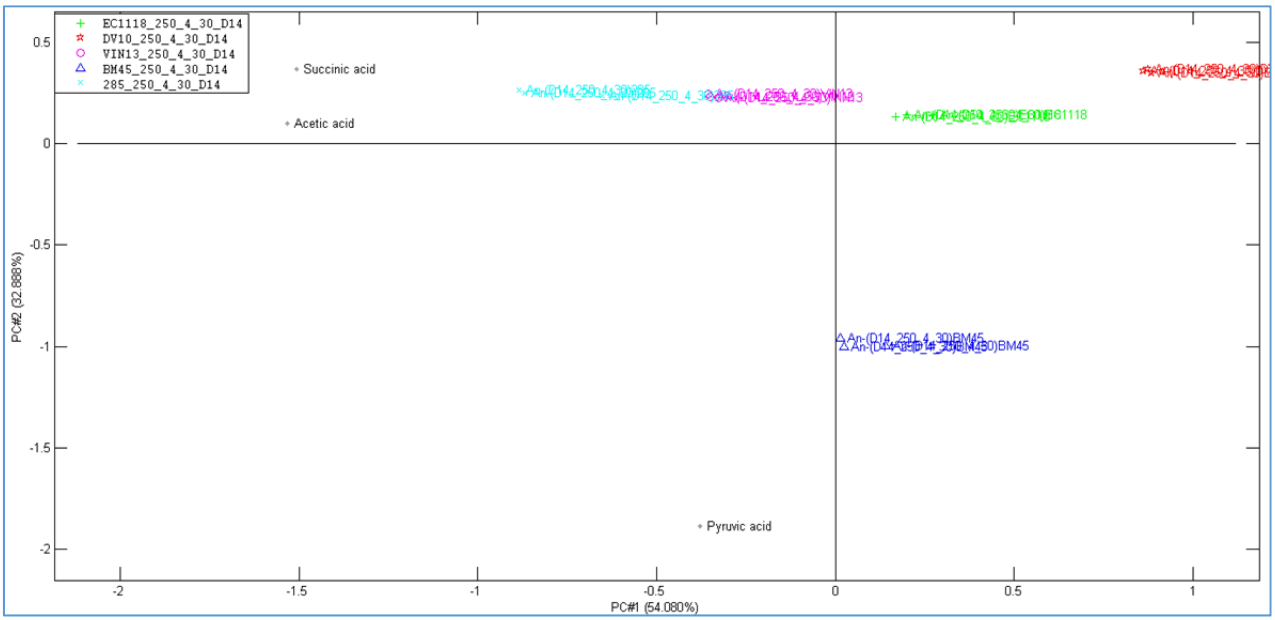

Figure A10. PCA bi-plot showing sample groupings for triplicate fermentations of strains DV10 (red-dataset), BM45 (blue), VIN13 (pink), 285 (sky blue) *and* EC1118 (green) at day 14 (D14) of fermentation. Fermentations were carried out in red wine –like anaerobic conditions (250 g/L, pH 4 and 30 ^{o}C).

Table A1. Amino acids, vitamins, anaerobic factors and oligoelements stock solutions

Stock of amino acids	Final concentration in synthetic must (mg/L)
Tyrosine	18.326
Tryptophan	179.333
Isoleucine	32.725
Aspartic acid	44.506
Glutamic acid	120.428
Arginine	374.374
Leucine	48.433
Threonine	75.922
Glycine	18.326
Glutamine	505.274
Alanine	145.299
Valine	44.506
Methionine	31.416
Phenylalanine	37.961
Serine	78.540
Histidine	32.725
Lysine	17.017
Cystein	13.090
Proline	612.612
Stock of vitamins	**Final concentration in synthetic must (mg/L)**
Myo-inositol	20
Calcium pantothenate	1.5
Thiamine, hydrochloride	0.25
Nicotinic acid	2
Pyridoxine	0.25
Biotine	0.003
Stock of anaerobic factors	**Final concentration in synthetic must (mg/L)**
Ergosterol	1.5
Oleic acid	0.5
Stock of oligoelements	**Final concentration in synthetic must (mg/L)**
$MnSO_4 \times H_2O$	4
$ZnSO_4 \times 7H_2O$	4
$CuSO_4 \times 5H_2O$	1
KI	1
$CoCl_2 \times 6H_2O$	0.4
H_3BO_3	1
$(NH_4)6Mo_7O_{24}$	1

Chapter 4

Research results

The impact of changes in environmental factors on organic acid production by commercial yeast strains

CHAPTER 4

The impact of changes in environmental conditions on organic acid production by commercial yeast strains

4.1 Abstract

Acidity in wine impacts the general balance of wine taste and colour. The sensory perception of wine is indeed dependent on the interplay between alcohol, sugars and acids, as well as many other secondary grape- and fermentation- derived metabolites such as higher alcohols and esters. Grape derived acids such as citric, tartaric and malic acid are dominant in grape juice and in wine, but fermentation derived acids such as pyruvic, acetic and succinic acid are also present in finished wines. Each acid specifically influences taste and acidity in a different manner, hence each can play a significant role in defining the sensory attributes of a wine. The current study aims to provide a better understanding of how individual or simultaneous changes in environmental parameters such as pH, nitrogen, sugar, aeration and temperature influence the production of individual organic acids during fermentation in synthetic must. Here, the impact of nitrogen on the production of organic acids was not observed, hence it was not reported. The study uses a multi-factorial experimental design to assess multiple environmental factors simultaneously, and monitors the variation in organic acid levels at three different stages of fermentation, namely the exponential phase of yeast growth, the early stationery phase and the late stationery phase. The data show that grape-derived acids (malic, tartaric and citric acid) were not significantly impacted by strains regardless of fermentation conditions. However, significant differences in the levels of pyruvic, acetic and succinic acids were observed for the different fermentation conditions. The study provides valuable information regarding the manner in which initial must parameters and environmental conditions throughout fermentation may affect wine acidity. Since many of these parameters can be at least in part controlled during the wine making process, the data provide important background information for the future improvement of oenological strategies which aim to optimise the acid balance of wines.

4.2 Introduction

Acidity and the level of individual acids in wine are crucial elements impacting on wine quality. The sensory impacts of acids have been reasonably well documented, with a sour and sharp taste being associated with too much acidity, while low acidity wines may be perceived as flat and generally results in a less well defined flavour profile (Mato *et al*., 2005). Furthermore, monitoring of certain acids during fermentation enables winemakers to effectively monitor aspects of wine ageing, alcoholic and malolactic fermentation (Bisson *et al*., 2002). Tartaric, malic and citric acid

are the major organic acids dominating in grapes, whereas other organic acids such as succinic, acetic and pyruvic acid are produced during the fermentation processes. All of these acids are known to specifically contribute to perceived and measurable acidity, as well as impacting other organoleptic properties of wines (Shiraishi *et al*., 2010). However, there is less information regarding the manner in which fermentation conditions affect the metabolism of both grape- and yeast derived acids under wine making conditions.

Several factors which individually influence the level and production of organic acids during fermentation have been identified in the past. Yeast and bacterial strains (wild or inoculated), fermentation temperature, initial sugar levels and pH have been identified as important factors. Most factors are at least in part manageable by the winemakers during fermentation, and a better understanding of their roles and interactions between them may provide better tools for fermentation and wine flavour management (Lafon-Lafourcade, 1983: Lambrechts and Pretorius, 2000; Agarwal *et al*., 2007; Kamzolova *et al*., 2009).

Generally, the organic acid responsible for the largest part of the change in titrable acidity during fermentation is succinic acid (Song, 2006). The influence of temperature, sugar levels and pH on succinic acid has been relatively well elucidated (Thoukis *et al*., 1965; Shimazu and Watanabe, 1981; Aragon *et al*., 1998; Torija *et al*., 2001). High succinic acid levels have been linked to high initial fermentation pH (Thoukis *et al*., 1965) and low fermentation temperature in rice wine (Liu *et al*., 2014). In most cases, aeration during fermentation appears to increase the production of succinic acid. This effect has been ascribed to the increase in the levels or activity of the TCA enzymes involved in the biosynthesis of secondary metabolites during aeration of which succinic acid is an intermediate (Boulton *et al*. 1996).

Acetic acid is a by-product of alcoholic fermentation formed *by S. cerevisiae* mostly at the beginning of alcoholic fermentation. Its production is also influenced by the yeast strain used (Shimazu and Watanabe 1981; Erasmus *et al*., 2004; Orlic *et al*. 2010), initial sugar concentration (Radler, 1993) and physical factors such as temperature and pH (Monk and Cowley 1984; Llauradó *et al*. 2005; Ribéreau-Gayon *et al*. 2006; Beltran *et al*., 2008). The correlation between high sugar levels (above 32 brix) in ice wine (Kontkanen *et al*., 2004, Pigeau and Inglis, 2005 and Pigeau and Inglis, 2007) and botrytized wine (Bely *et al*., 2005) with an increase in acetic acid production has also been reported. In addition, acetic acid levels in wine are significantly increased under aerobic conditions (Aceituno *et al*., 2012). Significant interactions between factors such as yeast strain, incubation temperature, and agitation time have also been reported (Fleet and Heard, 1992).

Pyruvic acid is generally present in wine as a secondary product of alcoholic fermentation and the amount of pyruvic acid in wine varies considerably, between 10 to 500 mg/L in dry wines (Usseglio,

1995). Pyruvic acid is mostly formed at the onset of fermentation and tends to decrease towards the end of fermentation. Several reports previously showed that pyruvic acid is affected by the pH of the must (Rankine, 1967; Samuelov *et al*., 1998). Furthermore, the degree of aeration and the sugar content of the grape juice have been reported to influence pyruvic acid concentrations (Lee *et al*., 1999).

While different fermentation parameters have been reported to significantly impact on acid production, no studies have been dedicated to understanding combined impacts. The current study holistically explores the multi-factorial interaction of pH, temperature, aeration and sugar contents at different fermentative stages. Due to the complex nature of our treatment combinations and variables, we could not adopt any previously reported experimental designs such as a Box-Behnken design (Box and Behnken, 1960). We omitted some of the intermediate conditions due to logistical limitations, we therefore opted for a simpler design that is aligned with conditions relevant to winemakers. A network-based evaluation of changes in organic acid concentrations across strains and time-points in 16 different fermentation conditions enabled us to gain a clearer picture of organic acid metabolism in yeast. The data contribute to our understanding of the integrated effects of these factors in fermentations conducted with different commercial wine yeast strains.

4.3 Materials and Methods

4.3.1 Strains, media and culture conditions

Yeast strains used as well as their characteristics are listed and described in Chapter 3. Methods used for cell cultivation, growth and preparation are described in Chapter 3.

4.3.2 Fermentation medium

Fermentation experiments were carried-out in synthetic must MS300 which approximates a natural must as described in detail in Chapter 3 (Bely *et al.,* 1990). As indicated in the experimental design (discussed in Chapter 3), the medium contained hexoses (equivalent amounts of glucose and fructose) of 150, 200 and 250 g/L. Temperature and pH were, respectively, set at 15, 25 or 30 ^{o}C, while pH settings of 3.0, 3.5 or 4.0 were used. Fermentations were carried out under anaerobic conditions in 250 ml Erlenmeyer flasks (containing 100 ml of the medium). Aerobic fermentations were agitated at 200 rpm while anaerobic fermentations were sealed with rubber stoppers with a CO_2 opening. Fermentations were complete after a period of 14 days. All fermentations were carried out in triplicate. Optical density and weight loss were monitored at an interval of 2 days while samples were collected at days 2, 5 and 14 for chemical analysis.

4.3.3 Growth measurement

Cell propagation (i.e. growth) was determined spectrophotometrically (PowerwaveX, Bio-Tek Instruments) by measuring the optical density (at 600 nm) of 200 µl samples of the suspensions during fermentation.

4.3.4 Experimental design

Different sets of fermentative conditions designed for this study incorporate different pH, sugar and temperature settings (table 1). All nine designs were duplicated under aerobic and anaerobic conditions. Four factors (pH, sugar level, temperature, aeration) were chosen as they are in principle manageable by winemakers to a greater or lesser extent. Five wine yeast strains (BM45, DV10, EC1118, 285 and VIN13) were inoculated to ferment under the different conditions. Samples were taken at three critical stages of fermentation, i.e. exponential (day 2), early stationary (day 5) and late stationary phase (day14). These time points were selected in order to investigate organic acid changes at distinct fermentation stages.

Table 1. Experimental design describing the composition of the nine different synthetic musts (fermented by five different wine yeast strains) under both anaerobic and aerobic conditions (thus 16 treatments in all). The must composition was varied in terms of the initial sugar concentration (150 or 250 g/L), initial pH (3, 4 or 3.5) and fermentation temperature (15, 25 or 30 ^{o}C).

EXPERIMENTAL DESIGN	Sugar	pH	Temperature	Sampling Time (Days)
An/aerobic_150_3_15	150	3	15	2/5/14
An/aerobic_150_4_15	150	4	15	2/5/14
An/aerobic_250_3_15	250	3	15	2/5/14
An/aerobic_250_4_30	250	4	30	2/5/14
An/aerobic_150_3_30	150	3	30	2/5/14
An/aerobic_150_4_30	150	4	30	2/5/14
An/aerobic_250_3_30	250	3	30	2/5/14
An/aerobic_250_4_30	250	4	30	2/5/14
An/aerobic_200_3.5_25	200	3.5	25	2/5/14

4.3.5 Chemical analysis

A capillary electrophoresis (CE) method adapted from Soga and Ross (1997) was used to analyse and quantify organic acid contents in synthetic must (MS300). Culture supernatants were obtained and analysed for sugars, ethanol and glycerol by high performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column at 55 ^{o}C using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 ml/min. Agilent RID and UV detectors were used for detection and quantification. Analyses were carried out using the HP Chemstation software package (Eyeghe *et al*., 2012).

4.3.6 Data analysis

4.3.6.1 Multivariate data analyses

Principal component analysis (PCA; Latentix 2.0, BRANDON GRAY INTERNET SERVICES, INC. DBA) was used to establish trends within various sets of data. In our study, the samples represent the different fermentations at different time points (three independent replicates for each of the five strains). Variables considered are the concentrations of different organic acids in the fermented synthetic must.

4.3.6.2 Network analyses

Cytoscape (Shannon *et al*., 2003) was used to visualize similar and dissimilar organic acid trends across various fermentative conditions, time points and strains. A database was generated for strains BM45 and VIN13 which independently produced different levels of organic acids (succinic, acetic and pyruvic acid) during anaerobic and aerobic fermentation conditions in different synthetic musts (table 1) and time points (day 2, 5 and 14). The ratio of organic acid levels between selected fermentation conditions were calculated for each organic acid produced and then imported by using table import version 0.7 of cytoscape. Annotations were created using Biomart client v 0.9 plugin (http://www.biomart.org; Zhang *et al*., 2011) and further integrated for visualization and analysis by using BIO PAX v07 (http://www.biopax.org/). In addition, an advanced network merger v1.16 (http://www.cytoscape.org/plugins2.php) was implemented to create the union, intersection, merging and differentiation of networks based on node identifiers. A complete interactive network of fermentation conditions was generated for interpretation. Several sub networks describing the relationship between fermentation conditions were also generated. Only statistically significant fold changes (cut-off fold change of +/- 1.5) are included in visualisations.

4.3.6.3 Statistical analysis

Analysis of variance (ANOVA) was performed to compare organic acid levels produced by VIN13, EC1118, DV10, BM45 AND 285 strains at different stages of fermentation. For significance tests, 95% confidence intervals were calculated. This was done using the STATISTICA 64 software.

4.4 Results and Discussion

4.4.1 The influence of environmental parameters on yeast growth

The impact of pH, temperature and sugars on all five industrial strains were assessed (as indicated in supplementary figs B1-B3) but since the growth and fermentation rates of all strains were similar in response to changing conditions, only data for strains EC1118 and DV10 are shown in figures 1

and 2 as representative of the general yeast response. Fermentations in the different synthetic musts were monitored until weight loss ceased (figs 1 and 2). In general, the growth of all yeast strains followed similar trends for the different fermentation conditions though the final biomass formation differed in many cases. These observations were an indication that the selected commercial wine yeast grow well over a wide range of pH, temperature and sugar levels. Generally the yeast strains grew faster at higher fermentation temperatures (fig 1). Fermentation and growth rates were slower at 15 ^{o}C compared to fermentations at 30 and 25 ^{o}C (fig 1). Regardless of other parameters, fermentations in both aerobic and anaerobic conditions showed faster growth at higher temperature (30 ^{o}C) as expected (Fleet and Heard, 1993).

Aeration increased the rate of fermentation and optical density (indicative of growth) for all five industrial strains investigated. Fermentations reached the stationary phase earlier (~5 days) with aeration (fig 1 and fig 2) and later (6 days) under anaerobic conditions. It has been previously observed that aeration, if appropriately controlled, can lead to increased yeast numbers and fermentation rates during alcoholic fermentation (Jones and Ingledew, 1994; Blateyron *et al*., 1998)**.** Nevertheless, the overall yeast growth patterns in anaerobic and aerobic fermentations remained similar for comparable culture conditions (fig 1 and 2).

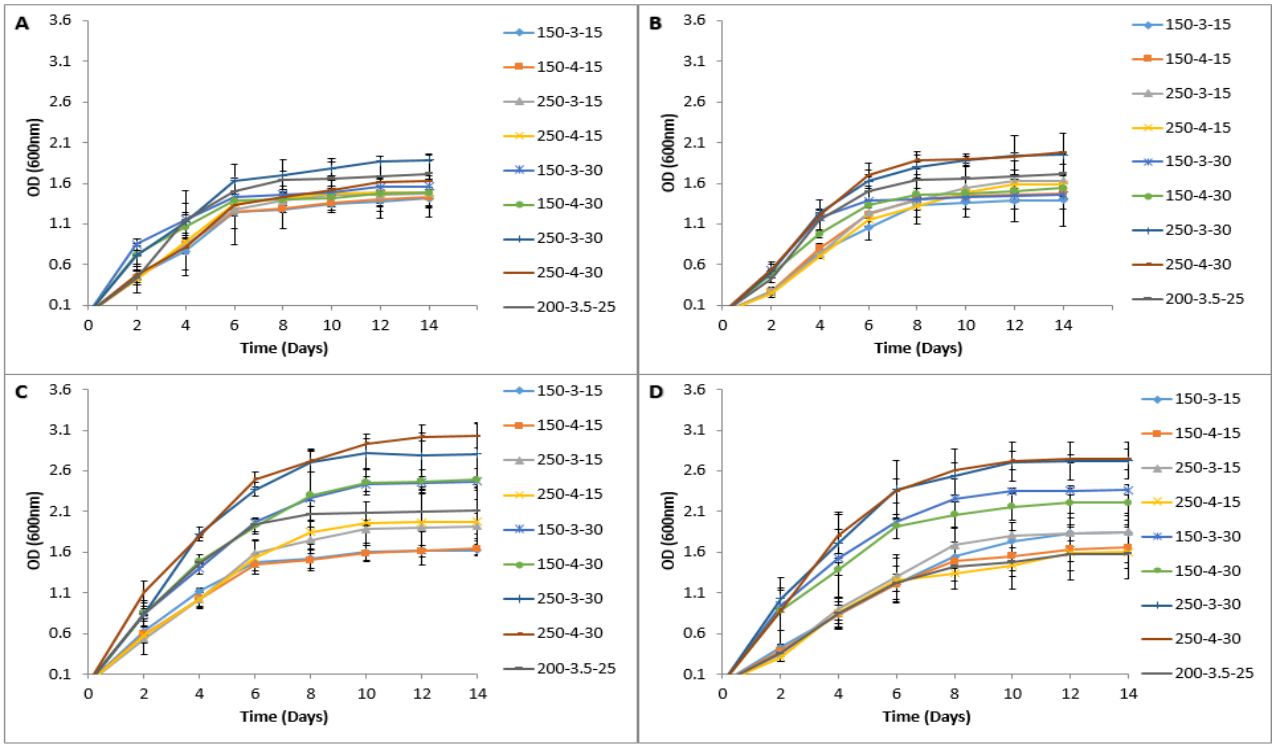

Figure 1: Anaerobic growth rates of EC1118 (A) and DV10 (B) and aerobic growth rates of EC1118 (C) and DV10 (D) under various must composition and environmental conditions, i.e. sugars (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30 ^{o}C). Results are the average of 3 biological repeats ± standard deviation.

The impact of the must sugar content on yeast growth and fermentation kinetics were clear and consistent with expectations: Higher initial sugar concentrations resulted in increased yeast growth (as represented by the OD_{600} values; fig 1C and D). This is due to the increased carbon availability which supports additional growth of the yeast regardless of other factors such as pH and temperature. While temperature clearly affects the rate of growth, particularly in the earlier stages of fermentation, the total sugar content is responsible for the final biomass attained. Previous studies have also highlighted the importance of temperature and sugar content of the must on yeast growth kinetics (Fleet and Heard, 1993; Charoenchai *et al*., 1998; D'Amato *et al*., 2006).

In contrast, the effect of changes to pH on fermentation kinetics of both strains were not as consistent under both aerobic and anaerobic fermentation conditions (fig 1): For instance, high pH value settings (pH 4) resulted in both the lowest and highest fermentation rates, as trends for these fermentations were driven by either low temperature or low sugar settings, or high sugars and temperatures, respectively. Several studies have previously established the impact of temperature and pH on wine yeast strains (Gao and Fleet, 1998; Serra, 2005; Yalcin and Osbaz, 2008). In these studies, the growth of *S. bayanus var. uvarum* was reported to be a function of both temperature and pH. However, pH did not play a significant role in determining yeast growth on its own.

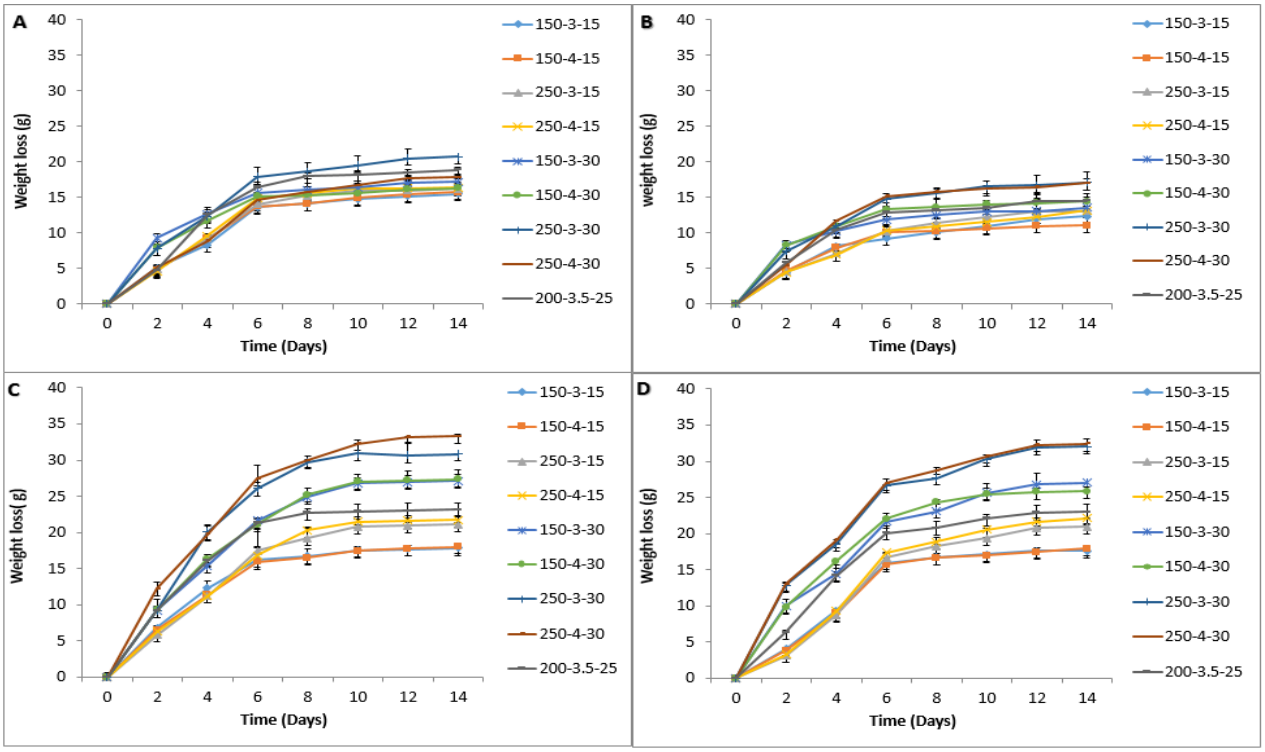

Figure 2: Anaerobic fermentation rates of EC1118 (A) and DV10 (B) and aerobic fermentation rates of EC1118 (C) and DV10 (D) under various must composition and environmental conditions, i.e. sugars (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30 ^{o}C). Results are the average of 3 biological repeats ± standard deviation.

4.4.2 The impact of environmental factors on grape derived acids

Although it is generally accepted that tartaric acid concentrations are largely unaffected by the metabolic processes of primary fermentation or secondary fermentation, minor variations in tartaric acid at the end of fermentation were observed when different fermentation conditions were tested. Here we present data of strain EC1118 as a representative example under both aerobic and anaerobic conditions (fig 3). Indeed, similar minor variations were observed for all strains (see supplementary figs B4 and B5) but no noteworthy trends could be observed. Final tartaric acid levels did varied slightly. Compared to EC1118 fermentations, tartaric acid levels were lower in some anaerobic conditions for strains BM45 (at 250 g/L_pH3_30 ^{o}C), VIN13 (at 150 g/L_pH 3_15 ^{o}C and at 250 g/L_pH 4_30 ^{o}C) and 285 (at 150 g/L_pH 3_30 ^{o}C). Slightly higher tartaric acid levels were also observed when BM45 (at 250 g/L_pH 3_15 ^{o}C) and 285 (at 150 g/L_pH 4_30 ^{o}C) were evaluated under aerobic conditions. Since no consistent trends were apparent for any of the strains or conditions tested, the observed variation may be linked to factors other than yeast metabolism, such as the possibility that different culture conditions (including aeration) affect tartaric acid solubility (Maujean *et al*., 1985; Margalit, 2004; Odageriu, 2006 and 2008).

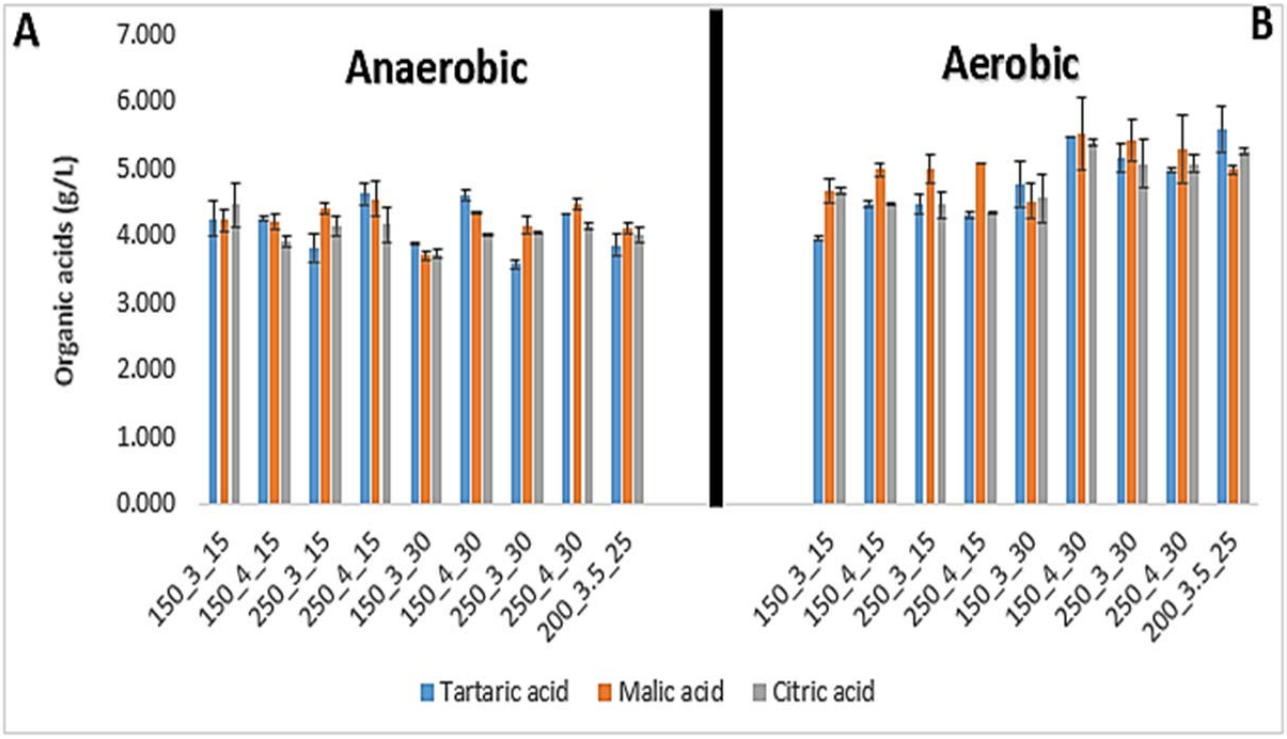

Figure 3: Grape derived acid variations (end-point) for EC1118 at different environmental settings under anaerobic and aerobic fermentation conditions i.e. sugar (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30 ^{o}C). Results are the average of 3 biological repeats ± standard deviation.

For the two other dominant grape-derived acids, malic and citric acid, the most pronounced differences were related to differences between yeast strains as reported in Chapter 3.

Some trends with regard to malic acid concentrations and changes to environmental conditions were observed. In particular, a statistically significant reduction was observed for several fermentations at low pH, such as for BM45 (at 250 g/L_pH 3_15 ^{o}C; supplementary fig B4) and 285 (at 150 g/L_pH 3_30 ^{o}C; supplementary fig B5). Indeed, a combination of lower pH and higher temperature resulted in a reduction in extracellular malic acid in both strains. Higher concentrations of the protonated form of malic acid at lower pH might result in easier diffusion across the cell membrane, a feature that would be reinforced by higher temperature which would increase membrane fluidity (Torija *et al*., 2003). As previously reported, malic acid is at least partially consumed when import of the acid can be effected (Volschenk *et al*., 1997).

