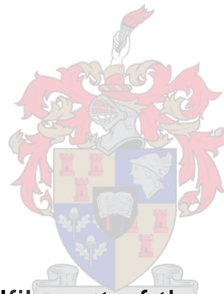
Characterisation of *Wickerhamomyces anomalus* and *Kazachstania aerobia*: Investigating fermentation kinetics and aroma production

by

Judith Lombard



Thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

at

Stellenbosch University

Institute for Wine Biotechnology, Faculty of AgriSciences

Supervisor: Prof Florian F Bauer

Co-supervisor: Dr Hannibal T Musarurwa

December 2016

Stellenbosch University https://scholar.sun.ac.za

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: December 2016

Copyright © 2016 Stellenbosch University
All rights reserved

Summary

Non-*Saccharomyces* yeasts have been studied extensively in the past two decades to use as catalysts for adjusting the aroma and chemical properties of wine. Many non-*Saccharomyces* yeasts dominate in grape must, but *Wickerhamomyces anomalus* and *Kazachstania aerobia* have recently been found to be more dominant in several musts in South Africa than what has been reported from other wine growing areas. It has been hypothesised that regional microflora can lead to a *terroir* specific wine. To further establish these claims, the impact of these non-*Saccharomyces* yeasts on the chemical profile and sensory perception of wine, in particular when present in high numbers, has yet to be fully elucidated. This study was designed to better characterise isolated strains of non-*Saccharomyces* species, determining its phenotypic space, as well as to assess their fermentation potential and volatile aroma compound production in synthetic and real grape must.

Eight *K. aerobia* and thirteen *W. anomalus* isolates were used for characterisation. DNA based taxonomic differences between isolates were investigated using the Random Amplification of Polymorphic DNA (RAPD) method and phenotypic heterogeneity was established using stress assays to determine heat, saline, osmotic and oxidative stress tolerance. Phenotypically diverse *K. aerobia* and *W. anomalus* strains were then selected for co- and sequential fermentations with two *S. cerevisiae* strains, VIN13 and EC1118, in synthetic grape must. In addition, sequential culture fermentations were conducted in Sauvignon blanc grape must by individually pairing two strains of *K. aerobia* and two strains of *W. anomalus* with *S. cerevisiae* EC1118. Wine aroma compounds were quantified using GC-FID.

RAPD analysis classified *W. anomalus* isolates into five distinct groups according to place of origin. Phenotypic variations were evident within and between the proposed strains as was exhibited by heterogeneous resistance to oxidative, saline and osmotic stresses compared to *S. cerevisiae*, VIN13. The *K. aerobia* isolates showed no marked genetic differences, although exhibiting slight variations in stress responses. During fermentation the non-*Saccharomyces* yeasts persisted for longer when *S. cerevisiae* was only inoculated after 48 hours, or at a lower density. The longer the non-*Saccharomyces* yeasts proliferated in the must the more pronounced was the effect on aroma production. *Kazachstania aerobia* yeasts did not achieve a high biomass compared to *W. anomalus*, but survived for longer in fermentation, especially in Sauvignon blanc grape must. Although *W. anomalus* displayed strong growth, it was inhibited by the growth of *S. cerevisiae.*

Kazachstania aerobia and *W. anomalus* gave a unique aroma profile to the wines. The latter yeast produced high concentrations of ethyl acetate, while *K. aerobia* was characterised by increased acetic acid concentration. Most aroma compounds were increased in mixed culture fermentations, especially higher alcohols, with a significant increase in the esters 2-phenylethyl acetate by *K. aerobia,* and ethyl caproate and caprylate by *W. anomalus*. Although, as single cultures these yeasts did not ferment wines to dryness in synthetic grape must and only completed fermentation after 28 days in Sauvignon blanc grape must, they are capable of conferring favourable wine aroma when in

Stellenbosch University https://scholar.sun.ac.za

association with *S. cerevisiae* strains with no risk of sluggish fermentation. This study provides a basis for future work on wine quality improvement through exploitation of non-*Saccharomyces* yeasts and gives insight to the possible impact of *K. aerobia* and *W. anomalus* present in grape must in a South African context.

Stellenbosch University https://scholar.sun.ac.za

Opsomming

Nie-*Saccharomyces* giste is in die afgelope twee dekades omvattend bestudeer om gebruik te word as katalisators vir die aanpassing van aroma en chemiese eienskappe van wyn. Baie nie-*Saccharomyces* giste domineer in druiwemos, maar onlangs is gevind dat *Wickerhamomyces anomalus* en *Kazachstania aerobia* meer dominant in verskeie druiwemos in Suid-Afrika is teenoor wat in ander wynbougebiede aangemeld is. Dit is voorgestel dat plaaslike mikroflora kan lei tot 'n *terroir* spesifieke wyn. Om hierdie stellings te evalueer, moet die impak van hierdie nie-*Saccharomyces* giste, veral wanneer hul in groot hoeveelhede teenwoordig is, op die chemiese profiel en sensoriese persepsie van wyn bepaal word. Hierdie studie is ontwerp om geïsoleerde gisrasse van nie-*Saccharomyces* spesies beter te karakteriseer, die fenotipiese ruimte te bepaal asook hul fermentasie potensiaal en aroma produksie in sintetiese en regte druiwemos vas te stel.

Vir karakterisering, is agt *K. aerobia* en dertien *W. anomalus* isolate gebruik. DNA-gebaseerde taksonomiese verskille is ondersoek met die gebruik van die “Random Amplified Polymorphic DNA” (RAPD) metode, waarna fenotipiese heterogeniteit bepaal is met behulp van stres toetse deur hitte, sout, osmotiese en oksidatiewe stres toleransie te bepaal. Fenotipies diverse *K. aerobia* en *W. anomalus* gisrasse is daarna gekies vir ko- en sekwensiële fermentasies met twee *S. cerevisiae* gisrasse, VIN13 en EC1118, in sintetiese druiwe mos. Daarna is sekwensiële fermentasies in Sauvignon blanc sap uitgevoer deur individuele paring van twee gisrasse van *K. aerobia* en twee gisrasse van *W. anomalus* met *S. cerevisiae* EC1118. Aroma komponente is gekwantifiseer met die gebruik van GC-FID.

RAPD-analise het *W. anomalus* isolate geklassifiseer in vyf afsonderlike groepe volgens plek van oorsprong. Fenotipiese variasies was duidelik waargeneem binne en tussen die voorgestelde gisrasse, soos voorgestel deur die heterogene weerstand teen oksidatiewe, sout en osmotiese spanning in vergelyking met *S. cerevisiae*, VIN13. Die *K. aerobia* isolate het geen merkbare genetiese verskille getoon nie, alhoewel effense variasies in stresreaksie waargeneem was. Gedurende fermentasie het die nie-*Saccharomyces* giste langer oorleef wanneer *S. cerevisiae* eers na 48 uur geïnokuleer was, of teen 'n laer digtheid. Hoe langer die nie-*Saccharomyces* giste oorleef het, hoe groter was die impak op aroma produksie. Alhoewel *K. aerobia* nie so ‘n hoë biomassa soos *W. anomalus* bereik het nie, het dit vir langer in fermentasie oorleef, veral in die Sauvignon blanc druiwe mos. Verder, alhoewel *W. anomalus* sterk gegroei het, was dit deur *S. cerevisiae* geïnhibeer.

Kazachstania aerobia en *W. anomalus* het 'n unieke aroma profiel aan die wyne verleen. Laasgenoemde gis het hoë konsentrasies etielasetaat vervaardig, terwyl *K. aerobia* gekenmerk was deur 'n toename in asynsuur produksie. Die meeste aroma komponente het in die gemengde fermentasies toegeneem, veral die produksie van hoër alkohole, met 'n beduidende toename in die esters 2-fenieletiel asetaat deur *K. aerobia,* en etielkaprylaat en etielkaproaat deur *W. anomalus*. Alhoewel die wyne nie droog gegis was deur die giste as enkel kulture in sintetiese druiwe mos nie en eers ná 28 dae in Sauvignon blanc druiwe mos fermentasie voltooi het, was dit in staat om

Stellenbosch University https://scholar.sun.ac.za

gunstige aromas aan die wyn te verleen en hou dit geen risiko vir slepende fermentasies in kombinasie met *S. cerevisiae* in nie. Hierdie studie bied 'n basis vir toekomstige werk oor die verbetering van wyngehalte deur die gebruik van nie-*Saccharomyces* giste en gee insig oor die moontlike impak van *K. aerobia* en *W. anomalus* wanneer teenwoordig in druiwe mos in 'n Suid-Afrikaanse konteks.

Stellenbosch University https://scholar.sun.ac.za

This thesis is dedicated to

Jo-Marí Basson

For her friendship and support during the years of my studies

Stellenbosch University https://scholar.sun.ac.za

Biographical sketch

Judith (Judy) was born in Cape Town on 13 January 1992. She started her University studies at Stellenbosch in 2011 and completed her BScAgric-degree in Viticulture and Oenology in 2014. In 2015 she enrolled for her postgraduate studies at the Institute for Wine Biotechnology to further her career in Science.

Stellenbosch University https://scholar.sun.ac.za

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- The Institute for Wine Biotechnology and my supervisors - Prof Bauer for all the scientific advice and Dr Hannibal Musarurwa for the many encouraging words.
- Christine du Toit for her valuable help during the first part of my research, and Hannah Muysers for taking over the baton from her.
- Dr Evodia Setati for inspiring me to further my post graduate studies.
- My fellow lab collegues for their advice and guidance.
- My family and friends for their support and encouragement.
- The Lord for His faithfulness.

Stellenbosch University https://scholar.sun.ac.za

Preface

This thesis is presented as a compilation of 5 chapters. Referencing is done to the style of the International Journal of Food Microbiology prescripts.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**

Non-*Saccharomyces* yeast in alcoholic fermentation

Chapter 3 **Research results**

Genetic and phenotypic characterisation of *Wickerhamomyces anomalus* and *Kazachstania aerobia*: investigating amino acid impact on growth and aroma production

Chapter 4 **Research results**

Determining the fermentation potential and aroma production of non-*Saccharomyces* yeast in mixed culture fermentations with *Saccharomyces cerevisiae*

Chapter 5 **General discussion and conclusions**

Stellenbosch University https://scholar.sun.ac.za

Table of Contents

Chapter 1. Introduction and project aims **1**

1.1 Introduction 2
1.2 Rationale 3
1.3 Aims and objectives 3
1.4 References 4

Chapter 2. Literature review: Non-*Saccharomyces* yeasts in alcoholic fermentation **7**

2.1 Introduction 8
2.2 Yeasts in alcoholic fermentation 9
2.3 Yeast identification 9
2.4 The wine yeast *S. cerevisiae* 10
2.5 Non-*Saccharomyces* yeast and fermentation properties 11
2.5.1 Non-*Saccharomyces* and its benefit to wine aroma 13
2.5.1.1 Esters 13
2.5.1.2 Higher alcohols 13
2.5.1.3 Acetic acid 14
2.5.1.4 Volatile phenols and sulphur compounds 14
2.5.2 Enzymatic activity 14
2.5.3 Lowering of ethanol concentration 15
2.6 Terroir specific yeasts 16
2.7 Non-*Saccharomyces* yeasts investigated in this study 17
2.7.1 *Kazachstania aerobia* 17
2.7.2 *Wickerhamomyces anomalus* 17
2.8 Mixed culture fermentations 18
2.8.1 Introduction 18
2.8.2 Impact on fermentation kinetics 19
2.8.3 Yeast interactions 22
2.8.3.1 Growth interactions 22
2.8.3.2 Metabolite interactions 23
2.8.4 Inoculation protocol 25
2.9 The role of nitrogen 26
2.10 Commercialisation of non-*Saccharomyces* yeasts 27
2.11 Conclusion 28
2.12 References 28

Chapter 3. Research results: Genetic and phenotypic characterisation of *Wickerhamomyces anomalus* and *Kazachstania aerobia*: investigating amino acid impact on growth and aroma production **43**

3.1 Introduction 44
3.2 Materials and methods 45
3.2.1 Yeast and culture conditions 45
3.2.2 Phenotypic characterisation 46
3.2.2.1 Plate assays 46
3.2.3 Genotypic characterisation 47
3.2.3.1 DNA extraction 47
3.2.3.2 Strain identification 48
3.2.4 Single culture fermentations 48
3.2.4.1 Inoculation strategy 48
3.2.4.2 Fermentation kinetics 49

Stellenbosch University https://scholar.sun.ac.za

3.2.4.3 Major volatile aroma production 49
3.2.4.4 Statistical analysis 49
3.3 Results 50
3.3.1 Phenotypic characterisation – plate assays 50
3.3.2 Genotypic characterisation – RAPD 54
3.3.3 *K. aerobia* and *W. anomalus* in single culture fermentations 55
3.3.3.1 Fermentation kinetics 55
3.3.3.2 Major volatile aroma production 64
3.4 Discussion 67
3.4.1 Phenotypic characterisation with stress assays 67
3.4.2 Genotypic characterisation with RAPD analysis 68
3.4.3 Impact of different nitrogen compositions on single culture fermentations of *K. aerobia* and *W. anomalus* 68
3.4.3.1 Fermentation kinetics 68
3.4.3.2 Major volatile aroma production 70
3.5 Conclusion 71
3.6 References 72
3.7 Appendix 77

Chapter 4. Research results: Determining the fermentation potential and aroma production of non-*Saccharomyces* yeast in mixed culture fermentations with *Saccharomyces cerevisiae* 79

4.1 Introduction 80
4.2 Materials and Methods 81
4.2.1 Mixed culture fermentations: *K. aerobia* and *W. anomalus* with *S. cerevisiae* in synthetic grape must 81
4.2.1.1 Inoculation strategy 81
4.2.1.2 Fermentation kinetics 82
4.2.1.3 Yeast enumeration 83
4.2.2 Mixed culture fermentations in Sauvignon blanc grape must 83
4.2.2.1 Microvinification procedure 83
4.2.2.2 Yeast species, isolates and strains 83
4.2.2.3 Inoculation strategy and culture conditions 83
4.2.2.4 Fermentation kinetics 84
4.2.2.5 Yeast enumeration 84
4.2.3 Major volatile aroma production 84
4.2.4 Statistical analysis 84
4.3 Results 85
4.3.1 Mixed culture fermentations: *K. aerobia* and *S. cerevisiae* 85
4.3.1.1 Fermentation kinetics 85
4.3.1.2 Yeast enumeration 88
4.3.1.3 Major volatile aroma production 90
4.3.2 Mixed culture fermentations: *W. anomalus* and *S. cerevisiae* 93
4.3.2.1 Fermentation kinetics 93
4.3.2.2 Yeast enumeration 95
4.3.2.3 Major volatile aroma production 98
4.3.3 Mixed culture fermentations in Sauvignon blanc grape must 101
4.3.3.1 Fermentation kinetics 101
4.3.3.2 Yeast enumeration 104
4.3.3.3 Major volatile aroma production 107
4.4 Discussion 111
4.4.1 Fermentation potential of *K. aerobia* and *W. anomalus* in single and mixed culture fermentations 111
4.4.2 Aroma production of *K. aerobia* and *W. anomalus* in single and mixed culture fermentations 113

Stellenbosch University https://scholar.sun.ac.za

4.5 Conclusion 116
4.6 References 117
4.7 Appendix 124

Chapter 5. General discussion and conclusions 127

5.1 Concluding remarks and future prospects 128
5.2 References 130

Stellenbosch University https://scholar.sun.ac.za

Chapter 1

Introduction and project aims

Stellenbosch University https://scholar.sun.ac.za

Chapter 1 – Introduction

1.1 Introduction

The earliest intentionally fermented beverage is thought to have been produced in the Neolithic period (10 000 – 5 000 BC), but it is only in the second half of the 19th century that yeasts were identified as the organisms responsible for alcoholic fermentation (Barnett, 2000). It is now known that alcoholic fermentation in grape juice is a biological process comprising of the conversion of sugars to ethanol and carbon dioxide by yeast; and also resulting in the production and biosynthesis of other primary as well as secondary metabolites. Primary metabolites (e.g. ethanol, glycerol, acetic acid, acetaldehyde) and secondary metabolites (e.g. esters, higher alcohols, fatty acids) determine the quality of wine and their production is influenced by viticultural and winemaking practices. Consequently, yeast species and the genetic background of individual strains are a key determinant of wine flavour and aroma (Ciani et al., 2010).