Previously, citric acid production has also been found to be reduced at lower pH values (Mattey, 1992). However, our data did not show any significant increase/decrease in this acid in any of the tested fermentation conditions (initial concentrations were 6 g/L). Based on our observations, the influence of pH on grape-derived acids, and in particular on malic acid, justifies the recommendation of a strain such as DV10 for the slight reduction of malic acid concentrations in wine. Despite the link between DV10 and reduced malic acid concentrations, no noteworthy trends were established regarding the influence of particular fermentation conditions and/or strains on the final levels of grape –derived organic acids in our study.

4.4.3 The impact of individual environmental factors on yeast –derived organic acid production across all conditions and physiological stages of yeast

For the purpose of this study, the impact of individual parameters on organic acid production was only assessed under anaerobic conditions because this condition is more relevant from a winemaking perspective and the condition and strain –dependent trends under aerobic conditions were roughly similar to those observed under anaerobic conditions. The absolute values of the changes in pyruvic, acetic and succinic acid for all strains are indicated in supplementary section (table B1 and B2).

In addition to graphs representations below, network analysis was carried out in order to create an integrative and user-friendly platform for interrogation of large-scale comparative datasets. Networks indeed present a visually simple means to assess multi-factorial impacts on metabolite levels across time-points. These networks also enabled the extraction of biological meaning from the complex data. In our analysis, only acids showing statistically significant changes (with a fold-change of greater than 1.5 or less than -1.5) between conditions were included in visualizations. For each of the three time-points, core nodes represent one-to-one comparisons of two conditions for each of the three acids. VIN13 and BM45 were selected as representative strains for the visualizations generated in this part of the study. Important observations arising from the network

analysis of these two strains were followed up in the remaining three strains in order to verify consistencies and differences among strains.

4.4.3.1 Pyruvic acid

In general, similar pyruvic acid trends were observed when temperature, sugar and pH were varied for each strain (refer to figure 4A, B and C). Pyruvic acid concentrations showed significant variations between different treatments, suggesting that pyruvic acid metabolism is impacted by one or more of the factors considered in this study. One notable effect was the response to pH, as a prominent increase in pyruvic acid production by strain 285 was observed at high initial pH (3.5 and 4.0) compared to lower pH 3.0 (fig 4A). VIN13 did the same only at pH 3.5, however other strains did not show any statistically significant responses ($p < 0.001$) to pH changes.

Although it is evident from this study that the effect of pH on pyruvic acid production is dependent on yeast strain (Chapter 3), we noted undetectable levels of pyruvic acid by BM45 and VIN13 under most fermentation conditions where pH was a variable (see supplementary materials; table B2 and B3). Earlier studies suggested a link between pH and pyruvic acid levels during fermentation (Rankine, 1967; Graham, 1979). The impact of pH on pyruvic acid levels is also confirmed by the dataset illustrated below in this chapter. Furthermore, the absolute values of the changes in pyruvic acid for strain 285 are shown in supplementary table B1. Here, a significant increase in pyruvic acid (undetected up to 0.314 g/L) was observed when pH was increased from pH 3 to pH 4 under anaerobic conditions (when other parameters were kept constant at 150 g/L and 15 ^{o}C at the end of fermentation). This is confirmation that pyruvic acid production is influenced by the combination of both strain and fermentation conditions. A useful recommendation would be the careful monitoring of must pH throughout fermentation, or at the different sampling times in future. This would enable a more thorough assessment of immediate pH impacts on pyruvic acid.

Different temperatures resulted in minor changes to overall pyruvic acid contents, with the exception of VIN13 which showed a significant increase in pyruvic acid production at higher temperatures (fig. 4B). This may well be linked to a high fermentation speed in high temperature conditions. In addition, higher temperatures (particularly when both sugar and pH were high) significantly increased pyruvic acid levels (up to 0.392 g/L) for strain 285 (see supplementary section; table B2).

Variations in sugar content of the must resulted in major changes in pyruvic acid levels of some strains. A notable increase ($p = 0.02908$) in pyruvic acid production by strain 285 and VIN13 was observed at 200 g/L compared to 150 g/L (fig 4C). However, other strains did not show any statistically significant responses to sugar variations. The increase in the initial sugar content of the

must (particularly when the pH and fermentation temperature were high) increased pyruvic acid levels for strain 285 (up to 0.392 g/L) and VIN13 (up to 0.378) (supplementary section; table B2). This outcome is not unexpected as increased sugar availability would lead to more biomass formation, which could be associated with an increase in key anabolic intermediates such as pyruvic acid. However, pyruvic acid levels in BM45 and VIN13 fermentations were below the detection threshold regardless of increases in the initial sugar levels (supplementary section; table B2). For these two strains, increased pyruvic acid levels were however observed when the fermentation temperature was high (30 ^{o}C). The influence of the initial sugar content of the must was not apparent for other strains (supplementary section; table B1 and B2).

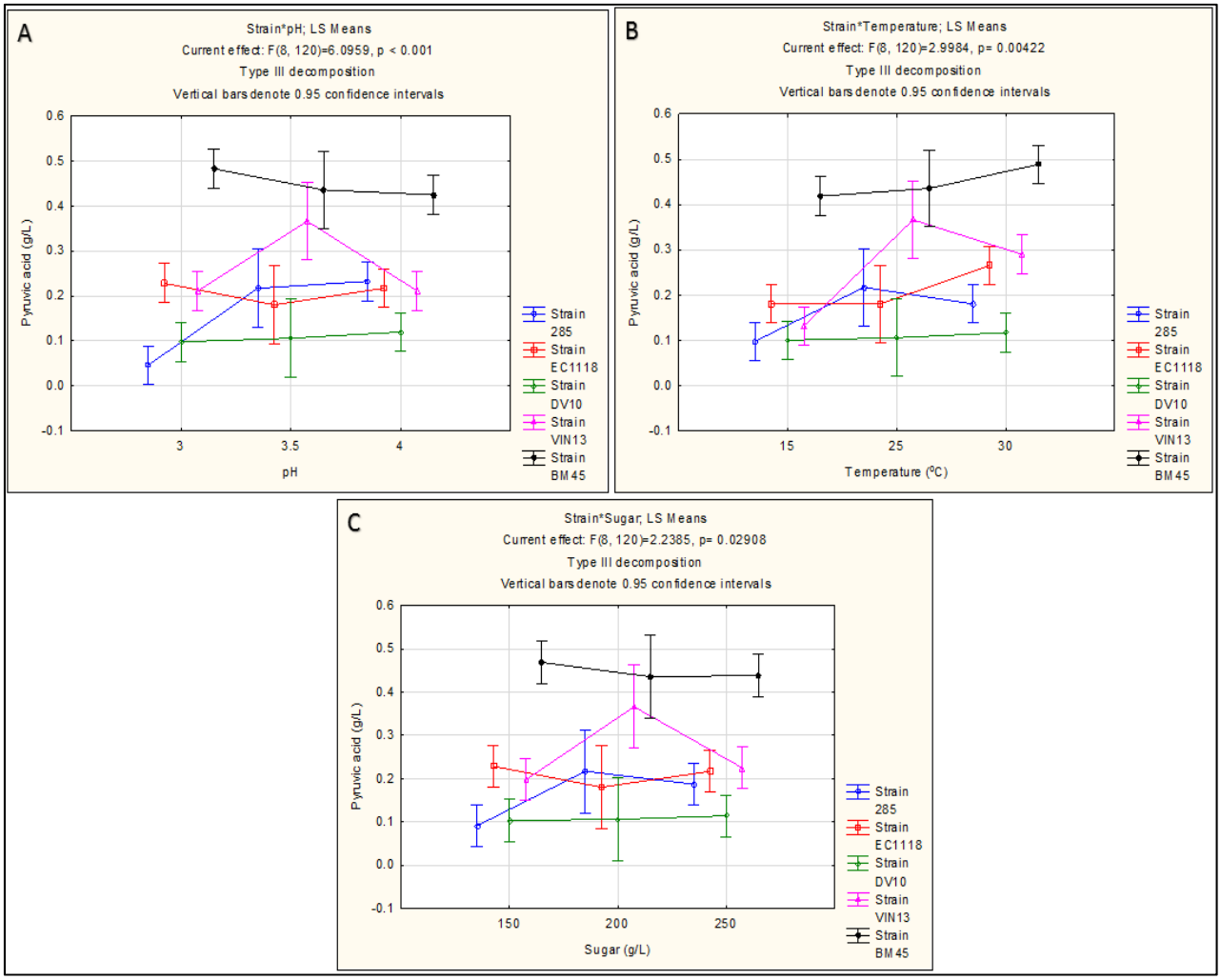

Figure 4: The impact of fermentation temperature, pH and sugar on pyruvic acid production across all experimentally designed conditions. All fermentations were performed in triplicate. For significance tests, 95% confidence intervals were calculated ($p < 0.05$).

While individual factors exert an influence on acid profiles in some cases, the network analysis highlights the combinatorial impact of some of the factors considered in this study (exemplified by strains VIN13 and BM45; supplementary fig B6). A significant increase in pyruvic acid was observed in both strains when conditions were changed as follows: (i) increased sugar (250 g/L) at

low pH (3) and high temperature (30 ^{o}C) at the exponential phase (supplementary fig B6 frame A2 and B2), (ii) increased sugar at low pH and temperature (15 ^{o}C) at early stationary phase (supplementary fig B6 frame A4 and B4), (iii) increased pH (4) at both low sugar and temperature and at early stationary phase (supplementary fig B7 frame A4 and B4), (v) increased temperature at both low sugar and pH at early stationary phase (supplementary fig B8 frame A4 and B4).

A significant decrease in pyruvic acid levels of both BM45 and VIN13 was noted for the following condition comparisons: (i) increasing sugar (250 g/L) at both high pH (4) and temperature at the stationary phase (supplementary fig B6- frame A6 and B6), (ii) increased pH at low sugar and high temperature (30 ^{o}C) and at late stationary phase (supplementary fig B7 frame A8 and B8), (iii) increased temperature at both low pH and sugar at exponential phase (supplementary fig B8- frame A1 and B1). All other changes were strain specific and no conserved impacts on pyruvic acid trends could be observed for multi-factorial condition shifts. In general, based on these observations, the combinatorial effects of lower pH, lower temperature and lower sugar were mostly responsible for high pyruvic acid levels, particularly at the exponential growth phase. These settings can be considered as representative of cool climate- white wine conditions. Once again, this is in line with previous studies which have indicated that pyruvic acid is normally produced at the onset of fermentation but may be reabsorbed and utilised as fermentation progresses (Usseglio, 1995).

4.4.3.2 Acetic acid

Changes to the prevailing fermentation conditions significantly influenced acetic acid production. Apart from the reported impact of sugar stress on acetic acid production in botrytized wines (Bely *et al*., 2005), the impact of individual/multiple parameters such as sugar, temperature and pH on acetic acid levels in wine have not been studied extensively. In the present study, the impact of the initial sugar content of the must was only apparent for a few strains (fig 5C). Surprisingly, VIN13 and 285 produced higher acetic levels when the sugar content of the must was at either very low (150 g/L) or very high (250 g/L) concentrations, while others did not show any statistically significant response to sugar changes (fig 5C). While it is not conclusive whether sugar alone plays a fundamental role in acetic acid concentration, a strong relationship between high fermentation sugars and high acetic acid levels in wine were previously reported for *S. cerevisiae* (Bely *et al*., 2005; Ferreira *et al*., 2006). Similarly, the initial pH (fig 5A) and fermentation temperature (fig 5B) exhibited a quadratic effect on acetic acid production by strains VIN13 and 285. High concentrations of acetic acid were observed at either low pH (3) and temperature (15 ^{o}C) or high pH (4) and temperature (30 ^{o}C). The influence of must sugar levels was however only observed when pH was high (4) and temperature low (15 ^{o}C) for other strains such as EC1118. Acetic acid

production decreased significantly (up to 0.094 g/L) at the end of fermentation when sugar levels dropped (supplementary data; table B1).

Although significant changes in end point acetic acid levels of other strains were observed when the initial sugar levels were raised from 150 to 250 g/L (compare supplementary table B1 and B2), strain EC1118 was the most dissimilar to the other four strains and showed a significant increase from 0.094 (supplementary table B1) to 0.209 g/L (supplementary table B2) when sugar levels were raised but when other parameters were set at high pH and low temperature at the end of fermentation. However, 0.2 g/L seemed to be the default/base amount of acetic acid produced by this strain under all fermentation conditions. It is not a surprising observation since variations in acetic acid production among yeast strains have been observed previously (Castellari *et al*., 1994).

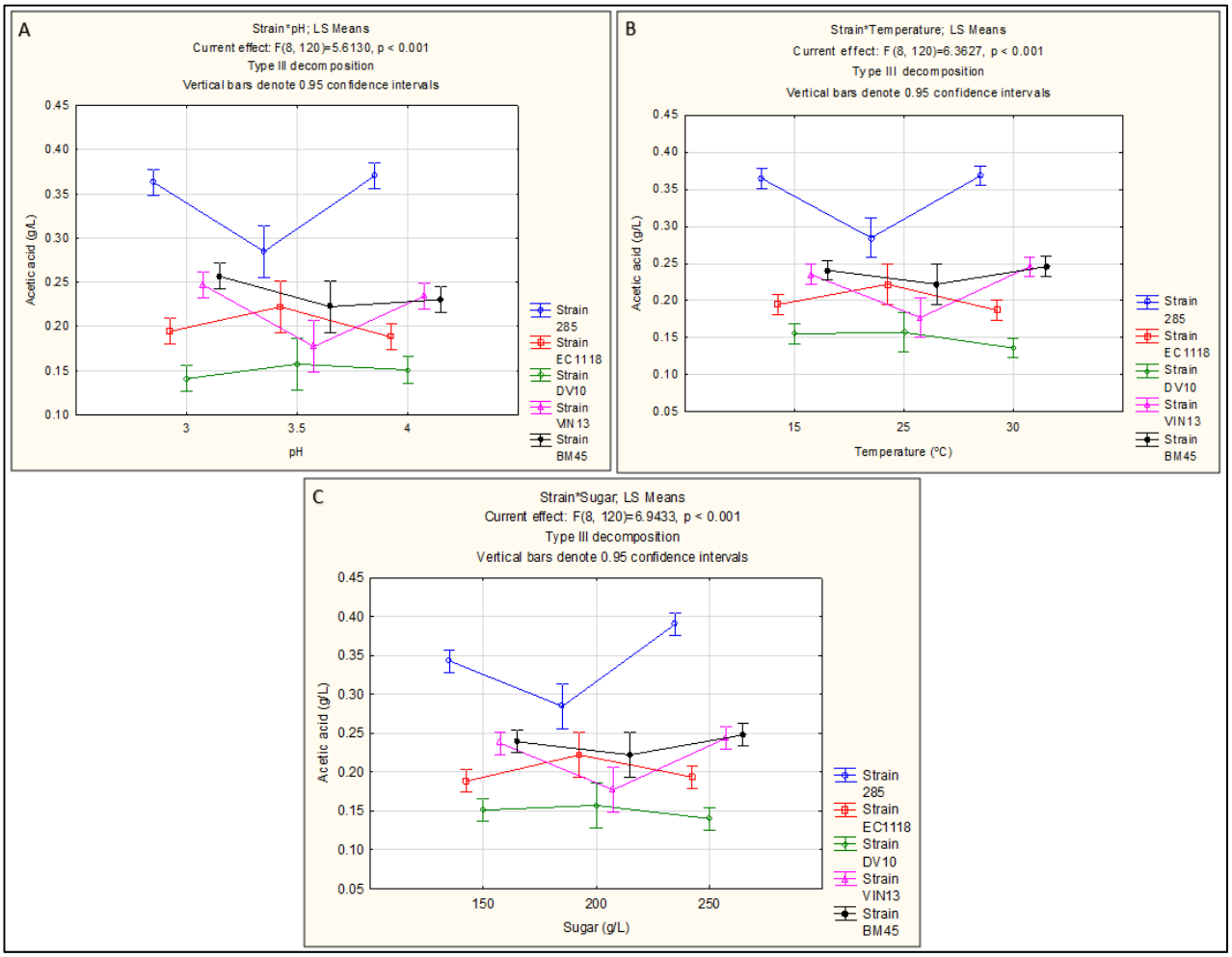

Figure 5: The impact of fermentation temperature, pH and sugar on acetic acid production across all experimentally designed conditions. All fermentations were performed in triplicate. For significance tests, 95% confidence intervals were calculated ($p < 0.05$).

Once again, network models were generated to futher explore the influences of multi-factorial changes in environmental parameters on acetic acid production of wine yeast. Changes in environmental factors affected VIN13 and BM45 differently in many cases. Some impacts were

however conserved among strains. For instance, similar increases in acetic acid production were noted for the following parameter shifts: (i) increased sugar, at high pH and temperature and at the early stationary phase (supplementary fig B6 frame A6 and B6), (ii) increased temperature at both low pH and sugar and at early stationary phase (supplementary fig B8 frame A4 and B4), (iii) increased temperature, at low sugar and high pH and at early stationary phase (supplementary fig B8- frame A5 and B5). Both high temperature and pH seemed to significantly increase acetic acid production by these two strains. High temperatures may result in membrane fluidity which allows metabolites to cross cell membranes more easily resulting in high extracellular acetic acid. In contrast, Muller *et al*. (1993) noted larger losses of volatile components such as acetic acid as a result of higher wine fermentation temperatures. This is most likely due to evaporation which would not play a significant role in sealed fermentation vessels.

On the other hand, simultaneous changes to the following factors decreased acetic acid levels at different stages of fermentation by VIN13 and BM45: (i) increased sugar, at low pH and temperature at the late stationary phase (supplementary fig B6 frame A7 and B7), (ii) increased pH, at low sugar and temperature at early stationary phase (supplementary fig B7 frame A4 and B4), (iii) increased pH, at low sugar and high temperature at early stationary phase (supplementary fig B7 frame A5 and B5), (iv) increased pH, at low sugar and temperature at late stationary phase (supplementary fig B7 frame A7 and B7). Fermentation settings at high initial sugar, low temperature and low pH seemed to significantly decrease acetic acid production by these strains. Although higher initial must sugar concentrations have been reported to increase acetic acid production (Erasmus *et al.,* 2004; Pigeau and Inglis, 2005), we observed a decrease in acetic acid levels as a result of the combinatorial effects of high pH, low temperature and high initial sugar content of the must.

4.4.3.3 Succinic acid

pH did not significantly ($p < 0.001$) affect succinic acid production by all tested strains (fig 6A). However, the initial must pH significantly affected succinic acid production/release. Strain dependent differences were also noted: succinic acid production by strain EC1118 was decreased from 0.625 to 0.417 g/L as a result of decreasing must acidity (pH changes from 3 to 4) when both temperature and sugar levels were low (see supplementary data; table B1). In contrast, a significant increase in succinic acid was observed under similar conditions for strain VIN13 from 0.686 to 0.829 g/L and 285 from 0.456 to 0.764 g/L (see supplementary data; table B1). For this strain there were no significant changes in succinic acid in response to pH changes under higher temperature and sugar fermentation conditions.

Succinic acid production by VIN13, EC1118 and 285 was significantly ($p < 0.001$) increased as a result of an increase in temperature (fig 6B). No statistically significant variations were observed for other strains. Temperature increases membrane fluidity in yeast hence diffusion rates of metabolites such as succinic acid into or out of the cell may be affected (Jarboe *et al*., 2013). The relationship between extracellular succinic acid and high temperature may therefore be diffusion and/or temperature related.

A notable decrease in succinic acid was observed at higher sugar concentrations (250 g/L) for VIN13, while other strains did not provide any statistically relevant conclusions (fig 6C). There were no conserved trends that could be established for succinic acid production across strains and conditions. Succinic acid production is clearly influenced by a combination of strain variation and fermentation conditions in a complex manner which does not allow the extrapolation of clear strain effects or predictable responses to changes in fermentation conditions. However, the influence of specific parameters were evident for individual strains.

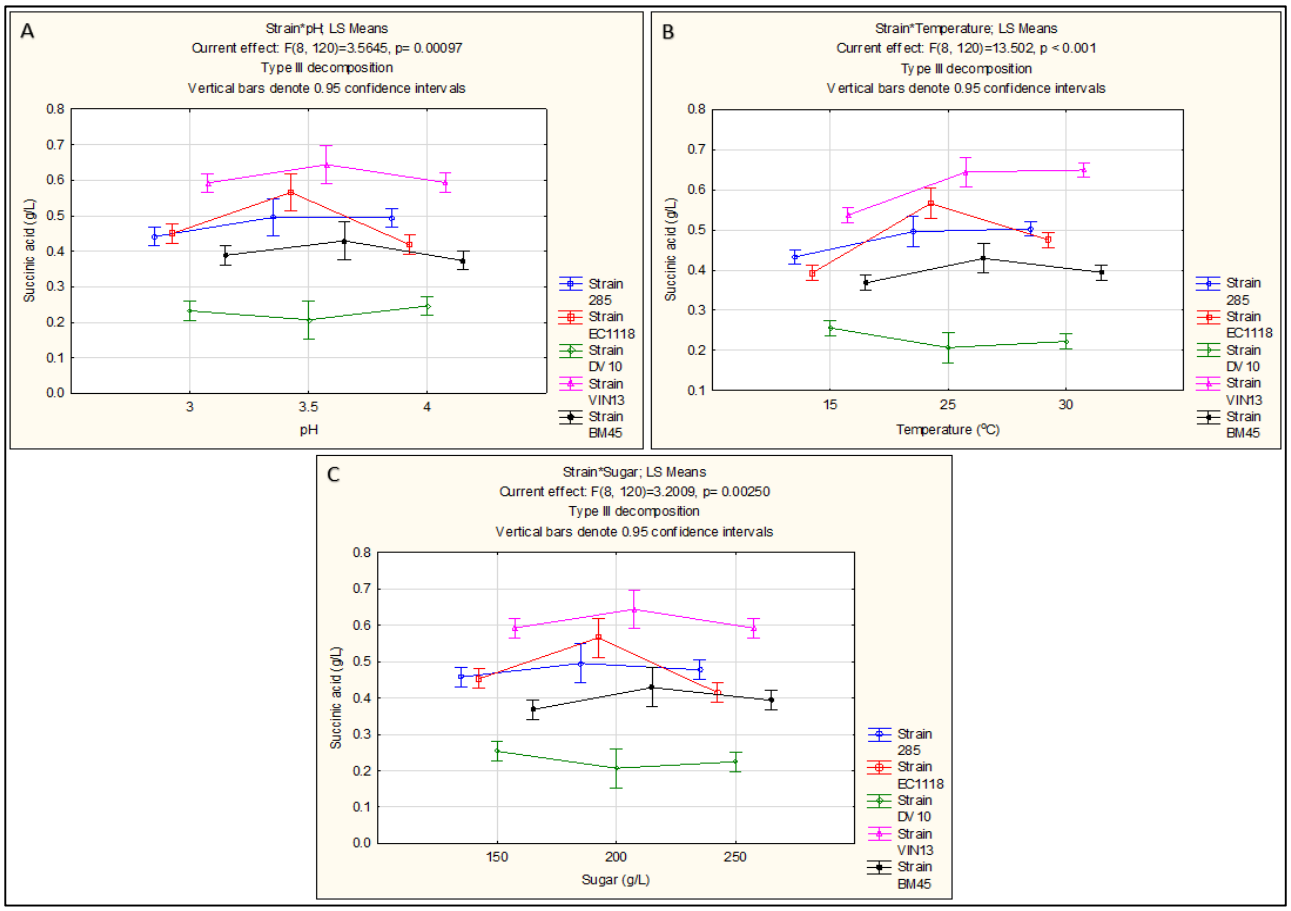

Figure 6: The impact of fermentation temperature, pH and sugar on pyruvic acid production across all experimentally designed conditions. All fermentations were performed in triplicate. For significance tests, 95% confidence intervals were calculated ($p < 0.05$).

Networks depicting one-to-one condition comparisons for BM45 and VIN13 were examined closely to delineate the impact of these changes on succinic acid concentrations at different fermentative stages. Condition impacts (or the lack thereof) on succinic acid were fairly conserved across strains. Succinic acid production by both VIN13 and BM45 was increased when the following condition shifts were implemented: (i) increased sugar, at low pH and high temperature at the exponential phase (supplementary fig B6 frame A2 and B2), (ii) increased sugar, at low pH and temperature at late stationary phase (supplementary fig B6 frame A7 and B7), (iii) increased temperature, at low sugar and high pH at early stationary phase (supplementary fig B8 frame A5 and B5), (iv) increased pH, at low sugar and high temperature at exponential phase (supplementary fig B7 frame A2 and B2), (v) increased pH, at high sugar and high temperature at early stationary phase (supplementary fig B7 frame A6 and B6).

In contrast, changes to the following factors decreased succinic acid levels at different physiological stages for VIN13 and BM45: (i) high sugar, at low pH and increased temperature at the late stationary phase (supplementary fig B8 frame A9 and B9), (ii) increased pH, at low sugar and high temperature at late stationary phase (supplementary fig B7 frame A8 and B8). There is no specific response in terms of succinic acid production by all the wine yeasts considered here. It does however seem as though combined temperature and pH effects are the main factors influencing succinic acid production in VIN13 and BM45. An unexpected finding was the decrease in succinic acid levels in VIN13 and BM45 fermentations upon an increase in temperature. These findings were not expected since high temperatures are associated with more biomass formation and the likelihood of eventual accumulation of TCA intermediates.

4.4.4. Organic acid profiles at different physiological stages of two wine yeast strains

Two wine yeast strains with divergent acid production patterns were further analysed by principal component analysis to assess the relationship between fermentation conditions and organic acids at the exponential (day 2), early stationary (day 5) and late stationary growth phase (day 14) of BM45 (fig 7A) and VIN13 (fig 7B). Two sets of fermentation conditions were selected to illustrate treatment differences when two very different starting musts and fermentation temperatures were used. The fermentation conditions were selected to reflect (in very broad terms) cool climate white wine fermentation (150 g/L_pH 3_15 ^{o}C) and warm climate red wine fermentation conditions (250 g/L_pH 4_30 ^{o}C). These conditions were selected in order to evaluate the impact of two very different fermentation settings (which could both be encountered by winemakers in industry) on different yeast strains in terms of organic acid metabolism.

Strains BM45 and VIN13 with different fermentation characteristics were selected for PCA analysis. Replicates clustered very well and reproducibility was satisfactory. The time point based comparison of organic acids on the PCA was different for the two wine yeast strains. Samples only

separated from one another based primarily on the stage of fermentation for strain BM45. Three distinct sample clusters (representing the three different sampling points and growth phases) could be clearly distinguished along the first component axis accounting for 67% of variation in the data in the case of BM45. Samples were clearly separated in timepoint clusters, with day 2 and 14 of different treatments (both white and red wine settings) exhibiting similar organic acid profiles (fig 7A1 and A2). Samples that were most disimilar in terms of organic acid profiles were those that were obtained at the early stationary growth phase (day 5).

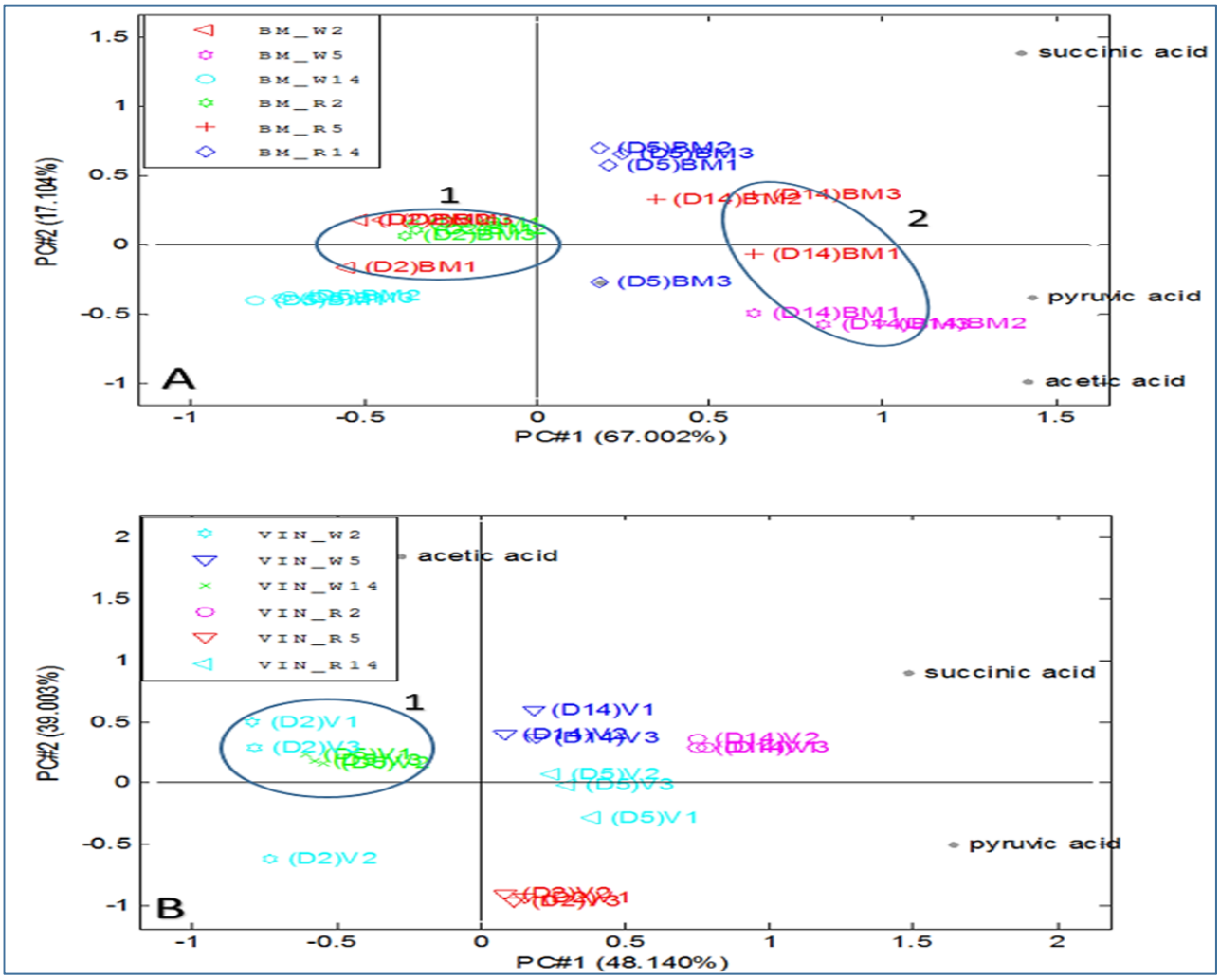

Figure 7: PCA bi-plot based on organic acid concentrations produced by strains BM45 (A) and VIN13 (B) at different time points (day 2, 5 and 14) under white and red wine-like conditions. Biological repeats of the same samples are differentiated as 1, 2 and 3. BM represents BM45 and VIN represents VIN13. The time points are described as 2 for day 2, 5 for day 5 and 14 for day14.

With regard to the VIN13 fermentations, the first principal component in fig 7B accounted for 48.14% of variation. Samples separated from one another based on the stage of fermentation and environmental conditions (fig 7B1). Major drivers of sample separation along the first principal component were succinic and pyruvic acid, while differences in acetic acid concentrations was the main contributor to separation of samples in the second component. Fermentation samples from day 2 and 5 in white wine conditions showed similar organic acid profiles. In the case of VIN13, the

impact of the fermentation treatment clearly exerted the strongest influence on sample separation, as samples from different timepoints for each condition individually clustered more closely together. Here, all samples from the 'red' setting were generally more similar across fermentation stages than the 'white' setting samples at corresponding timepoints. This is in contrast to BM45, where timepoint was the major source of variation and samples from the two conditions generally clustered close together for the different timepoints considered.

4.4.5. The global impact of environmental factors on organic acid profiles of different yeast towards the end of fermentation.

The broad impact of changes in fermentation conditions on organic acids released by BM45 and VIN13 is illustrated by the acid-centric network models below. The networks provide a general overview of the cumulative effects of condition changes on individual acids produced by these two strains under anaerobic conditions. Anaerobic conditions were selected for the analysis due to the applied relevance of anaerobic conditions to real wine fermentations. Here, the influence of pH, temperature, sugar or a combination of these factors were analysed comprehensively. Those changes in fermentation conditions which resulted in lower pyruvic (fig 8), acetic (fig 9) and succinic acid (fig 10) production are represented by blue shading of the nodes while those that resulted in higher acid levels are represented by red shading. In either case, the colour intensity of the shading represents the degree of the impact of the changed conditions on the acid in question.

4.4.5.1 Pyruvic acid

The groupings in figure 8 clearly shows the factors that resulted in the most significant increase (as identified by higher red colour intensity) in pyruvic acid production by VIN13. These were (i) simultaneous changes of pH (from 4 to 3) and temperature (from 15 to 30 ^{o}C), (ii) simultaneous changes of sugar content (from 150 to 250 g/L) and pH (from 4 to 3), (iii) simultaneous changes of sugar content (from 150 to 250 g/L) and temperature (from 15 to 30 ^{o}C). In case of BM45, a significant increases in pyruvic acid were mainly driven by a change in fermentation temperature from 15 to 30 ^{o}C, particularly in must with lower pH and sugar content (fig 8). This highlights once again the influence of strain genetic background on the impact of fermentation parameters on organic acid production. The impact of fermentation conditions on organic acid content is clearly not conserved across all strains.

In most case the reduced pyruvic acid production/release (as identified by high blue colour intensity) in VIN13 were mirror images of the increases described above. They were identified as (i) simultaneous changes in sugar (from 250 to 150 g/L), pH (from 3 to 4) and temperature (from 15 to 30 ^{o}C), (ii) simultaneous changes in pH (from 3 to 4) and temperature (from 15 to 30 ^{o}C), (iii) pH changes (from 3 to 4), particularly when the sugar content of the must was low.

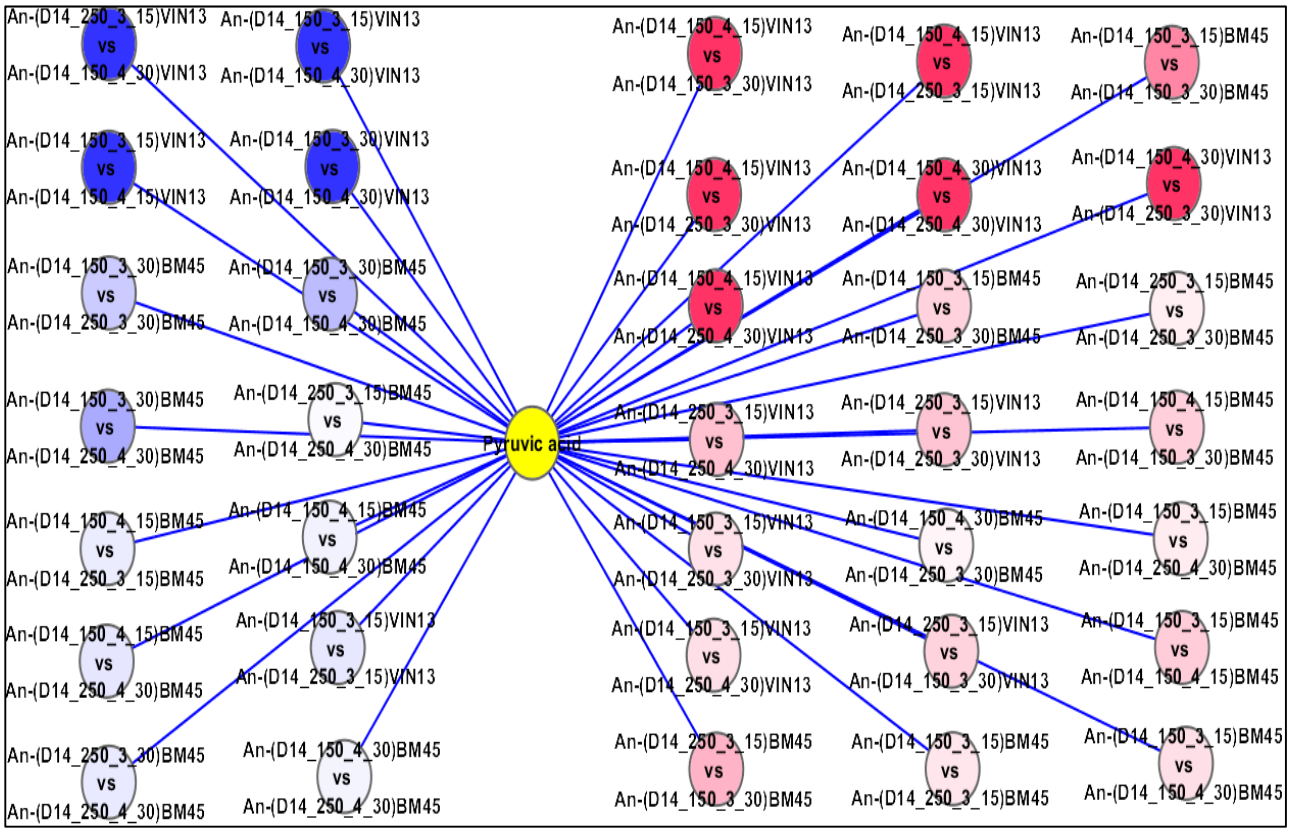

Figure 8: A network model indicating the relationship between changes in environmental conditions and pyruvic acid production of BM45 and VIN13 under anaerobic conditions at the end of fermentation. The red nodes indicate an increase in this organic acid between two conditions, whereas the blue nodes indicate a decrease. The colour intensity of each node represents the degree of change (either increase or decrease) between two conditions.

4.4.5.2 Acetic acid

Regarding the influence of different conditions on acetic acid production (fig 9), the combinatorial changes of sugars (from 150 to 250 g/L) and temperature (from 15 to 30 ^{o}C) showed the most impact on acetic acid production in both strains (red nodes), and these impacts appear to be conserved among all investigated wine yeast.

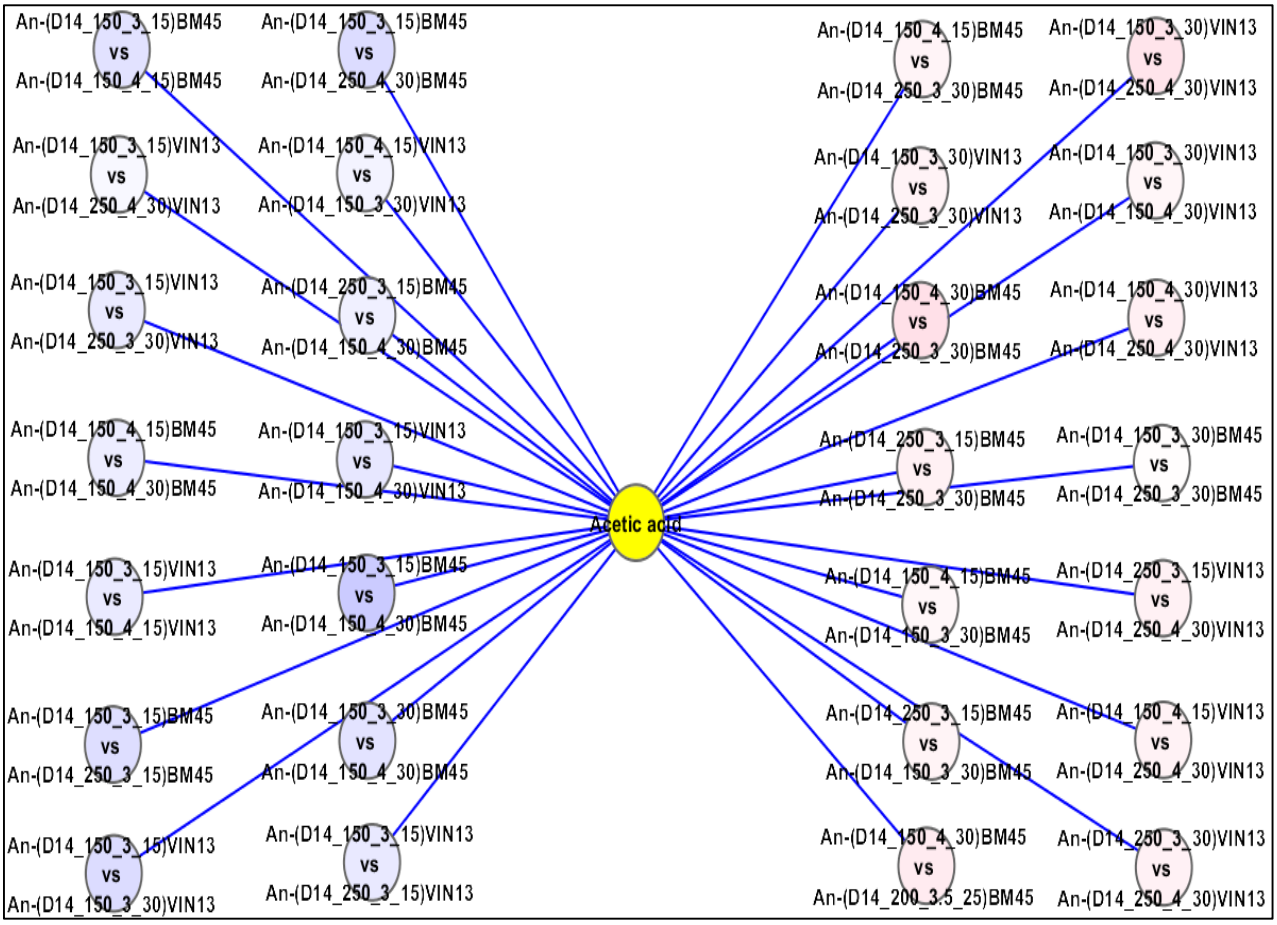

Figure 9: A network model indicating the relationship between changes in environmental conditions and acetic acid production of BM45 and VIN13 under anaerobic conditions at the end of fermentation. The red nodes indicate an increase in organic acid between two conditions, whereas the blue nodes show a decrease. The colour intensity of each node represents the degree of variation between two conditions.

4.4.5.3 Succinic acid

The current study investigated and generated networks to comprehensively establish links between multiple changes in environmental conditions and succinic acid production under anaerobic conditions. Multiple and/or individual parameters that resulted in higher succinic acid production for both VIN13 and BM45 were identified as the combinatorial changes of sugar content from 150 to 250 g/L, temperature from 15 to 30 ^{o}C and pH from 4 to 3 (red nodes, fig 10). Decreases in succinic acid levels are shown by blue nodes in both strains (fig 10). Acid-condition relationships were fairly conserved across strains, while changes in some conditions did not show any significant impact on succinic acid. These finding indicated a very strong impact of pH and sugar content of the must on succinic acid production. Higher succinic acid levels were mostly observed when pH was dropped and at higher sugar contents of the must.

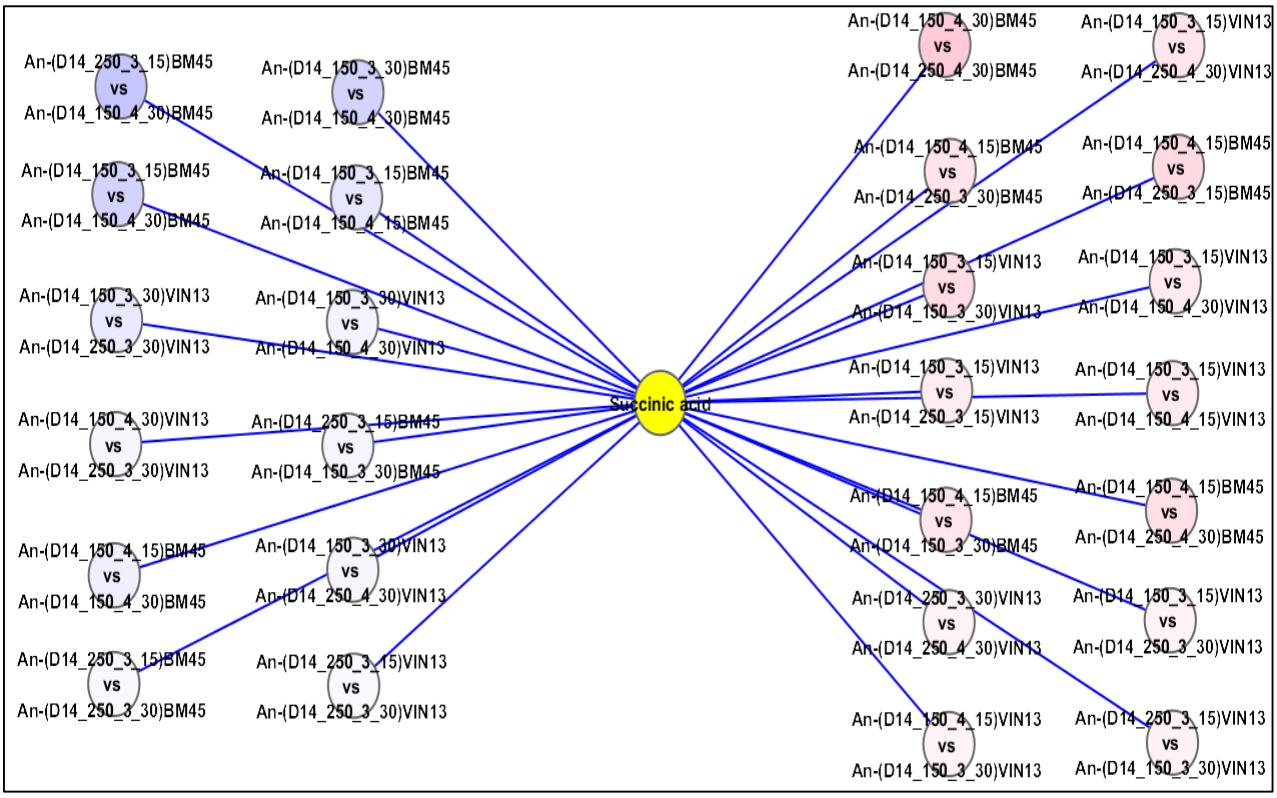

Figure 10: A network model indicating the relationship between changes in environmental conditions and succinic acid production of BM45 and VIN13 under anaerobic conditions at the end of fermentation. The red nodes indicate an increase in organic acid between two conditions, whereas the blue nodes show a decrease. The colour intensity of each node represents the degree of variation between two conditions.

Despite the fact that multi-factorial experiments are very complex due to the diverse nature of parameters investigated, we were able to pinpoint major drivers (individual and/or multiple) of organic acid production under fermentative conditions. Again these results highlight that combinatorial changes to fermentation parameters may have additive, inverse, or no impact on organic acid levels compared to single factorial trends. Combinatorial impacts were dependent on the specific strain employed. Thus, for a given strain, there appears to be specific trends which can be inferred from the network models. This provides a measure of predictability for a given strain where its response to multi-factorial changes have been determined experimentally.

4.5 Conclusion

The effect of nutritional and environmental factors on organic acid production was assessed using a multi-factorial design for different wine yeast strains with different characteristics. The data presented here clearly illustrate the importance of temperature, pH and sugar concentrations during fermentation. These factors proved to play a critical role in terms of impacting grape derived acids (though to a lesser extent) as well as yeast –derived acids which evolve during fermentation. The current study also enabled us to assess the impact of environmental conditions on several widely used commercial wine yeasts. Aeration, higher temperature and initial sugar increased the

rate of fermentation for all five industrial strains. A slight decrease in the initial tartaric, malic and citric acid concentrations were observed when anaerobic fermentation conditions were subjected to low initial sugar, low pH and high temperature for strains such as EC1118. All in all, pyruvic acid levels were significantly impacted by (i) simultaneous changes of pH and temperature (ii) simultaneous changes of sugar content and pH (iii) simultaneous changes of sugar and temperature. Acetic acid concentrations on the other hand, were affected by combinatorial changes of sugars and temperature. However, succinic acid production was mostly driven by the combinatorial changes of all the conditions tested.

Different organic acid profiles were observed among different treatments and strains. The modelling of the networks also enabled us to identify individual and/or multiple drivers of succinic, acetic and pyruvic acid production/release under different fermentation settings which could be encountered by winemakers in industry. To our knowledge, this is the first report highlighting the relationship between growth phase and organic acid levels during alcoholic fermentation by commercial wine yeasts under varying winemaking conditions. The study presents possibilities to better control and manages organic acid content without the use of traditional and labour intensive acid management methods. Moreover, from the data presented here, it is clear that other, non-evaluated parameters such as strain dependent differences are also major drivers of wine acidity, making it difficult to derive a presentable multivariate comparisons and associations.

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Appendix B

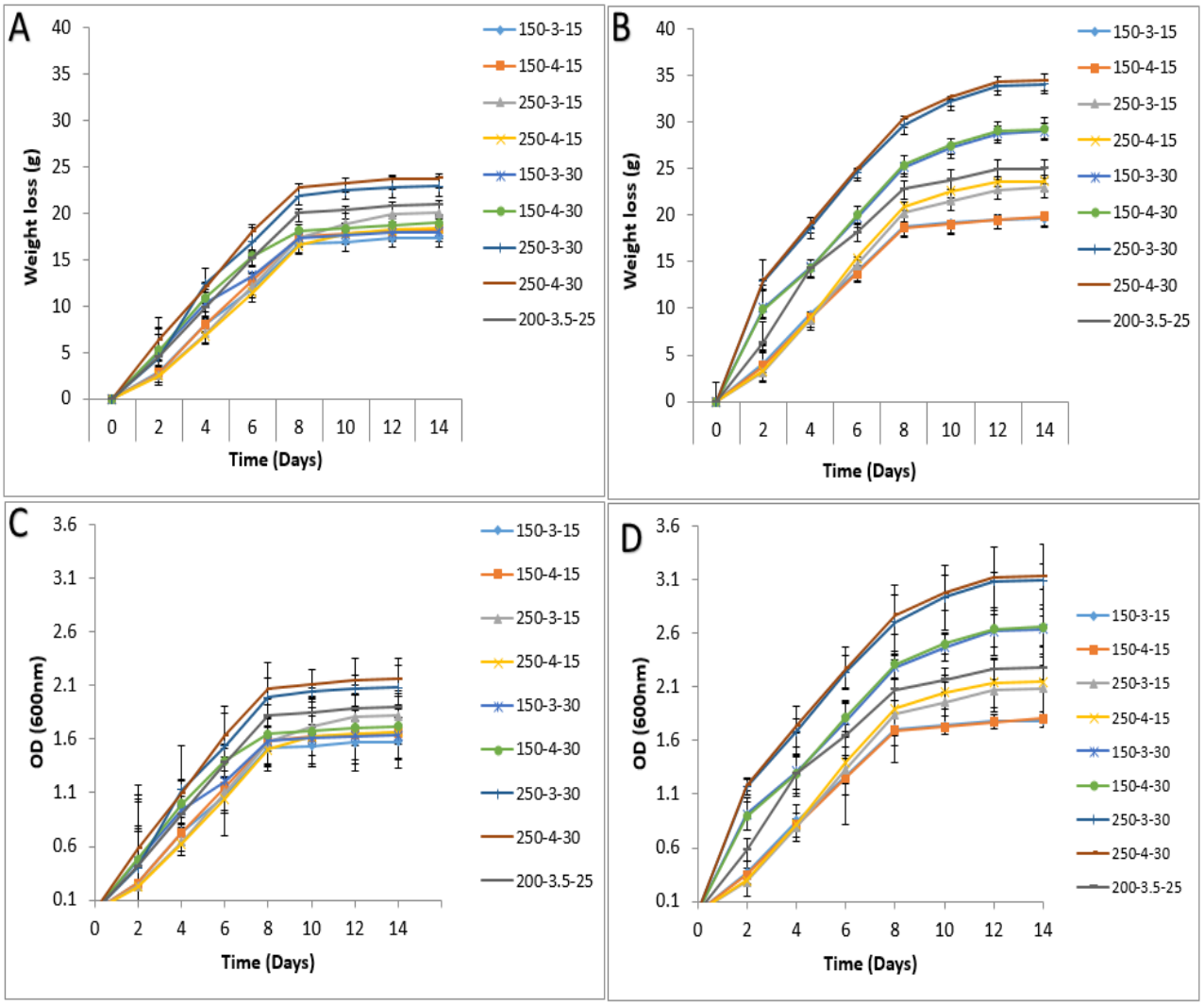

Figure B1. Anaerobic fermentation rates (frame A), aerobic fermentation rates (frame B), anaerobic growth rates (frame C) and aerobic growth rates(frame D) of 285 under various nutritional composition and environmental conditions i.e. sugar (150, 200, and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30^{o}C). Results are the average of 3 biological repeats ± standard deviations.

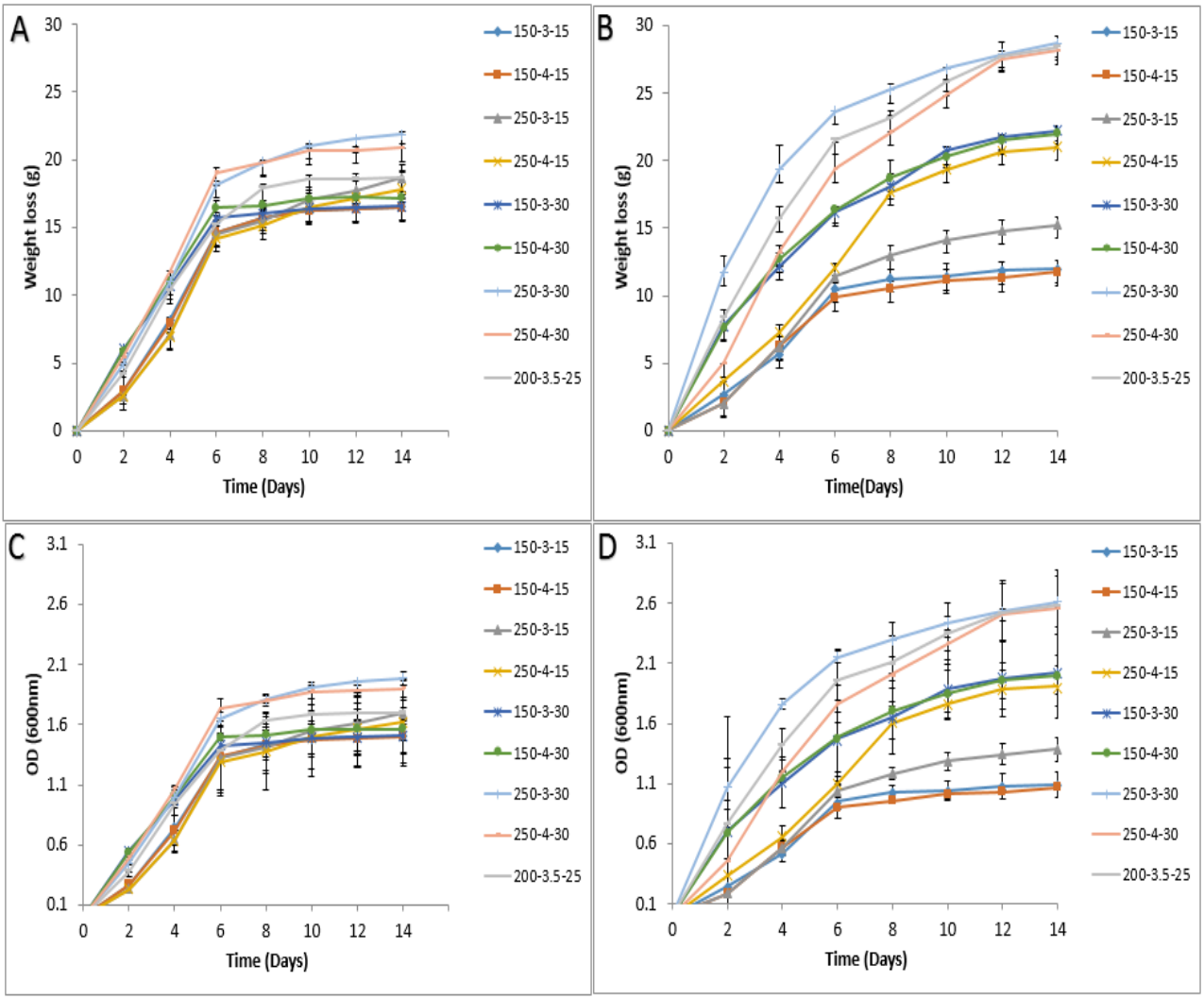

Figure B2. Anaerobic fermentation rates (frame A), aerobic fermentation rates (frame B), anaerobic growth rates (frame C) and aerobic growth rates (frame D) of BM45 under various nutritional composition and environmental conditions i.e. sugar (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30^{o}C). Results are the average of 3 biological repeats ± standard deviations.

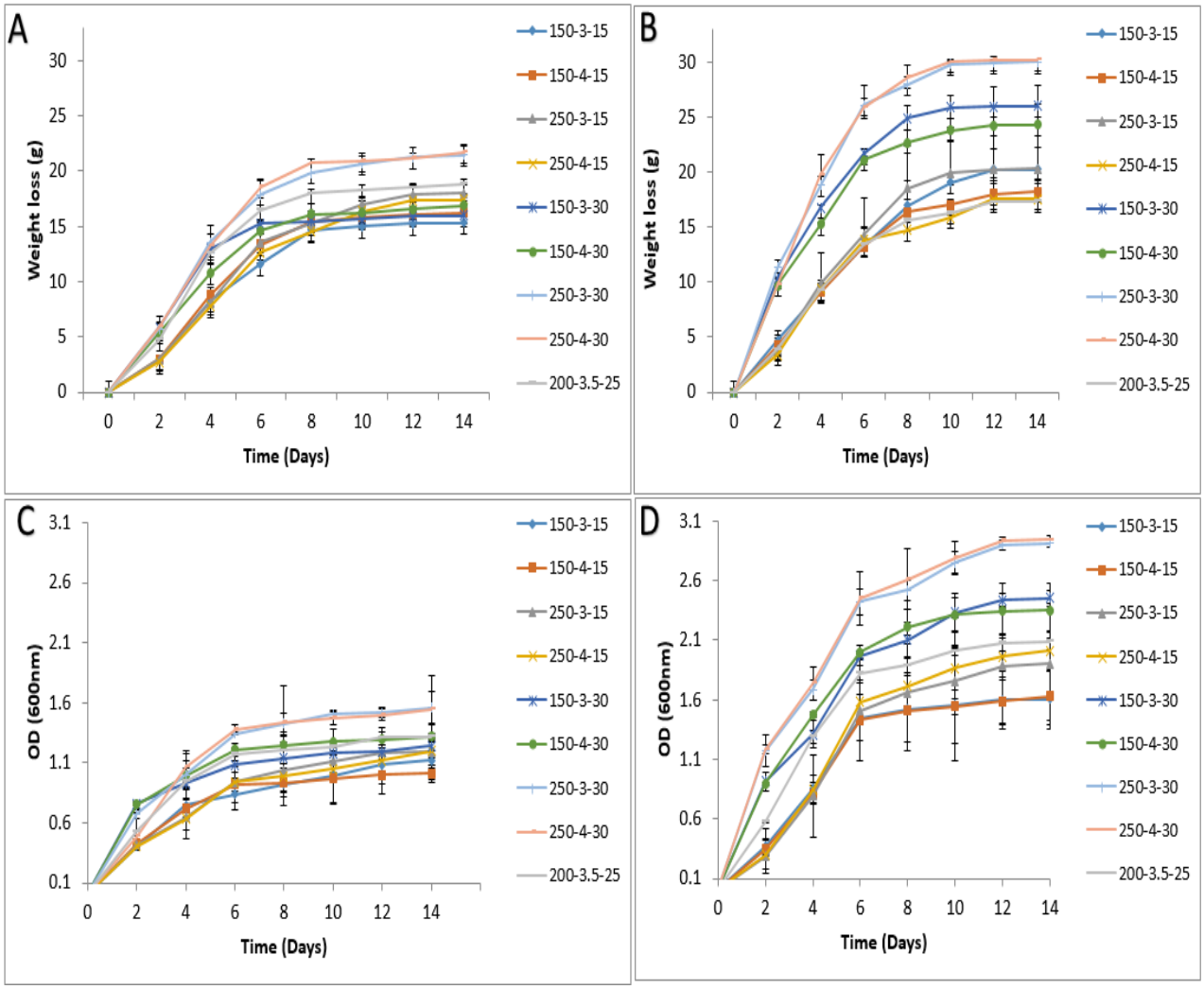

Figure B3. Anaerobic fermentation rates (frame A), aerobic fermentation rates (frame B), anaerobic growth rates (frame C) and aerobic growth rates (frame D) of VIN13 under various nutritional composition and environmental conditions i.e. sugar (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30^{o}C). Results are the average of 3 biological repeats ± standard deviations.