Different yeast species and strains are present at the onset of fermentation and these can be divided into two groups, non-*Saccharomyces* and *Saccharomyces* species (Boulton et al., 1996; Constantí et al., 1997). *Saccharomyces cerevisiae* is most frequently the dominant yeast conducting alcoholic fermentation and is capable of suppressing most non-*Saccharomyces* yeasts, at least in the latter stages of fermentation (Jackson, 2008). Until recently, it was thought that non-*Saccharomyces* only contribute negatively towards wine aroma by either being primarily spoilage organisms or insignificant during winemaking (Du Toit and Pretorius, 2000; Padilla et al., 2016). However, it is now well established that some non-*Saccharomyces* contribute positively towards wine quality (Lema et al., 1996; Soden et al., 2000). Nonetheless, due to various factors, such as low alcohol tolerance (Heard and Fleet, 1985), limited oxygen and increasing temperature (Fleet, 2003), most non-*Saccharomyces* yeasts struggle to complete alcoholic fermentation (Jolly, 2004). Combining *S. cerevisiae* and non-*Saccharomyces* species during fermentation, also known as a mixed culture fermentation, can bypass the challenges generally associated with single inoculation of non-*Saccharomyces* yeasts.

Globally, many studies have been undertaken that assess the impact of non-*Saccharomyces* yeasts in mixed culture fermentations with *S. cerevisiae* (Anfang et al., 2009; Azzolini et al., 2012; Benito et al., 2013; Canonico et al., 2016; Ciani et al., 2006; Comitini et al., 2011; Domizio et al., 2011; Gobbi et al., 2013; Jolly et al., 2014; Loira et al., 2014; Moreira et al., 2008, 2005; Soden et al., 2000; Viana et al., 2009). Mixed culture fermentations stimulate metabolic interactions between the yeasts that can alter the aromatic profile of wines (Ciani et al., 2010, 2006; Fleet, 2003; Luyt, 2015). These fermentations could potentially amplify the uniqueness of wines giving them more distinctive characteristics. Indeed, certain mixed culture fermentations have been found to be preferred by tasters (Izquierdo Cañas et al., 2014; Jolly et al., 2003a; Viana et al., 2009). Nonetheless, more

Stellenbosch University https://scholar.sun.ac.za

knowledge is needed of the interactions between specific non-*Saccharomyces* strains and *S. cerevisiae* yeast (Ciani and Comitini, 2015; Ciani et al., 2010).

Recently different strains have been isolated from vineyards in Stellenbosch, South Africa (Bagheri, 2014; Setati et al., 2012) and of these strains *Kazachstania aerobia* and *Wickerhamomyces anomalus* showed promising fermentative characteristics. *Kazachstania aerobia* was found to be dominant in grape must from a biodynamic as well as from a conventional farm. *Wickerhamomyces anomalus* was isolated in 2013 from grape must and fermenting wine sourced from an integrated farming system. This yeast was one of the few non-*Saccharomyces* yeasts still present after 50% sugar consumption (Bagheri, 2014). These species were chosen for further characterisation in the current study, as little research had been done on them previously. According to our understanding, *K. aerobia* was only recently used in mixed culture fermentations (Beckner Whitener et al., 2016), although *W. anomalus* (formerly *Hansenula anomala* and *Pichia anomala*) has been used successfully in sequential inoculation with *S. cerevisiae* in a recent study (Izquierdo Cañas et al., 2014, 2011).

1.2 Rationale

Non-*Saccharomyces* yeasts, even when only present initially in fermentation, can contribute to the distinctiveness of the wine. Each yeast species indeed has distinct properties such as characteristic aroma profiles that may be beneficial to wine in general or specific wine styles in particular (Pretorius, 2000). It has been suggested that these local yeasts impart a specific *terroir* character to wine. Some yeast strains and isolates exhibit more favourable characteristics than others and prominent variations between strains can occur (Fleet, 2008). It is thus required to characterise and identify these isolates genotypically and phenotypically. Furthermore, to fully understand the impact of these yeasts, it is essential to determine their fermentation potential in single and mixed culture fermentations with *S. cerevisiae* and the subsequent aroma production.

1.3 Aims and objectives

The initial aim of this project is to characterise the *K. aerobia* and *W. anomalus* yeasts that have been isolated mainly from South African vineyards and secondly to determine the potential of these yeasts to ferment synthetic grape must and their impact on the aroma profile of wine, using both synthetic and Sauvignon blanc grape must.

To achieve the above mentioned aims, the following objectives were pursued.

1. Characterise the phenotypic variation of different *Kazachstania aerobia* and *Wickerhamomyces anomalus* strains and isolates by using salt, osmotic, oxidative and heat stress tests.

Stellenbosch University https://scholar.sun.ac.za

2. Assess the genotypic variation between the isolates using Random Amplified Polymorphic DNA (RAPD) analysis.
3. Investigate the fermentation dynamics and aroma production potential of selected *K. aerobia* and *W. anomalus* as mono- and mixed culture fermentations with *S. cerevisiae* in synthetic grape must.
4. Determine the fermentation dynamics and aroma production of *K. aerobia* and *W. anomalus* in mono- and sequential culture fermentations in Sauvignon blanc grape must.

1.4 References

Anfang, N., Brajkovich, M., Goddard, M.R., 2009. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon blanc. Aust. J. Grape Wine Res. 15, 1–8.

Azzolini, M., Fedrizzi, B., Tosi, E., Finato, F., Vagnoli, P., Scrinzi, C., Zapparoli, G., 2012. Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. Eur. Food Res. Technol. 235, 303–313.

Bagheri, B., 2014. Comparative analysis of fermentative yeasts during spontaneous fermentation of grapes from different management systems. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Barnett, J.A., 2000. A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850-1880. Yeast 16, 755–771.

Beckner Whitener, M.E., Stanstrup, J., Panzeri, V., Carlin, S., Divol, B., Du Toit, M., Vrhovsek, U., 2016. Untangling the wine metabolome by combining untargeted SPME – GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. Metabolomics 12. doi:10.1007/s11306-016-0962-4

Benito, S., Palomero, F., Morata, A., Calderón, F., Palmero, D., Suárez-Lepe, J.A., 2013. Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. Eur. Food Res. Technol. 236, 29–36.

Boulton, R.B., Singleton, V.L., Bisson, L.F., Kunkee, R.E., 1996. Principles and practices of winemaking, 1st ed. Springer US, New York.

Canonico, L., Comitini, F., Oro, L., Ciani, M., 2016. Sequential fermentation with selected immobilized non-*Saccharomyces* yeast for reduction of ethanol content in wine. Front. Microbiol. 7, 1–11.

Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. 108, 239–245.

Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation. Curr. Opin. Food Sci. 1, 1–6.

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

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol. 28, 873–882.

Constantí, M., Poblet, M., Arola, L., Mas, A., Guillamón, J.M., 1997. Analysis of yeast populations during alcoholic fermentation in a newly established winery. Am. J. Enol. Vitic. 48, 339–344.

Domizio, P., Romani, C., Comitini, F., Gobbi, M., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Ann. Microbiol. 61, 137–144.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal- A review. S. Afr. J. Enol. Vitic. 21, 74–96.

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

Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11–22.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. Food Microbiol. 33, 271–281.

Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microbiol. 50, 727–728.

Izquierdo Cañas, P.M., Esteban, C., Romero, G., Heras, J.M., Mónica, M., González, F., 2014. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. Eur. Food Res. Technol. 239, 279–286.

Izquierdo Cañas, P.M., Palacios Garcia, A.T., Garcia Romero, E., 2011. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starter. Vitis 50, 177–182.

Jackson, R.S., 2008. Fermentation - Biochemistry of alcoholic fermentation, in: Wine Science Principles and Applications. Elsevier, London, pp. 358–372.

Jolly, N.P., 2004. Characterisation, evaluation and use of non-*Saccharomyces* yeast strains isolated from vineyards and must. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Jolly, N.P., Augustyn, O.H.P., Pretorius, I.S., 2003. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55–62.

Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: Non-S*accharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14, 215–237.

Lema, C., Garcia-Jares, C., Orriols, I., Angulo, L., 1996. Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albarino wine aroma. Am. J. Enol. Vitic. 47, 206–216.

Stellenbosch University https://scholar.sun.ac.za

Loira, I., Vejarano, R., Bañuelos, M.A., Morata, A., Tesfaye, W., Uthurry, C., Villa, A., Cintora, I., Suárez-Lepe, J.A., 2014. Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. LWT - Food Sci. Technol. 59, 915–922.

Luyt, N.A., 2015. Interaction of multiple yeast species during fermentation. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., Vasconcelos, I., 2008. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. Int. J. Food Microbiol. 124, 231–238.

Moreira, N., Mendes, F., Hogg, T., Vasconcelos, I., 2005. Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. Int. J. Food Microbiol. 103, 285–294.

Padilla, B., Gil, J. V., Manzanares, P., 2016. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Front. Microbiol. 7, 1–20.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

Setati, M.E., Jacobson, D., Andong, U., Bauer, F., 2012. The vineyard yeast microbiome, a mixed model microbial map. PLoS One 7, e52609.

Soden, A., Francis, I.L., Oakey, H., Henschke, P.A., 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape Wine Res. 6, 21–30.

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

Stellenbosch University https://scholar.sun.ac.za

Chapter 2

Literature review

Non-*Saccharomyces* yeasts in alcoholic fermentation

Chapter 2 – Non-*Saccharomyces* yeast in alcoholic fermentation

2.1 Introduction

Grape must is a complex ecosystem consisting of a variety of yeasts, filamentous fungi and bacterial species, constantly interacting with one another (Setati et al., 2012). However, yeast species are predominantly responsible for conducting the alcoholic fermentation (Fleet and Heard, 1993). Yeasts originate from the grape berries, as well as from cellar equipment and may also include commercial strains added by the winemaker to conduct alcoholic fermentation (Boulton et al., 1996; Fleet and Heard, 1993). The yeast most commonly used in wine production is *Saccharomyces cerevisiae*. Other wine yeasts that are part of the *Saccharomyces* genera include *S. paradoxus* and *S. bayanus*. However, the majority of yeasts that are naturally present in the wine environment are not part of this genera and are commonly referred to as non-*Saccharomyces* yeasts (Jolly et al., 2014).

Saccharomyces cerevisiae is usually the dominant species conducting alcoholic fermentation due to its strong fermentative abilities (Jackson, 2008). In addition, this yeast produces a desirable aroma profile. Consequently *S. cerevisiae* strains were commercialised and are now used as inoculation starter culture for wine fermentations. Most non-*Saccharomyces* yeasts were previously seen as spoilage organisms (Fleet and Heard, 1993; Jolly et al., 2014; Moreno-Arribas and Polo, 2005). However, there is growing evidence that certain metabolites produced by non-*Saccharomyces* yeasts contribute positively to wine complexity (Andorrà et al., 2012; Ciani et al., 2010; Fleet, 2008, 2003; Jolly et al., 2006; Lambrechts and Pretorius, 2000; Lema et al., 1996; Rooyen and Tracey, 1987; Soden et al., 2000). These yeasts yield maximal benefits when used in conjunction with *S. cerevisiae* in order to ensure a complete fermentation and some have already been commercialised as inoculum cultures (Azzolini et al., 2015; Ciani et al., 2010). The interactions between some non-*Saccharomyces* yeast species and *S. cerevisiae* have been investigated with regards to population dynamics, fermentation kinetics, and the resulting aroma profiles (Albergaria et al., 2010; Bely et al., 2008; Ciani et al., 2006; Fleet, 2003; Pérez-Nevado et al., 2006; Sadoudi et al., 2012). A specific focus has been directed on the use of such yeast to reduce ethanol concentrations (Ciani and Comitini, 2011; Fleet, 2008).

Data suggest that non-*Saccharomyces* yeast populations, species or strains may be specific to a region or terroir, and may promote a particular style of wine (Fleet, 2003). Numerous yeast strains are present on grapes and musts, and strain diversity has been well documented for *S. cerevisiae*. However, similar information on specific non-*Saccharomyces* yeasts is lacking (Jolly et al., 2014). Studies have looked in depth at the variation that occurs between strains of *S. cerevisiae* and have found the genotypic and phenotypic differences to be prominent and noteworthy (Camarasa et al., 2011; Knight and Goddard, 2015; Kvitek et al., 2008; Liti et al., 2009; Mendes et al., 2013; Vilanova et al., 2007). However, studies on the phenotypic space of non-*Saccharomyces* species remain

limited. Strain differences have been described for some species (Albertin et al., 2016; Rossouw and Bauer, 2016; Tofalo et al., 2012), but the full phenotypic space of many non-*Saccharomyces* species has yet to be determined. This review focusses on non-*Saccharomyces* yeasts occurring in grape must and its role and impact on alcoholic fermentation.

2.2 Yeasts in alcoholic fermentation

During alcoholic fermentation primary (*e.g.* ethanol, glycerol, acetic acid, acetaldehyde) and secondary metabolites (*e.g.* esters, higher alcohols, fatty acids) determine the ultimate chemical and sensory quality of wine (Fleet and Heard, 1993). Production of these metabolites is influenced by environmental factors, grape cultivar, viticultural practices, fruit condition and pH as well as winemaking practices (*e.g.* sulphur dioxide addition, malolactic fermentation) (Ciani et al., 2010; Lilly et al., 2000). Consequently, the yeast strains contributing to fermentation determine the amount of metabolites generated and utilised, and the chemical and sensory bouquet of the final product (Bisson and Joseph, 2009; Fleet and Heard, 1993).

At the start of fermentation apiculate yeasts are primarily responsible for conducting the fermentation and dominate the grape must for the first 3-4 days (Fleet and Heard, 1993). In most cases, *S. cerevisiae* is present in low quantities during the initial stages, but tends to take over once ethanol percentage rises and oxygen levels decrease (Fleet and Heard, 1993; Lema et al., 1996). This spontaneous or natural fermentation is thus a sequential process of different yeasts dominant at various intervals (Beltran et al., 2002; Mendoza et al., 2007). *Saccharomyces cerevisiae* is not the only yeast present during the middle and end stages of fermentation, and species from other non-*Saccharomyces* genera such as *Candida, Pichia, Zygosaccharomyces, Schizosaccharomyces, Torulaspora, Lachancea* (previously *Kluyveromyces*)*, Metschnikowia, Hanseniaspora, Rhodotorula, Starmarella* and *Issatchenkia* can be identified (Combina et al., 2005; Ghosh et al., 2015; Heard and Fleet, 1985; Setati et al., 2012) and survive during fermentation (Fleet et al., 1984; Heard and Fleet, 1985). From grape must, more than 40 yeast species have been isolated (Ciani et al., 2010; Jolly et al., 2006; Kurtzman et al., 2011). DNA based techniques have improved the accuracy and efficiency of classification, and older literature has to be carefully evaluated to establish which specific species is referred to (Jackson, 2008; Jolly et al., 2014).

2.3 Yeast identification

Identification and correct classification of different species and strains within a species enables researchers to characterise yeasts. Non-molecular techniques involve the use of physiological and biochemical tests investigating colony morphology and fermentative ability (in terms of growth and sugar assimilation) (Lodder and Kreger-van Rij, 1952).

Stellenbosch University https://scholar.sun.ac.za

Modern taxonomic methods rely on DNA-based technologies (Bokulich et al., 2012) and can be either culture dependent or independent. These approaches comprise polymerase chain reaction (PCR) based techniques; pulsed-field gel electrophoresis (PFGE) and restriction fragment length polymorphism analysis (RFLP), amongst others (Deák, 1993; Pretorius, 2000). The most popular culture dependent method for the identification of isolates is analysis of the 5.8S ITS rDNA region by using PCR amplified fragments in restriction fragment length polymorphism analysis (PCR-RFLP) (Combina et al., 2005; Guillamón et al., 1998; Wang and Liu, 2013). RFLP uses restriction enzymes to cleave DNA at specific nucleotide sequences. These fragments can then be separated electrophoretically on agarose gels. However, direct methods to analyse the microbial population (*e.g.* denaturing gradient gel electrophoresis (DGGE)) are faster and able to identify non-culturable microorganisms (Ivey and Phister, 2011; Mills et al., 2002; Renouf et al., 2007). Random Amplified Polymorphic DNA (RAPD) PCR has been employed as an effective and fast way to differentiate between strains and have been applied in taxonomic identification of different yeasts, including *Saccharomyces, Torulaspora, Hansenula, Candida, Pichia,* and *Rhodotorula* (Capece et al., 2003; Quesada and Cenis, 1995).

In light of this, the best results are obtained when using a wider range of strains and incorporating more than one method of identification (Khan et al., 2000; Van der Westhuizen et al., 2000). Time, cost and instrument availability plays an important role in choice of method for characterisation (Bokulich et al., 2012). Techniques are usually based on *S. cerevisiae* as model due to its role as the primary "wine yeast", but, with adaptions, it can also be utilised for non-*Saccharomyces* yeasts.

2.4 The wine yeast *S. cerevisiae*

Saccharomyces cerevisiae (as the primary representative for the *Saccharomyces* genus) dominates spontaneous fermentations due to its strong fermentative abilities, being able to complete fermentations rapidly (Fleet and Heard, 1993). This yeast is also characterised by relatively high sulphur dioxide tolerance and can withstand high ethanol concentrations (Arroyo-López et al., 2010; Fleet, 2003; Ludovico et al., 2001), in addition to being tolerant to temperature fluctuations (Goddard, 2008; Salvadó et al., 2011). Furthermore, *S. cerevisiae* produces many aromatic secondary metabolites which mostly positively impact the sensory profile of wine (Swiegers and Pretorius, 2005). Strains of *S. cerevisiae* differ regarding the formation of these metabolites (Fleet et al., 1984; Herjavec et al., 2003; Lema et al., 1996). Aromas range from oxidized, paper and sweaty (strain K-1M) (Henick-Kling et al., 1998) to vegetative and astringent characters (strain EC1118) (Egli et al., 1998), while others were identified as fruity, floral, pear or spicy (strain Assmannshausen) (Egli et al., 1998), or lime and tropical fruit (strain AWRI 838) (Soden et al., 2000).

In 1890 the concept of inoculating grape must with a selected pure yeast culture to achieve successful alcoholic fermentation was introduced by Hermann Müller-Thurgau (Pretorius, 2000). Active dried wine yeast (ADWY) was first commercialised in 1965 (Chambers and Pretorius, 2010)

and it is now standard practice that most winemakers inoculate grape must with *S. cerevisiae* not only to complete fermentation but also sometimes to compete with and suppress indigenous yeasts (Fleet and Heard, 1993; García-Ríos et al., 2014; Pretorius et al., 1999).

2.5 Non-*Saccharomyces* yeast and fermentation properties

Approximately twenty non-*Saccharomyces* yeast genera have been described in fermenting grape must, including *Candida, Metschnikowia, Kluyveromyces, Hanseniaspora* (anamorph *Kloeckera*) and *Pichia*, and less frequently those from the genera's *Torulaspora, Dekkera, Zygosaccharomyces, Saccharomycodes*, and *Schizosaccharomyces* (Fleet and Heard, 1993; Fleet, 2003; Johnson and Echavarri-Erasun, 2011).

Experiments regarding non-*Saccharomyces* yeasts are frequently conducted in mixed culture fermentations with *S. cerevisiae*. Subsequently it is not always clear if the impact on fermentation or metabolites produced is due to the inherent property of the non-*Saccharomyces* yeast or the result of an interaction between the yeasts. Many have reviewed the resulting wine produced by mixed culture fermentations, but few document the specific contribution of the non-*Saccharomyces* yeast (Ciani and Comitini, 2011). Table 2.1 is a summary of some of the major non-*Saccharomyces* yeasts and their oenologically relevant properties.

Table 2.1 Fermentation behaviour of non-*Saccharomyces* yeast in pure culture (adapted from Ciani & Comitini, 2011)

Non-*Saccharomyces* yeast species	**Characteristic behaviour of pure culture**	**References**
Debaryomyces variji	High level of β-glucosidase activity	Garcia et al. (2002)
Hanseniaspora guilliermondii	High ethyl acetate producer	Moreira et al. (2008); Rojas et al. (2003); Viana et al. (2008)
Hanseniaspora osmophila	High 2-phenyl ethyl acetate producer	Viana et al. (2009)
Hanseniaspora uvarum (anamorph *Kloeckera apiculata)*	High ethyl acetate producer	Ciani and Maccarelli (1998); Ciani et al. (2006); Moreira et al. (2008); Plata et al. (2003)
	High acetic acid producer	Ciani and Comitini (2011); Romano et al. (1992)
	High acetoin producer	Ciani and Maccarelli (1998)
	High glycerol production	Clemente-Jimenez et al. (2004)
Issatchenkia orientalis	Utilise malic acid	Seo et al. (2007)
	Low ethyl acetate producer	Clemente-Jimenez et al. (2004)
Issatchenkia terricola	High ethyl acetate	Clemente-Jimenez et al. (2004)

Stellenbosch University https://scholar.sun.ac.za

Non-*Saccharomyces* yeast species	**Characteristic behaviour of pure culture**	**References**
Lachancea thermotolerans (Kluyveromyces thermotolerans)	Low acetaldehyde producer	Ciani et al. (2006)
	High acid producer	Gobbi et al. (2013)
	Lactic acid producer (some strains)	Kapsopoulou et al. (2005)
Metschnikowia pulcherrima	High producer of 2-Methoxy-4-vinylphenol	Beckner Whitener et al. (2015)
	High glycerol production	Clemente-Jimenez et al. (2004)
Pichia anomala	High producer of isoamyl acetate (EAHase) or low producer	Rojas et al. (2003)
	High producer of acetic acid	Rojas et al. (2003)
	High producer of ethyl acetate	Rojas et al. (2003)
Pichia fermentans	High glycerol production	Clemente-Jimenez et al. (2004)
	High acetoin production or no production– fermentation condition dependent	Clemente-Jimenez et al. (2005, 2004)
Pichia kluyveri	High producer of 3-mercaptohexyl acetate (3MHA)	Anfang et al. (2009)
Pichia membranifaciens	High ethyl acetate	Viana et al. (2008)
Saccharomycodes ludwigii	High acetoin	Ciani and Maccarelli (1998)
	High ethyl acetate	Ciani and Maccarelli (1998)
Schizosaccharomyces spp.	High rate of malic acid degradation	Benito et al. (2014); Yokotsuka et al. (1993)
Starmarella bacillaris (Candida zemplinina)	High producer of 3-mercaptohexan-1-ol (3MH)	Anfang et al. (2009)
	Low acetic acid producer	Rantsiou et al. (2012); Tofalo et al. (2012)
	Fructophilic yeast	Tofalo et al. (2012)
Starmerella bombicola (Candida stellata)	High glycerol producer	Ciani & Ferraro (1996, 1998); Ciani & Maccarelli (1998)
	High succinic acid producer	Ciani & Maccarelli (1998)
	High acetaldehyde producer	Ciani & Ferraro (1998)
	High acetoin producer	Ciani & Ferraro (1998)
	Low ethanol yield	Contreras et al. (2014)
Torulaspora delbrueckii	Low acetic acid producer	Bely et al. (2008); Comitini et al. (2011); Renault et al. (2009)

Stellenbosch University https://scholar.sun.ac.za

2.5.1 Non-*Saccharomyces* and its benefit to wine aroma

Over 680 volatile aroma compounds have been identified in wine, mainly categorised into higher alcohols, fatty acids, esters, carbonyl and sulphur compounds. Non-*Saccharomyces* yeasts produce as wide a range of compounds as *S. cerevisiae* (Jolly et al., 2014; Manzanares et al., 2011), although relatively little data regarding the metabolism of these yeasts are available (Lambrechts and Pretorius, 2000; Moreira et al., 2005; Nykänen, 1986). Nevertheless, many studies have shown the significant impact of non-*Saccharomyces* yeasts such as *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii*, *P. kluyveri*, *W. anomalus*, *H. uvarum* (anamorph *K. apiculata*) and *Candida* spp., on aroma in wine fermentations (Andorrà et al., 2012; Anfang et al., 2009; Gobbi et al., 2013; Izquierdo Cañas et al., 2011; Jolly et al., 2014; Sadoudi et al., 2012).

2.5.1.1 Esters

Some of the most desirable aromatic features of wine are due to compounds known as esters, of which more than 160 have been identified in wine (Jackson, 2008). Generally non-*Saccharomyces* yeasts produce lower amounts of ethyl esters than *S. cerevisiae*, although production of ethyl acetate is frequently increased (Rojas et al., 2003, 2001). Data showed that the *Pichia* genus generally had a high production of ethyl acetate, whereas *Candida*, *Saccharomyces*, *Torulaspora* and *Zygosaccharomyces* produced significantly lower levels (Viana et al., 2008). This is the main ester in wine and is undesirable at levels of above 150–200 mg/L (Lambrechts and Pretorius, 2000). The *Hanseniaspora* genus is a good producer of esters, especially 2-phenylethyl acetate and isoamyl acetate (Moreira et al., 2008, 2005; Plata et al., 2003; Rojas et al., 2003), although strain differences was notable (Viana et al., 2008) A relatively unknown yeast, *Kazachstania gamospora*, has been found to produce high amounts of esters, especially phenylethyl propionate, compared to *S. cerevisiae* and other non-*Saccharomyces* yeasts (Beckner Whitener et al., 2015).

2.5.1.2 Higher alcohols

Production of higher alcohols have a significant influence on the quality and aroma composition of wines (Beckner Whitener et al., 2015; Gil et al., 1996; Herraiz et al., 1990) and can enhance complexity in wine aroma at concentrations below 300 mg/L (Lambrechts and Pretorius, 2000; Moreira et al., 2005). Similar to *S. cerevisiae*, non-*Saccharomyces* yeasts produce higher alcohols such as active amyl alcohol, isobutanol and n-propanol (Lambrechts & Pretorius, 2000). Although compared to *S. cerevisiae*, production by non-*Saccharomyces* yeasts in monoculture is typically lower, in particular *Hanseniaspora* spp., *Pichia membranifaciens, P. fermentans* and *W. anomalus* (Clemente-Jimenez et al., 2004; Gil et al., 1996; Moreira et al., 2008; Rojas et al., 2003; Viana et al., 2008). However, higher alcohols are usually increased in mixed culture fermentations (Manzanares et al., 2011). Contrary, *Starmerella bacillaris* exhibited an increased production of higher alcohols compared to *S. cerevisiae* as monoculture, although with a lower concentration in mixed culture

Stellenbosch University https://scholar.sun.ac.za

fermentations (Andorrà et al., 2012). With regards to specific higher alcohols, *L. thermotolerans* and *P. fermentans* produced high concentrations of butanol (Clemente-Jimenez et al., 2005; Mains, 2014), while *M. pulcherrima* produced high concentrations of 2-phenyl ethanol (Clemente-Jimenez et al., 2004).

2.5.1.3 Acetic acid

Acetic acid comprises 90% of volatile acidity, making this compound a large determinant of wine quality (Padilla et al., 2016). Apiculate yeast, such as *C. cantarellii, C. zemplinina, P. guillermondii, H, uvarum* and *W. anomalus* have been found to produce high levels of acetic acid (Benito et al., 2011; Fleet and Heard, 1993; Rojas et al., 2003; Sadoudi et al., 2012; Toro and Vazquez, 2002). Many strain differences occur, for instance between strains of *C. zemplinina* (Rantsiou et al., 2012), *H. uvarum* (Mendoza et al., 2007; Romano et al., 2003, 1992) and *T. delbrueckii* (Renault et al., 2009). *Schizosaccharomyces pombe* (Benito et al., 2013) and *M. pulcherrima* (Sadoudi et al., 2012) have been documented to produce low levels of acetic acid.

2.5.1.4 Volatile phenols and sulphur compounds

Disagreeable aromas produced by non-*Saccharomyces* yeasts remain a major cause for concern, specifically production of volatile phenols and sulphur compounds. Due to its low perception threshold, vinyl- and ethylphenols contribute negatively to wine aroma, even at low concentrations (Manzanares et al., 2011; Padilla et al., 2016). *Brettanomyces* spp. is known for its high production of ethylphenols, although other non-*Saccharomyces* yeast, such as *Candida* spp., *T. delbrueckii*, *M. pulcherrima* and *P. guilliermondii* can also produce volatile phenols (Beckner Whitener et al., 2015; Dias et al., 2003; Loureiro and Malfeito-Ferreira, 2003; Padilla et al., 2016). Hydrogen sulphide is produced in medium to high amounts by *Candida* spp., *T. delbrueckii, H. uvarum, H. guilliermondii* and *H. osmophila* (Renault et al., 2009; Strauss et al., 2001; Viana et al., 2008), although *P. guillermondii* produce no hydrogen sulphide (Viana et al., 2008). Furthermore, *H. guilliermondii* and *H. osmophila* have been found to excrete high amounts of heavy sulphur compounds (Moreira et al., 2008).

2.5.2 Enzymatic activity

In addition to non-*Saccharomyces* yeast's contribution to secondary aroma metabolites, some of these yeasts have been reported to be able to produce oenologically relevant amounts of certain extracellular enzymes (Manzanares et al., 2011). In general, several enzymes with primarily hydrolytic catalytic activities are secreted by yeast during fermentation; and may support aroma release (through glycosidases), wine processing and clarification (proteases, xylanases, pectinases, glucanases) and ethyl carbamate reduction (urease) (Van Rensburg and Pretorius, 2000). Frequently, these enzymatic activities are not active under wine conditions, although it has been

found that it is more often exhibited by certain non-*Saccharomyces* yeasts compared to *S. cerevisiae* (Jolly et al., 2014; Manzanares et al., 2011; Maturano et al., 2015; Mendes Ferreira et al., 2001; Pérez et al., 2011). Glycosidase activity consists of β-glucosidase, β-D-xylosidase, α-arabinofuranosidase and α-rhamnosidase and its activity in non-*Saccharomyces* yeasts have recently been reviewed in Manzanares et al. (2011). Yeasts such as *H. vineae*, *H. uvarum*, *W. anomalus*, *M. pulcherrima*, *T. delbrueckii, Kluyveromyces fragilis*, *Pachysolen tannophilus*, *Pichia stipites*, *Candida railenensis*, and *Cryptococcus flavescens* can enable hydrolysis of terpenyl-glycosides (Ciani et al., 2010). This process is conducted through β-glucosidase activity in order to release aroma precursors, increasing the aromatic profile of wines (Fernández et al., 2000; Maturano et al., 2012; Mendes Ferreira et al., 2001; Pérez et al., 2011). Extracellular esterases, responsible for cleavage of esters (degrading esters) and sometimes formation of ester bonds, occur in some strains of *M. pulcherrima, L. thermotolerans, T. delbrueckii* and many *Candida* spp. (Comitini et al., 2011; Swiegers and Pretorius, 2005; Swiegers et al., 2005). In addition, β-D-xylosidase excreted by *H. uvarum, H. osmophila, W. anomalus* (Manzanares et al., 1999) and *Candida utilis* (Yanai and Sato, 2001), is also involved in releasing aroma compounds.

Moreover, protease enzymes responsible for the breakdown of proteins are produced by *Starmarella bombicola, H. uvarum, H. vinae*, *P. membranifaciens, M. pulcherrima, T. delbrueckii* and *Zygoascus meyerae* (Divol and Setati, 2015; Fernández et al., 2000; Jolly, 2004; Maturano et al., 2012). These yeasts and the non-*Saccharomyces* yeasts - *K. thermotolerans*, *W. anomalus*, *Brettanomyces clausenii* and *Candida stellata* - exhibit polygalacturonase activity (Fernández et al., 2000; Jolly, 2004). Indeed, pectinase activity, more rare in wine yeasts, has been detected in species of *Candida, Kluyveromyces, Rhodotorula and Cryptococcus* (Benítez and Codón, 2002; Charoenchai et al., 1997). Additionally, urease activity has been detected in *Shizosaccharomyces pombe* (Benito et al., 2013; Lubbers et al., 1996).

2.5.3 Lowering of ethanol concentration

A prime advantage of many non-*Saccharomyces* yeasts is their potential to lower ethanol yields, which is sometimes favoured by consumers and have been reported to consequently enhance fruit, flower, and acidic aromas (Styger et al., 2011). The non-*Saccharomyces* yeasts *Zygosaccharomyces bisporus, Z. bailii, Z. sapae, H. uvarum, K. marxianus, W. subpelliculosus, Dekkera bruxellensis, Pichia ciferrii, P. fermentans, I. orientalis, T. delbrueckii, Shizosaccharomyces pombe* and many other lesser known non-*Saccharomyces* yeasts has a lower ethanol yield (ethanol per sugar consumed) compared to *S. cerevisiae* (Contreras et al., 2014; Gobbi et al., 2014). However, these yeasts need to be used in a mixed culture fermentation with *S. cerevisiae* to ensure complete consumption of sugars. Lower ethanol wines have been produced in mixed culture fermentations of *S. cerevisiae* yeasts with *Starmarella bacillaris* (Sadoudi et al., 2012), *M. pulcherrima* (Canonico et al., 2016; Sadoudi et al., 2012), *L. thermotolerans* (Gobbi et al., 2013), *H. osmophila*, *H. uvarum* (Canonico et al., 2016) and *Starmerella bombicola* (Canonico et al., 2016;

Stellenbosch University https://scholar.sun.ac.za

Milanovic et al., 2012; Ferraro et al., 2000; Soden et al., 2000) amongst others. However, some non-*Saccharomyces* yeasts can ferment wines to dryness as monocultures and simultaneously produce lower ethanol wines *e.g. Shizosaccharomyces pombe* (Benito et al., 2013), *C. zemplinina, M. pulcherrima* and *T. delbrueckii* (Sadoudi et al., 2012). Cautiously, mixed culture fermentations or spontaneous fermentations can have ethanol levels slightly higher than *S. cerevisiae* monoculture fermentations (Erten et al., 2006; Toro and Vazquez, 2002; Yokotsuka et al., 1993).