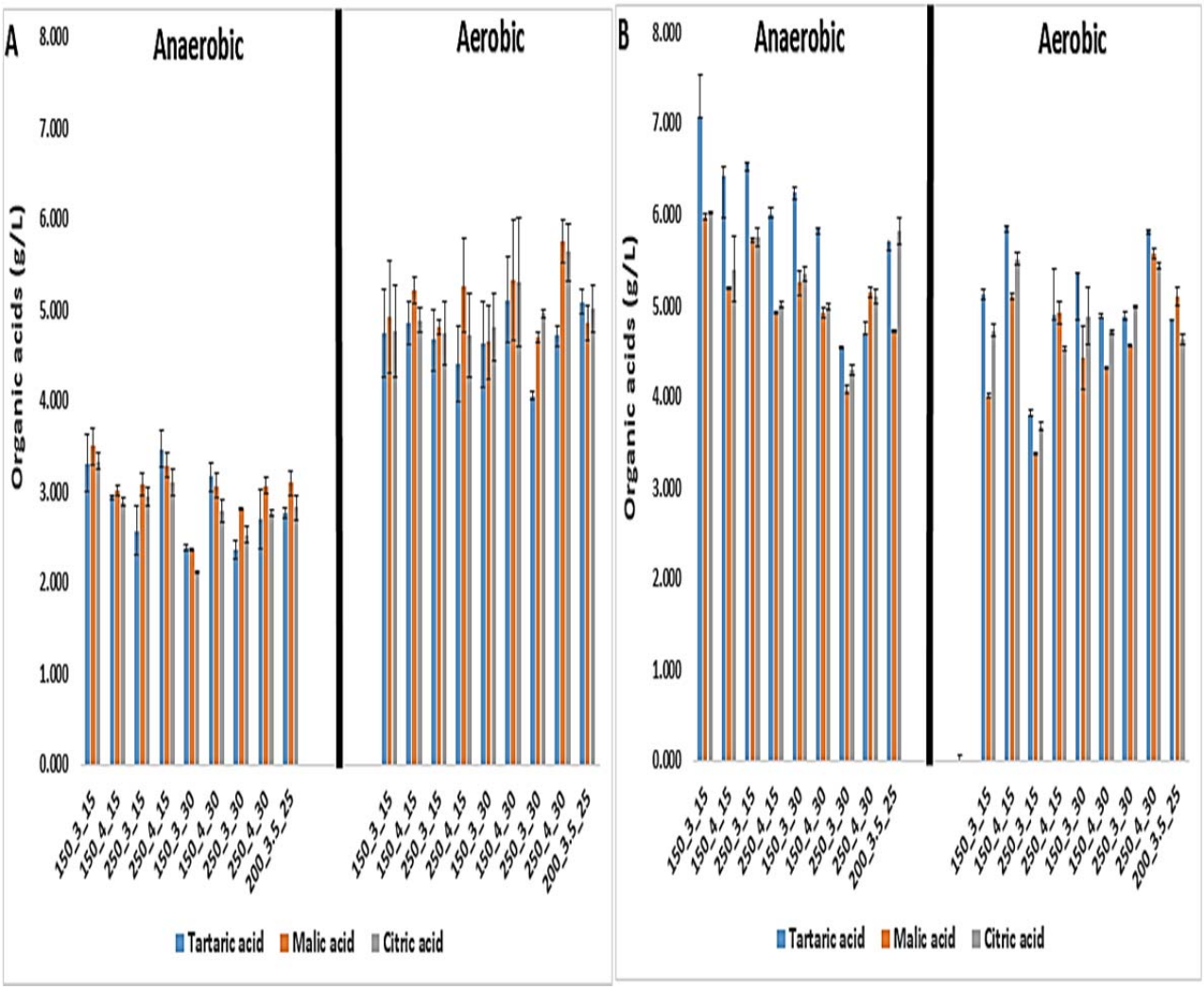

Figure B4. Grape derived acids variations (end-point) for DV10 (A) and BM45 (B) at different environmental conditions under anaerobic and aerobic fermentation conditions, i.e. sugar (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30^{o}C). Results are the average of 3 biological repeats ± standard deviation.

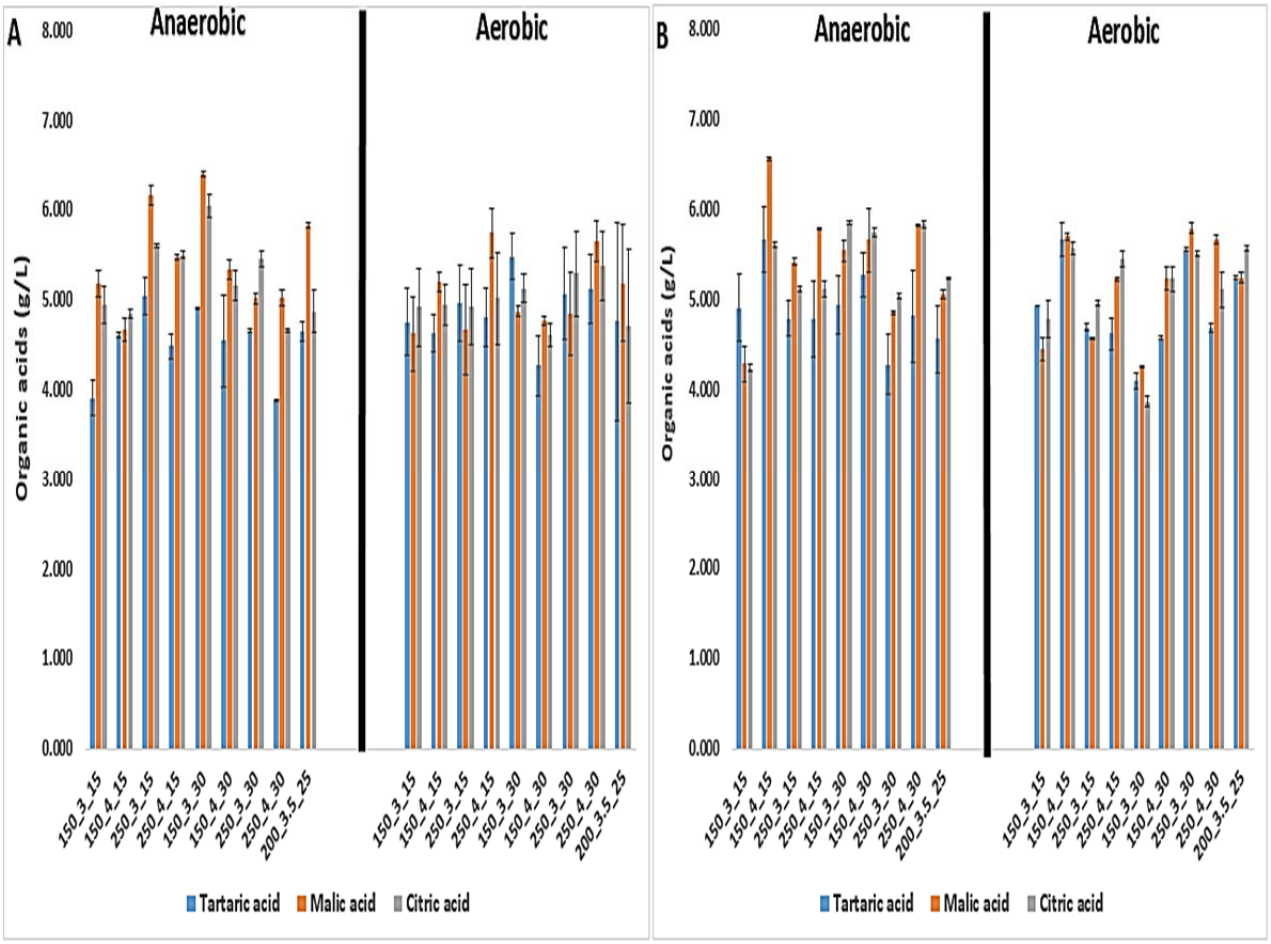

Figure B5. Grape derived acids variations (end-point) for VIN13 (A) and 285 (B) at different environmental conditions under anaerobic and aerobic fermentation conditions, i.e. sugar (150, 200 and 250 g/L), pH (3.0, 3.5 and 4.0) and temperature (15, 25 and 30^{o}C). Results are the average of 3 biological repeats ± standard deviation.

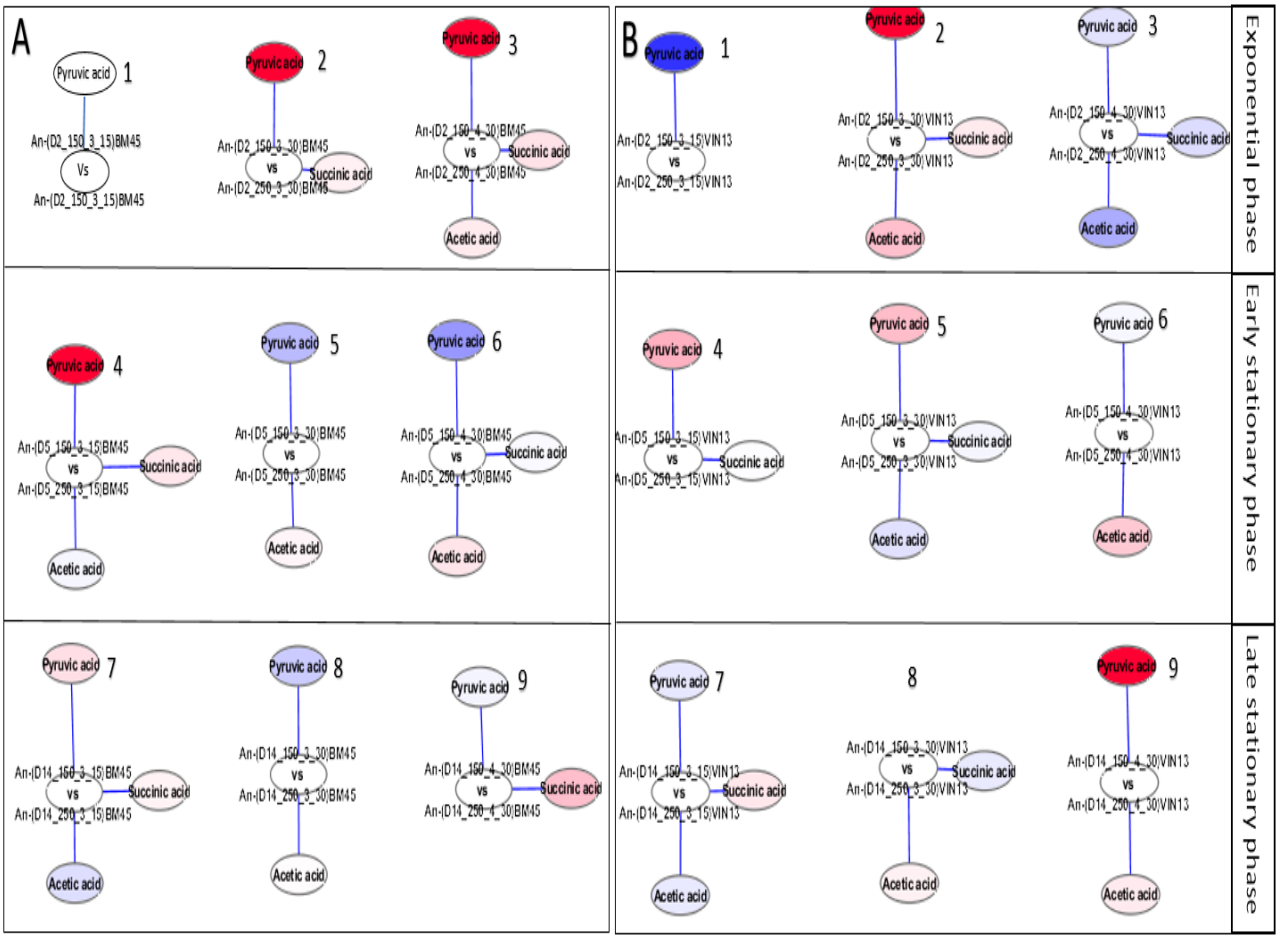

Figure B6. A network model indicating the relationship between the initial sugar content of the must at two varying possible wine making conditions and organic acids produced at different time points for BM45 (A) and VIN13 (B) under anaerobic conditions. The influence of the initial sugar on pyruvic, acetic and succinic acid was compared at 150 and 250 g/L while other parameters (i.e. temperature at 15 or 30°C, pH at 3 or 4) were kept constant. The red nodes indicate an increase in organic acid between two conditions, whereas the blue nodes show a decrease. The colour intensity of each node represents the degree of variation between two conditions.

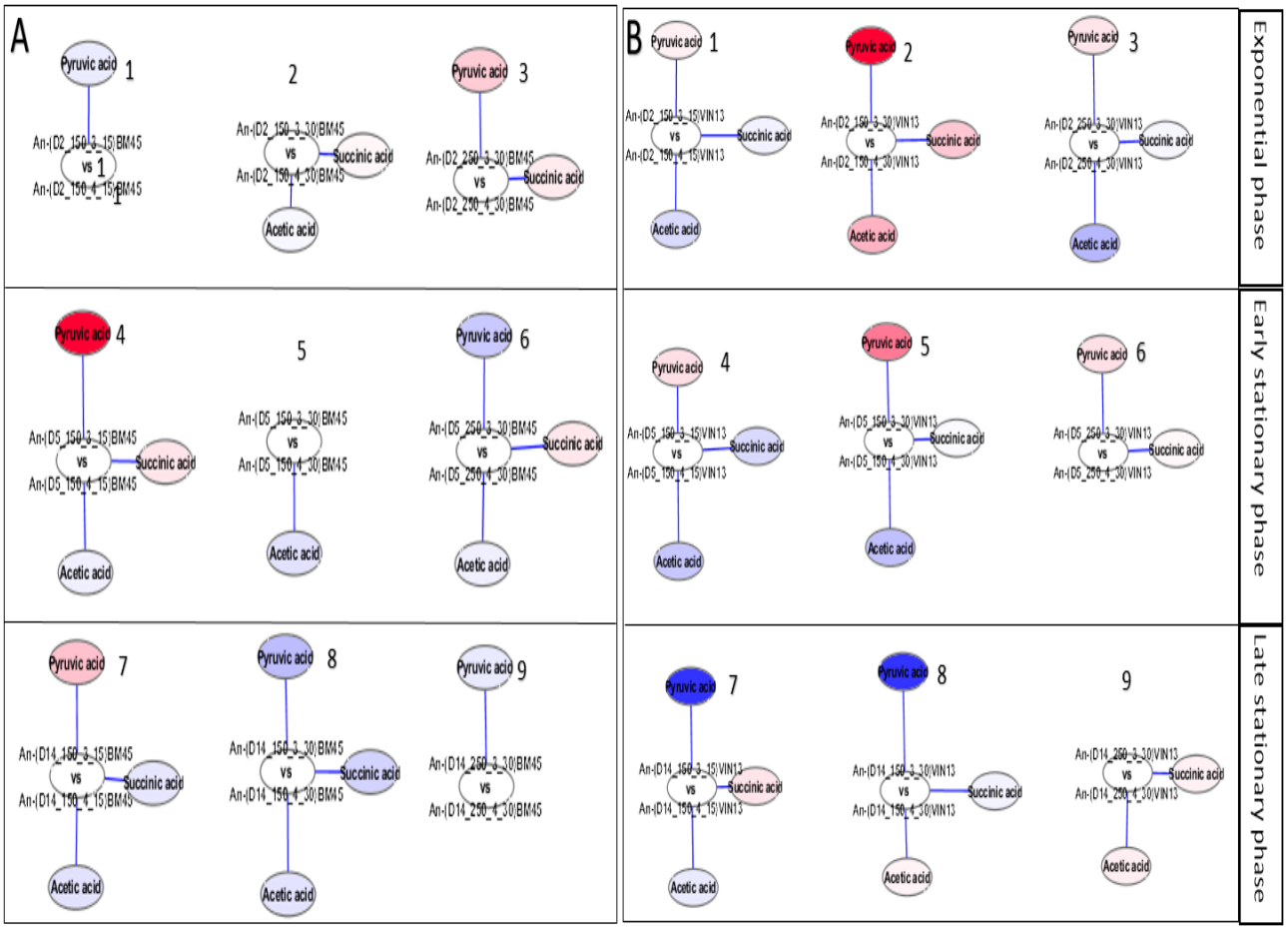

Figure B7. A network model indicating the relationship between the initial pH of the must at two varying possible wine making conditions and organic acids produced at different time points for BM45 (A) and VIN13 (B) under anerobic conditions. The influence of the initial pH on pyruvic, acetic and succinic acid was compared at pH 3.0 and 4.0 while other parameters (i.e. temperature at 15 or 30^{o}C and sugar at 150 or 250 g/L) were kept constant. The red nodes indicate an increase in organic acid between two conditions, whereas the blue nodes show a decrease. The colour intensity of each node represents the degree of variation between two conditions.

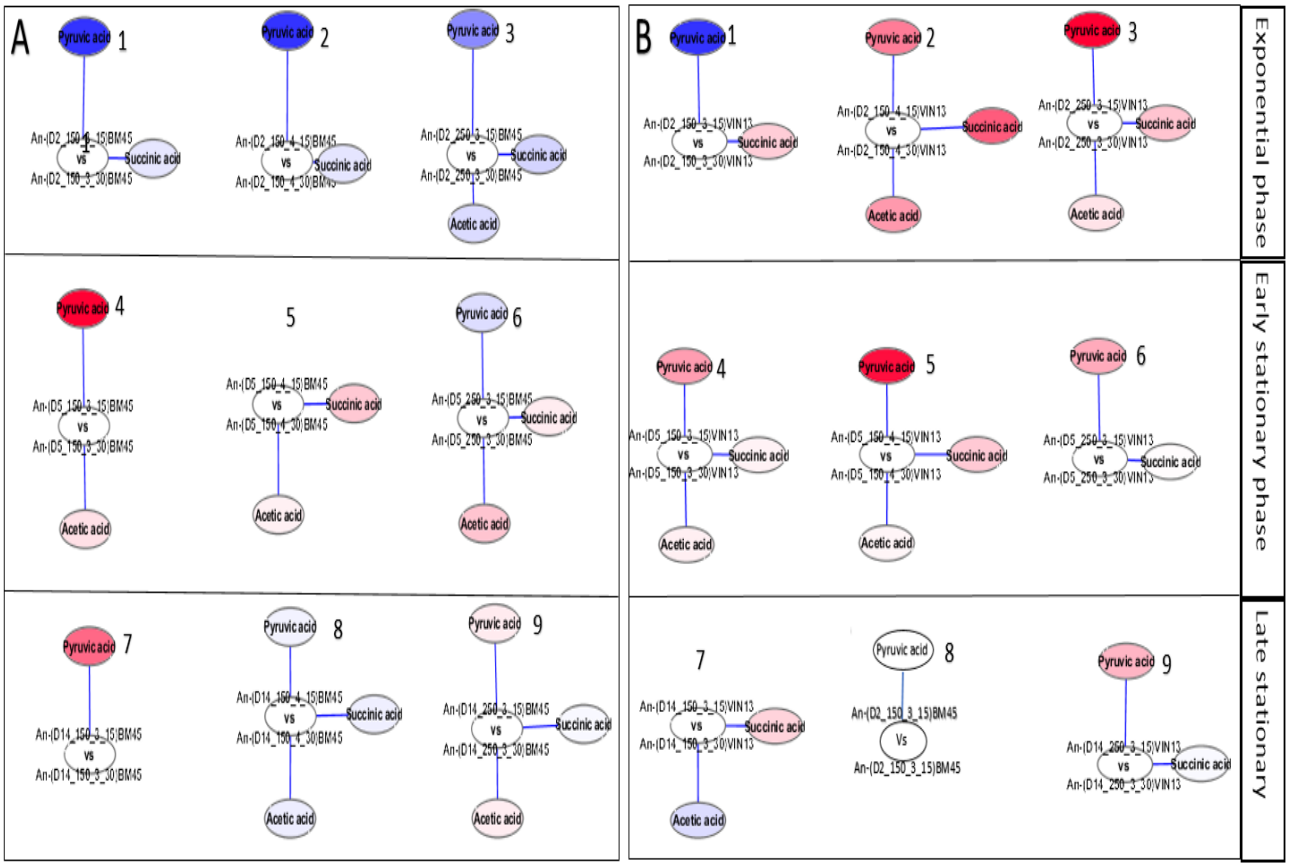

Figure B8. A network model indicating the relationship between fermentation temperature at two varying possible wine making conditions and organic acids produced at different time points for BM45 (A) and VIN13 (B) under anaerobic conditions. The influence of temperature on pyruvic, acetic and succinic acid was compared at 15 and 30 ^{o}C while other parameters (i.e. sugar at 150 or 250 g/L and pH 3 or 4) were kept constant. The red nodes indicate an increase in organic acid between two conditions, whereas the blue nodes show a decrease. The colour intensity of each node represents the degree of variation between two conditions.

Table B1. Organic acid concentrations at the end of fermentation conducted by five commercially available wine yeast strains under anaerobic conditions at lower initial sugar (150 g/L) content, varying pH and temperature. Values are the average of three repeats ± standard deviation.

	Succinic acid	Pyruvic acid	Acetic acid	Anaerobic Conditions
EC1118	0.625±0.077	0.146±0.009	0.217±0.003	150 g/L pH 3 15^{0}C
DV10	0.337±0.009	0.095±0.008	0.150±0.003	
BM45	0.630±0.041	0.219±0.011	0.339±0.019	
VIN13	0.686±0.009	0.295±0.022	0.276±0.008	
285	0.456±0.007	ND	0.313±0.034	
EC1118	0.417±0.006	0.135±0.005	0.094±0.004	150 g/L pH 4 15^{0}C
DV10	0.279±0.005	0.109±0.005	0.155±0.003	
BM45	0.687±0.004	ND	0.265±0.005	
VIN13	0.829±0.002	ND	0.228±0.010	
285	0.764±0.011	0.314±0.035	0.404±0.139	
EC1118	0.673±0.012	0.201±0.008	0.209±0.002	150 g/L pH 3 30^{0}C
DV10	0.246±0.006	0.065±0.002	0.136±0.002	
BM45	0.565±0.007	0.284±0.030	0.286±0.003	
VIN13	0.925±0.032	0.341±0.04	0.205±0.005	
285	0.738±0.061	0.113±0.001	0.561±0.063	
EC1118	0.688±0.025	0.262±0.019	0.230±0.006	150 g/L pH 4 30^{0}C
DV10	0.258±0.003	0.084±0.009	0.157±0.003	
BM45	0.596±0.007	ND	0.225±0.007	
VIN13	0.830±0.020	ND	0.223±0.006	
285	0.814±0.023	0.326±0.025	0.476±0.045	

ND-not detected. Method detection limits: 0.0394 mg/l.

Table B2. Organic acids production at the end of fermentation by five commercially available wine yeast as well as their response to varying anaerobic culture conditions at higher initial sugar (≥200 g/L) content and varying pH and temperature. Values are the average of three repeats ± standard deviation.

	Succinic acid	Pyruvic acid	Acetic acid	Anaerobic Conditions
EC1118	0.467±0.018	0.147±0.007	0.218±0.003	250 g/L pH 3 15°C
DV10	0.275±0.008	0.102±0.006	0.160±0.005	
BM45	0.615±0.004	0.316±0.019	0.257±0.004	
VIN13	0.814±0.026	0.239±0.014	0.226±0.003	
285	0.601±0.054	0.114±0.005	0.389±0.110	
EC1118	0.537±0.026	0.206±0.007	0.209±0.004	250 g/L pH 4 15°C
DV10	0.334±0.012	0.154±0.014	0.162±0.005	
BM45	0.470±0.017	ND	0.261±0.003	
VIN13	0.780±0.011	ND	0.207±0.006	
285	0676±0.009	0.302±0.027	0.459±0.149	
EC1118	0.622±0.013	0.192±0.013	0.215±0.013	250 g/L pH 3 30°C
DV10	0.242±0.014	0.105±0.007	0.136±0.003	
BM45	0.643±0.017	0.230±0.010	0.293±0.004	
VIN13	0.765±0.007	0.378±0.009	0.228±0.001	
285	0.653±0.023	0.207±0.011	0.413±0.055	
EC1118	0.576±0.004	0.367±0.005	0.185±0.018	250 g/L pH 4 30°C
DV10	0.218±0.004	0.178±0.004	0.138±0.003	
BM45	0.598±0.008	0.297±0.029	0.253±0.025	
VIN13	0.854±0.009	0.387±0.004	0.258±0.002	
285	0.793±0.013	0.392±0.025	0.510±0.073	
EC1118	0.859±0.005	0.181±0.007	0.247±0.002	200 g/L pH3.5 25°C
DV10	0.225±0.006	0.125±0.007	0.194±0.006	
BM45	0.651±0.039	0.296±0.018	0.268±0.015	
VIN13	0.829±0.008	0.373±0.006	0.185±0.003	
285	0.748±0.007	0.151±0.003	0.243±0.039	

ND-not detected. Method detection limits: 0.0394 mg/l.

Chapter 5

Research results

Assessment of wine acid related genes: A model based approach

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Current Genetics

2016

Current Genetics, 62(1), 149-164

CHAPTER 5

Title: Assessment of wine acid related genes in yeast: A model based approach

5.1 Abstract

Alcoholic fermentation of grape must is carried out by various wine yeast strains (most often of the species *Saccharomyces cerevisiae*) with different fermentation characteristics and metabolic profiles. These variations often contribute significantly to differences in the organoleptic properties of the wine produced by different strains. Such differences between strains have been shown to also significantly impact on the organic acid composition of wine, an important quality parameter. However, little is known about the genes (or their regulation) which may impact on organic acids during grape must fermentation. To pursue novel insights into the genetic factors which may impact organic acid metabolism, a subset of genes (*ADH3, AAD6, SER33, ICL1, GLY1, SFC1, SER1, KGD1, AGX1, OSM1* and *GPD2*) was selected based on the inter-strain differences in transcription of these genes and their likely potential to influence organic acid metabolism based on functional annotation. Many of these genes have been known to play a significant role either directly in organic acid synthesis and/or degradation, or indirectly in central carbon metabolism. The metabolic impact of these genes was predicted based on a comparative analysis of the transcriptomes and organic acid profiles of different yeast strains showing different production levels of organic acids. Yeast strains carrying deletions for these genes were used to conduct fermentations and determine organic acid profiles to further confirm their potential role in organic acid metabolism. Organic acid levels were determined at various stages during alcoholic fermentation and changes in organic acid levels were assessed and aligned with prediction models based on the original gene expression and organic acid correlations. The alignment of predicted and actual organic acid concentrations produced by mutants was consistent for certain of the deletion strains and/or specific time points but in several cases did not lead to the expected outcome. The data however contribute to our understanding of the roles of selected genes in yeast metabolism in general, and of organic acid metabolism in particular. Several additional genes (*SPG4, ATF2, HXT4, SDH1, FBP26, IDP3, LSC2* and *MEP1*) were further identified in an unbiased manner (without enrichment based on functional annotation) by our differential gene expression and metabolic analyses as candidates for further investigation.

5.2 Introduction

Wine acidity is influenced by the level of organic acids in grape berries at harvest, as well as the organic acids produced or utilised during alcoholic fermentation. The yeast–derived organic acids, primarily succinic, acetic and pyruvic acid, contribute to define the sensorial properties and organoleptic characteristics of wines. There are several genes that play a critical role in organic acid metabolism, but only limited information exists regarding their potential impact on, and role in the regulation of, wine acidity. Understanding the roles of these genes could aid the production of wines with well-balanced organic acids levels to achieve desirable sensory outcomes in a given scenario.

In *S. cerevisiae* central carbon metabolism has been studied extensively, however organic acid metabolism under fermentative conditions as well as the metabolic networks and gene regulation involved are not fully understood. Metabolic pathways such as glycolysis, the glyoxylate cycle and Krebs cycle (TCA) all have organic acids as intermediates or by-products, and therefore have the potential to impact on the concentration of organic acids in wine. Besides carbon metabolism, nitrogen metabolism-related pathways may also act as a major source of acids such as pyruvic and succinic acid (Camarasa *et al*., 2003; Torrea *et al*., 2004; Vilanova *et al*., 2007; Magyar *et al*., 2014; fig. 1).