2.6 Terroir specific yeasts

It has been proposed that indigenous yeast, naturally occurring in grape must, may be specific to an area or terroir, with characteristic differences in population profiles (Amerine, 1966; Knight et al., 2015). Studies have mainly focused on the distribution of *S. cerevisiae* (Barata et al., 2011; Khan et al., 2000; Knight et al., 2015), found to be due to climatic and viticultural factors (Barata et al., 2011). More recent studies have focussed on the distribution of other microorganisms, including non-*Saccharomyces* yeasts (Bokulich et al., 2013; Setati et al., 2012). However, the scientific question remains whether microbial terroirs exist, that could subsequently lead to a typical aromatic or chemical feature of the wine end product.

In South Africa studies have been performed on terroir specific yeasts, although also more focussed on *S. cerevisiae* (Khan et al., 2000; Pretorius et al., 1999; Setati et al., 2012; Van der Westhuizen et al., 2000). Following these studies Jolly et al. (2004), found four different non-*Saccharomyces* yeast species to be dominant before the start of fermentation – *H. uvarum, Starmarella bombicola, T. delbrueckii* and *C. pulcherrima*. However, these yeasts are found globally in other wine regions as well (Combina et al., 2005; Cordero-Bueso et al., 2011; Díaz et al., 2013; Heard and Fleet, 1985; Zohre and Erten, 2002). More recently yeasts were isolated from spontaneous fermentations in Stellenbosch originating from different farming practises – conventional, integrated and biodynamic farming – exhibiting a large diversity in yeast species (Bagheri, 2014). Shared yeasts were found in all three farming practises (*e.g. M. pulcherrima* and *H. uvarum*) and yeasts not so commonly found in grape must present in high numbers – *e.g. Kazachstania aerobia* and *Wickerhamomyces anomalus* (Bagheri, 2014). In this study the latter yeasts are investigated more in depth with regards to its impact on fermentation and flavour biosynthesis. Although scarce, *W. anomalus* has been detected in other areas - Spain (Cordero-Bueso et al., 2011; Mora and Mulet, 1991; Regueiro et al., 1993), Slovenia (Zagorc et al., 2001) and Switzerland (Díaz et al., 2013). According to our knowledge, *K. aerobia* has never before been isolated from a wine environment.

2.7 Non-*Saccharomyces* yeasts investigated in this study

2.7.1 *Kazachstania aerobia*

Kazachstania spp. is part of the family *Saccharomycetaceae* and the first species to be described was *K. viticola* (Vaughan-Martini et al., 2011). Multigene sequence analysis led to the reclassifying of some species of *Saccharomyces, Kluyveromyces, Arxiozyma* and *Pachytichospora* to the *Kazachstania* family (Kurtzman, 2003; Kurtzman & Robnett, 2003). As a whole this genus is evolutionarily the most related to *S. cerevisiae* (Hagman et al., 2013). *Kazachstania aerobia* was first isolated in Tochigi, Japan, from corn silage deteriorating under aerobic conditions (Lu et al., 2004). Through molecular techniques, it was found that this novel species is phylogenetically closely related to *K. servazzii* and *K. unispora*. In recent years *K. aerobia* was dominantly found in sugary kefir (Magalhães et al., 2010), cereal barley grain (Olstorpe et al., 2010) and detected in tibico grains (Miguel et al., 2011).

After isolation of *K. aerobia* from healthy grapes in Stellenbosch (Bagheri, 2014); this yeast was used for the first time in wine fermentations conducted sequentially with *S. cerevisiae* (Beckner Whitener, 2016). Sensory analysis showed that the wine had a more dried or stewed fruit aromatic profile with bitter, solvent characteristics. Chemical analysis revealed that the later characteristics were most probably due to high ethyl acetate and volatile acidity concentrations. Furthermore, these fermentations had significantly higher terpene concentrations. Interestingly, the *K. aerobia* aromatic profile had many peaks that could not be identified by untargeted GC×GC-TOF-MS analysis. In light of these findings it is still not yet known how this yeast performs as single culture and its dominance and impact on aroma in other fermentation setups.

2.7.2 *Wickerhamomyces anomalus*

Wickerhamomyces anomalus, previously known as *Hansenula anomala*, *Candida pelliculosa*, and *Pichia anomala* (Kurtzman, 2011) naturally occurs in grape must (Cordero-Bueso et al., 2013, 2011; Díaz et al., 2013; Mora and Mulet, 1991; Regueiro et al., 1993; Ribéreau-Gayon et al., 2006; Zagorc et al., 2001; Zott et al., 2008). This yeast is active early in fermentation (Renouf et al., 2007) and can lead to wine spoilage when high levels of acetic acid and ethyl acetate are produced (Plata et al., 2003; Rojas et al., 2003); although strain differences occur (Romano et al., 1997).

In monoculture fermentations of *W. anomalus* it has been found that yeast populations exceeded 10^7 cfu/mL for the duration of fermentation, whereas *S. cerevisiae* populations started declining after three days (Kurita, 2008). In contrast, others found that *W. anomalus* died off immediately after addition of *S. cerevisiae* (Zott et al., 2008). High acetate esters formed by *W. anomalus* lends a fruity character to wine (Rojas et al., 2003) and was seen as the main benefit in red wine aroma (Izquierdo Cañas et al., 2014). These wines were preferred by tasters compared to wines fermented with only *S. cerevisiae* (Izquierdo Cañas et al., 2014). More recently, a *W. anomalus* strain (DBVPG 3003)

Stellenbosch University https://scholar.sun.ac.za

was found secreting a killer toxin, named Pikt, active against *Dekkera/Brettanomyces* spp. (Comitini et al., 2004). Cautiously, it has been reported that *W. anomalus* has a low resistance to SO_2 (Izquierdo Cañas et al., 2011).

High ethyl acetate production is a probable cause for concern as well as the decline in population after addition of *S. cerevisiae*. However it still has potential to be used in mixed culture fermentations with *S. cerevisiae*, if the correct strains can be identified. This yeast, as well as *K. aerobia*, is not able to complete alcoholic fermentation as a single culture in wine fermentations and needs to be inoculated with *S. cerevisiae* in order to ensure an efficient fermentation. In grape must, *S. cerevisiae* is naturally present and will gradually take over the fermentation (Fleet and Heard, 1993; Lema et al., 1996). It is thus necessary to understand the interaction and effect of these yeasts in a mixed culture fermentation setup.

2.8 Mixed culture fermentations

2.8.1 Introduction

The inability of most non-*Saccharomyces* yeasts to complete alcoholic fermentation in the absence of *S. cerevisiae* can lead to spoilage or re-fermentation of wines during aging (Jolly et al., 2003a). Inoculating both *S. cerevisiae* and non-*Saccharomyces* to conduct a mixed culture fermentations alleviates the shortcomings of single inoculated non-*Saccharomyces* yeasts. Single culture fermentations, also known as pure or monoculture, are conducted with a high concentration of a single inoculated yeast strain, although indigenous microflora is still present in the must. A mixed culture or multistarter fermentation is where more than one microorganism is involved (Hesseltine, 1992). In this review the focus is only on mixed cultures performed with yeasts and not any other microorganisms. Generally, two different inoculation strategies can be followed when using a mixed culture setup and are referred to as co- and sequential inoculation. Co-inoculation (also known as simultaneous inoculation) is when yeasts are added at the same time to the grape must (Comitini et al., 2011; Jolly et al., 2006; Soden et al., 2000). Sequential inoculation is conducted by inoculating the one yeast after the other at different time points (Clemente-Jimenez et al., 2005; Contreras et al., 2015; Gobbi et al., 2013; Herraiz et al., 1990; Toro and Vazquez, 2002). *Saccharomyces cerevisiae* can be inoculated sequentially from 1 hour up to a week or longer after the non-*Saccharomyces* yeast have been inoculated, allowing the non-*Saccharomyces* to proliferate, increasing its contribution to the wine making process.

Mixed culture fermentations can have several advantages, depending on the yeast strain and its presence in the fermentation. Specific pairings of *S. cerevisiae* and non-*Saccharomyces* yeasts can lead to wines with an improved complexity, in addition to enhancing particular and specific characteristics of the wine (Ciani et al., 2010). Undesirable aromas produced by non-*Saccharomyces* yeasts can be minimised with the correct inoculation timing of *S. cerevisiae*, to suppress or modify

Stellenbosch University https://scholar.sun.ac.za

the metabolic activity of the yeast (Ciani and Comitini, 2011). Nonetheless, mixed culture fermentations can yield varying amounts of fermentation products at unpredictable rates. It is thus necessary to further investigate the impact of the non-*Saccharomyces* yeasts on fermentation and the interactions between yeasts to improve the practical application of mixed culture fermentations (Ciani et al., 2010).

2.8.2 Impact on fermentation kinetics

The inherent characteristics of specific non-*Saccharomyces* yeasts, as mentioned in Table 2.1, are in most cases also observed in mixed culture fermentation setups with *S. cerevisiae*. However, in some cases, mixed culture fermentations can lead to the reduction of acetic acid, ethyl acetate, acetoin and acetaldehyde levels, compared to high levels in monoculture fermentations (Ciani and Comitini, 2011; Ciani and Ferraro, 1998; Ciani et al., 2006; Clemente-Jimenez et al., 2005; Moreira et al., 2008; Rojas et al., 2003). Such results interestingly suggest that interactions between yeast species impact directly on metabolic activities.

Many studies documenting production of fermentation metabolites in mixed culture fermentations often do not report on the non-*Saccharomyces* yeasts performance as single culture. This creates uncertainty on whether the effect was due to an increase in biomass, an interaction between yeasts or if it is a characteristic of the non-*Saccharomyces* yeast. For example, mixed cultures of either *W. anomalus* or *T. delbrueckii* with *S. cerevisiae* showed an increase in total acetates and ethyl acetate, but the cause was uncertain as no single culture fermentations were performed (Izquierdo Cañas et al., 2011). Kapsopoulou et al. (2007) reported a significant increase in lactic acid concentration observed in mixed cultures of *K. thermotolerans* and *S. cerevisiae*. Although no single cultures of the non-*Saccharomyces* yeasts were used as a control, previous reports suggested that this increase was due to the non-*Saccharomyces* yeast present in the fermentations (Kapsopoulou et al., 2005). Many similar studies have been conducted as outlined in Table 2.2 below. To thoroughly understand the impact of non-*Saccharomyces* yeast in mixed culture fermentations it is necessary to determine the physiological and metabolic interactions between yeasts when present in the same media (Ciani and Comitini, 2015; Ciani et al., 2010).

Stellenbosch University https://scholar.sun.ac.za

Table 2.2 Mixed fermentation processes that have been proposed in winemaking, using *Saccharomyces cerevisiae* and non-*Saccharomyces* (NS) yeasts (adapted from Ciani *et al.*, 2010)

Species used with *S. cerevisiae*	**Aim**	**Process**	**Cause**	**References**
C. cantarellii	Enhancement of glycerol content	Co- and sequential cultures	NS yeast	Toro & Vazquez (2002)
C. pulcherrima	Improve wine aroma profile	Co- and sequential cultures	NS yeast	Jolly et al. (2003a); Zohre & Erten (2002)
D. vanriji	Increase in geraniol concentration	Sequential cultures	NS yeast	Garcia et al. (2002)
H. guilliermondii	Improvement of aroma complexity	Co-cultures	NS yeast and/ or interaction	Moreira et al. (2005, 2008)
H. osmophila	Increased 2-phenyl ethyl acetate	Co-cultures	NS yeast and/ or interaction	Viana et al. (2009)
H. uvarum	Improvement of aroma complexity	Co- or sequential cultures	NS yeast	Andorrà et al. (2012); Herraiz et al. (1990); Jolly et al. (2003a); Moreira et al. (2005, 2008); Zohre & Erten (2002)
	Unacceptable increase in ethyl acetate	Sequential cultures	NS yeast	Ciani et al. (2006)
	Lowering of ethanol	Immobilised cells, sequential-cultures	NS yeast and/ or interaction	Canonico et al. (2016)
H. guilliermondii	Improvement of aroma complexity	Co-cultures	NS yeast and/ or interaction	Moreira et al. (2008, 2005)
I. orientalis	Reduction of malic acid content	Co-cultures	NS yeast	Kim et al. (2008)
L. thermotolerans	Reduction of acetic acid production	Co- and sequential cultures	NS yeast	Ciani et al. (2006)
	Increased acidity	Co- and sequential cultures	NS yeast	Gobbi et al. (2013)
	Enhancement of titratable acidity	Co- and sequential cultures	NS yeast	Gobbi et al. (2013); Mora et al. (1990)
M. pulcherrima	Lowering of ethanol	Sequential cultures	NS yeast	Contreras et al. (2014)
P. fermentans	Increased and more complex aroma, increased glycerol	Sequential cultures	NS yeast	Clemente-Jimenez et al. (2005)
	Increased polysaccharides	Co-cultures	NS yeast and/ or interaction	Domizio et al. (2011)

Species used with *S. cerevisiae*	**Aim**	**Process**	**Cause**	**References**
P. kluyveri	Increased varietal thiol (3MHA)	Co-cultures	NS and/or interaction	Anfang et al. (2009)
Saccharomycodes ludwigii	Increased polysaccharides	Co-cultures	NS yeast and/ or interaction	Domizio et al. (2011)
Schizosaccharomyces spp. *Saccharomycodes* spp. *Pichia* spp.	Influence on sensorial and physico-chemical properties of wines	Ageing over the lees during wine maturation	NS yeast	Palomero et al. (2009)
Starmarella bacillaris	Increased varietal thiol (3MH)	Co-cultures	NS yeast and/ or interaction	Anfang et al. (2009)
	Reduced acetic acid	Co- and sequential cultures	NS yeast	Rantsiou et al. (2012)
Starmarella bombicola	Improve wine aroma profile	Co- or sequential cultures	NS yeast and/ or interaction	Soden et al. (2000)
Shizosaccharomyces pombe	Malic acid degradation	Immobilised cells (continuous process)	NS yeast	Yokotsuka et al. (1993)
T. delbrueckii	Reduction of acetic acid production	Sequential cultures	NS yeast	Bely et al. (2008); Ciani et al. (2006)
	Reduction of acetaldehyde and VA	Sequential cultures	NS yeast and/ or interaction	Izquierdo Cañas et al. (2011)
	Increased aromatic complexity	Co- and sequential cultures	NS yeast	Azzolini et al. (2012); Loira et al. (2014)
	Increased polysaccharides	Co- cultures	NS yeast and/ or interaction	Comitini et al. (2011)
W. anomalus	Increased aromatic qualities	Sequential cultures	NS yeast and/ or interaction	Izquierdo Cañas et al. (2014, 2011)

Stellenbosch University https://scholar.sun.ac.za

2.8.3 Yeast interactions

Interactions between microorganisms are categorised as competitive, neutralistic and mutualistic (Rayner and Webber, 1984). It has been described that in yeasts, these interactions mainly impact growth and metabolite production (Ciani and Comitini, 2015; Ciani et al., 2010); observed by numerous studies focussing on mixed culture fermentations (Ciani and Comitini, 2015; Ciani et al., 2006; Comitini et al., 2011; Domizio et al., 2007; Gobbi et al., 2013). Additional evidence is seen in a study that found a blend of wines fermented with single cultures of different *S. cerevisiae* strains to not have the same effect as co-culture fermentations with the same strains (Howell et al., 2006). Furthermore, metabolic, chemical and sensory profiles of yeasts in mixed cultures differ from when it is only fermented as monocultures (King et al., 2008; Ciani et al., 2010).

2.8.3.1 Growth interactions

The main growth interactions between yeasts are due to competing for nutrients (oxygen, vitamins, nitrogen) and the toxic effect of certain metabolites (ethanol, killer proteins, short peptides, fatty acids) (Ciani and Comitini, 2015). Studies have reported positive and negative interactions between yeasts regarding nitrogen use and limitation (Ciani and Comitini, 2015; Oro et al., 2014). Non-*Saccharomyces* yeasts utilise nutrients (*i.e.* vitamins, amino acids, and ammonium) in the initial stages of fermentation before *S. cerevisiae* takes over (Medina et al., 2012). Furthermore, the proteolytic activity of these yeasts can add to the nutrients in grape must (Ciani and Comitini, 2015). Indeed, complimentary consumption of amino acids in mixed cultures by different yeasts can cause synergistic relationships between species (Ciani and Comitini, 2015). Furthermore, oxygen limitation, during fermentation, drastically impacted the viable cell counts of, amongst others, *T. delbrueckii* and *K. thermotolerans* (Hansen et al., 2001). Reductive environments can cause competition between sensitive strains such as *K. thermotolerans* and *T. delbrueckii* in the presence of *S. cerevisiae* (Hansen et al., 2001).

As for toxicity, many data sets point to ethanol as a significant factor (Ciani and Comitini, 2015). Most non-*Saccharomyces* yeasts cannot withstand the high ethanol concentrations produced by *S. cerevisiae* (Pretorius, 2000). In addition, medium chain fatty acids produced by yeast inhibit growth, and are especially prevalent in mixed culture fermentations (Bisson, 1999). At higher ethanol concentrations these compounds are more toxic (Viegas et al., 1989). Moreover, the production of antimicrobial cationic peptides by *S. cerevisiae* are additional toxic compounds, affecting certain non-*Saccharomyces* such as *T. delbrueckii*, *K. thermotolerans*, *K. marxianus, D. bruxellensis* and *H. guillermondii* (Albergaria et al., 2010; Branco et al., 2014). Killer toxins are furthermore secreted by different species (Meinhardt and Klassen, 2009; Van Vuuren and Jacobs, 1992; Zagorc et al., 2001) and killer activity of *S. cerevisiae* can reduce the sensitive indigenous species present in musts. In this regard non-*Saccharomyces* yeasts have the competitive advantage as more strains

Stellenbosch University https://scholar.sun.ac.za

(belonging to the *Candida, Hansenula, Pichia* and *Hanseniaspora* genus) secrete proteinaceous compounds that are toxic to other species, whereas *S. cerevisiae* only has killer activity against yeasts of the same species (El-Banna et al., 2011; Fleet and Heard, 1993).

Recently a cell-to-cell contact mechanism has been investigated with regards to *T. delbrueckii* and *L. thermotolerans* in a mixed culture setup with *S. cerevisiae*. It was found that these non-*Saccharomyces* yeasts interact with each other on a physical level – in such a way that mainly the non-*Saccharomyces* yeasts viability decreased (Luyt, 2015; Nissen and Arneborg, 2003; Nissen et al., 2004, 2003). Not all interactions lead to decreased cell growth and synergistic interactions have been observed between yeast species. In a mixed culture fermentation with *H. uvarum* and *S. cerevisiae* the non-*Saccharomyces* yeast had a lower production of biomass, but persisted for longer during fermentation (Mendoza et al., 2007). The co-flocculation of one flocculent (usually non-*Saccharomyces*) and one non-flocculent strain (*S. cerevisiae*) has also been reported (Ciani et al., 2010; Sosa et al., 2008).

2.8.3.2 Metabolite interactions

Metabolic interactions either result in an additive, synergistic or negative effect (Ciani and Comitini, 2015). Additive interactions are defined as a production or reduction in metabolites where the persistence of both strains determine the quantity of the metabolite. When metabolites are exchanged or enhanced it is known as a synergistic effect, compared to a negative effect where metabolites are reduced (Ciani and Comitini, 2015).

In mixed culture fermentations, the redox status of cells can possibly be impacted by the yeasts, enabling the exchange of metabolites (Cheraiti et al., 2005). Metabolic interactions have found to possibly increase higher alcohols and esters while simultaneously decreasing volatile acidity (Ciani et al., 2006; Moreira et al., 2005; Viana et al., 2008). This impact on wine aroma in mixed culture fermentations has frequently been studied (Andorrà et al., 2012; Comitini et al., 2011; Gobbi et al., 2013; Loira et al., 2014; Sadoudi et al., 2012).

Negative interactions, leading to a decrease in undesirable compounds can favourably impact wine quality. For instance, excessive concentrations of acetaldehyde produced by *Starmarella bacillaris* can be metabolised by *S. cerevisiae* (Ciani and Ferraro, 1998). Similarly, volatile acidity was reduced in fermentations with *S. cerevisiae* and *K. thermotolerans* (Ciani et al., 2006), *Starmarella bacillaris* (Rantsiou et al., 2012) and *T. delbrueckii* (Azzolini et al., 2015). Some non-*Saccharomyces* yeast can improve ester production and, at the same time, specifically reduce the production of ethyl acetate (Kurita, 2008; Moreira et al., 2008).

An additive interaction was observed in mixed culture fermentations with *S. cerevisiae* and either *Starmarella bacillaris* or *L. thermotolerans*, where glycerol levels (and for the latter yeast also total acidity) depended on the duration of the viability of the non-*Saccharomyces* yeasts (Comitini et al.,

Stellenbosch University https://scholar.sun.ac.za

2011). In this way ethanol concentration can be reduced when fermenting with a low producing non-*Saccharomyces* strain (Contreras et al., 2014; Gobbi et al., 2014; Quirós et al., 2014).

Other interactions include glucose, fructose, ethyl acetate, esters, isoamyl acetate, volatile compounds (Ciani et al., 2010) and 3-mercaptohexyl acetate (Anfang et al., 2009). Sadoudi et al. (2012) showed a positive aromatic effect with mixed cultures of *M. pulcherrima* and *S. cerevisiae*, in contrast to *Starmarella bacillaris* and *S. cerevisiae* which exhibited a negative interaction. Table 2.3 describes some known interactions and the results thereof. Although mixed culture fermentations can exhibit unique characteristics, the interactions between yeasts are not all yet well understood (Ciani et al., 2010). To optimise favourable interactions resulting in increased aromatic complexity, controlled inoculations are essential and protocols are needed for specific species (Ciani et al., 2006).

Table 2.3 Interactions described in mixed fermentation of wines (adapted from Ciani et al., 2010, 2015)

Species used	Interactions	References
S. cerevisiae *H. uvarum/guillermondii*	Reduced ethyl acetate Increased esters*	Moreira et al. (2008)
S. cerevisiae *H. uvarum*	Persistence of non-*Saccharomyces*	Ciani et al. (2006); Mendoza et al. (2007)
S. cerevisiae *H. uvarum*	Decreased ethanol	Mendoza et al. (2007)
S. cerevisiae *L. thermotolerans*	Increased glycerol content*	Gobbi et al. (2013)
S. cerevisiae *M. pulcherrima*	Increased medium chain fatty acids	Mains (2014)
S. cerevisiae *M. pulcherrima*	Increased aroma profile in mixed culture fermentations	Comitini et al. (2011); Sadoudi et al. (2012)
S. cerevisiae *P. anomala*	Increased isoamyl acetate (EAHase) by *S. cerevisiae*	Kurita (2008)
S. cerevisiae *P. kluyveri*	Increased 3-Mercaptohexyl acetate*	Anfang et al. (2009)
S. cerevisiae *Starmarella bacillaris*	Decreased terpene and lactone concentration	Sadoudi et al. (2012)
S. cerevisiae *Starmarella bacillaris*	Increased glycerol*	Zara et al. (2014)
S. cerevisiae *Starmarella bacillaris*	Reduced acetic acid	Rantsiou et al. (2012)
S. cerevisiae *Starmarella bombicola*	Complementary consumption of acetaldehyde, acetoin, glucose and fructose	Ciani and Ferraro (1998)
S. cerevisiae *Starmarella bombicola*	Modification of ADH1 and PDC1 gene expression in *S. cerevisiae*	Milanovic et al. (2012)

Stellenbosch University https://scholar.sun.ac.za

Species used	Interactions	References
S. cerevisiae *T. delbrueckii*	Increased death rate of non-*Saccharomyces* due to cell-to-cell contact	Nissen and Arneborg (2003); Nissen et al. (2003)
S. cerevisiae *T. delbrueckii*	Reduced acetic acid	Taillandier et al. (2014)
Mixed indigenous yeasts	Increased and more complex aroma (volatile compounds)	Garde-Cerdán and Ancín-Azpilicueta (2006); Varela et al. (2009)

EAHase, ethyl acetate-hydrolysing esterase.
*Possibly due to an additive effect

2.8.4 Inoculation protocol

Inoculation of *S. cerevisiae* in fermentations can be controlled to either suppress non-*Saccharomyces* yeast growth partially or completely by variation in inoculum levels, timing of inoculation, winemaking practices and the specific *S. cerevisiae* strain used (Ciani et al., 2010). Co-inoculation strategies have been thoroughly studied for specific species; however, commercially, yeast strains are inoculated sequentially. In mixed culture fermentations a waiting period of one hour to fifteen days between the inoculation of the non-*Saccharomyces* yeast and *S. cerevisiae* is usually followed, depending on the species and the type of interactions between the yeast (Ferraro et al., 2000; Herraiz et al., 1990; Jolly et al., 2014, 2003b). By delaying the inoculation of *S. cerevisiae* or increasing the ratio of non-*Saccharomyces* to *S. cerevisiae*, the growth of non-*Saccharomyces* yeasts can be promoted (Anfang et al., 2009; Ciani et al., 2010). This allows the non-*Saccharomyces* yeasts to grow and proliferate in the grape must, while some can even survive until the end of fermentation, *e.g. L. thermotolerans*, *I. orientalis* and *Candida* spp. (Mains, 2014; Mills et al., 2002).

Consequently, the establishment of the correct inoculation level for each yeast species is of great importance (Andorrà et al., 2012; Ciani et al., 2006). The inoculation of *Starmarella bombicola* at 10 times the concentration of *S. cerevisiae* still suppressed the growth and metabolism of *Starmarella bombicola,* and no change in the aroma profile was observed compared to the *S. cerevisiae* monoculture (Soden et al., 2000). However, when *S. cerevisiae* was inoculated sequentially (after 15 days), the aroma profile was an intermediate between that of the monocultures of *Starmarella bombicola* and *S. cerevisiae* with a reduction in ethanol concentration (Soden et al., 2000). Other studies have investigated the impact of different waiting periods (inoculation of *S. cerevisiae* after 2, 3, 4, 6 or 8 days) and found that the longer the delay in inoculation of *S. cerevisiae*, the more intense the impact of *P. fermentans* on the aroma profile was (Clemente-Jimenez et al., 2005). The same increasing effect was observed when the non-*Saccharomyces* inoculum was increased. These findings are comparable to a study on co-inoculation of *T. delbrueckii* and *S. cerevisiae* at different inoculation ratios varying from 5:1 to 100:1 (Bely et al., 2008). A decrease in volatile acidity and acetaldehyde was seen with a delay in *S. cerevisiae* inoculation or when increasing the inoculum

Stellenbosch University https://scholar.sun.ac.za

level of non-Saccharomyces yeasts (Bely et al., 2008). Commercial protocols advise inoculation of *S. cerevisiae* after 24-72 hours (for *M. pulcherrima* and *T. delbrueckii*) or according to fermentation progress, after 1.5-3°B or 6-8°B have been used by the non-*Saccharomyces* yeasts (Chr. Hansen, Denmark; Laffort, France; Lallemand, Canada). Similar approaches have been followed to inoculate *S. cerevisiae* after the non-*Saccharomyces* yeast has consumed 50% of the sugar (Contreras et al., 2014) or 15 units (Izquierdo Cañas et al., 2011).

Several different inoculation strategies have been used with varying results, highlighting the importance of finding the correct inoculation timing and density for each non-*Saccharomyces* yeast species during mixed culture fermentations (Fleet and Heard, 1993). The impact of yeast growth will also affect nutrient consumption. This differ between yeast species, although little research has been done on nutrient consumption in mixed culture fermentations (Medina et al., 2012).

2.9 The role of nitrogen

During fermentation nitrogen is secondary only to carbon in its importance as nutrient assimilated by yeast (Henschke and Jiranek, 1993) as it is needed for cell metabolism and protein biosynthesis (Bell and Henschke, 2005). Yeast assimilable nitrogen (YAN), consisting of ammonia, free alpha amino acids and small peptides, is used by yeast during fermentation and the concentrations in grape must varies depending on various viticultural factors. Levels lower than 150 mg/L can result in poor yeast growth and stuck fermentations (Pretorius, 2000).

Nitrogen compounds are not equally preferred by wine yeast and subsequently ammonia will be utilised first, followed by the amino acids according to the yeast's requirements for biosynthesis and the total nitrogen available in the grape must (Salmon and Barre, 1998). Recent studies have begun investigating nitrogen use by non-*Saccharomyces* yeasts, as most earlier research has been conducted on *S. cerevisiae* (Llungdahl and Daignan-Fornier, 2012). Mendoza et al., (2007) found that in mixed culture fermentations with *S. cerevisiae* and *H. uvarum* less assimilable nitrogen compounds were consumed compared to fermentations with only *S. cerevisiae*. In single culture fermentations with *H. uvarum* even less nitrogen was consumed. Furthermore it has been found that indigenous *Saccharomyces* yeast is slow to take up amino acids compared to commercial *S. cerevisiae* strains (Barrajón-Simancas et al., 2011). Additional knowledge on utilisation of ammonia and amino acids by non-*Saccharomyces* yeasts and yeast in mixed culture fermentations is still needed (Medina et al., 2012).

Furthermore, the relationship between addition of nitrogen to grape juice or must and formation of volatile compounds has been studied in recent years (Mckinnon, 2013; Smit, 2013; Ugliano et al., 2007). Branched chain and aromatic amino acids (BCAA's), consisting of valine, leucine, isoleucine and tryptophan, tyrosine, phenylalanine, are precursors for aromatic compounds and have been shown to increase higher alcohols (Dickinson et al., 2000, 1998, 1997; Smit, 2013). In addition, strain

differences between *S. cerevisiae* yeasts have been found regarding nitrogen utilisation (Carrau et al., 2008; Vilanova et al., 2007). In general, high nitrogen demanding strains synthesised less higher alcohols and more esters (Barrajón-Simancas et al., 2011). However, the use of BCAA's and its influence on aroma compounds is unknown for non-*Saccharomyces* yeasts.

2.10 Commercialisation of non-*Saccharomyces* yeasts

In view of these findings, several non-*Saccharomyces* yeasts have been commercialised in the past decade. *Torulaspora delbrueckii* was the first non-*Saccharomyces* yeast to be produced industrially and today different strains of this species are available to inoculate grape must (Azzolini et al., 2015). In commercialisation of yeast, parameters are measured to establish guidelines for optimal fermentation and yeast viability (Ciani et al., 2010; Mendoza et al., 2007). These parameters include sensitivity to SO_2, temperature fluctuations and nutrient requirements amongst others. Currently all commercial non-*Saccharomyces* yeasts are used in conjunction with *S. cerevisiae* to ensure complete fermentations.

Non-*Saccharomyces* yeasts are all commercialised for their improvement of aroma complexity and many promise a smooth and rounder mouthfeel. *Starmerella bombicola* is produced for the enhanced production of glycerol (Ciani and Ferraro, 1998; Comitini et al., 2011) and *Shizosaccharomyces pombe* to reduce malic acid (ProMalic®, Lallemand, USA) (Ciani et al., 2010). *Torulaspora* delbrueckii (Prelude™, Chr. Hansen, Denmark; Zymaflore® Alpha^{TD}, Laffort, France; Biodiva™ TD291, Lallemand, Canada) and *Lachancea thermotolerans* (Viniflora® Concerto™, Chr. Hansen, Denmark) is commercialised and promoted for lowering acetate levels, increasing higher alcohols, with a general improvement of aroma. *Metschnikowia pulcherrima* (Flavia™ Mp346, Lallemand, Canada) is shown to increase medium chain fatty acids and lower alcohol, acetate and glycerol levels. *Pichia kluyveri* (Frootzen™, Chr. Hansen, Denmark) reduces medium chain fatty acids and increases esters and acetates when used in combination with *S. cerevisiae*. In addition, a multi-yeast starter culture has been developed consisting of *L. thermotolerans, T. delbrueckii* and *S. cerevisiae* (Melody™, Chr. Hansen, Denmark) for optimal fermentation to produce high end Chardonnay. In South Africa yeasts other than *S. cerevisiae* have been commercialised for instance the hybrid between *S. cerevisiae* and *S. paradoxus* (Exotics, Anchor Yeast, South Africa) and a co-inoculant of *T. delbrueckii* and *S. cerevisiae* (Level^{2}TD™, Lallemand, South Africa).