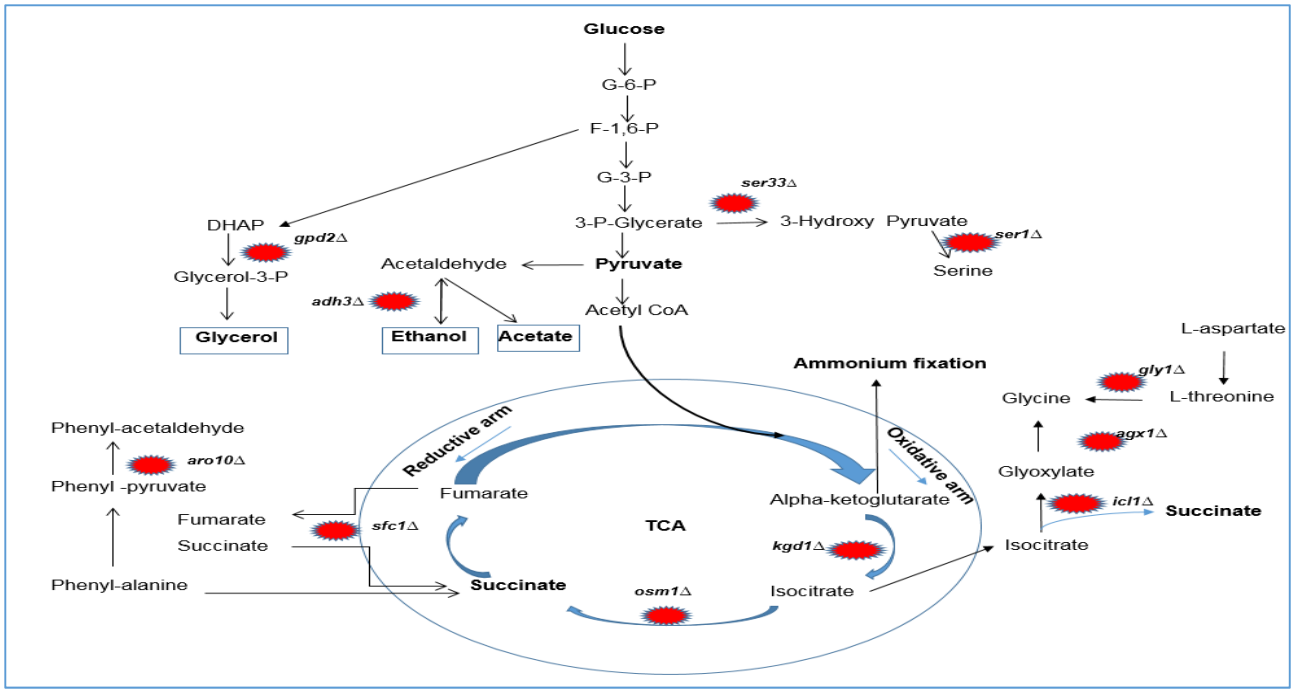

Figure 1: Diagrammatic representation of pathways associated with organic acid production under anaerobic conditions. (Gene names encoding the relevant enzymes are indicated in bold italics and only those genes that were targeted in the deletion study are indicated on the metabolic pathway maps). The diagram also shows specific points that are required for organic nitrogen fixation as well as metabolic pathways that are discussed in this study.

Transcriptomic studies provide useful information regarding the specific function of certain genes or groups of genes and their characteristics (Hirasawa *et al*., 2010). Transcriptomic studies of wine yeast have been employed successfully to identify genes that influence the production of volatile aroma compounds during fermentation (Rossouw *et al*., 2008). The aim of our study was to identify candidate genes which may have an impact on organic acid profiles during alcoholic fermentation using a transcriptomics approach. In our study, the transcriptomic data of five industrial yeast strains were analysed at three different time-points in order to identify differentially expressed genes linked to changes in organic acid profiles. From the list of candidate genes identified in this unbiased manner, target genes for further experimentation were selected based on their known roles in, or association with carbon and/or nitrogen metabolism.

Genes that were selected in this manner include those encoding enzymes involved in maintaining redox balance or in catalysing key reactions linked to organic acid flux in central carbon metabolism. A number of genes associated with redox reactions and redox balancing were selected in this manner, including *SER33* (3-phosphoglycerate dehydrogenase), *GDP2* (NAD-dependent glycerol 3-phosphate dehydrogenase), *ARO10* (phenylpyruvate decarboxylase), *AAD6* (Putative aryl-alcohol dehydrogenase) and *ADH3* (alcohol dehydrogenase isozyme III). In addition, genes which play a role in glucose transport (*HXT4*), glucose metabolism (*FBP26*) and growth (*SPG4*) were also identified as potential organic acid role-players based on comparative gene expression and metabolite analysis across strains. Other genes including *KGD1* (alpha-ketoglutarate dehydrogenase), *AGX1* (glyoxylate aminotransferase) and *GLY1* (Threonine aldolase) have previously been shown to play a significant role in organic acid metabolism in yeast (Arikawa *et al*., 1999; Porro *et al*., 1995).

Earlier studies have investigated the physiological and metabolic roles of some of the genes identified in our study. For instance, *GLY1* (Threonine aldolase, involved in glycine biosynthesis) deletion resulted in reduced growth of *Saccharomyces cerevisiae* (DSM70452) when glucose was used as sole carbon source (Monschau *et al*., 1997) and *SFC1* gene disruption results in cells unable to grow on ethanol and acetate carbon sources (Fernandez *et al*., 1994). In a study by Otero *et al*. (2013) it was shown that *SER33* disruption (see table 1 for annotation) increased succinate formation due to the interruption of glycolysis through serine metabolism (Otero *et al.*, 2013). Albers *et al*. (2003) indicated a decrease in acetic acid, biomass and glycerol formation when *SER33* mutant strains were tested with glutamate as the sole nitrogen source. In addition, a substantial increase in pyruvic acid and fumaric acid and a decrease in succinic acid were also observed.

In another study, Arikawa *et al*. (1999) demonstrated that the disruption of the fumarate reductase gene (*OSM1*) resulted in 1.5-fold higher levels of succinate compared to the wild-type while alpha-

ketoglutarate dehydrogenase (*KGD1*) deletion reduced succinate productivity in Japanese alcohol beverages. In contrast, succinic acid production by *S. cerevisiae* was shown to increase when genes encoding succinate (*SDH1*, *SDH2*) dehydrogenase and isocitrate dehydrogenase (*IDH1, IDP1*) were deleted (Raab *et al*., 2010).

Several gene deletion/over expression studies have reported significant effects on yeast growth as well as glycerol metabolism. For example (table 1), the deletion of the ADH genes (*ADH3* and *ADH4*) encoding alcohol dehydrogenase isozymes (involved in the shuttling of mitochondrial NADH to the cytosol under anaerobic conditions and ethanol production) displayed a significantly increased glycerol formation and sluggish growth (Drewke *et al*., 1990). Overexpresson of *GPD2*, a NAD-dependent glycerol 3-phosphate dehydrogenase, results in an increase in both glycerol and acetic acid production in *S. cerevisiae* (de Barros *et al*., 2000; Michnick *et al*., 1997).

An important biochemical relationship exists between glycerol and acetic acid production (Michnick *et al*., 1997 and Remize *et al*., 1999). Glycerol is formed in order to reoxidize NADH which is formed during glycolysis (Jackson, 2008). *S. cerevisiae* is capable of utilizing acetic acid as a redox sink to convert NAD+ back to NADH). Alterations in glycerol metabolism, such as increased glycerol production, is generally accompanied by an increase in acetic acid production to compensate for changes to the cellular redox balance (de Barros Lopes *et al*., 2000; Prior *et al*., 2000).

The impact of the selected differentially expressed genes involved in redox balance and organic acid metabolism were evaluated in our study using the corresponding knockout strains from the EUROSCARF deletion library (accession numbers provided in table 1). Fermentations were carried out using these strains to determine whether the changes in one or more of the organic acids were in line with predictions based on the transcriptome-metabolome correlation matrices. Here we describe the application of a systems biology approach, linking comparative transcriptomics and organic acid analysis in wine in order to identify and further evaluate genes which may influence organic acid levels in wine.

5.3 Materials and methods

5.3.1 Strains, media and culture conditions

Five commercial wine yeast strains were selected based on their divergent fermentation properties (see chapter 3). The deletion mutants used in this study were obtained from the EUROSCARF deletion library. All the deletion mutants used as well as description of the function/s of deleted genes are listed in table 1. These genes were selected based on the transcriptome data that was generated previously (Rossouw *et al*., 2008). Only those genes that showed differential expression

between strains at one or more time points during fermentation and which are also directly/indirectly related to either glycerol or organic acid metabolism were investigated.

Cells were maintained on solid medium YPD which was supplemented with 2% agar (Biolab, South Africa). Pre-cultures were carried out in 50 ml shake-flasks at 30 ^{o}C, 250 rpm in YPD synthetic media containing 1% yeast extract (Biolab, South Africa), 2% peptone (Fluka, Germany), 2% dextrose (Sigma, Germany). Fermentations were inoculated at an OD_{600} of 0.1 (i.e. a final cell density of approximately 10^6 cfu/ml) after washing pre-culture cells with sterile distilled water.

Table 1. Subset of genes selected for their potential roles in acid balance (gene descriptions were obtained from Saccharomyces Genome Database).

Target Gene	Accession numbers	Description
SFC1	Y16907	Mitochondrial succinate-fumarate transporter; transports succinate into and fumarate out of the mitochondrion; required for ethanol and acetate utilization.
OSM1	Y14216	Fumarate reductase; catalyzes the reduction of fumarate to succinate, required for the reoxidation of intracellular NADH under anaerobic conditions; mutations cause osmotic sensitivity.
ICL1	Y10202	Isocitrate lyase; catalyzes the formation of succinate and glyoxylate from isocitrate, a key reaction of the glyoxylate cycle; expression of *ICL1* is induced by growth on ethanol and repressed by growth on glucose.
KGD1	Y12284	Component of the mitochondrial alpha-ketoglutarate dehydrogenase complex, which catalyzes a key step in the tricarboxylic acid (TCA) cycle, namely the oxidative decarboxylation of alpha-ketoglutarate to form succinyl-CoA.
AGX1	Y15649	Alanine: glyoxylate aminotransferase; catalyzes the synthesis of glycine from glyoxylate, which is one of three pathways for glycine biosynthesis in yeast; has similarity to mammalian and plant alanine: glyoxylate aminotransferases.
GLY1	Y10287	Threonine aldolase; catalyzes the cleavage of L-allo-threonine and L-threonine to glycine; involved in glycine biosynthesis.
SER33	Y11467	3-phosphoglycerate dehydrogenase; catalyzes the first step in serine and glycine biosynthesis; isozyme of Ser3p.
GPD2	Y11751	NAD-dependent glycerol 3-phosphate dehydrogenase; homolog of Gpd1p, expression is controlled by an oxygen-independent signalling pathway required to regulate metabolism under anoxic conditions; located in cytosol and mitochondria.
ARO10	Y14216	Phenylpyruvate decarboxylase; catalyzes decarboxylation of phenylpyruvate to phenylacetaldehyde, which is the first specific step in the Ehrlich pathway.
AAD6	Y15677	Putative aryl-alcohol dehydrogenase with similarity to P. chrysosporium aryl-alcohol dehydrogenase, involved in the oxidative stress response.
SER1	Y12440	3-phosphoserine aminotransferase; catalyzes the formation of phosphoSerine from 3-phosphohydroxypyruvate, required for serine and glycine biosynthesis; regulated by the general control of amino acid biosynthesis mediated by Gcn4p.

Table 1. (cont.)

Target Gene	Accession numbers	Description
ADH3	Y16217	Mitochondrial alcohol dehydrogenase isozyme III; involved in the shuttling of mitochondrial NADH to the cytosol under anaerobic conditions and ethanol production.
SPG4	--------	Protein required for survival at high temperature during stationary phase; not required for growth on non-fermentable carbon sources.
FBP26	--------	Fructose-2,6-bisphosphatase; required for glucose metabolism. Protein abundance increases in response to DNA replication stress
ATF2	---------	Alcohol acetyltransferase; may play a role in steroid detoxification, forms volatile esters during fermentation, which is important in brewing.
SDH1	---------	Minor succinate dehydrogenase isozyme; homologous to Sdh1p, the major isozyme responsible for the oxidation of succinate and transfer of electrons to ubiquinone; induced during the diauxic shift in a Cat8p-dependent manner.
HXT4	---------	High-affinity glucose transporter of the major facilitator superfamily, expression is induced by low levels of glucose and repressed by high levels of glucose.
IDP3	---------	Peroxisomal NADP-dependent isocitrate dehydrogenase; catalyzes oxidation of isocitrate to alpha-ketoglutarate with the formation of NADP (H+), required for growth on unsaturated fatty acids.
LSC2	---------	Beta subunit of succinyl-CoA ligase; a mitochondrial enzyme of the TCA cycle that catalyzes the nucleotide-dependent conversion of succinyl-CoA to succinate.
MEP1	---------	Ammonium permease; belongs to a ubiquitous family of cytoplasmic membrane proteins that transport only ammonium (NH4+); expression is under nitrogen catabolite repression regulation.
BY4742	---------	Haploid laboratory strain (control)

5.3.2 Fermentation medium

Fermentation experiments of BM45, EC1118, 285, DV10 and VIN13 were conducted in synthetic must MS300 which resembles a natural must as previously described (Bely *et al*., 1990). The medium contained equivalent amounts of 100 g/L each of glucose and fructose (total sugars of 200 g/L), and the pH was set to 3.5 using sodium hydroxide. Fermentations were carried out under anaerobic conditions in 250 ml Erlenmeyer flasks (containing 100 ml of the medium) at a temperature of 25 ^{o}C. Anaerobic fermentations were sealed with rubber stoppers with a CO_2 opening and no stirring was done during the course of the fermentation for a period of 30 days. These batch fermentations were carried out in triplicate. The fermentation progress was monitored by daily weight measurements to determine CO_2 loss and samples from these fermentations were taken at days 2 (exponential phase), 5 (early stationary) and 14 (late stationary phase). Likewise, fermentations inoculated with deletion strains were conducted in synthetic must MS300 at 200 g/L, pH 3.5 and 25 ^{o}C. Fermentations were monitored by weight loss and samples were taken at days 3, 6, 12, 16, 22, and 30 for chemical analysis. These sampling days were selected to cover the range of growth phases of the yeast (exponential, early and late stationary), which in the case of the deletion strains followed a more extended cycle across a longer period of time. For most of the

deletion strains, fermentations were complete by day 30 as opposed to day 14 as was the case for the industrial yeasts. This is due to the lower fermentative capacity of the haploid laboratory strain BY4742 which serves as the genetic background for all the gene knockouts in our study. Cell growth was monitored at two day intervals by measuring the optical density (at 600 nm) using a spectrophotometer (PowerwaveX, Bio-Tek Instruments).

5.3.3 Analyses

5.3.3.1 Analytical methods for sugars – HPLC

Culture supernatants were obtained and analysed for sugars by high performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column at 55 ^{o}C using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.5 ml/min. Agilent RID and UV detectors were used for detection and quantification. Analyses were carried out using the HP Chemstation software package (Eyeghe *et al*., 2012).

5.3.3.2 Enzyme robot

Culture supernatants were filtered through 0.45 µM nylon membranes and analyzed by an enzyme robot (Arena 20XT; Thermo Electron, Finland) using the Enzytec™ Fluid Acetic acid test kit (Thermo Fisher Scientific, Finland) for acetic acid quantification, Boehringer Mannheim/R-Biopharm Succinic acid kit (R-Biopharm AG, Darmstadt) for succinic acid determination as well as the Megazyme Pyruvic acid kit (Megazyme International Ireland) for pyruvic acid quantification. NADH consumption was measured by the decrease in absorbance at 340 nm.

5.3.4 Microarray analysis and data processing

Microarray data generated for the five yeast strains under similar fermentation conditions were generated by Rossouw *et al*. (2008). The transcript data can be obtained from the Gene Expression Omnibus (GEO) repository under accession number GSE11651.

5.3.5 Principal component analysis

Principal component analysis (PCA; Latentix 2.0, BRANDON GRAY INTERNET SERVICES, INC. DBA) was used in order to evaluate the impact of gene deletions on glycerol, succinic, acetic and pyruvic acid production at different physiological stages of yeast during fermentation (day 3, 6, 12, 16, 22 and 30). The transformation of the data was carried out using the autoscale function with no validation.

5.3.6 Statistical analysis

SAM (Significance Analysis of Microarrays) version 2 was used to determine the differential gene expression between experimental parameters as described by Rossouw *et al*. (2008). Genes with a Q-value less than 0.5 were regarded as differentially expressed by using the two-class, unpaired setting. Consideration was given to those genes with a fold change higher than 2 (positive or negative) for inter- and intra- strain comparisons. Statistical analysis of differences in organic acid concentrations was performed by means of T-tests (Excel); tail 1 (directional test/one tailed distribution) and type 1 (repeated measure design/paired test).

5.3.7 Alignment model approach

The model was based on the expectation that the deletion of genes may directly or indirectly affect organic acid metabolism in yeast. Our alignment model was formulated by taking into consideration the correlation co-efficiencies of the selected genes and acids for the five industrial strains (BM45, VIN13, 285, DV10 and EC1118) for both intra- and inter-strain comparisons. Although, the study compared transcriptomic and organic acid data of the must with 20% difference in sugar concentration, these are not major differences in sugar levels as they would not affect the physiology of the yeast differently at comparable time points. The sampling points selected present defined points of yeast growth during fermentation, and these were appropriately synchronised as can be seen by comparing the growth and fermentation kinetics of the five strains in the two studies. The actual to measured comparisons were then carried out by comparing these gene-acid correlations with the actual ratio change in organic acid concentrations produced by deletion mutants for the gene in question at the exponential (day 6) and early stationery phase (day 12). The models were aligned by firstly normalising the actual organic acid concentrations relative to the wild type (BY4742). In order to normalise the dimensions of the plot; the inverse of the actual values was applied and the scale of the predicted Vs measured plots were further normalised between -1 and +1.

5.4 Results

5.4.1. Fermentation kinetics and organic acid profiles of wine yeast

The selected commercial wine yeast strains were inoculated to ferment synthetic must under representative winemaking conditions. The strains show some variation in their fermentation kinetics (fig 2), which is in line with previous observations (Rossouw *et al*., 2008). For the purpose of this study, fermentation conditions similar to those of Rossouw *et al*. (2008) were selected for the acid analysis and subsequent identification and modelling of key genes. As reported earlier on, the only difference between the fermentation conditions used in our study and those of Rossouw *et*

al. (2008) was that the initial sugar concentration was 200 g/L as opposed to 250 g/L. However, the fermentation kinetics (and total duration of fermentation) were similar in our fermentations suggesting that the alignment of transcriptome and organic acid production was feasible. In the current study, the fermentations of all strains reached the stationery phase by day 6. Carbon dioxide release (indicative of the rate of fermentation) was similar in fermentations with BM45, 285, VIN13 and EC1118, while DV10 released less carbon dioxide (fig 2A). Higher biomass formation (measured as optical density) was observed for strain 285 while less biomass formation was observed for strain DV10 (fig 2B). Growth profiles and biomass formation of the other three strains were similar in the conditions used.

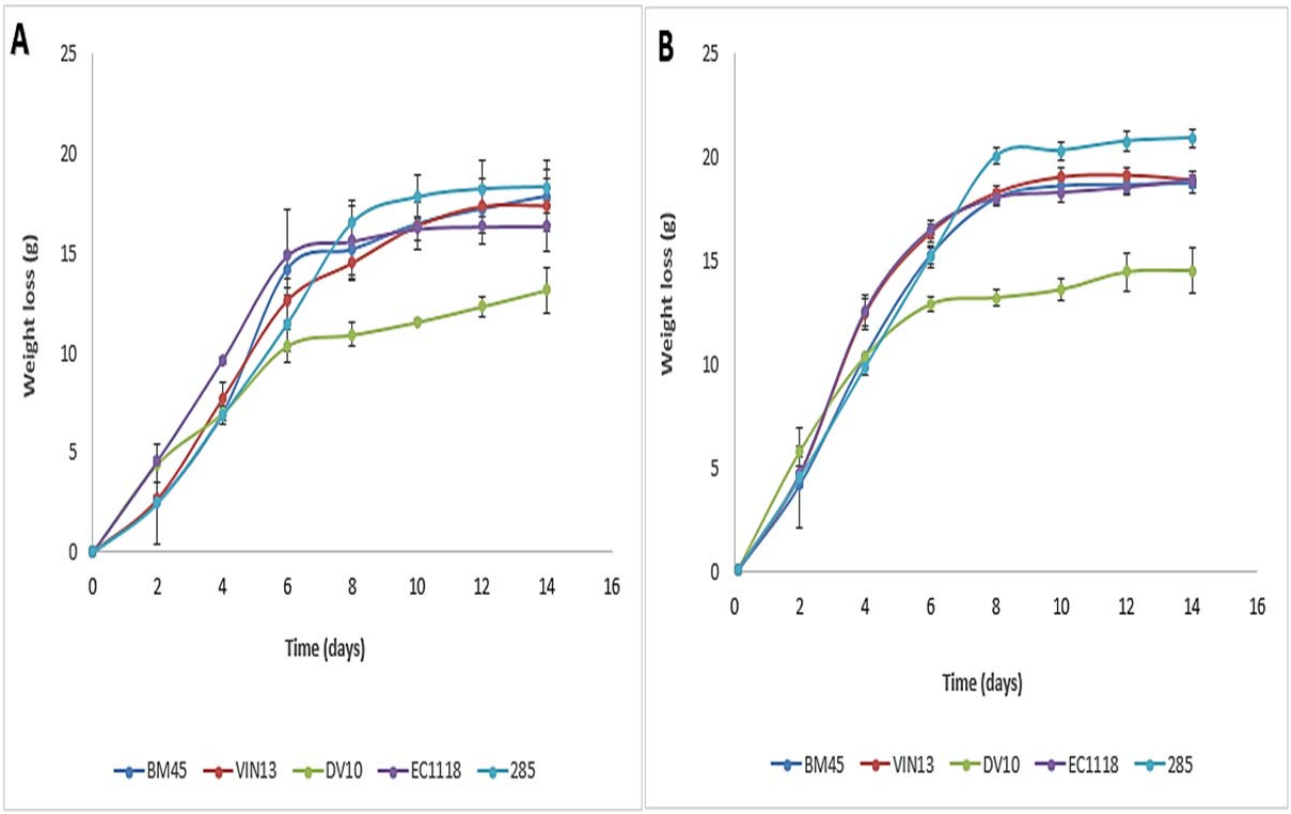

Figure 2: Anaerobic fermentation weight loss (frame A) and growth profiles (frame B) of five strains under simulated wine making conditions, i.e. sugar (200 g/L), pH (3.5) and temperature (25 ^{o}C). Results are the average of 3 biological repeats ± standard deviations.

Differences in organic acid concentrations produced by the five strains at different time points were investigated (fig 3). The concentration of fermentation-derived organic acids (succinic, pyruvic and acetic acid) was evaluated at day 2 (exponential phase), 5 (early logarithmic) and 14 (early logarithmic). All strains behaved similarly with regard to acetic, succinic and pyruvic acid production trends though the absolute concentrations of acids produced varied between strains. A continuous increase in succinic acid throughout fermentation was noted in all strains. The levels of pyruvic and acetic acid remained more or less constant across time points for all strains indicating that acetic and pyruvic acid production by these strains occurred mostly at the onset of fermentation.

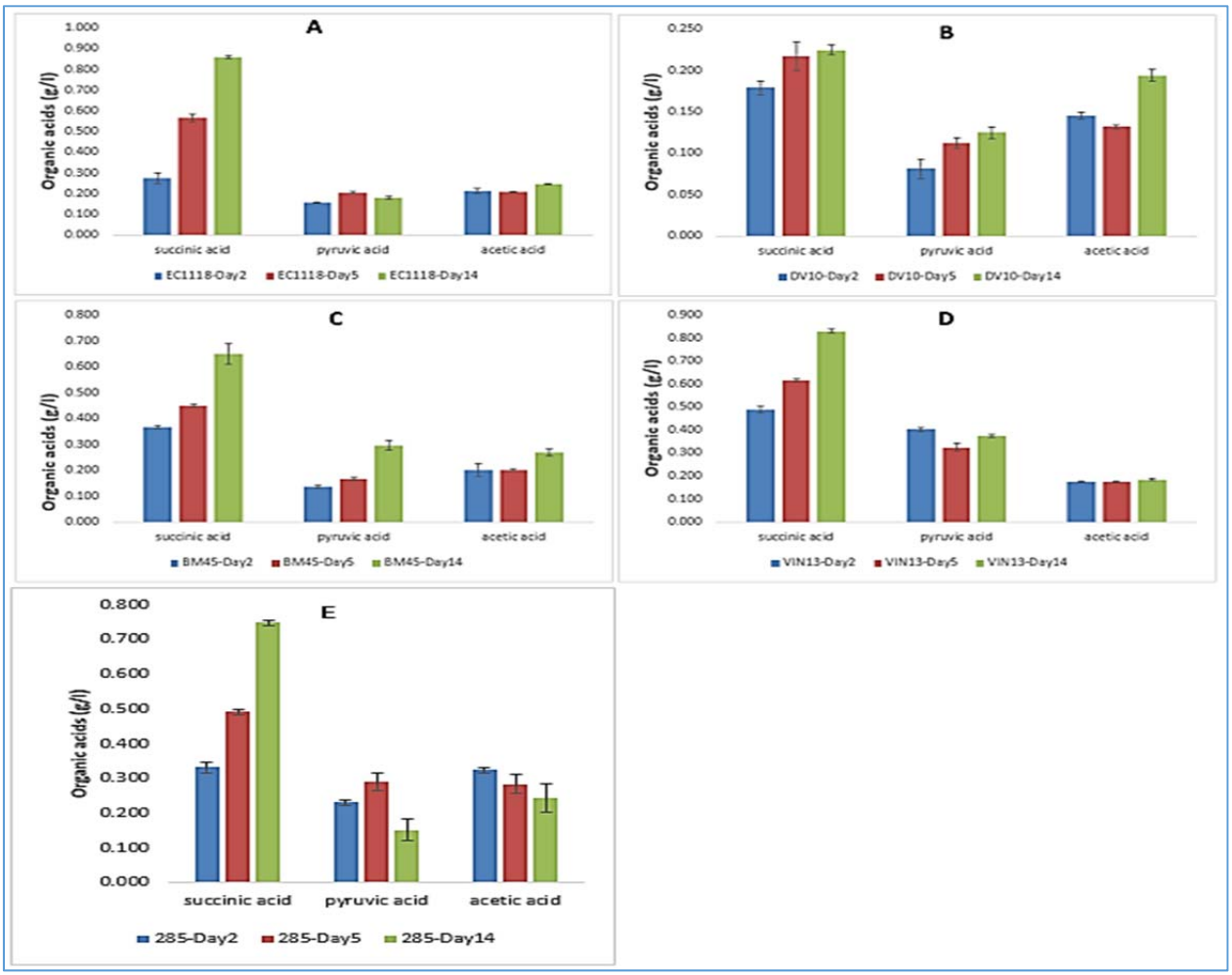

Figure 3: Organic acid profiles of EC1118 (A), DV10 (B), BM45 (C), VIN13 (D) and 285 (E) strains at the exponential (day 2), early (day 5) and late stationary phase (day14). Fermentation conditions were set at 200 g/L, pH 3.5 and 25 ^{o}C. Results are the average of 3 biological repeats ± standard deviations.

Strain identity significantly affected organic acid production (fig 4): As reported previously in chapter 3, VIN13 produced significantly higher succinic acid levels throughout fermentation. As fermentation progressed, succinic acid concentrations in the EC1118, BM45 and 285 fermentations also increased from day 2 to day 5, as well as from day 5 to day 14 (fig 4B and C). Strain DV10 produced significantly lower succinic acid concentrations across all time points. VIN13 and BM45 showed a significant increase in pyruvic acid levels throughout fermentation while concentrations in DV10 fermentations were the lowest compared to other strains. Strain 285 also produced significantly higher pyruvic acid levels compared to DV10, EC1118 and BM45 at day 2 (fig 4A) and 5 (fig 4B). However, similar to DV10, the final concentration of pyruvic acid at day 14 was the lowest for strain 285. Strain EC1118 did not show any significant variation in pyruvic acid across time points.

The influence of strain identity on acetic acid was also evident throughout fermentation. The levels of acetic acid at day 2 and 5 were higher for fermentations conducted by strain 285 compared to other strains tested. DV10 showed slightly lower acetic acid levels at all three time points

considered. It is evident from the current study that organic acid profiles of the fermenting wines are both strain and time-point dependent. These variations present the opportunity to establish genetic and metabolic relationships between relevant genes and organic acids for the yeast at different time points.

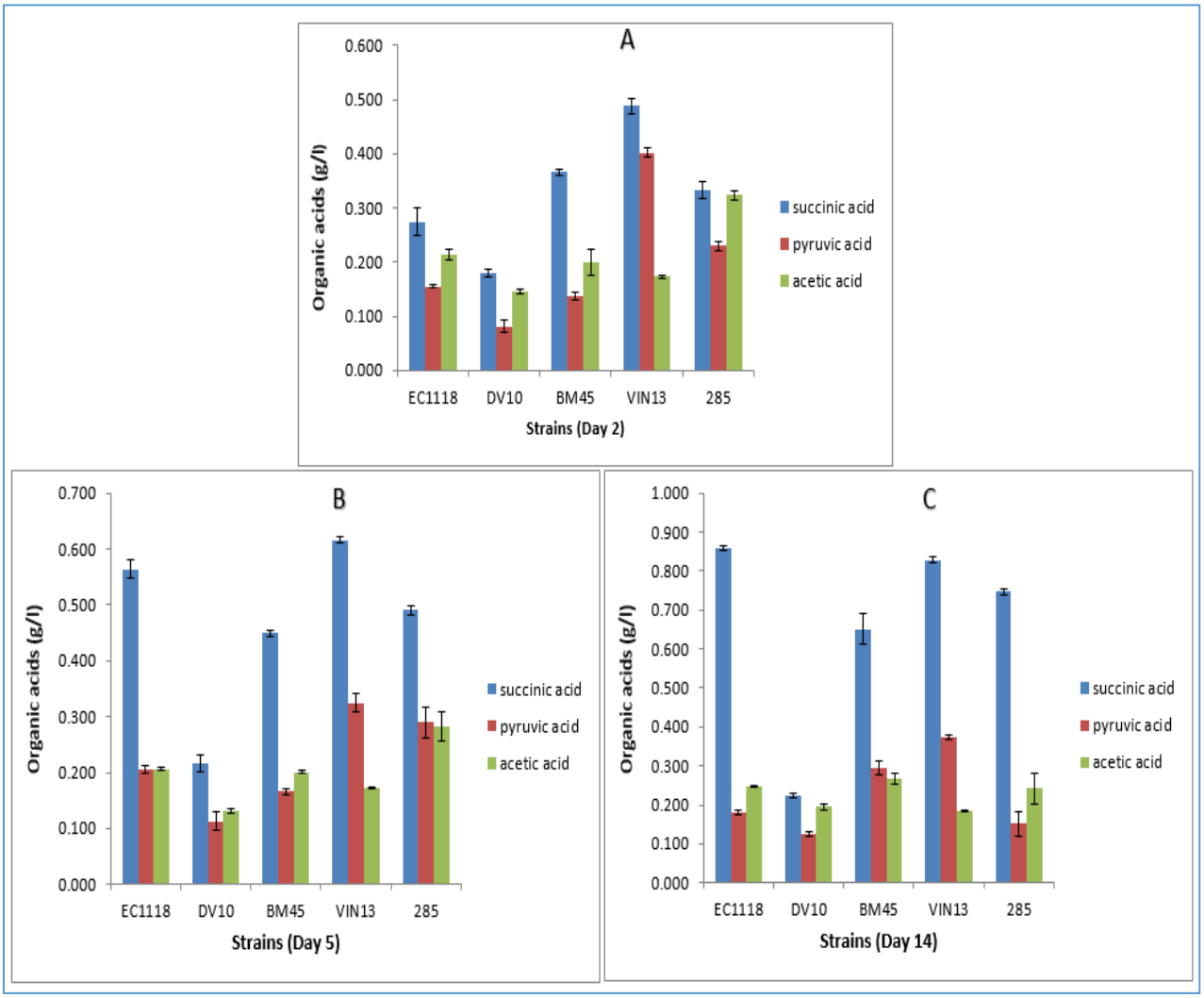

Figure 4: Organic acid profiles of five strains under wine making conditions, i.e. sugar (200 g/L), pH (3.5), temperature (25 ^{o}C) at day 2 (Frame A), day 5 (Frame B) and day14 (Frame C). Results are the average of 3 biological repeats ± standard deviations.