Starter cultures ensure reliable and fast fermentations with a more consistent end product, enabling the use of the same yeast in consecutive vintages (Fleet and Heard, 1993; Sadoudi et al., 2012). Although fermentation is more active, dry wine yeast (ADWY) generalises the use of these yeasts globally and simplifies the microbial communities that produces a more predicted, standardised aromatic profile (Ciani et al., 2010). Using different strains of *S. cerevisiae* and non-*Saccharomyces*

Stellenbosch University https://scholar.sun.ac.za

yeasts solves this problem to a degree and further research is currently conducted on a consortium approach, using multiple non-*Saccharomyces* species.

2.11 Conclusion

Past studies have explored the microflora of vineyards and grape musts globally and in South Africa, and shown that yeast population structures and dynamics are diverse and frequently changing (Jolly et al., 2003b; Setati et al., 2012; Van Zyl and Du Plessis, 1961). The data also suggest that much of the yeast biodiversity in the wine ecosystem has not yet been properly investigated or exploited, offering seemingly endless possibilities for further investigation. The wine industry has recently started to realise this hidden potential, and a shift towards usage of non-*Saccharomyces* yeasts to produce aromatically unique and complex wines has been one of the major oenological developments in the past decade. Considering the limit of currently available data, it remains paramount to further investigate yeast ecosystems and the interaction of non-*Saccharomyces* species with *S. cerevisiae* and each other to better understand and control their contribution to alcoholic fermentation (Fleet, 2008).

Furthermore, mixed culture fermentations with the deliberate inoculation of non-*Saccharomyces* yeasts and *S. cerevisiae* can possibly improve the uniqueness of wines by altering the chemical and sensory matrix of the wine, moving away from seemingly monotone wines fermented with traditional *S. cerevisiae* starter cultures (Pretorius, 2000). Such strategies will ensure the presence of non-*Saccharomyces* yeasts and improve their impact on wine (Bagheri, 2014), resembling a spontaneous fermentation without the associated risks (Ciani et al., 2006; Jolly et al., 2006; Rojas et al., 2001; Romano et al., 2003). However there is still a need to further characterise individual non-*Saccharomyces* – *S. cerevisiae* combinations and many more steps need to be taken to enable winemakers to make informed decisions.

2.12 References

Albergaria, H., Francisco, D., Gori, K., Arneborg, N., Gírio, F., 2010. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. Appl. Microbiol. Biotechnol. 86, 965–972. doi:10.1007/s00253-009-2409-6

Albertin, W., Setati, M.E., Miot-Sertier, C., Mostert, T.T., Colonna-Ceccaldi, B., Coulon, J., Girard, P., Moine, V., Pillet, M., Salin, F., Bely, M., Divol, B., Masneuf-Pomarede, I., 2016. *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. Front. Microbiol. 6, doi: 10.3389/fmicb.2015.01569.

Amerine, M.A., 1966. The Search for Good Wine. Science. 154, 1621–1628.

Stellenbosch University https://scholar.sun.ac.za

Andorrà, I., Berradre, M., Mas, A., Esteve-Zarzoso, B., Guillamón, J.M., 2012. Effect of mixed culture fermentations on yeast populations and aroma profile. LWT - Food Sci. Technol. 49, 8–13.

Anfang, N., Brajkovich, M., Goddard, M.R., 2009. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon blanc. Aust. J. Grape Wine Res. 15, doi:10.1111/j.1755-0238.2008.00031.x

Arroyo-López, F.N., Salvadó, Z., Tronchoni, J., Guillamón, J.M., Barrio, E., Quero, A., 2010. Susceptability and resistance to ethanol in *Saccharomyces* strains isolated from wild and fermentative environments. Yeast 27, 1005–1015.

Azzolini, M., Fedrizzi, B., Tosi, E., Finato, F., Vagnoli, P., Scrinzi, C., Zapparoli, G., 2012. Effects of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* mixed cultures on fermentation and aroma of Amarone wine. Eur. Food Res. Technol. 235, 303–313.

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., Zapparoli, G., 2015. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 31, 277–293.

Bagheri, B., 2014. Comparative analysis of fermentative yeasts during spontaneous fermentation of grapes from different management systems. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2011. The microbial ecology of wine grape berries. Int. J. Food Microbiol. 153, 243–259.

Barrajón-Simancas, N., Giese, E., Arévalo-Villena, M., Úbeda, J., Briones, A., 2011. Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. Food Chem. 127, 441–446.

Beckner Whitener, M.E., 2016. Metabolomic profiling of non-*Saccharomyces* yeasts in wine. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Beckner Whitener, M.E., Carlin, S., Jacobson, D., Weighill, D., Divol, B., Conterno, L., Du Toit, M., Vrhovsek, U., 2015. Early fermentation volatile metabolite profile of non-*Saccharomyces* yeasts in red and white grape must: A targeted approach. LWT - Food Sci. Technol. 64, 412–422.

Bell, S.J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242–295.

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

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii*-*Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122, 312–320.

Benítez, T., Codón, A.C., 2002. Genetic diversity of yeasts in wine production, in: Khachatourians, G.G., Arora, D.K. (Eds.), Applied Mycology and Biotechnology. Elsevier, pp. 19–44.

Stellenbosch University https://scholar.sun.ac.za

Benito, S., Morata, A., Palomero, F., González, M.C., Suárez-Lepe, J.A., 2011. Formation of vinylphenolic pyranoanthocyanins by *Saccharomyces cerevisiae* and *Pichia guillermondii* in red wines produced following different fermentation strategies. Food Chem. 124, 15–23.

Benito, S., Palomero, F., Calderón, F., Palmero, D., Suárez-Lepe, J.A., 2014. Selection of appropriate *Schizosaccharomyces* strains for winemaking. Food Microbiol. 42, 218–224.

Benito, S., Palomero, F., Morata, A., Calderón, F., Palmero, D., Suárez-Lepe, J.A., 2013. Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. Eur. Food Res. Technol. 236, 29–36.

Bisson, L.F., 1999. Stuck and sluggish fermentations. Am. J. Enol. Vitic. 50, 107–119.

Bisson, L.F., Joseph, C.M.L., 2009. Yeasts, in: Koning, H., Unden, G., Frohlich, J. (Eds.), Biology of Microorganisms on Grapes, in Must and in Wine. Springer, Germany, pp. 47–60.

Bokulich, N.A., Bamforth, C.W., Mills, D.A., 2012. A review of molecular methods for microbial community profiling of beer and wine. Am. Soc. Brew. Chem. 70, 150–162.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2013. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. U. S. A. 111, 139–148.

Boulton, R.B., Singleton, V.L., Bisson, L.F., Kunkee, R.E., 1996. Principles and practices of winemaking, 1st ed. Springer US, New York.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M.G., Caldeira, J., Albergaria, H., 2014. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl. Microbiol. Biotechnol. 98, 843–853.

Camarasa, C., Sanchez, I., Brial, P., Bigey, F., Dequin, S., 2011. Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: Evidence for origin-dependent metabolic traits. PLoS One 6, e25147.

Canonico, L., Comitini, F., Oro, L., Ciani, M., 2016. Sequential fermentation with selected immobilized non-*Saccharomyces* yeast for reduction of ethanol content in wine. Front. Microbiol. 7, doi:10.3389/fmicb.2016.00278

Capece, A., Salzano, G., Romano, P., 2003. Molecular typing techniques as a tool to differentiate non-*Saccharomyces* wine species. Int. J. Food Microbiol. 84, 33–39.

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. FEMS Yeast Res. 8, 1196–1207.

Chambers, P.J., Pretorius, I.S., 2010. Fermenting knowledge: the history of winemaking, science and yeast research. Eur. Mol. Biol. Organ. EMBO reports 11, 914–920.

Charoenchai, C., Fleet, G.H., Henschke, P.A., Todd, B.E.N., 1997. Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. Aust. J. Grape Wine Res. 3, 2–8.

Cheraiti, N., Guezenec, S., Salmon, J.-M., 2005. Redox interactions between *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in mixed culture under enological conditions. Appl. Environ. Microbiol. 71, 255–260.

Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. 108, 239–245. doi:10.1016/j.ijfoodmicro.2005.11.012

Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation. Curr. Opin. Food Sci. 1, 1–6.

Ciani, M., Comitini, F., 2011. Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. Ann. Microbiol. 61, 25–32.

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

Ciani, M., Ferraro, L., 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. J. Appl. Microbiol. 85, 247–254.

Ciani, M., Ferraro, L., 1996. Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. Appl. Environ. Microbiol. 62, 128–132.

Ciani, M., Maccarelli, F., 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World J. Microbiol. Biotechnol. 14, 199–204.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J., Rodríguez-Vico, F., 2005. Influence of sequential yeast mixtures on wine fermentation. Int. J. Food Microbiol. 98, 301–308.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J., Rodríguez-Vico, F., 2004. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. Food Microbiol. 21, 149–155.

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

Comitini, F., De, J.I., Pepe, L., Mannazzu, I., Ciani, M., 2004. *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/ Brettanomyces* spoilage yeasts. FEMS Mircobiology Lett. 238, 235–240.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. Food Microbiol. 28, 873–882.

Contreras, A., Curtin, C., Varela, C., 2015. Yeast population dynamics reveal a potential “collaboration” between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. Appl. Microbiol. Biotechnol. 99, 1885–1895.

Stellenbosch University https://scholar.sun.ac.za

Contreras, A., Hidalgo, C., Henschke, P.A., Chambers, P.J., Curtin, C., Varela, C., 2014. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. Appl. Environ. Microbiol. 80, 1670–1678.

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M.D., Valero, E., 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. Int. J. Food Microbiol. 145, 132–139.

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

Deák, T., 1993. Simplified techniques for identifying foodborne yeasts. Int. J. Food Microbiol. 19, 15–26.

Dias, L., Dias, S., Sancho, T., Stender, H., Querol, A., Malfeito-Ferreira, M., Loureiro, V., 2003. Identification of yeasts isolated from wine-related environments and capable of producing 4-ethylphenol. Food Microbiol. 20, 567–574.

Díaz, C., Molina, A.M., Nähring, J., Fischer, R., 2013. Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. Biomed Res. Int. Article ID 540465

Dickinson, J.R., Harrison, S.J., Dickinson, J.A., Hewlins, M.J.E., 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 275, 10937–10942.

Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 273, 25751–25756.

Dickinson, J.R., Lanterman, M.M., Danner, D.J., Pearson, B.M., Sanz, P., Harrison, S.J., Hewlins, M.J., 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in Saccharomyces cerevisiae. J. Biol. Chem. 272, 26871–26878.

Divol, B., Setati, M., 2015. Secretion of hydrolytic enzymes by non-*Saccharomyces* yeasts – a relevant trait for winemaking? Wineland 78–80.

Domizio, P., Lencioni, L., Ciani, M., Di Blasi, S., Pontremolesi, C., Sabatelli, M.P., 2007. Spontaneous and inoculated yeast populations dynamics and their effect on organoleptic characters of Vinsanto wine under different process conditions. Int. J. Food Microbiol. 115, 281–289.

Domizio, P., Romani, C., Comitini, F., Gobbi, M., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Ann. Microbiol. 61, 137–144.

Egli, C.M., Edinger, W.D., Mitrakul, C.M., Henick-Kling, T., 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. J. Appl. Microbiol. 85, 779–789.

El-Banna, A.A., El-Sahn, M.A., Shehata, M.G., 2011. Yeasts producing killer toxins: An overview. Alex. J. Fd. Sci. Technol 8, 41–53.

Stellenbosch University https://scholar.sun.ac.za

Erten, H., Tanguler, H., Cabaroglu, T., Canbas, A., 2006. The influence of inoculum level on fermentation and flavour compounds of white wines made from cv. Emir. J. Inst. Brew. 112, 232–236.

Fernández, M., Úbeda, J.F., Briones, A.I., 2000. Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. Int. J. Food Microbiol. 59, 29–36.

Ferraro, L., Fatichenti, F., Ciani, M., 2000. Pilot scale vinification process using immobilized *Candida stellata* cells and *Saccharomyces cerevisiae*. Process Biochem. 35, 1125–1129.

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

Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11–22.

Fleet, G.H., Heard, G.M., 1993. Yeasts – growth during fermentation, in: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. pp. 27–46.

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

Garcia, A., Carcel, C., Dulau, L., Samson, A., Aguera, E., Agosin, E., Gunata, Z., 2002. Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles of a Muscat wine. J. Food Sci. 67, 1138–1143.

García-Ríos, E., Gutiérrez, A., Salvadó, Z., Arroyo-López, F.N., Guillamon, J.M., 2014. The fitness advantage of commercial wine yeasts in relation to the nitrogen concentration, temperature, and ethanol content under microvinification conditions. Appl. Environ. Microbiol. 80, 704–713.

Garde-Cerdán, T., Ancín-Azpilicueta, C., 2006. Contribution of wild yeasts to the formation of volatile compounds in inoculated wine fermentations. Eur. Food Res. Technol. 222, 15–25.

Ghosh, S., Bagheri, B., Morgan, H.H., Divol, B., Setati, M.E., 2015. Assessment of wine microbial diversity using ARISA and cultivation-based methods. Ann. Microbiol. 65, 1833–1840.

Gil, J. V, Mateo, J.J., Jiménez, M., Pastor, A., Huerta, T., 1996. Aroma compounds in wine as influenced by apiculate yeasts. J. Food Sci. 61, 1247–1250.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. Food Microbiol. 33, 271–281.

Gobbi, M., De Vero, L., Solieri, L., Comitini, F., Oro, L., Giudici, P., Ciani, M., 2014. Fermentative aptitude of non-*Saccharomyces* wine yeast for reduction in the ethanol content in wine. Eur. Food Res. Technol. 239, 41–48.

Goddard, M.R., 2008. Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. Ecology 89, 2077–2082.

Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J., Querol, a, 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch. Microbiol. 169, 387–392.

Stellenbosch University https://scholar.sun.ac.za

Hagman, A., Säll, T., Compagno, C., Piskur, J., 2013. Yeast “make-accumulate-consume” life strategy evolved as a multi-step process that predates the whole genome duplication. PLoS One 8, e68734.

Hansen, H., Nissen, P., Sommer, P., Nielsen, J.C., Arneborg, N., 2001. The effect of oxygen on the survival of non-Saccharomyces yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. J. Appl. Microbiol. 91, 541–7.

Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microbiol. 50, 727–728.

Henick-Kling, T., Edinger, W., Daniel, P., Monk, P., 1998. Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J. Appl. Microbiol. 84, 865–876.

Henschke, P.A., Jiranek, V., 1993. Yeasts-metabolism of nitrogen compounds, in: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Howard Academic Publishers, pp. 77–164.

Herjavec, S., Podgorski, V., Redžepović, S., Mirošević, N., 2003. The influence of some commercial *Saccharomyces cerevisiae* strains on the quality of Chardonnay wines. Food Technol. Biotechnol. 41, 77–81.

Herraiz, T., Reglero, G., Herraiz, M., Martin-Alvarez, P.J., Cabezudo, M.D., 1990. The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. Am. J. Enol. Vitic. 41, 313–318.

Hesseltine, C.W., 1992. Mixed-culture fermentations. National Academies Press (US), Washington (DC).

Howell, K.S., Cozzolino, D., Bartowsky, E.J., Fleet, G.H., Henschke, P.A., 2006. Metabolic profiling as a tool for revealing *Saccharomyces* interactions during wine fermentation. FEMS Yeast Res. 6, 91–101.

Ivey, M.L., Phister, T.G., 2011. Detection and identification of microorganisms in wine: A review of molecular techniques. J. Ind. Microbiol. Biotechnol. 38, 1619–1634.

Izquierdo Cañas, P.M., Esteban, C., Romero, G., Heras, J.M., Mónica, M., González, F., 2014. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. Eur. Food Res. Technol. 239, 279–286.

Izquierdo Cañas, P.M., Palacios Garcia, A.T., Garcia Romero, E., 2011. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and Saccharomyces yeast starter. Vitis 50, 177–182.

Jackson, R.S., 2008. Fermentation - Biochemistry of alcoholic fermentation, in: Wine Science Principles and Applications. Elsevier, London, pp. 358–372.

Johnson, E.A., Echavarri-Erasun, C., 2011. Yeast biotechnology, in: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), The Yeasts, A Taxonomic Study. Elsevier B.V., Amsterdam, pp. 21–44.

Jolly, N.P., 2004. Characterisation, evaluation and use of non-*Saccharomyces* yeast strains isolated from vineyards and must. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Jolly, N.P., Augustyn, O.H.P., Pretorius, I.S., 2003a. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55–62.

Jolly, N.P., Augustyn, O.H.P.H., Pretorius, I.S., 2003b. The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S. Afr. J. Enol. Vitic. 24, 63–69.

Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2006. The role and use of non-*Saccharomyces* yeasts in wine production. S. Afr. J. Enol. Vitic. 27, 15–39.

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

Kapsopoulou, K., Kapaklis, A., Spyropoulos, H., 2005. Growth and fermentation characteristics of a strain of the wine yeast *Kluyveromyces thermotolerans* isolated in Greece. World J. Microbiol. Biotechnol. 21, 1599–1602.

Kapsopoulou, K., Mourtzini, A., Anthoulas, M., Nerantzis, E., 2007. Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 23, 735–739.

Khan, W., Augustyn, O.P.H., Westhuizen, T.J. Van Der, Lambrechts, M.G., Pretorius, I.S., 2000. Geographic distribution and evaluation of *Saccharomyces cerevisiae* strains isolated from vineyards in the warmer, inland regions of the Western Cape in South Africa. S. Afr. J. Enol. Vitic. 21, 17–31.

Kim, D.H., Hong, Y.A., Park, H.D., 2008. Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic acid content in wine. Biotechnol. Lett. 30, 1633–1638.

Knight, S., Goddard, M.R., 2015. Quantifying separation and similarity in a *Saccharomyces cerevisiae* metapopulation. ISME J. 9, 361–70.

Knight, S., Klaere, S., Fedrizzi, B., Goddard, M.R., 2015. Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. Nature, Sci. Reports 5.

Kurita, O., 2008. Increase of acetate ester-hydrolysing esterase activity in mixed cultures of *Saccharomyces cerevisiae* and *Pichia anomala*. J. Appl. Microbiol. 104, 1051–1058.

Kurtzman, C.P., 2011. *Wickerhamomyces*; Kurtzman, Robnett & Basehoar-Powers (2008), in: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), The Yeasts, A Taxonomic Study. Elsevier B.V., London, UK, pp. 902–903.

Kurtzman, C.P., Fell, J.W., Boekhout, T., 2011. The Yeasts, A Taxonomic Study, Fifth. ed. Elsevier Science Pulishers, Amsterdam.

Kvitek, D.J., Will, J.L., Gasch, A.P., 2008. Variations in stress sensitivity and genomic expression in diverse *S. cerevisiae* isolates. PLoS Genet. 4, e1000223.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its importance to wine aroma - A review. S. Afr. J. Enol. Vitic. 21, 97–129.

Lema, C., Garcia-Jares, C., Orriols, I., Angulo, L., 1996. Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albarino wine aroma. Am. J. Enol. Vitic. 47, 206–216.

Lilly, M., Lambrechts, M.G., Pretorius, I.S., 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. Appl. Environ. Microbiol. 66, 744–753.

Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., Davey, R.P., Roberts, I.N., Burt, A., Tsai, I.J., Bergman, C.M., Bensasson, D., Michael, J.T., Kelly, O., van Oudenaarden, A., Barton, D.B.H., Bailes, E., Ba, A.N.N., Jones, M., Quail, M.A., Goodhead, I., Sims, S., Blomberg, A., Durbin, R., Louis, E.J., 2009. Population genomics of domestic and wild yeasts. Eur. PMC Funders Gr. 458, 337–341.

Llungdahl, P.O., Daignan-Fornier, B., 2012. Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. Genetics 190, 885–929.

Lodder, J., Kreger-van Rij, N., 1952. The yeasts-a taxonomic study. North-Holland Publishing Company, Amsterdam.

Loira, I., Vejarano, R., Bañuelos, M.A., Morata, A., Tesfaye, W., Uthurry, C., Villa, A., Cintora, I., Suárez-Lepe, J.A., 2014. Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. LWT - Food Sci. Technol. 59, 915–922.

Loureiro, V., Malfeito-Ferreira, M., 2003. Spoilage yeasts in the wine industry. Int. J. Food Microbiol. 86, 23–50.

Lu, H.Z., Cai, Y., Wu, Z.W., Jia, J.H., Bai, F.Y., 2004. *Kazachstania aerobia* sp. nov., an ascomycetous yeast species from aerobically deteriorating corn silage. Int. J. Syst. Evol. Microbiol. 54, 2431–2435.

Lubbers, M.W., Rodriguez, S.B., Honey, N.K., Thornton, R.J., 1996. Purification and characterization of urease from *Schizosaccharomyces pombe*. Can. J. Microbiol. 42, 132–140.

Ludovico, P., Sousa, M.J., Silva, M.T., Leão, C., Côrte-Real, M., 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology 147, 2409–2415.

Luyt, N.A., 2015. Interaction of multiple yeast species during fermentation. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Magalhães, K.T., de Pereira, G.V.M., Dias, D.R., Schwan, R.F., 2010. Microbial communities and chemical changes during fermentation of sugary Brazilian kefir. World J. Microbiol. Biotechnol. 26, 1241–1250.

Mains, A.O., 2014. Evaluating the impact of yeast co-inoculation on individual yeast metabolism and wine composition. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Manzanares, P., Ramón, D., Querol, A., 1999. Screening of non-*Saccharomyces* yeasts for the production of ß-D-xylosidase activity. Int. J. Food Microbiol. 46, 105–112. X

Manzanares, P., Vallés, S., Viana, F., 2011. Non-*Saccharomyces* yeasts in the winemaking process, in: Carrascosa, A. V., Munoz, R., Gonzalez, R. (Eds.), Molecular Wine Microbiology. Elsevier Inc., London, pp. 85–110.

Maturano, Y.P., Assof, M., Fabani, M.P., Nally, M.C., Jofré, V., Rodríguez Assaf, L.A., Toro, M.E., Castellanos de Figueroa, L.I., Vazquez, F., 2015. Enzymatic activities produced by mixed *Saccharomyces* and non-*Saccharomyces* cultures: relationship with wine volatile composition. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 108, 1239–1256.

Maturano, Y.P., Rodríguez Assaf, L.A., Toro, M.E., Nally, M.C., Vallejo, M., Castellanos de Figueroa, L.I., Combina, M., Vazquez, F., 2012. Multi-enzyme production by pure and mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts during wine fermentation. Int. J. Food Microbiol. 155, 43–50.

Mckinnon, A., 2013. The impact of amino acids on growth performance and major volatile compound formation by industrial wine yeast. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Medina, K., Boido, E., Dellacassa, E., Carrau, F., 2012. Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. Int. J. Food Microbiol. 157, 245–250.

Meinhardt, F., Klassen, R., 2009. Yeast killer toxins: Fundamentals and applications, in: Anke, T., Weber, D. (Eds.), Physiology and Genetics, The Mycota. Springer, Heidelberg, Germany, pp. 107– 130.

Mendes Ferreira, A., Clímaco, M.C., Mendes Faia, A., 2001. The role of non-*Saccharomyces* species in releasing glycosidic bound fraction of grape aroma components - A preliminary study. J. Appl. Microbiol. 91, 67–71.

Mendes, I., Franco-Duarte, R., Umek, L., Fonseca, E., Drumonde-Neves, J., Dequin, S., Zupan, B., Schuller, D., 2013. Computational models for prediction of yeast strain potential for winemaking from phenotypic profiles. PLoS One 8, e66523.

Mendoza, L.M., De Nadra, M.C.M., Farías, M.E., 2007. Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. Biotechnol. Lett. 29, 1057–1063.

Miguel, M.G.D.C.P., Cardoso, P.G., Magalhães, K.T., Schwan, R.F., 2011. Profile of microbial communities present in tibico (sugary kefir) grains from different Brazilian States. World J. Microbiol. Biotechnol. 27, 1875–1884.

Milanovic, V., Ciani, M., Oro, L., Comitini, F., 2012. *Starmerella bombicola* influences the metabolism of *Saccharomyces cerevisiae* at pyruvate decarboxylase and alcohol dehydrogenase level during mixed wine fermentation. Microb. Cell Fact. 11, 1-18.

Mills, D.A., Johannsen, E.A., Cocolin, L., 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Appl. Environ. Microbiol. 68, 4884–4893.

Mora, J., Barbas, J., Mulet, A., 1990. Growth of yeast species during the fermentation of musts inoculated with *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. 41, 156–159.

Mora, J., Mulet, A., 1991. Effects of some treatments of grape juice on the population of growth of yeast species during fermentation. Am. J. Enol. Vitic. 42, 133–136.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., Vasconcelos, I., 2008. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. Int. J. Food Microbiol. 124, 231–238.

Moreira, N., Mendes, F., Hogg, T., Vasconcelos, I., 2005. Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. Int. J. Food Microbiol. 103, 285–294.

Moreno-Arribas, V.M., Polo, C.M., 2005. Winemaking biochemistry and microbiology: current knowledge and future trends. Crit. Rev. Food Sci. Nutr. 45, 265–286.

Nissen, P., Arneborg, N., 2003. Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Arch. Microbiol. 180, 257–263.

Nissen, P., Neilsen, D., Arneborg, N., 2004. The relative glucose uptake abilities of non-*Saccharomyces* yeasts play a role in their coexistence with *Saccharomyces cerevisiae* in mixed cultures. Appl. Microbiol. Biotechnol. 64, 543–550.

Nissen, P., Nielsen, D., Arneborg, N., 2003. Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell – cell contact-mediated mechanism. Yeast 20, 331–341.

Nykänen, L., 1986. Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. Am. J. Enol. Vitic. 37, 84–96.

Olstorpe, M., Schnürer, J., Passoth, V., 2010. Microbial changes during storage of moist crimped cereal barley grain under Swedish farm conditions. Anim. Feed Sci. Technol. 156, 37–46.

Oro, L., Ciani, M., Comitini, F., 2014. Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. J. Appl. Microbiol. 116, 1209–1217.

Padilla, B., Gil, J. V., Manzanares, P., 2016. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. Front. Microbiol. 7, doi: 10.3389/fmicb.2016.00411.

Palomero, F., Morata, A., Benito, S., Calderón, F., Suárez-Lepe, J.A., 2009. New genera of yeasts for over-lees aging of red wine. Food Chem. 112, 432–441.

Pérez, G., Fariña, L., Barquet, M., Boido, E., Gaggero, C., Dellacassa, E., Carrau, F., 2011. A quick screening method to identify β-glucosidase activity in native wine yeast strains: Application of Esculin Glycerol Agar (EGA) medium. World J. Microbiol. Biotechnol. 27, 47–55.

Pérez-Nevado, F., Albergaria, H., Hogg, T., Girio, F., 2006. Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 108, 336–345.

Plata, C., Millán, C., Mauricio, J.C., Ortega, J.M., 2003. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. Food Microbiol. 20, 217–224.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

Stellenbosch University https://scholar.sun.ac.za

Pretorius, S., Westhuizen, T.J. Van Der, Augustyn, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. A review. S. Afr. J. Enol. Vitic. 20, 61–70.

Quesada, M., Cenis, J., 1995. Use of random amplified polymorphic DNA (RAPD-PCR) in the characterization of wine yeasts. Am. J. Enol. Vitic. 46, 204–208.

Quirós, M., Rojas, V., Gonzalez, R., Morales, P., 2014. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. Int. J. Food Microbiol. 181, 85–91.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolina, L., 2012. *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. Appl. Environ. Microbiol. 78, 1987–1994.

Rayner, A.D.M., Webber, J.F., 1984. Interspecific mycelial interactions: An overview, in: Jennings, D.H., Rayner, A.D.M. (Eds.), The Ecology and Physiology of the Fungal Mycelium. Cambridge University Press, Cambridge, United Kingdom, p. 388.

Regueiro, L.A., Costas, C.L., Rubio, J.E.L., 1993. Influence of viticultural and enological practices on the development of yeast populations during winemaking. Am. J. Enol. Vitic. 44, 405–408.

Renault, P., Miot-Sertier, C., Marullo, P., Hernández-Orte, P., Lagarrigue, L., Lonvaud-Funel, A., Bely, M., 2009. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: Potential applications in the wine industry. Int. J. Food Microbiol. 134, 201–210.

Renouf, V., Claisse, O., Lonvaud-Funel, A., 2007. Inventory and monitoring of wine microbial consortia. Appl. Microbiol. Biotechnol. 75, 149–164.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2006. The microbiology of wine and vinifications. Handbook of Enology, volume 1, 2nd ed. John Wiley & Sons, Ltd, West Sussex, England.

Rojas, V., Gil, J. V., Piaga, F., Manzanares, P., 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int. J. Food Microbiol. 70, 283–289.

Rojas, V., Gil, J. V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int. J. Food Microbiol. 86, 181–188.

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A., 2003. Function of yeast species and strains in wine flavour. Int. J. Food Microbiol. 86, 169–180.

Romano, P., Suzzi, G., Comi, G., Zironi, R., 1992. Higher alcohol and acetic acid production by apiculate wine yeasts. J. Appl. Bacteriol. 73, 126–130.

Romano, P., Suzzi, G., Domizio, P., Fatichenti, F., 1997. Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. Antonie Van Leeuwenhoek 71, 239–242.

Rooyen, T. Van, Tracey, R.P., 1987. Biological deacidification of musts induced by yeasts or malolactic bacteria and the effect on wine quality. S. Afr. J. Enol. Vitic. 8, 60–69.

Rossouw, D., Bauer, F.F., 2016. Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. Food Microbiol. 55, 32–46.

Stellenbosch University https://scholar.sun.ac.za

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.-J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H., Alexandre, H., 2012. Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol. 32, 243–253.

Salmon, J.M., Barre, P., 1998. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial *Saccharomyces cerevisiae* strain. Appl. Environ. Microbiol. 64, 3831–3837.

Salvadó, Z., Arroyo-López, F.N., Guillamón, J.M., Salazar, G., Quero, A., Barrio, E., 2011. Temperature adaptation markedly determines evolution within the genus *Saccharomyces*. Appl. Environ. Microbiol. 77, 2292–2302.

Seo, S.-H., Rhee, C.-H., Park, H.-D., 2007. Degradation of malic acid by Issatchenkia orientalis KMBL 5774, an acidophilic yeast strain isolated from Korean grape wine pomace. J. Microbiol. 45, 521–527.

Setati, M.E., Jacobson, D., Andong, U., Bauer, F., 2012. The vineyard yeast microbiome, a mixed model microbial map. PLoS One 7, e52609.

Smit, A.Y., 2013. The impact of nutrients on aroma and flavour production during wine fermentation. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Soden, A., Francis, I.L., Oakey, H., Henschke, P.A., 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape Wine Res. 6, 21–30.

Sosa, O.A., Manca de Nadra, M.C., Farías, M.E., 2008. Modifcation by glucose of the flocculent phenotype of a *Kloeckera apiculata* wine strain. J. Ind. Microbiol. Biotechnol. 35, 851–857.

Strauss, M.L.A., Jolly, N.P., Lambrechts, M.G., van Rensburg, P., 2001. Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. J. Appl. Microbiol. 91, 182–190.

Styger, G., Prior, B., Bauer, F.F., 2011. Wine flavor and aroma. J. Ind. Microbiol. Biotechnol. 38, 1145–1159.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139–173.

Swiegers, J.H., Pretorius, I.S., 2005. Yeast modulation of wine flavor. Adv. Appl. Microbiol. 57, 131–175.

Taillandier, P., Lai, Q.P., Julien-Ortiz, A., Brandam, C., 2014. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. World J. Microbiol. Biotechnol. 30, 1959–1967.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. Food Microbiol. 29, 18–26.

Toro, M.E., Vazquez, F., 2002. Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. World J. Microbiol. Biotechnol. 18, 347–354. Ugliano, M., Henschke, P.A., Herderich, M.J., Pretorius, I.S., 2007. Nitrogen management is critical for wine flavour and style. Wine Ind. J. 22, 24–30.

Stellenbosch University https://scholar.sun.ac.za

Van der Westhuizen, T.J., Augustyn, O.P.H., Pretorius, I.S., 2000. Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the Western Cape in South Africa. S. Afr. J. Enol. Vitic. 21, 3–9.

Van Rensburg, P., Pretorius, I.S., 2000. Enzymes in winemaking: Harnessing natural catalysts for effiecent biotransformations - A review. S. Afr. J. Enol. Vitic. 21, 52–73.

Van Vuuren, H.J.J., Jacobs, C.J., 1992. Killer yeasts in the wine industry: A review. Am. J. Enol. Vitic. 43, 119–128.

Van Zyl, J.A., Du Plessis, L. de W., 1961. The microbiology of South African winemaking. I. The yeasts occurring in vineyards, must and wines. S. Afr. J. Agric. Sci. 4, 393–403.