5.4.2 Transcriptional analysis of organic acid metabolism

Genes were first selected based on the intra- and inter-strain comparisons regarding expression levels obtained from the transcriptome data of BM45, VIN13, 285, DV10 and EC1118 (Rossouw *et al*., 2008). Secondly, a subset of genes were selected (from the differentially expressed gene list) based on their known link to organic acid metabolism, organic acid transportation, amino acid metabolism and redox balance. Twelve organic acid related transcripts/genes that were significantly up/down regulated based on the microarray analysis at days 2 (table 2A) and 5 (table

2B) of fermentation were selected in this manner for further investigation and experimentation. The inter-strain differential expressions (table 2) highlight those genes that were significantly increased or decreased in expression between strains and table 4 shows genes that were significantly increased or decreased in expression (2-fold cut-off) between time points (i.e. day 5 vs 2 or day 14 vs day 5; supplementary table C3). Some of the genes with the greatest up/down regulation between the five different industrial yeast were *ARO10* (-19.7 fold for 285 versus BM45) and *AAD6* (24.7 fold for BM45 versus VIN13) at day2 of fermentation (table 2A). Genes such as *GPD2* (7.99-fold increase for DV10 compared to 285) and *AAD6* (6.89-fold increase for BM45 versus VIN13) also showed high fold change differences in the inter-strain comparisons at day 5 (table 2B). The huge transcriptional responses in *AAD6* were noteworthy since the Aad6p enzyme may play a pivotal role in oxidative responses and redox balance which could impact on acid formation in yeast. Of interest to the current study, *ARO10* exhibited the highest fold change between day 5 and 2 for VIN13 (-9.42), 285 (14.27), BM45 (-12.15), DV10 (-3.94) and EC1118 (-9.86 fold change; table 3). *ARO10* encodes phenyl-pyruvate decarboxylase, which catalyses a key step in the Ehrlich pathway, namely the deamination of amino acids into keto-acids.

Although the alignment models are based on the active growth phase (i.e. day 2 and 5) of yeast, the lists of organic acid compound -related transcripts that were significantly up/down regulated between different strains at day 14 (Supplementary table C1) as well as between day 14 and 5 (Supplementary table C3) are also provided. At day 14 some of the genes with the highest differential expression between strains are *AAD6* (-19.97 fold change for DV10 versus BM45), *ARO10* (-5.04 fold change for DV10 versus VIN13) and *GLY1* (3.17 (for DV10 versus 285 and EC1118; Supplementary table C1).

Table 2. List of organic acid compound -related transcripts significantly up/down regulated between different strains at day 2 (A) and day 5 (B) (Rossouw *et al*.,2008). Positive fold changes greater than 2 (increase in expression) are indicated by bold-highlighted font and negative fold changes less than -2 (decrease in expression) by bold font.

A	DAY 2 FOLD CHANGE									
Gene name	BM45 vs EC1118	BM45 vs VIN13	EC1118 vs VIN13	DV10 vs VIN13	285 vs VIN13	DV10 vs EC1118	DV10 vs BM45	285 vs EC1118	285 vs BM45	DV10 vs 285
SFC1	1.14	1.26	1.11	-1.06	1.93	-1.18	-1.34	1.75	1.53	**-2.05**
OSM1	-1.06	1.08	1.15	1.14	1.09	-1.00	1.06	-1.25	-1.177	1.24
ICL1	-1.39	1.19	1.65	1.07	**2.66**	-1.54	-1.11	1.62	**2.24**	**-2.49**
KGD1	-1.39	-1.19	1.17	-1.13	1.95	-1.32	1.05	1.67	**2.32**	**-2.20**
AGX1	1.19	**2.48**	**2.08**	**2.05**	**3.01**	-1.01	-1.21	1.45	1.21	-1.47
GLY1	**2.27**	**2.56**	1.13	1.09	1.66	-1.03	**-2.33**	1.48	-1.54	-1.52
SER33	1.13	1.12	-1.01	1.02	-1.10	1.03	-1.10	-1.09	-1.23	1.12
GPD2	1.23	**4.23**	**3.44**	1.20	1.16	**-2.86**	**-3.51**	**-2.97**	**-3.65**	1.04
ARO10	**2.18**	1.31	-1.67	-1.60	**-15.11**	1.04	**-2.09**	**-9.05**	**-19.78**	**9.45**
AAD6	**9.35**	**24.71**	**2.64**	1.21	**11.64**	**-2.18**	**-20.35**	**4.41**	**-2.12**	**-9.59**
SER1	-1.15	-1.03	1.11	-1.07	-1.24	-1.19	-1.04	-1.38	-1.2	1.15
ADH3	-1.06	-1.05	1.01	-1.12	-1.66	-1.13	-1.07	-1.68	-1.58	1.48
B	**DAY 5 FOLD CHANGE**									
Gene name	BM45 vs EC1118	BM45 vs VIN13	EC1118 vs VIN13	DV10 vs VIN13	285 vs VIN13	DV10 vs EC1118	DV10 vs BM45	285 vs EC1118	285 vs BM45	DV10 vs 285
SFC1	-1.22	1.26	1.54	1.87	-1.20	1.21	1.48	-1.85	-1.51	**2.24**
OSM1	1.14	-1.10	-1.25	-1.05	-1.15	1.19	1.04	1.09	-1.05	1.09
ICL1	-1.10	1.02	1.11	1.04	-1.09	-1.07	1.03	-1.21	-1.11	1.13
KGD1	-1.03	1.10	1.13	1.26	-1.17	1.11	1.15	-1.33	-1.29	1.48
AGX1	-1.13	1.04	1.18	1.65	-1.02	1.40	1.58	-1.20	-1.06	1.68
GLY1	1.49	1.60	1.08	-1.03	1.16	-1.11	-1.66	1.08	-1.38	-1.20
SER33	1.33	1.38	1.04	-1.06	1.34	-1.10	-1.46	1.29	-1.03	-1.42
GPD2	-1.42	-1.58	-1.11	1.71	**-4.66**	1.89	**2.70**	**-4.22**	**-2.96**	**7.99**
ARO10	1.77	1.02	-1.74	-1.92	-1.60	-1.11	-1.96	1.09	-1.63	-1.21
AAD6	**6.03**	**6.89**	1.14	1.11	**5.82**	-1.03	**-6.19**	**5.09**	-1.18	**-5.22**
SER1	1.53	1.13	-1.35	-1.07	-1.01	1.25	-1.22	1.33	-1.15	-1.06
ADH3	1.02	1.03	1.01	1.20	-1.16	1.19	1.17	-1.17	-1.19	1.38

Table 3: List of organic acid compound -related transcripts significantly up/down regulated within each strain between days 2 and 5 of fermentation. Positive fold changes greater than 2 (increase in expression) are indicated by bold-highlighted font and negative fold changes less than -2 (decrease in expression) by bold font.

DAY 5 vs DAY 2					
Gene name	***VIN13***	***285***	***BM45***	***DV10***	***EC1118***
SFC1	**2.32**	***-2.70***	**2.31**	**5.86**	**3.22**
OSM1	1.06	1.29	-1.12	-1.16	-1.35
ICL1	**2.90**	***-2.68***	**2.48**	**2.74**	1.96
KGD1	**2.28**	***-2.58***	**2.99**	**3.06**	**2.22**
AGX1	**3.06**	-1.94	1.29	**2.19**	1.73
GLY1	1.43	1.17	-1.12	1.27	1.37
SER33	-1.47	1.13	-1.19	-1.47	-1.41
GPD2	**5.41**	-1.06	-1.23	**11.21**	1.42
ARO10	***-9.42***	**14.27**	***-12.15***	***-3.94***	***-9.86***
AAD6	**2.00**	1.82	-1.79	1.60	-1.16
SER1	-1.23	1.15	-1.05	-1.12	-1.83
ADH3	-1.44	1.63	-1.33	1.01	-1.44

The current study managed to identify genes that showed the most inter and intra -strain differential gene expression between strains and/or time points using a previously generated transcriptomic data of five commercial yeast strains. Yeast growth, fermentation kinetics and sampling points were carefully synchronised to avert differences in the physiology of the yeast at comparable time points. The correlation and predictions generated here were therefore very essential in hypothesising the roles of some of the selected genes that were either selected based on the intra- and inter-strain comparisons or based on their known link to organic acid metabolism, organic acid transportation, amino acid metabolism and redox balance (from the differentially expressed gene list). The alignment models used in this current study proved to be practicable since they were confirmed by a follow-up deletion studies which showed changes in acid production levels. For this reasons, inter- and intra-strain analysis strategies were very useful in providing information about several genes that were previously not known to play a direct/indirect role in organic acid metabolism.

5.4.3 Deletion studies

5.4.3.1 Fermentation kinetics of selected deletion strains

In order to further investigate the metabolic roles of potentially important acid-related genes, the corresponding deletion strains were selected to conduct fermentations in synthetic must. The aim was to evaluate the impact of gene deletion on pyruvic, succinic and acetic acid production during

fermentation. Fermentation kinetics (growth and carbon dioxide) and metabolite production (ethanol and glycerol) of all deletion mutants under fermentative conditions showed similar patterns and all fermented to dryness with the exception of the *gpd2*Δ mutant which exhibited significantly lower carbon dioxide release (fig 5A), glucose/fructose utilisation, glycerol and ethanol production (fig 6).

BY4742 (the laboratory strain which serves as the genetic background for all the deletions investigated in this study) is a slow growing and fermenting strain under wine-like anaerobic fermentation conditions. As previously explained the fermentation timescales for this strain are different to those of commercial wine yeast strains. Based on growth curves, day 6 of BY4742 fermentation is similar to day 2 (exponential phase) of commercial yeast fermentations, and both day 12 and 16 fall within the early stationary range (comparable to day 5 in commercial yeast). As a result, we used these physiological stages for the alignment of data between the deletion strain fermentations and those conducted by the original five wine yeast strains. Time points towards the end of stationary phase were not evaluated in this regard due to the known difficulties with correlating gene expression and metabolite levels at the end of fermentation when metabolic activity slows rapidly and cell viability begins to decline.

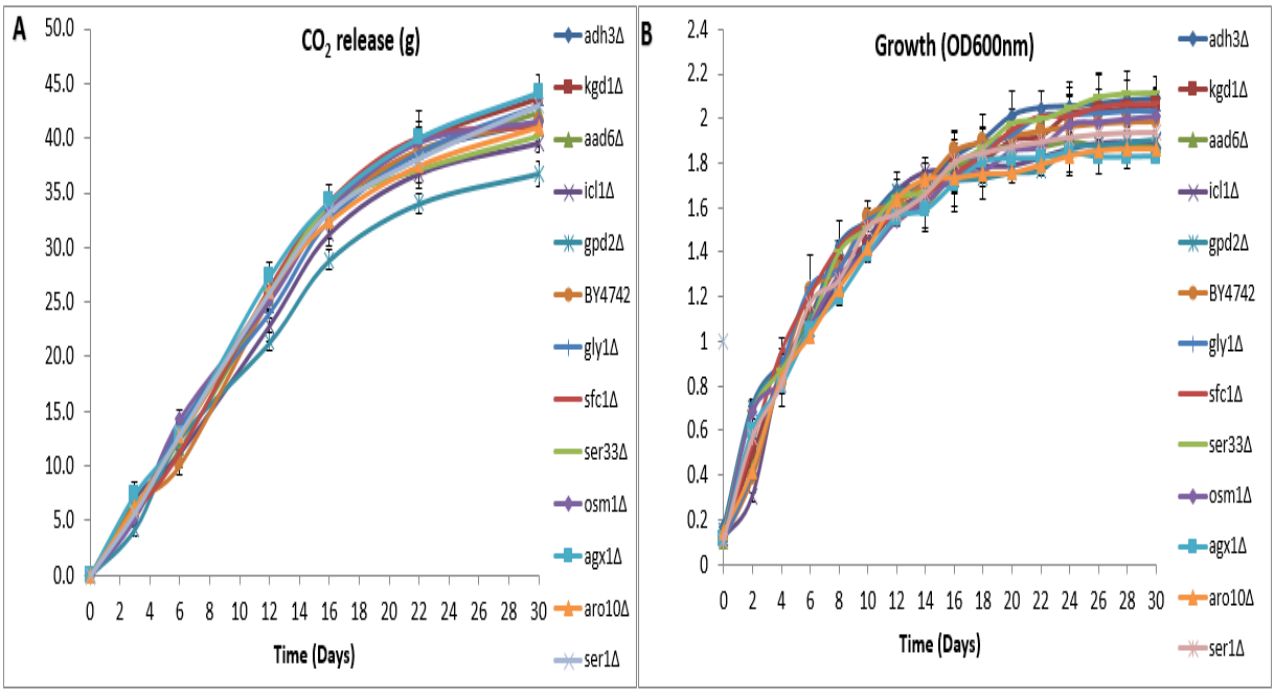

Figure 5: CO_2 release (frame A) and growth (frame B) of the deletion strains during alcoholic fermentation. Values are the average of 3 biological repeats ± standard deviation.

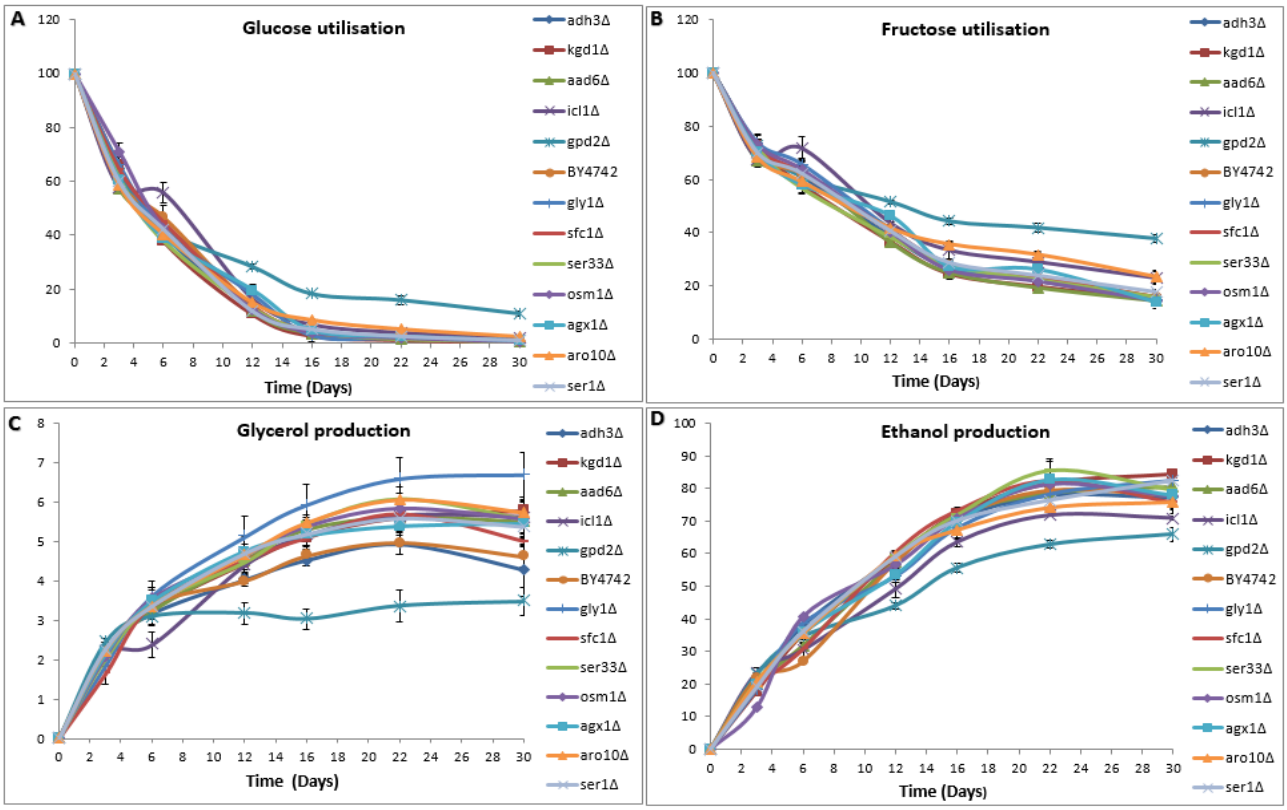

Figure 6: Fermentation kinetics of deletion strains: Glucose utilization (A), fructose utilization (B), glycerol production (C) and ethanol production (D) in g/L. Values are the average of 3 biological repeats ± standard deviation.

5.4.3.2 Organic acid profiles of the deletion mutants

Compared to the control strain (BY4742), some deletion mutants exhibited significant changes to their glycerol and organic acid profiles, while others did not show any notable differences by the end of fermentation. As a product of fermentation, pyruvic acid can be found in small quantities in wine. Compared to other acids, pyruvic acid production was significantly influenced by the deletion of most genes (fig 7C). The deletion of redox related genes such as *KGD1*, *AAD6*, *ARO10* and *SER33* resulted in less pyruvic acid production compared to the control at the end of fermentation. Other deletion strains which showed a significant decrease in pyruvic acid production during fermentation were *icl1*Δ (isocitrate lyase) and *AGX1* (alanine: glyoxylate aminotransferase). These are genes that are directly involved in the glyoxylate pathway during yeast growth. *GLY1* (amino acid biosynthesis related gene) and *OSM1* (fumarate reductase) deletion significantly increased pyruvic acid production at the end of fermentation, as well as at the earlier time points of fermentation (Supplementary fig C3).

Variations in acetic acid levels at the end of fermentation are shown in fig 7A. Compared to the reference strain, *osm1*Δ, *kgd1*Δ, *aad6*Δ, *icl1*Δ, *agx1*Δ and *aro10*Δ mutants resulted in fermentations with slightly higher acetic acid levels at the end of fermentation. Similar trends were also observed at other time points (i.e. days 12, 16, 22; Supplementary fig C2).

The impact of gene deletion on succinic acid production was most obvious for *ser33*Δ and *kgd1*Δ mutants. The deletion of these genes resulted in a remarkable increase in succinic acid production at the end of fermentation (fig 7B). Mutant strains *icl1*Δ and *ser1*Δ (Supplementary fig C1-c) resulted in fermentations with increased succinic acid production at day 12 of fermentation while *gly1*Δ mutants led to increased concentrations of this acid at day 6 (Supplementary fig C1-b).

The impact of gene deletion on glycerol levels in wine is shown in fig 7D. Though several deletion strains (i.e. *adh3*Δ, *sfc1*Δ and *ser1*Δ) appeared to produce slightly higher concentrations of glycerol compared to the control, these differences were not statistically significant. Only the deletion of *GLY1* led to significantly increased glycerol levels while *GPD2* deletion led to the expected decrease in glycerol concentrations.

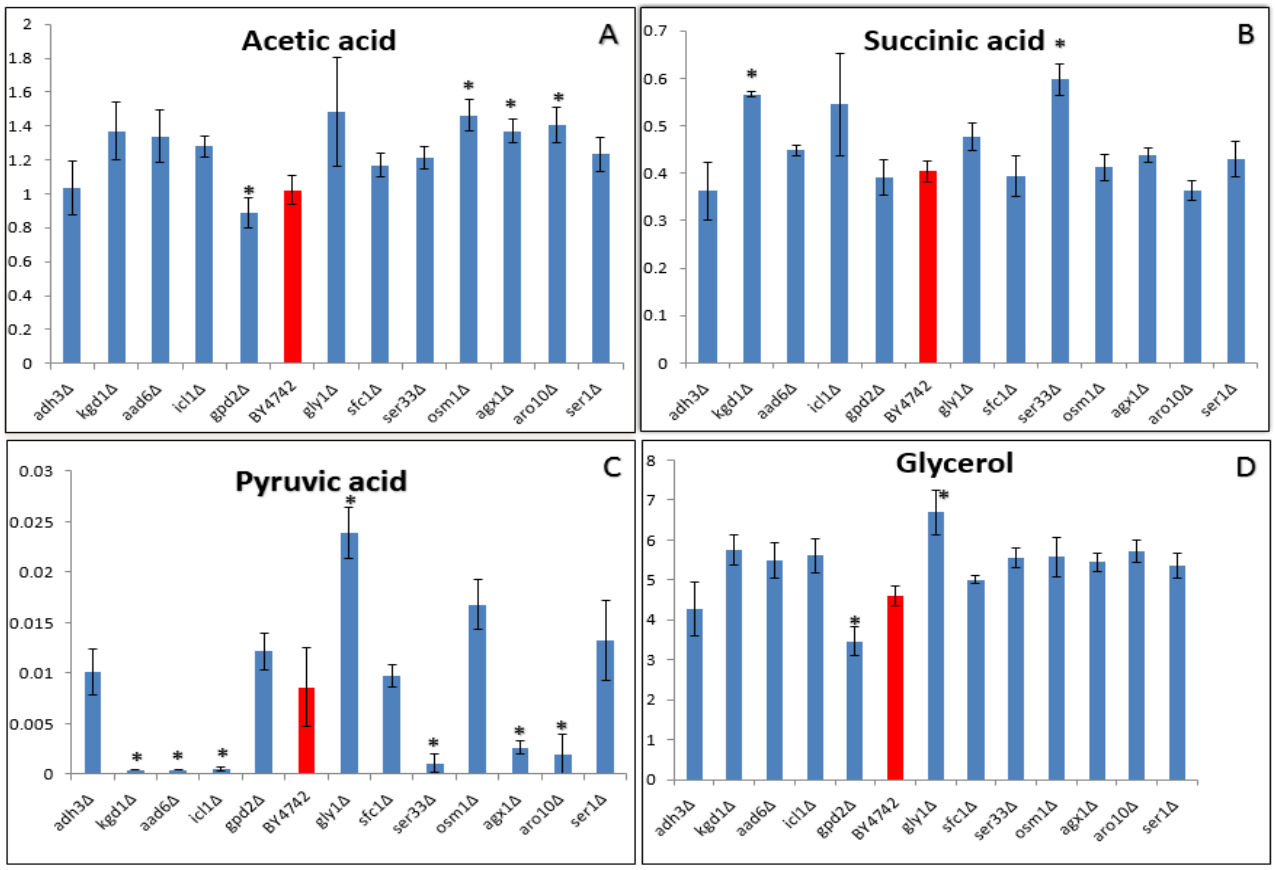

Figure 7: Acetic acid (frame A), succinic acid (frame B), pyruvic acid (frame C) and glycerol (frame D) production (g/L) by deletion mutants at the end of fermentation. BY4742 (control) bars are indicated in red. Values are the average of 3 independent repeats ± standard deviation. The asterix (*) indicates those values that are statistically significantly different from the control ($p<0.05$).

5.4.3.3 Principal component analysis

Principal component analysis was carried out using organic acid data for the different strains at different time-points (days 6, 12, 16, 22, and 30). The results show a clear separation of samples

into time-point clusters (data not shown). To highlight strain-dependent groupings, the PCA plot below (fig 8) shows sample groupings based on organic acid concentrations at two time-points only (day 6 and 16) for all 12 deletion strains. Groupings were observed based on the two different stages of fermentation as well as acid-gene relationships.

The PCA in figure 8 accounts for 91.55% of the total explained variance in the dataset. Separation along the first principal component axis was dominated by differences in glycerol, succinic and acetic acid concentrations. As expected the time point, or stage of fermentation was the main factor driving the separation of clusters in the first principal component, which accounts for the majority (73%) of explained variance. Differences in pyruvic acid between treatments was clearly a strong contributor to variance explained by both the first and second principal components.

In terms of strain impacts, *gly1*Δ, *icl1*Δ, *gpd2*Δ and *osm1*Δ mutants formed clearly distinct groupings at the exponential phase (day 6) while *adh3*Δ, aro10Δ and *ser33*Δ mutants were separated from the rest of the strains based on organic acid profiles at early stationery phase (day 16). The other deletion strains showed similar organic acid profiles at these two time points. The influence of physiological stage and strain type were clearly evident and the main drivers of variance in the dataset were highlighted by the principal component analysis.

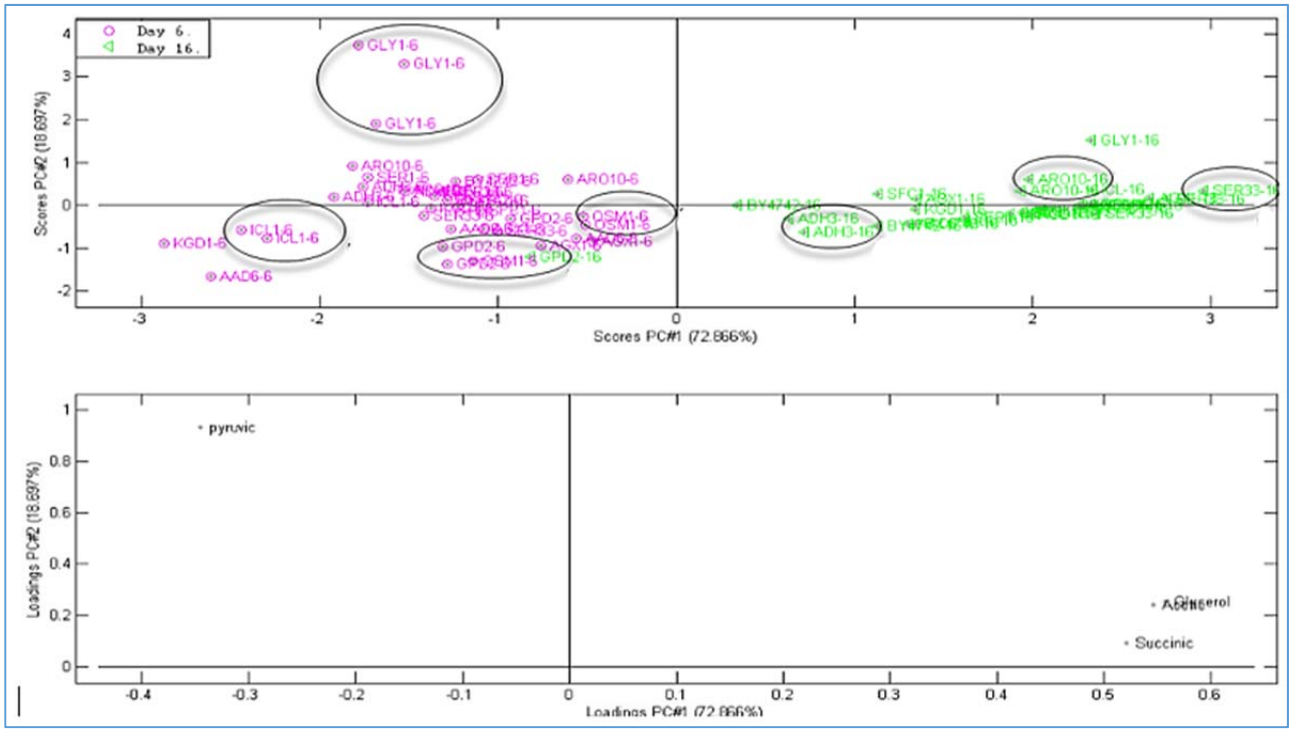

Figure 8: Principal component analysis of succinic, acetic, pyruvic acid and glycerol data at different time points (day 6; purple and day 16; green). Samples are labelled based on the gene name and day of sampling (e.g. GLY1-6 represents the GLY1 deletion strain at day 6 of fermentation). Biological repeats (in triplicate) are shown.

5.4.3.4 Correlation between predicted and observed impacts of genes involved in glycerol and organic acid metabolism

The expression data used to generate the correlation coefficients were not generated from yeast strains grown under similar conditions in which the organic acid levels were determined (reasons stated in the materials and methods). Transcriptomic data of the five industrial yeast strains were used to determine the correlation coefficients of genes and organic acid concentrations at different time points. The coefficients were used to establish a predictive framework for identifying the impact of genes with strong links to specific acids (succinic and acetic acid). The impact of genes on pyruvic acid was also considered in the current study. However, it was not feasible to generate a reliable alignment model for pyruvic acid because of (i) undetectable levels for most of the strains at one or more points during fermentation, (ii) extremely low pyruvic acid levels (0-0.025 g/L) which could possibly project biased or heavily weighted/skewed predictions.

The alignment models for succinic and acetic acid were based on two critical time points (exponential growth and early stationary phase) as these points represent distinct physiological phases of the yeast. Once again, day 6 of BY4742 fermentation is similar to day 2 (exponential phase) of commercial yeast fermentations, and both day 12 and 16 fall within the early stationary range (comparable to day 5 in commercial yeast). The expected changes in organic acid levels for each gene (predicted) and the measured changes when such genes were deleted (actual measured) are depicted in fig 9 and fig 10. The alignment model was based on the assumption that intra-strain transcriptional responses for given organic acid -related genes which correspond to changes in the organic acid profiles are likely to affect organic acid trends, either negatively or positively. Thus deletion mutants (carrying null mutations for the target genes) would show changes to the production levels if indeed the identified genes do play a direct role, or have an indirect impact on one/more of the acids. Mutant responses were classed into three groups; (i) those genes that did not significantly influence acid production after deletion, (ii) those that increased acid production and (iii) those that decreased acid production after deletion.

The model showed a strong alignment based on the directionality of the changes of the predicted and measured levels of succinic acid at the exponential growth phase in *gpd2*Δ, *icl1*Δ, *aad6*Δ, *ser1*Δ, *osm1* Δ and *sfc1*Δ mutants (fig 9A). The model did not align (based on opposite directionality of changes between predicted and measured values) in *aro10*Δ (predicted -0.6 and measured 0.6), *agx1*Δ (predicted 0.2 and measured -0.2), *ser33*Δ (predicted -0.7 and measured 0.4), *kgd1*Δ (predicted -0.8 and measured 0.2), *gly1*Δ (predicted 0.6 and measured -0.4) and *adh3*Δ (predicted -0.1 and measured 0.5) mutants at the exponential phase. On the other hand, a strong alignment was also observed at the early stationary phase (either day 12 or 16) in *adh3*Δ, *sfc1*Δ, *agx1*Δ, *ser33*Δ (aligned only at day 12), *osm1*Δ, *gpd2*Δ *and icl1*Δ mutant (fig 9B). Models of other mutants (*kgd1*Δ, *ser1*Δ, *aro10*Δ, gly1Δ and *aad6*Δ) did not align at the early stationary phase.

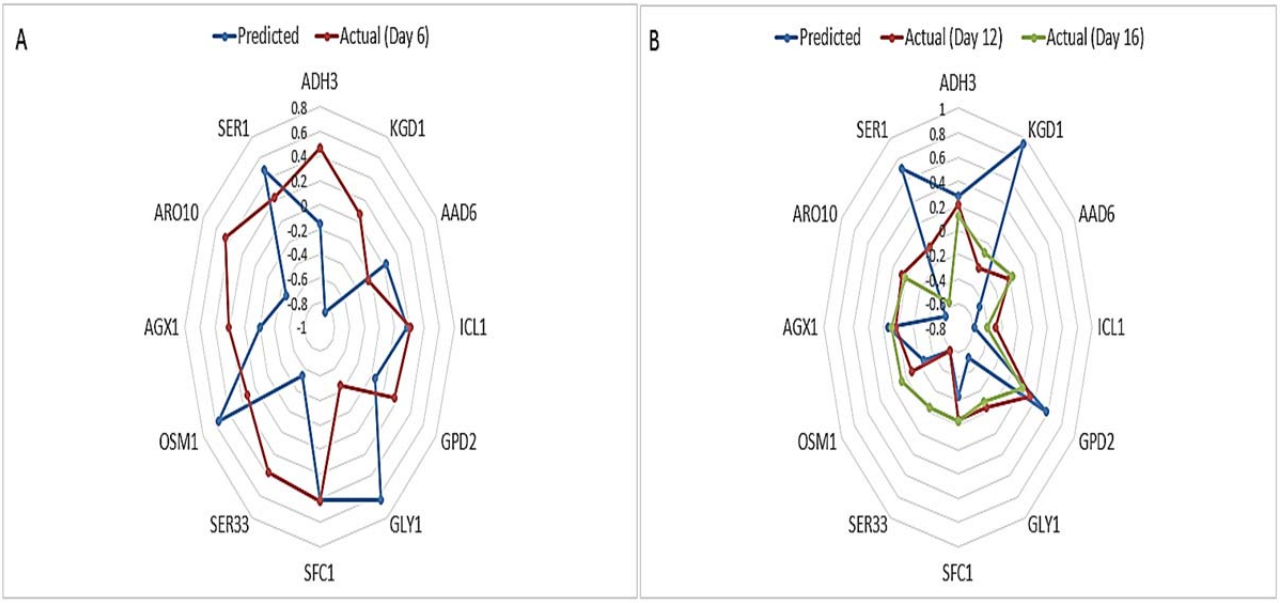

Figure 9: Predicted vs measured succinic acid changes at the exponential (day 6) (A) and early stationary growth phases (day 12 and 16) (B). Predicted correlations for gene expression and succinic acid concentrations are indicated by the blue lines while the observed ratio of change (change in succinic acid concentrations in deletion strain versus control) are indicated by the red (day 12) and green lines (day 16).

As in succinic acid models, similar comparisons were also made for acetic acid. Here, the predicted and measured levels of acetic acid for *agx1*Δ, *aad6*Δ, *icl1*Δ, *aro10*Δ and *gpd2*Δ mutants aligned quite well at the exponential phase (fig 10A). A partial alignment for *ser1*Δ (predicted 0.5 and measured 0.0), *gly1*Δ (predicted 0.6 and measured 0.0), *sfc1*Δ (predicted 0.5 and measured 0.0) and *osm1*Δ (predicted 0.6 and measured 0.0) was observed. However, *adh3*Δ (predicted -0.8 and measured 0.1), *ser33*Δ (predicted -0.7 and measured 0.0), and *kgd1*Δ (predicted -0.8 and measured 0.2) mutants did not align at this growth stage (fig 10A). Furthermore, the models for *gpd2*Δ, *gly1*Δ, *sfc1*Δ, *aad6*Δ, *aro10*Δ, *agx1*Δ ser33Δ and *osm1*Δ mutants aligned quite well at the early stationary phase (both day 12 and 16) but the model for *kgd1*Δ (predicted 1 and measured -0.2), *icl1*Δ (predicted -0.4 and measured 0.1), *adh3*Δ (predicted -0.6 and measured 0.0) and *ser1*Δ (predicted 0.8 and measured -0.2) mutants showed a significant variation in the predicted and measured values (fig 10B).

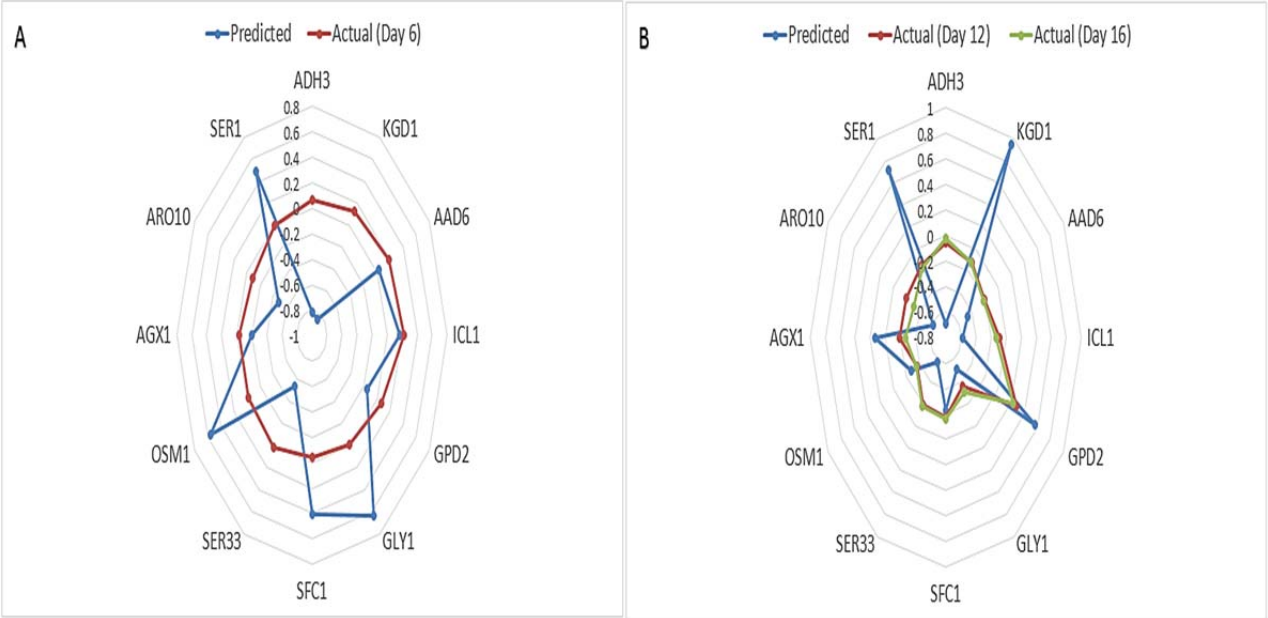

Figure 10: Predicted correlations for gene expression and acetic acid concentrations are indicated by the blue lines while the observed ratio of change (change in acetic acid concentrations in deletion strain versus control) are indicated by the red (day 12) and green lines (day 16).

5.4.4 Other potential genes of interest

The identification of other genes showing significantly changed expression levels (greater than 2-fold increase or decrease) between strains at different stages of alcoholic fermentation was also undertaken. Genes were identified in an unbiased manner by analysing yeast transcriptional data (of a subset of approximately 800 metabolic genes) in conjunction with organic acid variations. Based on this analysis, the genes *HXT4*, *LSC2*, *FBP26*, *ATF2*, *SDH1*, *IDP3*, *SPG4* and *MEP1* were identified since they were among the genes that showed the most inter and intra -strain differential gene expression between strains (table 4) or time points (table 5) and correlated to observed differences in organic acid profiles. Their descriptions and potential roles in organic acid metabolism are also listed in table 1.

SPG4 showed the highest fold change between day 5 and 2 for VIN13 (4.89), 285 (-7.49), BM45 (10.69), DV10 (4.88) and EC1118 (6.10) (table 5A). Another interesting gene which showed higher expression levels (4.72 fold higher) in 285 versus VIN13 at the exponential phase was *IDP3* (table 4A. Interesting genes that were differentially expressed between two or more strains at day 5 and/or day 14 are listed in table 5B and supplementary table C2. Although these genes were not further investigated experimentally in deletion studies future work could be done to further explore the relationship between the enzymes encoded by these genes and organic acid levels.

Table 4: List of organic acid compound-related transcripts significantly up/down regulated between different strains at day 2 (A) and day 5 (B). Positive fold changes greater than 2 (bold-highlighted) are indicative of increased expression and negative fold changes less than -2 (bold) of decreased gene expression.

A	DAY 2 FOLD CHANGE									
Gene name	***BM45 vs EC1118***	***BM45 vs VIN13***	***EC1118 vs VIN13***	***DV10 vs VIN13***	***285 vs VIN13***	***DV10 vs EC1118***	***DV10 vs BM45***	***285 vs EC1118***	***285 vs BM45***	***DV10 vs 285***
SPG4	**-2.45**	**-2.87**	-1.17	1.29	**2.18**	1.51	**3.69**	**2.56**	**6.27**	-1.70
ATF2	1.62	-1.02	-1.65	-1.50	**-2.86**	1.10	-1.47	-1.74	**-2.81**	1.90
HXT4	1.06	-1.00	-1.07	-1.12	-1.49	-1.05	-1.12	-1.40	-1.49	1.33
YJL045W	-1.50	-1.48	1.01	1.10	**2.44**	1.08	1.62	**2.41**	**3.60**	**-2.22**
FBP26	-1.05	1.01	1.06	1.28	**2.99**	1.21	1.28	**2.82**	**2.97**	**-2.33**
IDP3	-1.15	1.38	1.59	1.68	**4.72**	1.06	1.22	**2.97**	**3.43**	**-2.81**
LSC2	1.07	1.29	1.20	-1.14	**-2.45**	-1.37	-1.47	**-2.95**	**-3.16**	**2.15**
MEP1	1.91	1.66	-1.15	-1.25	-1.73	-1.08	**-2.06**	-1.50	**-2.86**	1.39
B	DAY 5 FOLD CHANGE									
Gene name	***BM45 vs EC1118***	***BM45 vs VIN13***	***EC1118 vs VIN13***	***DV10 vs VIN13***	***285 vs VIN13***	***DV10 vs EC1118***	***DV10 vs BM45***	***285 vs EC1118***	***285 vs BM45***	***DV10 vs 285***
SPG4	-1.40	-1.31	1.06	1.50	**-2.24**	1.41	1.97	**-2.38**	-1.71	**3.36**
ATF2	1.77	**2.46**	1.39	1.42	**2.39**	1.02	-1.73	1.72	-1.03	-1.68
HXT4	1.38	**2.35**	1.71	-1.05	**2.35**	-1.79	**-2.47**	1.37	-1.00	**-2.46**
YJL045W	-1.25	-1.33	-1.07	1.38	-1.70	1.47	1.84	-1.60	-1.28	**2.35**
FBP26	1.16	1.09	-1.06	1.08	-1.17	1.14	-1.02	-1.10	-1.28	1.25
IDP3	1.34	1.16	-1.15	-1.06	1.01	1.09	-1.23	1.17	-1.15	-1.07
LSC2	1.03	-1.31	-1.35	-1.83	-1.88	-1.36	-1.39	-1.39	-1.43	1.03
MEP1	-1.08	-1.21	-1.13	-1.34	**-2.10**	-1.19	-1.11	-1.87	-1.73	1.57

Table 5: List of organic acid compound -related transcripts significantly up/down regulated within each strain between days 5 and 2 (A) and between day14 and 5 (B) of fermentation. Positive fold changes greater than 2 (bold-highlighted) are indicative of increased expression and negative fold changes less than -2 (bold) of decreased gene expression.

A	DAY 5 vs DAY 2				
Gene name	***VIN13***	***285***	***BM45***	***DV10***	***EC1118***
SPG4	**4.89**	***-7.49***	**10.69**	**4.88**	**6.10**
ATF2	***-6.87***	**3.90**	***-2.72***	***-3.29***	***-2.98***
HXT4	***-3.51***	*1.92*	-1.49	***-3.42***	-1.93
YJL045W	**4.15**	***-2.67***	**4.60**	**5.61**	**3.84**
FBP26	**3.49**	***-2.98***	**3.79**	**2.79**	**3.10**
IDP3	**4.66**	***-4.21***	**3.93**	**2.50**	**2.54**
LSC2	-1.31	**2.99**	***-2.21***	***-2.17***	***-2.13***
MEP1	1.22	**2.67**	-1.65	1.28	1.25
B	**DAY 14 vs DAY 5**				
Gene name	***VIN13***	***285***	***BM45***	***DV10***	***EC1118***
SPG4	**17.13**	***93.46***	1.53	**7.06**	**8.01**
ATF2	-1.02	***-16.43***	1.94	***-2.14***	***-2.99***
HXT4	***-13.09***	***-11.56***	***-3.77***	-1.93	***-6.39***
YJL045W	**3.67**	***12.05***	1.59	1.83	1.98
FBP26	**2.05**	**6.35**	-1.08	**2.11**	**2.30**
IDP3	**2.44**	**9.14**	-1.13	**2.01**	**2.45**
LSC2	***-5.09***	***-9.14***	1.18	***-5.94***	***-3.08***
MEP1	**3.39**	**2.49**	1.23	**2.66**	**2.19**

5.5 Discussion

In the current study, efforts have been applied to understand organic acid metabolism in yeast through exploration of yeast strains carrying deletions for genes which might contribute towards acidity in wine. These genes were identified through a combination of biased and un-biased methods. Firstly, we analysed transcriptional differences between strains displaying differences in acid production, but focused on genes with known or expected impacts on carbon and nitrogen metabolism. Genes selected in this manner were *ARO10*, *SER1*, *SER33*, *GPD2*, *OSM1*, *AGX1*, *KGD1*, *GLY1*, *ADH3*, *SFC1*, *ICL1* and *AAD6*. The impact of the deletion of these 12 differentially expressed organic acid–related genes on organic acid metabolism was investigated. The deletion of several genes affected organic acid metabolism while a few did not appear to impact acid production during fermentation. A summary of the genes that showed a significant impact on organic acid production throughout fermentation are described in figure 11 below. Only those genes that showed a significant impact on glycerol and/or organic acid at one or more stages of fermentation (see supplementary fig C1 and C2) are discussed below.

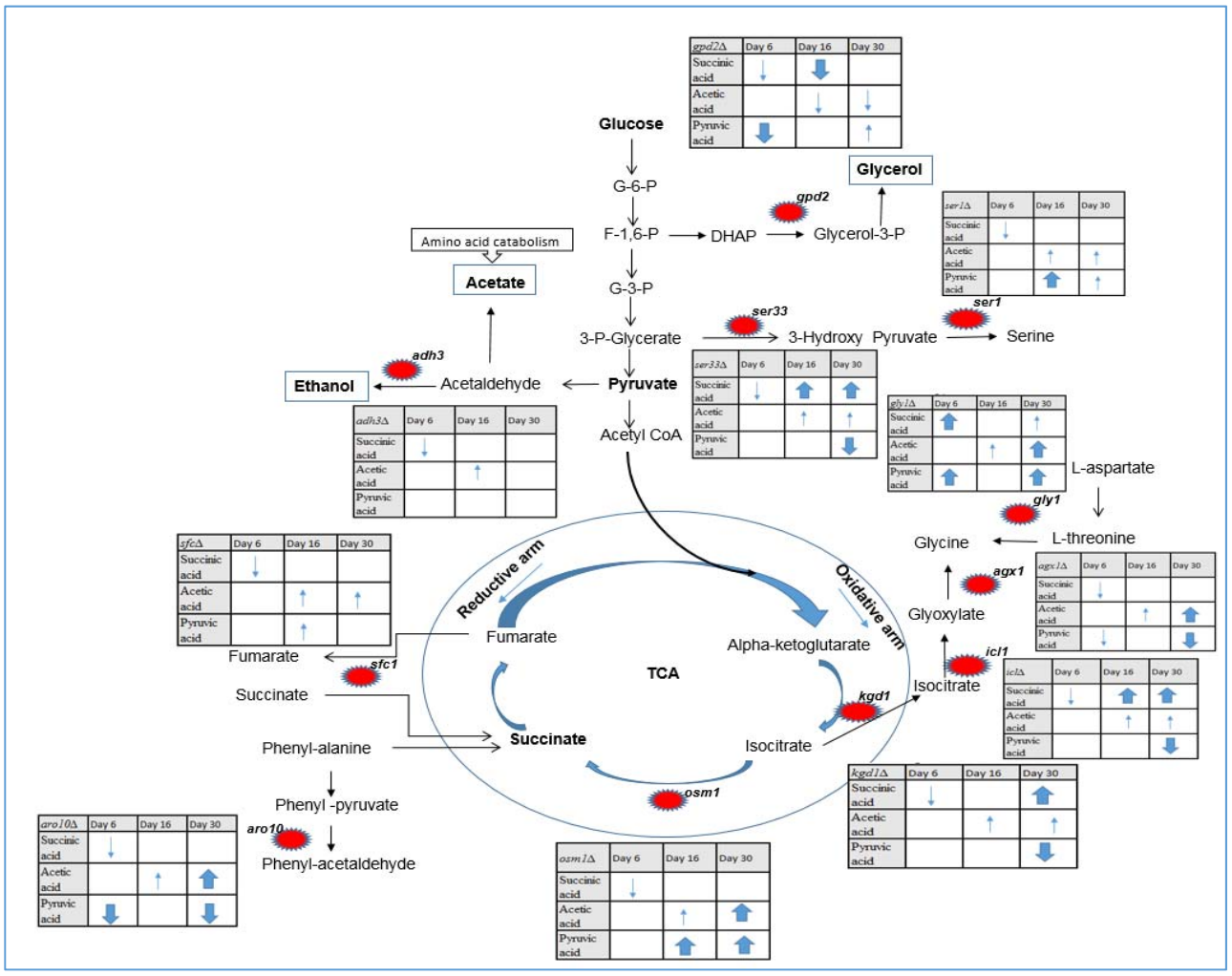

Figure 11: A pathway representation showing the involvement in organic acid metabolism of the genes which were absent in the deletion strains used to conduct fermentations. The data boxes for each gene highlight statistically significant changes (up arrow for an increase and down arrow for a decrease) in the levels of the organic acids at the exponential phase (day 6), early stationary phase (day 16) and end of fermentation (day 30) for the deletion strain compared to the control (BY4742). The width of the arrows represent the magnitude of the increase/decrease relative to the control.

5.5.1 Impact of deletion of redox-related genes on growth, organic acids and glycerol production

The fermentation kinetics of the deletion strains that were selected in this study showed similar trends under the fermentation conditions used. However, the *gpd2*Δ mutant showed reduced glucose/fructose consumption and ethanol and glycerol production (fig 6). This is contrary to previous observations reported by Styger *et al*. (2011), but these differences might be explained by different fermentation conditions. In particular, our experimental set-up ensured close-to anaerobic conditions, while Styger *et al*. (2011) did not control access to oxygen. Our conditions may have resulted in a more stringent reliance on glycerol production for NAD+ regeneration since no oxygen is available for re-oxidation of NADH. Another possibility is a growth-inhibiting accumulation of glycerol-3-phosphate in the cells in the absence of *GPD2*, as proposed by Pahlman *et al*. (2001).

Relative to the reference (BY4742) strain, the *gpd2*Δ deletion mutants showed lower glycerol production (0.34 g/L less) at the end of fermentation (fig 7D) confirming the importance of *GPD2* for glycerol production. Overexpression of this gene is indeed well characterised in terms of increased glycerol production in *S. cerevisiae* (Michnick *et al*., 1997; Remize *et al*., 1999; de Barros Lopes *et al*., 2000). These authors also reported significant increases in acetic acid when *GPD2* is over-expressed. *GPD2* deletion, on the other hand, only led to a small, though statistically insignificant, decrease in acetic acid levels at day 12 (supplementary fig C2-C) and 16 (supplementary fig C2-D), suggesting that the production of acetic acid in this mutant may not be stringently linked to redox-balancing. Otherwise, the *GPD2* mutation did not significantly affect any of the other organic acids considered. These observations are in line with the prediction model which showed no significant impact of *GPD2* on acetic and succinic acid.

5.5.2 The role of *SER33* and *SER1* on organic acid metabolism in yeast

SER33 and *SER1* deletion led to an increase in succinic acid levels at the end of fermentation (fig 7B). This is in line with the results of a previous study attempting to increase succinic acid concentrations. Otero *et al*. (2003) noted higher yields of succinic acid in a chemically defined minimal medium in shake flask cultures when *ser33*Δ, *ser3*Δ and *sdh1*Δ deletion mutants were tested. *SER33* plays a significant role in the biosynthesis of amino acids and is one of the essential genes in the glyoxylate pathway. A possible explanation for this increase in succinic acid in our study revolves around the disruption of serine and glycine biosynthesis in *ser33*Δ mutants. Under these conditions, cells will use the alternative pathway from isocitrate to produce glycine and serine. In this pathway isocitrate is converted to glyoxylate and succinate by Icl1p. Succinate is thus indirectly produced as a by-product of the reaction, which accounts for the increased succinate concentrations when serine and glycine biosynthesis are forced to proceed via isocitrate (fig 1). Ser1p is likewise responsible for catalysing one of the final reactions in serine biosynthesis Disruption of this gene would be expected to have the same metabolic impact as *SER33* deletion in terms of increasing succinic acid production as a by-product of glyoxylate and ultimate serine biosynthesis from isocitrate.

SER33 disruption resulted in very low levels of pyruvic acid at the end of fermentation while the disruption of *SER1* increased production of this acid (fig 7C). Both genes operate in serine biosynthesis, and it might be expected that the disruption of either of the two should channel carbon away from glyoxylate and towards pyruvate. The metabolic pathways illustrating this hypothesis are shown in figure 1. The potential impact of *SER1* and *SER33* on pyruvic acid metabolism has not been previously investigated elsewhere, hence these two phenotypes cannot be explained.

5.5.3 The role of *KGD1* on organic acid metabolism in yeast

KGD1 (alpha ketoglutarate dehydrogenase) encodes a key enzyme of the TCA cycle. Some work has been done to assess the potential roles of *KGD1* on succinic acid production under fermentative conditions. Arikawa (1999) showed 1.5 fold higher succinic acid production by *kgd1*Δ mutants compared to the wild type strain K901 during sake (Japanese alcohol beverage) fermentation. The increased succinic acid observed in our study confirms this observation (fig 7B).

Our findings may be linked to the fact that *KGD1* catalyses the conversion of alpha ketoglutarate to isocitrate and deletion results in the interruption of the oxidative branch of the TCA cycle. This means that carbon entering the TCA cycle would likely be channelled to succinate via the reductive branch of the TCA cycle as an alternative pathway. Alpha ketoglutarate is required for ammonium fixation during fermentation, the primary nitrogen source in our conditions and is therefore necessary for biomass formation. This would mean that *KGD1* disruption during the exponential growth phase of the yeast would result in a build-up of alpha-ketoglutarate, which could be partially channelled to ammonium fixation and growth during the earlier stages of fermentation. This would explain why no increase in succinate (via the reductive branch of the TCA cycle) was observed during the early stages of fermentation (supplementary fig C1). However when active biomass formation has ceased and no ammonium fixation takes place, carbon entering the TCA from glycolysis might be redirected to succinate via the reductive branch to avoid accumulation of alpha-ketoglutarate (fig 1). This again accounts for the increase in succinate concentrations towards the later stages of fermentation by the *KGD1* deletion strain (fig 7B)

5.5.4 The impact of other several genes on growth and organic acid production

GLY1 deletion (Threonine aldolase, a key enzyme involved in glycine biosynthesis) significantly increased pyruvic acid production throughout fermentation (supplementary fig C3). The link between pyruvic acid production and *GLY1* gene disruption has not received much consideration in literature. Glycine is primarily synthesised from threonine, which is derived from aspartate, which is derived from oxaloacetate and which is in turn derived from pyruvate. The disruption of this chain of events could have led to a build-up of pyruvic acid due to the disruption of glycine biosynthesis. This will explain why fermentations inoculated with *gly1*Δ deletion mutants resulted in higher levels of pyruvic acid. This and the previous examples highlight the complexity of the metabolic interplay between reaction networks involved in central carbon metabolism and amino acid biosynthesis.

In the current study, other genes (*ARO10*, *SER1*, *AGX1*, *SFC1* and *OSM1*) significantly affected pyruvic acid metabolism throughout fermentation. Although their deletion mutants showed different

levels of pyruvic acid at different physiological stages, the deletion of *AGX1*, *SFC1* and *ARO10* genes resulted in significantly higher pyruvic acid levels at the early stages of fermentation but these differences diminished as fermentation progressed. These differences at the later stages of fermentation were however still statistically significant compared to the wild type.

There is no information available regarding how these genes impact on pyruvic acid metabolism during fermentation, however, previous reports have shown a 58.6% decrease in pyruvic acid yield when *S. cerevisiae sfc1*Δ deletion mutants were tested under micro-aerobic conditions (Zhang *et al*., 2007). Interestingly, *aro10*Δ mutants showed significantly lower pyruvic acid levels at all stages of fermentation when compared to the wild type (supplementary fig C3). *ARO10* is primarily responsible for the decarboxylation of phenyl-pyruvate to phenyl acetaldehyde during fermentation; no plausible hypothesis for the possible indirect role of Aro10p in pyruvic acid metabolism could be formulated. The disruption of *OSM1* (Osm1p is involved in the re-oxidation of intracellular NADH under anaerobic conditions) resulted in a remarkable increase in pyruvic acid at the end of fermentation. This is not surprising considering that Osm1p plays a central role in TCA cycle, which accounts for the build-up of pyruvate from glycolysis as the TCA cycle is disrupted.

Apart from *GPD2*, the deletion of other NADH requiring genes (*OSM1*, *KGD1* and *ICL1*) did not significantly affect growth in this study. Famili *et al*. (2003) reported increased growth on a defined complete glucose media when *osm1*Δ, *kgd1*Δ and *icl1*Δ mutants were evaluated. In a different study, the deletion of *OSM1* did not affect the anaerobic cell growth (Camarasa and Faucet, 2007). However, our data showed a slight decrease in optical density throughout fermentation by *osm1*Δ mutants (fig 5). *OSM1* is one of the genes required for the reoxidation of intracellular NADH under anaerobic conditions. The disruption of this gene, therefore, would result in an NAD^+/NADH imbalance which could eventually lead to reduced cell growth as a result of the lack of ATP generation.

5.5.5 Other genes of interest

Other genes (*SPG4*, *ATF2*, *HXT4*, *SDH1*, *FBP26*, *IDP3*, *LSC2* and *MEP1*) also showed a significant variation in expression between different strains and/or time points and therefore future deletion studies should also take these into consideration. These genes cover a wide range of functions, including organic acid, glucose and nitrogen metabolism, all of which are connected to organic acid production (Large, 1986).

5.6 General Conclusions

The current study is the first that explores comparative transcriptomic and metabolomic linkages to further improve our understanding of acid evolution during fermentation. There were several occasions when the model predictions based on gene expression and organic acid correlations did not align with observed changes in acid levels following gene deletion. However, the correlation of intracellular mRNA expression values with extracellular metabolites is fraught with obvious limitations such as being unable to precisely correlate mRNA activity with the secondary metabolite levels, which could explain the mismatch between predicted versus actual acid levels for certain genes. Moreover, the regulation of carbon flux through the various interconnected pathways which together influence the levels of organic acids is highly complex and integrated. This means that model predictions based on single gene considerations are likely to underestimate the integrated network response to the genetic perturbation introduced by deletion of the target gene/s. Central carbon metabolism is tightly regulated by external and internal factors. Organic acids, being mostly by-products or intermediates of these pathways are therefore likely to be subject to the same level of regulation. In contrast, secondary metabolite levels can be predicted with greater accuracy in genetic models as these are often end products produced by pathways in which flux is not as tightly regulated. We also considered the possibilities that, variations pertaining to the industrial yeast genetic backgrounds were likely to impact significantly on their fermentative capacity and central carbon metabolism which may eventually impact on the transcript-acid correlation studies. However, yeast growth, fermentation kinetics and sampling points were appropriately synchronised to represent similar yeast physiological state. Synchronisation was also made since variation in sugar concentrations can also affect carbon metabolism, gene expression, flux and organic acid production,

The study also focused experimental attention on only three definitive time points during fermentation (representing three different physiological stages of yeast growth) for the investigation of gene expression and acid relationships. Essential information regarding organic acid regulation in yeast could thus have been overlooked as the datasets are not continuous, and valuable information may be found between the discrete time points selected in our study. The possibility of finding correlations that are merely artefacts is also a high probability. Given the large number of expressed transcripts in yeast (approximately 6000) many genes may be correlated linearly with organic acid trends without any real biological significance; being some of the problems encountered in comparative transcriptomic and metabolomics studies.

In summary, the impact of genes involved in glycerol and organic acid metabolism have been comprehensively investigated in this study. The use of an alignment model -based approach

incorporating both transcriptomic and organic acid data aided our identification of genes which play important roles in acid evolution in synthetic must under fermentative conditions. Rather than seeking to produce precise mathematical models we were hoping to create visualisations to contextualise the observed changes in organic acids.

For several of the genes selected we were unable to successfully correlate individual acids and individual genes, which could be due to the polygenic nature of acid-related traits for particular strains and the lack of predictability based on transcriptional data at only three time points. In other instances, the protein/enzyme produced by genes of interest may be stable and remain active while the transcription of gene is either low or not induced. Other factors that may also affect the correlation studies may depend on the half-life of some specific mRNA molecules which may be short or exceptionally long. However, for several genes, interesting changes in organic acid levels (which were to some degree predicted based on the transcriptional-metabolic models) were observed in fermentations conducted with the deletion strains. The influences of *GPD2* on glycerol was confirmed, and deletion of *KGD1* and *SER33* genes resulted in the strongest changes in overall acid production. This study presents, for the first time, a comparative analysis to link transcriptional data of yeast and organic acid profiles in wine. This provides a useful platform for further investigations into the genetic factors which are responsible for differences in acid evolution between distinct wine yeast strains. Based on the inter- and intra-strain analysis presented here, we do not propose to put forth any strong conclusions on individual gene function based on our results. We do highlight the shortcomings of our approach, however the data still provide the basis for scientifically sound argumentation of likely impacts of genes on organic acid production in wine, from an arguably novel angle/approach.

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Appendix C

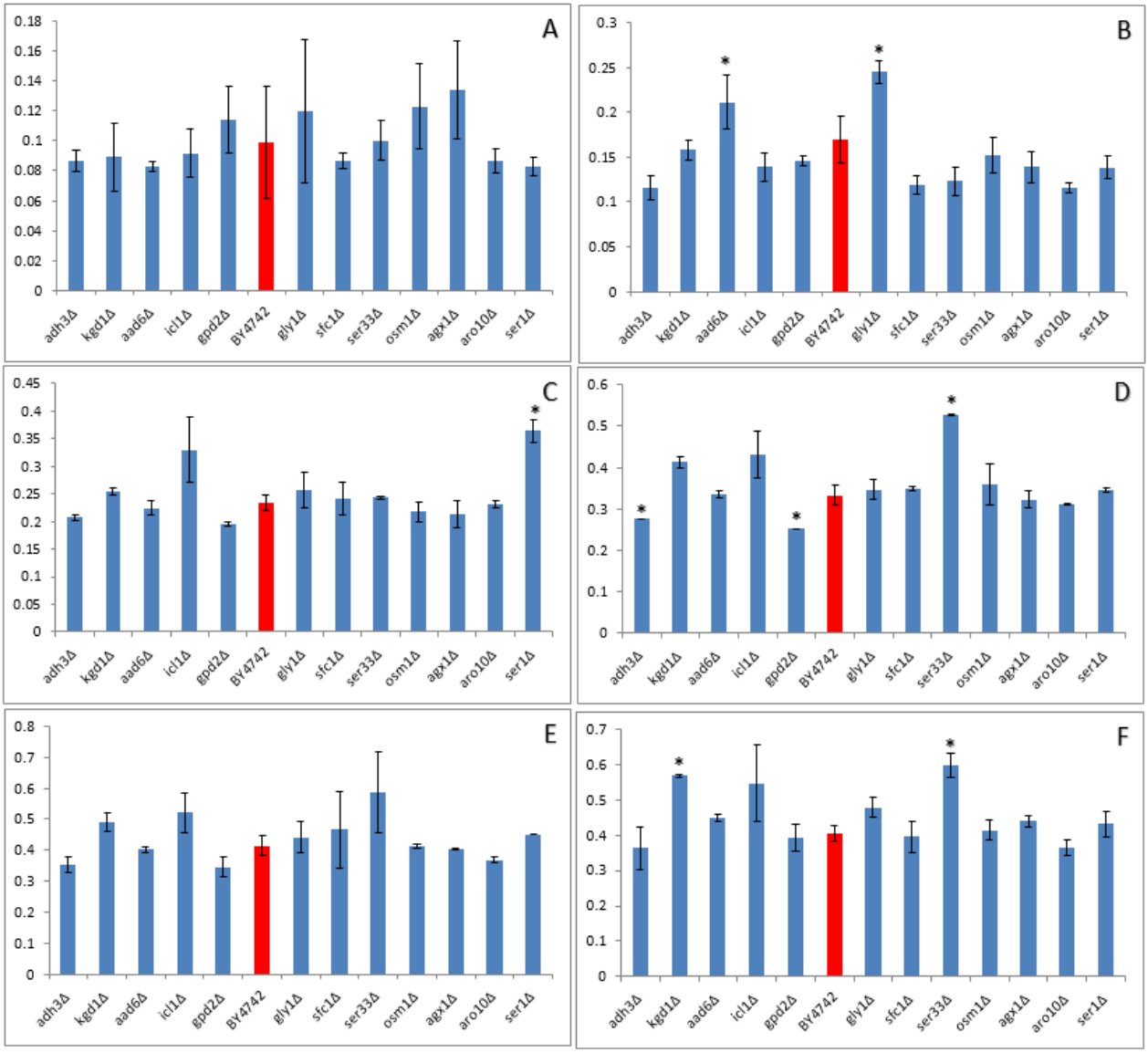

Figure C1. Succinic acid production at day 3 (Frame A), day 6 (Frame B), day 12 (Frame C), day 16 (Frame D), day 22 (Frame E) and day 30 (Frame F). BY4742 (highlighted in red) was a control Values are the average of 3 biological repeats ± standard deviation. The asterix (*) indicates those values that are statistically significantly different from the control ($p<0.05$).

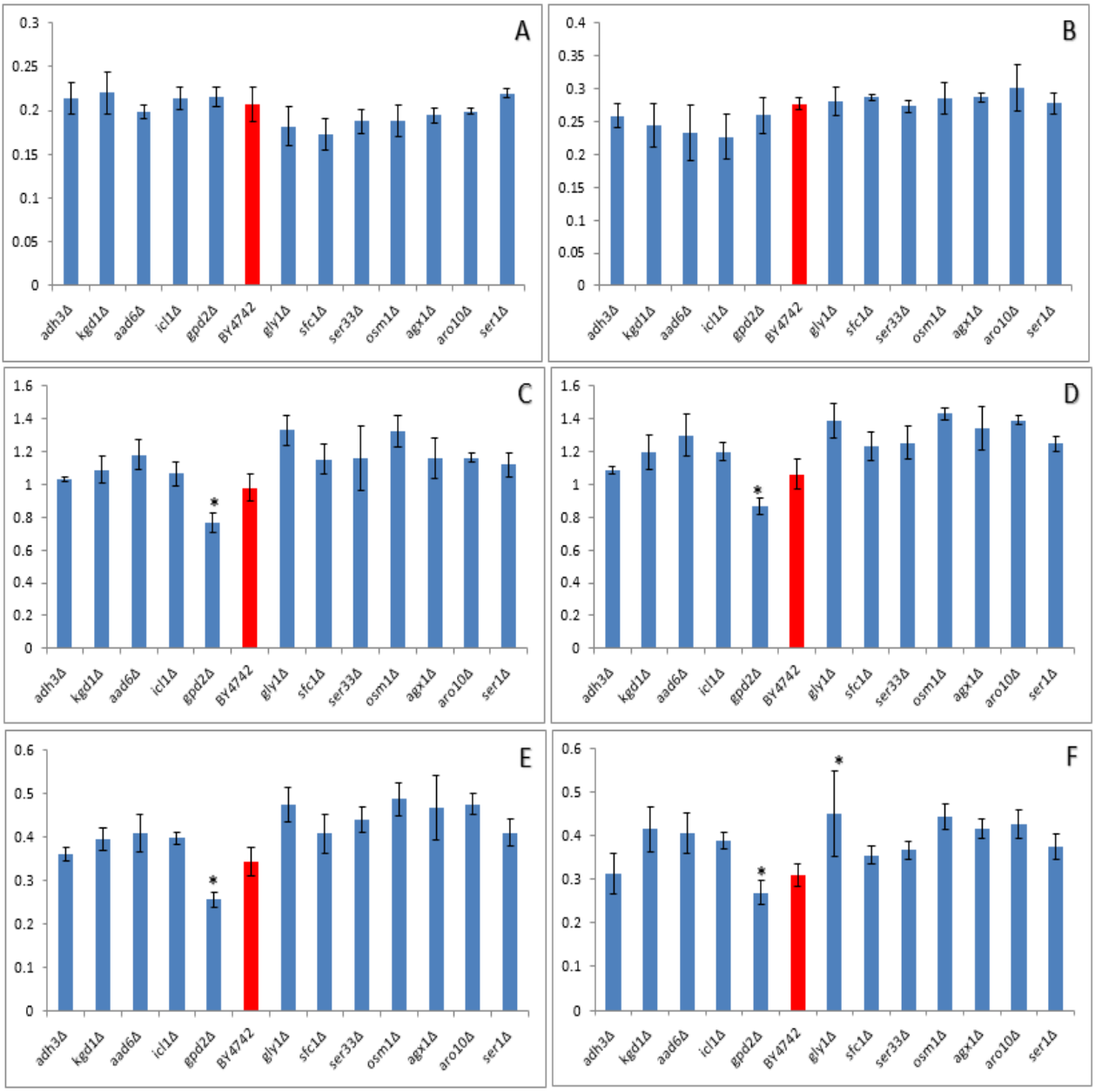

Figure C2. Acetic acid production at day 3 (Frame A), day 6 (Frame B), day 12 (Frame C), day 16 (Frame D), day 22 (Frame E) and day 30 (Frame F). BY4742 (highlighted in red) was a control. Values are the average of 3 biological repeats ± standard deviation. The asterix (*) indicates those values that are statistically significantly different from the control ($p<0.05$).

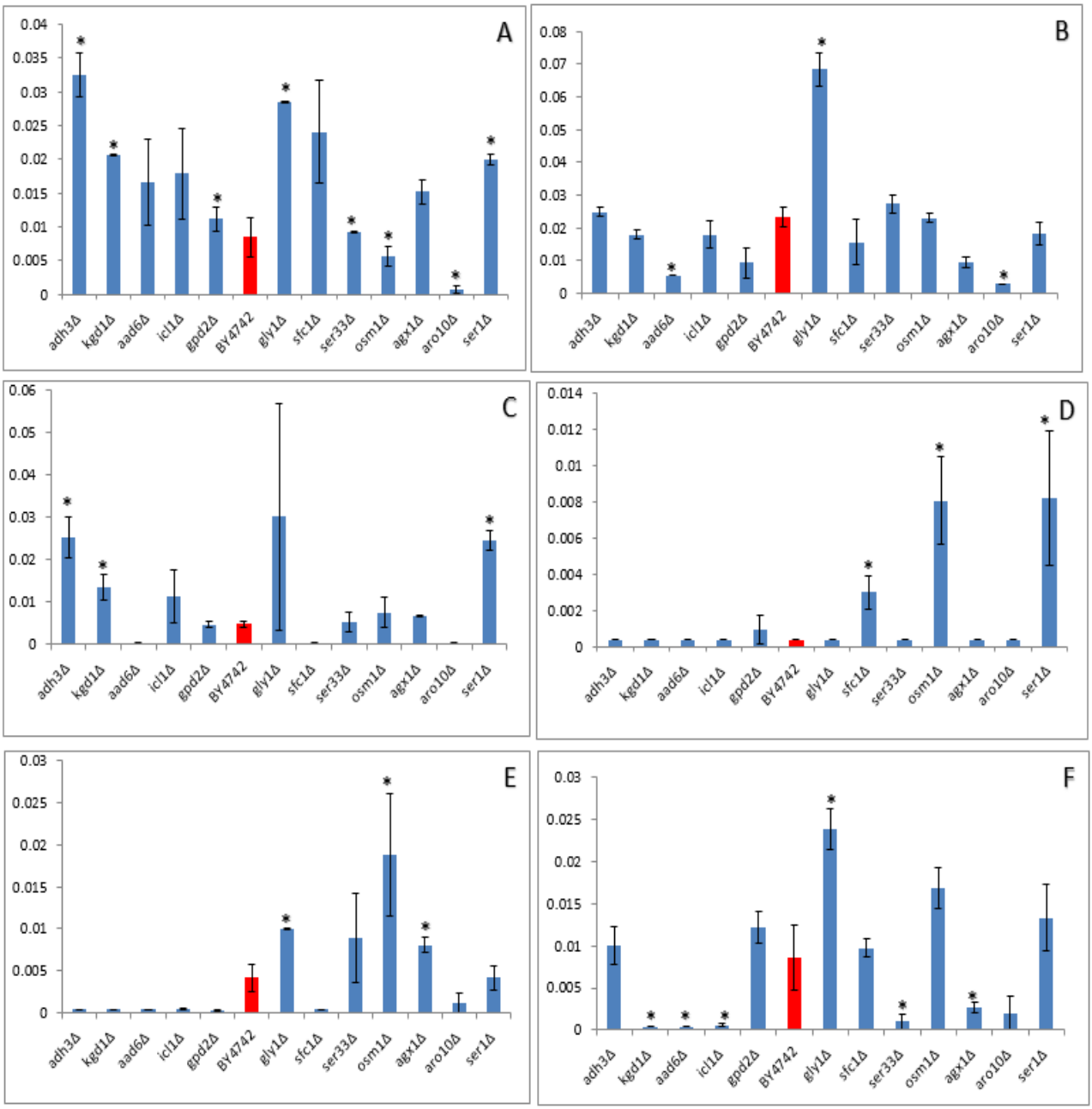

Figure C3. Pyruvic acid production at day 3 (Frame A), day 6 (Frame B), day 12 (Frame C), day 16 (Frame D), day 22 (Frame E) and day 30 (Frame F). BY4742 (highlighted in red) was a control. Values are the average of 3 biological repeats ± standard deviation. The asterix (*) indicates those values that are statistically significantly different from the control ($p<0.05$).

Table C1. List of organic acid compound-related transcripts significantly up/down regulated between different strains at day 14. Positive fold changes (bold-highlighted) are indicative of increased expression and negative fold changes (bold) of decreased gene expression

DAY 14 FOLD CHANGE										
Gene name	BM45 vs EC1118	BM45 vs VIN13	EC1118 vs VIN13	DV10 vs VIN13	285 vs VIN13	DV10 vs EC1118	DV10 vs BM45	285 vs EC1118	285 vs BM45	DV10 vs 285
SFC1	-1.25	1.41	1.76	**2.00**	1.12	1.14	1.42	-1.57	-1.26	1.79
OSM1	-1.62	-1.72	-1.06	-1.09	-1.01	-1.03	1.57	1.05	1.70	-1.08
ICL1	-1.49	-1.16	1.29	-1.10	-1.02	-1.43	1.05	-1.31	1.14	-1.08
KGD1	1.50	1.44	-1.04	1.15	1.17	1.19	-1.26	1.22	-1.23	-1.02
AGX1	1.66	1.33	-1.24	-1.23	-1.01	1.01	-1.64	1.24	-1.34	-1.22
GLY1	1.71	1.08	-1.58	1.30	**2.01**	**2.05**	1.20	**3.17**	1.86	-1.55
SER33	1.36	1.24	-1.10	-1.18	-1.10	-1.07	-1.46	1.00	-1.36	-1.07
GPD2	-1.60	-1.69	-1.05	1.43	-1.18	1.51	**2.42**	-1.12	1.44	1.68
ARO10	**-2.55**	**-5.07**	-1.99	**-5.04**	-1.02	**-2.54**	1.01	1.94	**4.94**	**-4.92**
AAD6	**6.23**	**8.04**	1.29	**-2.48**	1.04	**-3.21**	**-19.97**	-1.24	**-7.72**	**-2.59**
SER1	1.52	-1.34	**-2.03**	-1.68	1.02	1.21	-1.26	**2.06**	1.36	-1.70
ADH3	1.17	1.08	-1.08	-1.01	-1.06	1.08	-1.09	1.03	-1.14	1.05

Table C2. List of organic acid compound-related transcripts significantly up/down regulated between different strains at day 14. Positive fold changes (bold-highlighted) are indicative of increased expression and negative fold changes (bold) of decreased gene expression

DAY 14 FOLD CHANGE										
Gene name	BM45 vs EC1118	BM45 vs VIN13	EC1118 vs VIN13	DV10 vs VIN13	285 vs VIN13	DV10 vs EC1118	DV10 vs BM45	285 vs EC1118	285 vs BM45	DV10 vs 285
SPG4	1.46	-1.38	**-2.01**	-1.62	**-3.08**	1.24	-1.17	-1.53	**-2.23**	1.90
ATF2	1.19	-1.77	**-2.10**	-1.47	-1.72	1.43	1.21	1.22	1.03	1.17
HXT4	**2.62**	**9.17**	**3.50**	**6.45**	**5.09**	1.84	-1.42	1.45	-1.80	1.27
YJL045W	**3.02**	1.47	**-2.05**	-1.45	-1.38	1.41	**-2.14**	1.48	**-2.04**	-1.05
FBP26	1.19	1.26	1.06	1.11	-1.12	1.04	-1.14	-1.19	-1.42	1.24
IDP3	-1.01	-1.16	-1.15	-1.28	-1.11	-1.11	-1.10	1.03	1.05	-1.15
LSC2	-1.27	-1.04	1.22	**-2.13**	-1.13	**-2.61**	**-2.06**	-1.38	-1.09	-1.90
MEP1	1.32	-1.32	-1.74	-1.71	-1.07	1.02	-1.29	1.63	1.24	-1.60

Table C3. List of organic acid compound -related transcripts significantly up/down regulated within each strain between days 14 and 5 of fermentation. Positive fold changes (bold-highlighted) are indicative of increased expression and negative fold changes (bold) of decreased gene expression.

DAY 14 vs DAY 5					
Gene name	***VIN13***	***285***	***BM45***	***DV10***	***EC1118***
SFC1	1.02	**3.71**	-1.14	1.09	1.17
OSM1	**-2.01**	**-2.28**	1.01	**-2.09**	-1.71
ICL1	1.22	**3.50**	-1.01	1.06	1.42
KGD1	1.14	4.07	-1.19	1.04	-1.02
AGX1	1.08	**2.12**	-1.01	-1.87	-1.34
GLY1	1.24	1.83	**-2.09**	1.66	-1.37
SER33	**-3.70**	**-6.16**	-1.65	**-4.11**	**-4.23**
GPD2	-1.97	**2.13**	-1.57	**-2.36**	-1.88
ARO10	**2.46**	**-3.71**	-1.05	-1.06	**2.16**
AAD6	**2.57**	**-3.94**	**-5.84**	-1.07	**2.90**
SER1	1.26	1.12	-1.39	-1.23	-1.19
ADH3	1.31	-1.13	-1.16	1.09	1.20

Chapter 6

General discussion and conclusions

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General Discussion and conclusion

The overarching aim of the projects described in this dissertation was to investigate the changes in organic acid composition during fermentation by five widely used and phenotypically distinct wine yeast strains under simulated wine making conditions. These strains were subjected to different initial temperature, pH and sugar concentrations in a multi-factorial experimental design under anaerobic and aerobic fermentation conditions. The influence of fermentation conditions and strain identity at different physiological and fermentative stages on organic acid degradation/evolution was assessed. Organic acid contents were quantified at the exponential phase, early stationary phase and late stationary growth phase, and compared with gene expression patterns to identify specific genetic elements involved in organic acid metabolism.

The three main objectives of the work therefore were (i) to assess the impact of yeast strain on acid profiles, (ii) to assess the impact of different fermentation parameters (and combinations thereof) on organic acids during fermentation and (iii) to investigate the genetic framework of organic acid metabolism in yeast.

To address the first objective, five yeast strains were used to ferment in two very different grape musts, reflecting ‘red wine’ and ‘white wine’ fermentation conditions. All strains fermented to dryness in the fermentation settings selected in our study. This is not surprising considering that these strains are all commercial wine yeast strains which have been selected due to their high fermentation competencies and desirable characteristics. However, changes in environmental factors did in some cases affect the growth of certain strains.

In the context of specific acids, succinic acid levels generally increased throughout fermentation for all strains and in all conditions. However in many cases the absolute concentrations of this acid varied significantly across strains at one or more stages of fermentation. These observations are in line with findings reported elsewhere (Ribéreau-Gayon *et al*., 2006; Magyar *et al*., 2014).

The impact of strain identity on acetic acid is an important topic in the wine industry as excess acetic acid is an undesirable fermentation outcome from a sensory and consumer perspective. The current study identified yeast strains that are capable of producing different

acetic acid levels under simulated wine making conditions. However, apart from strain dependent variation in acetic acid metabolism, changes in fermentation conditions as well as the time point of fermentation also played a notable role. Previously, widespread variation in acetic acid formation by *S. cerevisiae* has been observed (Romano *et al*., 2003), however, these studies did not follow acetic acid evolution across fermentation time points and under different conditions.

Key points to consider regarding pyruvic acid include (i) pyruvic acid metabolism has not been thoroughly examined in wine, (ii) it is usually present in rather lower quantities in wine (Usseglio, 1995) and (iii) it is generally produced at the onset of fermentation. Some of the strains in our study produced only undetectable pyruvic acid levels at one or more stages of fermentation. It was very interesting to see a highly diverse pyruvic acid profile among different strains under both aerobic and anaerobic conditions. Follow-up work could incorporate additional strains to gain a clearer understanding of the significant influence of strain variability on pyruvic acid evolution in wine.

To summarise the outcomes of this part of the investigation, organic acid profiling of the five industrial strains showed clear differences in acid trends between strains at different simulated wine making conditions. Of interest to the study, lower, moderate and higher producers of individual acids were noted for the varying fermentation conditions. However, no clear influence of strain identity on the metabolism of grape derived acids (tartaric, malic acid and citric acid) was evident. Despite the need for acid profiling using a larger set of yeast strains in future, the outcomes of our study may already assist winemakers to appropriately select specific yeast strains based on requirements regarding wine acidity and flavour.

Apart from strain variability, a totally different approach was instigated to further explore wine acidity (the second study objective). This part of the project was accomplished by investigating the influence of individual and/or combined environmental parameters (aeration, pH, temperature and sugar) on acid degradation/formation. This approach helped to holistically identify specific environmental factors that accounted for organic acid variations. Apart from the fact that these parameters are known to affect yeast metabolism, they were specifically selected because they can be managed and controlled during wine making.

Currently, there is little published ‘’wine acid related studies’’ which explore organic acid trends in wine; (i) under different fermentation conditions (multi-factorial designed

strategies), (ii) at the three critical growth phases and (iii) in the presence and/or absence of oxygen. Studies have been limited to exploration of individual parameters and acids, with no special attention as to how the physiological stages of yeast affect acid trends in wine. However, several attempts have been made to investigate the impact of single/multiple genes on fermentation properties (Zhang *et al*., 2007), glycerol (Michnick *et al*., 1997 de Barros Lopes et al., 2000; Pahlman *et al*., 2001), aroma profiles (Rossouw *et al*., 2008; Styger *et al*., 2011) and organic acid production (Arikawa *et al*., 1999; Camarasa *et al*., 2003; Otero *et al*., 2013) mostly under a single growth phase and condition.

Our data also provided some insight into the combinatorial effect of several environmental factors on wine yeast growth. Not surprisingly, faster growth was observed under aerobic conditions, lower initial sugar content of the must and at higher fermentation temperature conditions. All three conditions acted additively regardless of the changes in other parameters. Similar growth patterns were also noted previously (Fleet and Heard, 1993; Blateyron *et al*., 1998; Serra, 2005).

As expected, significant changes in yeast acid profiles were noted when different fermentation conditions were tested. Again these results highlight the fact that combinatorial changes to fermentation parameters may have additive or subtractive impacts on organic acid levels compared to single factorial trends. These combinatorial impacts are also dependent on the specific strain employed. However, for a given strain, there are clear trends which can be inferred from the network models. This provides a measure of predictability for a given strain with regards to its response to multi-factorial changes.

In particular, the data show the direct proportional relationship between (i) acetic acid and the sugar content of the must, (ii) temperature and succinic acid levels and (iii) pyruvic acid and pH under fermentative conditions. This information is important as it will enable winemakers to make informed decisions regarding how environmental factors should be controlled in order to manage acidity and the organoleptic characters of wines.

There are several key points that the current study revealed about succinic acid production during alcoholic fermentation. (i) Strain identity was one of the strongest contributing factors that resulted in variations in succinic acid throughout fermentation since varying levels of succinic acid levels were observed among wine yeast strains at different fermentation conditions and time points, (ii) succinic acid production was cumulative in most fermentation conditions as has been reported previously (Ribéreau-Gayon *et al*., 2006) (iii) the importance of aeration was also clear as succinic acid levels were increased in aerobic

versus anaerobic conditions for all strains tested, as reported elsewhere (Wiebel *et al*., 2008; Aceituno *et al*., 2012). As described in chapter 4, succinic acid production by wine yeast strains was mainly affected by (i) increased sugar, at low pH and high temperature at the exponential phase, (ii) increased sugar, at low pH and low temperature at late stationary phase, (iii) increased temperature, at low sugar and high pH at early stationary phase, (iv) increased pH, at low sugar and high temperature at exponential phase (v) increased pH, at high sugar and high temperature at early stationary phase.

Significantly different levels of production of pyruvic acid were identified in different strains and different fermentation conditions. The study in particular revealed conditions which increased pyruvic acid production in all strains as (i) increased sugar (250 g/L) at low pH (3), high temperature (30 ^{o}C) during the exponential phase of growth, (ii) increased sugar at low pH and temperature (15 ^{o}C) at early stationary phase, (iii) increased pH (4) at both low sugar and temperature at early stationary phase and (v) increased temperature at both low sugar and pH at early stationary phase.

Strain identity was one of the most relevant factors that affected acetic acid production under varying anaerobic and aerobic fermentation conditions. Most strains indeed showed different production trends at different time points and under both aerobic and anaerobic conditions. Nevertheless, a high initial sugar, low temperature and low pH seemed to significantly decrease acetic acid content in the final wine. For most strains, acetic acid production was increased under both anaerobic and aerobic fermentation conditions when grown on (i) high initial sugar at a low pH and at low temperature, (ii) increased pH at low sugar and low temperature (iii) increased pH at low sugar and high temperature at early stationary phase, (iv) increased pH, at low sugar and temperature at late stationary phase.

While the novelty of our approach lies in the multifactorial framework employed, there are several shortfalls and problems associated with this approach which need to be taken into consideration. It has been previously indicated that a multi-factorial approach sometimes makes it difficult to identify critical factors and their interactions with a minimal number of experiments (Ray *et al*., 2009). The challenge in experimentally implementing this framework is also complicated by obvious interpretational complexities due to the non-linear nature of many of the changes observed when several parameters are changed together. However, if well applied, multi-factorial experiments can provide novel insights that would be missed by simpler experimental strategies. This was clearly the case in our work, where network – based analyses of the acid data aided identification of parameters which together lead to differences in the concentrations of certain acids.

The third and final objective of the current project was to identify genes that may play a direct/indirect role in organic acid metabolism in yeast. Genes were identified that were significantly different in expression between strains and at either of the three fermentative stages tested. The selection of a subset of genes for further investigation was based on functional annotation: A subset of genes that may potentially play a role in organic acid synthesis, growth, nitrogen metabolism and transportation of acid/sugars were selected from the differential gene sets and the impact of these genes on acid evolution was assessed using strains carrying deletions of the target genes. A total of 12 differentially expressed organic acid–related genes (*ARO10, SER1, SER33, GPD2, OSM1, AGX1, KGD1, GLY1, ADH3, SFC1, ICL1* and *AAD6)* were selected in this manner.

Prediction models were subsequently generated based on correlations between differential gene expression values and the concentrations of individual organic acids. Experimentally observed changes in organic acid levels in fermentations conducted with the deletion strains were then compared with model predictions. The alignment of the predicted vs measured acid levels proved to be a very useful tool for discovering the mechanisms by which the enzymes (encoded by the target genes) may impact specific organic acids in yeast. As hypothesised, the deletion of several genes either increased/decreased acid production while a few did not significantly affect acid production throughout fermentation. The data also showed that the deletion of some genes which showed strong correlations between their expression levels and acid levels across strains did not always lead to the expected outcome. However, the use of deletion mutants contributed to our understanding of the roles played by some of the genes selected in our study and their potential role in regulating acidity in wine. Several other potential genes of interest in this regard (*SPG4, ATF2, HXT4, YJL045W, FBP26, IDP3, LSC2* and *MEP1)* were also identified by our combined transcriptional and metabolic analysis.

Although the current study paid a special attention to a limited number of important factors (strain type, aeration, initial sugar of the must, initial pH and fermentation temperature) that can be at least to some degree controlled and managed by winemakers, future work should also focus on investigating more strains and more physical parameters that may potentially play a direct or indirect role in acid evolution in wines. However, such analysis may require more complex multivariate data analysis and a large number of individual fermentations, creating logistical problems. The current study already involved more than 3000 individual fermentations. Future work should also not be limited to just three stages of fermentations

since more relevant information regarding metabolic regulations may also be discovered at other points along the fermentation time course.

Furthermore, we have only focused our attention on the extracellular organic acids (as it reflects the organic acid profiles of the wines), but future work should also evaluate the influence of these factors on the intracellular levels of these organic acids in order to understand the *in vivo* metabolic production. The relationship between genes and acid production levels has been demonstrated by the use of prediction models and deletion strategies. However, it is possible that the deletion or overexpression of many other genes (particularly those that did not show any transcriptional responses among strains and/or growth stages) may also have real biological significance. In light of these prospects, more recommendations can still be offered to wine makers in order to improve wine acidity.

While there were few previous attempts to understand how wine acidity evolves, the current study is the first that comprehensively explored the influences of individual and/or multiple changes in environmental factors (fermentation temperature, pH and sugars levels of the must) and strain identity on the production of three important organic acids which play a significant role in defining the organoleptic characteristics of wine. Such influences were investigated at three critical stages of fermentation (i.e. exponential, early and late stationary growth phase) and under both aerobic and anaerobic conditions. Organic acid trends and profiles were established among strains and different fermentation conditions. In addition, prediction models were also generated in order to identify genes (verified by deletion studies) that are responsible for organic acid regulation in wine. The current study will influence current winemaking practices by providing valuable information regarding wine acidity management by simply controlling “easily” manageable parameters such as pH, temperature, initial sugar levels of the must and the genetic background of the wine yeast.

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