Varela, C., Siebert, T., Cozzolino, D., Rose, L., McLean, H., Henschke, P.A., 2009. Discovering a chemical basis for differentiating wines made by fermentation with “wild” indigenous and inoculated yeasts: Role of yeast volatile compounds. Aust. J. Grape Wine Res. 15, 238–248.

Vaughan-Martini, A., Lachance, M.-A., Kurtzman, C.P., 2011. *Kazachstania Zubkova* (1971), in: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), The Yeasts, A Taxonomic Study. Elsevier B.V., London, UK, pp. 443–444.

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

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

Viegas, C.A., Rosa, M.F., Correia, I.S., Novais, J.M., 1989. Inhibition of yeast growth by octanoic and decanoic acids produced during ethanolic fermentation. Appl. Environ. Microbiol. 55, 21–28.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Appl. Microbiol. Biotechnol. 77, 145–157.

Wang, C., Liu, Y., 2013. Dynamic study of yeast species and *Saccharomyces cerevisiae* strains during the spontaneous fermentations of Muscat blanc in Jingyang, China. Food Microbiol. 33, 172–177.

Yanai, T., Sato, M., 2001. Purification and characterization of an beta-D-xylosidase from *Candida utilis* IFO 0639. Biosci. Biotechnol. Biochem. 65, 527–533.

Yokotsuka, K., Otaki, A., Naitoh, A., Tanaka, H., 1993. Controlled simultaneous deacidification and alcohol fermentation of high-acid grape must using two immobilized yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. 44, 371–377.

Zagorc, T., Maráz, A., Cadez, N., Povhe Jemec, K., Péter, G., Resnik, M., Nemanič, J., Raspor, P., 2001. Indigenous wine killer yeasts and their application as a starter culture in wine fermentation. Food Microbiol. 18, 441–451.

Stellenbosch University https://scholar.sun.ac.za

Zara, G., Mannazzu, I., Del Caro, A., Budroni, M., Pinna, M.B., Murru, M., Farris, G.A., Zara, S., 2014. Wine quality improvement through the combined utilisation of yeast hulls and *Candida zemplinina/Saccharomyces cerevisiae* mixed starter cultures. Aust. J. Grape Wine Res. 20, 199–207.

Zohre, D.E., Erten, H., 2002. The influence of *Kloeckera apiculata* and *Candida pulcherrima* yeasts on wine fermentation. Process Biochem. 38, 319–324.

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., Masneuf-Pomarede, I., 2008. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. Int. J. Food Microbiol. 125, 197–203.

Stellenbosch University https://scholar.sun.ac.za

Chapter 3

Research results

Genetic and phenotypic characterisation of *Wickerhamomyces anomalus* and *Kazachstania aerobia*: investigating amino acid impact on growth and aroma production

Stellenbosch University https://scholar.sun.ac.za

Chapter 3 – Genetic and phenotypic characterisation of *Wickerhamomyces anomalus* and *Kazachstania aerobia*: investigating amino acid impact on growth and aroma production

3.1 Introduction

The wine yeast, *Saccharomyces cerevisiae,* and some closely related *Saccharomyces* species, are the main drivers of alcoholic fermentation and extensive research has characterised this species at both genetic and phenotypic levels (Camarasa et al., 2011; Dunn et al., 2012; Kvitek et al., 2008; Liti et al., 2009). Contrary, similar comprehensive studies have yet to be conducted on most other yeast genera and species that are present in a wine environment and are broadly classified as non-*Saccharomyces* yeasts. Identification of the species and strains present in wine is an obvious prerequisite for understanding their impact. In yeast taxonomy, numerous methods have been used for characterisation at species and strain levels (Jolly, 2004). Traditionally, phenotypic approaches were primarily used, investigating bio-chemical characteristics, morphology and physiology (Agustini et al., 2014). Traits such as osmotolerance, temperature and ethanol tolerance, growth and fermentation kinetics and consumption rate of specific compounds can enable researchers to categorise species into different strains (Ali and Khan, 2014; Camarasa et al., 2011). Furthermore, researchers have used the presence of toxins, nutrient limitations and nutrient sources when characterising and differentiating *S. cerevisiae* strains (Camarasa et al., 2011; Kvitek et al., 2008; Nikolaou et al., 2006; Zuzuarregui and del Olmo, 2004). However, modern technology has now made genetic characterisation the method of choice, exploiting culture dependent or independent methods. Indeed, Random Amplified Polymorphic DNA (RAPD) PCR has been employed as an effective and fast way to differentiate between strains (Zahavi et al., 2002).

Non-*Saccharomyces* yeasts has a prominent impact on the wine aroma profile, even when only present at the onset of fermentation (Jolly et al., 2014). It is thus necessary to evaluate various metabolic pathways to better characterise their contribution, as have extensively been done for *S. cerevisiae* (Carrau et al., 2008; Llungdahl and Daignan-Fornier, 2012; Vilanova et al., 2007). In addition, the nitrogen content of grape must - consisting of mainly ammonium and amino acids – has a significant effect on aroma production (Bell and Henschke, 2005; Ugliano et al., 2007; Vilanova et al., 2007). Branched chain and aromatic amino acids are of especial importance as these are the precursors for various aroma compounds, synthesised via the Ehrlich pathway (Dickinson et al., 2000, 1998, 1997; Hazelwood et al., 2008; Lambrechts and Pretorius, 2000; Smit, 2013). We can therefore assume that non-*Saccharomyces* yeast will affect wine aroma either by their own metabolic conversion of amino acids to aromatic compounds or by competing with *S. cerevisiae* for these

nutrients (thereby changing *S. cerevisiae*'s ability to produce these compounds). It is therefore important to better understand the amino acid utilisation of specific non-*Saccharomyces* yeasts.

Recently, two yeast species - *Kazachstania aerobia* and *Wickerhamomyces anomalus* - have been isolated in Stellenbosch, South Africa, that had not yet been extensively investigated (Bagheri, 2014). *Kazachstania aerobia* had only been used in wine fermentations in a study on sequential culture fermentations in real grape must and was found to release higher amounts of ethyl acetate, esters and terpenes compared to *S. cerevisiae* (Beckner Whitener, 2016). *Wickerhamomyces anomalus* has been identified as a high producer of ethyl acetate (Rojas et al., 2003) but has been used successfully to improve aroma of both white and red wines (Izquierdo Cañas et al., 2014, 2011). This study was thus designed to genetically and phenotypically characterise *K. aerobia* and *W. anomalus* isolates, in addition to determining their impact on the chemical and aromatic properties of wines after fermenting synthetic grape must. The study was our first attempt to identify strains from the two non-*Saccharomyces* yeast species with wine making potential and favourable amino acid and ammonia utilisation.

3.2 Materials and methods

3.2.1 Yeast and culture conditions

Eight *Kazachstania aerobia* and thirteen *Wickerhamomyces anomalus* isolates from South Africa and France were used in this study. The *W. anomalus* isolates ARC (ARC 40/20, ARC 40/8, ARC 40/10, ARC 40/10, ARC 40/10, ARC 19/17, ARC 19/22 and ARC 25/12) were obtained from the Agricultural Research Council (ARC) Nietvoorbij collection, situated in Stellenbosch, South Africa. The remaining isolates, including those of the *K. aerobia* species, were obtained from the collection at the Institute for Wine Biotechnology (IWBT), Stellenbosch University. *Saccharomyces cerevisiae* VIN13 (Anchor Yeast, South Africa) were used as the control yeast. Table 3.1 below is a list of the *K. aerobia* and *W. anomalus* isolates used for this study and also indicates their place of origin. Growths of all yeasts were maintained on Yeast Peptone Dextrose (YPD) agar (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar), purchased from Biolab, SA.

Stellenbosch University https://scholar.sun.ac.za

Table 3.1 Local and international *K. aerobia* and *W. anomalus* yeast isolates compared in this study

Yeast species	Isolate	Origin
Kazachstania aerobia	Y837-A	Stellenbosch, South Africa
Kazachstania aerobia	Y837-B	Stellenbosch, South Africa
Kazachstania aerobia	Y845-A	Stellenbosch, South Africa
Kazachstania aerobia	Y845-B	Stellenbosch, South Africa
Kazachstania aerobia	Y965	Stellenbosch, South Africa
Kazachstania aerobia	Y895-A	Stellenbosch, South Africa
Kazachstania aerobia	Y895-B	Stellenbosch, South Africa
Kazachstania aerobia	CBS 9918	CBS culture collection*
Wickerhamomyces anomalus	Y934-1	Elgin, South Africa
Wickerhamomyces anomalus	Y934-2	Elgin, South Africa
Wickerhamomyces anomalus	Y934-A	Elgin, South Africa
Wickerhamomyces anomalus	Y934-B	Elgin, South Africa
Wickerhamomyces anomalus	Y934-C	Elgin, South Africa
Wickerhamomyces anomalus	LO632	France
Wickerhamomyces anomalus	LO633	France
Wickerhamomyces anomalus	ARC 40/8	Paarl, South Africa
Wickerhamomyces anomalus	ARC 40/10	Paarl, South Africa
Wickerhamomyces anomalus	ARC 40/20	Paarl, South Africa
Wickerhamomyces anomalus	ARC 25/12	Constantia, South Africa
Wickerhamomyces anomalus	ARC 19/17	Stellenbosch, South Africa
Wickerhamomyces anomalus	ARC 19/22	Stellenbosch, South Africa

* The Centraalbureau voor Schimmelcultures (CBS), Utrecht (The Netherlands)

3.2.2 Phenotypic characterisation

3.2.2.1 Plate assays

Isolates of *K. aerobia* and *W. anomalus* were exposed to different stresses and their responses were qualitatively evaluated following methods described by Rossouw et al. (2009). Oxidative, osmotic, hypersaline and heat stresses were investigated. Cells were grown overnight to the exponential growth phase in YPD broth incubated at 30°C with shaking. Cells were washed with sterile distilled

water and suspended in 0.9% NaCl solution to make a saline cell suspension with an OD_{600nm} of 1. Cultures were then treated as specified below and 5 µl of each dilution was spotted on agar plates. Impact of stress was determined by visually evaluating growth on plates, after sufficient incubation (24-48 hours) at 30°C

Oxidative stress

Yeast cells were serially diluted by a factor of 10^{-1} and spotted on YPD plates supplemented with hydrogen peroxide (H_2O_2) at the following concentrations: 3 mM and 4 mM for *K. aerobia* isolates and 5 mM, 6 mM, 7 mM, 7.5 mM, and 8 mM for *W. anomalus* isolates.

Osmotic and hypersaline stress

Yeast cells were serially diluted by a factor of 10^{-1} and spotted on YPD agar plates containing 1 M, 1.5 M, 2 M, 2.5 M, 3.5 M, and 4 M sorbitol and 1 M, 1.2 M, 1.5 M, and 2 M NaCl each. Additional YPD plates with a concentration of 0.5 M sorbitol and 0.1 and 0.5 M NaCl were included for the *K. aerobia* isolates.

Heat shock

Heat shock was tested by resuspending cells in distilled water heated to a temperature of 55°C. Samples were then incubated at 55°C for respectively 15, 30 and 45 min before being serially diluted by a factor of 10^{-1} and spotted on normal YPD plates.

3.2.3 Genotypic characterisation

3.2.3.1 DNA extraction

A single colony of each isolate was inoculated respectively in YPD broth and cultured for 24 hours with agitation at 30˚C after which 2 mL of the samples were centrifuged at 6000 rpm for 5 minutes and the supernatant discarded. The cells were resuspended in 500 µl distilled water followed by another centrifugation step at 6000 rpm for 5 minutes and the supernatant discarded. Thereafter, 300 µl breaking buffer, containing 2% (w/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), was added, followed by addition of 300 µl glass beads and 300 µl PCI (phenol: chloroform: isoamylalcohol; in the ratio of 25:24:1). This mixture was vortexed for 3 min after which 300 µl TE buffer (pH 7.6) was added. Following centrifugation at 12000 rpm for 5 min, the top phase was aspirated into a microcentrifuge tube and 1 ml 100% (v/v) ethanol was added and mixed briefly by vortexing the tube for 5 seconds. The sample was incubated at -80°C for 10 minutes and then centrifuged at 12000 rpm for another 10 minutes. After discarding the supernatant, 500 µl 70% (v/v) ethanol was added and again centrifuged at 12000 rpm for 2 minutes. The supernatant was discarded and the samples dried in a Savant SpeedVac® DNA110 (Thermo Scientific). The

Stellenbosch University https://scholar.sun.ac.za

pellet was resuspended in 90 µl distilled water and 10 µl RNase A (10 mg/ml; Macherey-Nagel, Düren, Germany) and stored at -20°C until used.

3.2.3.2 Strain identification

Random amplified polymorphic DNA (RAPD) PCR was used to determine differences between isolates and the clusters are then considered as different strains. The PCR reactions were performed in 25 µL reaction mixtures containing 1 µL of DNA template, 10.9 µL of milli-Q water, 0.1 µL of 2.5 U/µl GoTaq®DNA Polymerase (Promega), 0.4 µL of 100 mM primer, 5 µL of ColorlessGoTaq®Flexi Buffer (Promega, Madison, U.S.A.), 2 µL of 2.5 mM deoxynucleoside triphosphate (dNTP) mixture and 2 µL of 25 mM $MgCl_2$ (Promega). Three reactions were performed with three different primers: OPA-01 (5´-CAGGCCCTTC-3´), OPA-05 (5´-AGGGGTCTTG-3´) and OPA-09 (5´-GGGTAACGCC-3´). DNA amplification was executed by using the Applied Biosystems® 2720 Thermal Cycler. PCR conditions were as follows: initial denaturation at 94°C for 1 min; 45 cycles of denaturing at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 2 min; and a final extension at 72°C for 8 min (Bujdoso et al., 2011). The PCR products were separated on 1.5% agarose gels prepared in 1X Tris-Acetic acid-EDTA (TAE) buffer, stained with GelRed™. Electrophoresis was conducted for 2 hours at 70 V and afterwards gels were visualised under UV light and photographed. Sizes were estimated by comparison against a GeneRuler™ 100bp plus DNA Ladder (Fermentas, South Africa). Random segments of the isolates were amplified, allowing differentiation and grouping into strains according to different banding patterns.

3.2.4 Single culture fermentations

3.2.4.1 Inoculation strategy

Fermentations were conducted with selected isolates; *K. aerobia* Y837-B, Y965, CBS 9918 and *W. anomalus* Y934-C, LO632, LO633, ARC 40/20, ARC 19/22; in 100 mL spice bottles containing 80 mL synthetic grape must (SGM) fitted with fermentation locks. Fermentations were done in triplicate with *S. cerevisiae* VIN13 as the control. The SGM was prepared as described by Henschke and Jiranek (1993) with minor adjustments (Smit, 2013). The pH of the must was adjusted to 3.5 using KOH with an initial sugar content of 200 g/L (100 g/L glucose and 100 g/L fructose) and yeast assimilable nitrogen (YAN) content of 300 mg N/L (the only exception from the SGM described in Smith (2013)).

The YAN component of the must was adjusted to form three different nitrogen treatments as defined in Table 3.2 and identified as Treatment A, Treatment B and Treatment C as follows:

- Treatment A served as the control with only ammonium (provided as ammonium chloride) as nitrogen source (300 mg N/L).

Stellenbosch University https://scholar.sun.ac.za

- Treatment B consisted of all 20 amino acids that contributed in equal amounts to a total of 150 mg N/L as well as ammonium chloride providing the remaining 150 mg N/L.
- Treatment C only had the branched-chained and aromatic amino acids (BCAA) - isoleucine, leucine, valine, phenylalanine and tyrosine – providing equal amounts of nitrogen contributing 150 mg N/L as well as ammonium chloride providing the remaining 150 mg N/L.

All isolates were cultured as described previously in section 3.2.1 and grown overnight in YPD broth, incubated at 30°C. Thereafter, the yeast isolates were inoculated at an OD_{600} of 0.1 into the SGM. Fermentations were incubated at 30°C and conducted in static conditions with the exception of being shaken once a day just before weighing. Fermentations were conducted for three weeks.

3.2.4.2 Fermentation kinetics

Carbon dioxide release, change in optical density (OD) and sugar consumption were used to determine the growth kinetics and fermentation potential of the isolates under study. Samples were obtained for the first three days and thereafter every second day to measure the OD at 600 nm wavelength in order to determine biomass formation. At these time points sugar (glucose and fructose), ammonia and alpha amino nitrogen concentrations were determined using the Arena 20XT Photometric Analyzer (Thermo Electron Oy, Finland). Doubling times of yeast isolates were calculated with the formula $Td = \log(2)/\log(1+r)$, where "Td" indicates doubling time and "r" the linear correlation coefficient calculated from three OD measurements during exponential growth phase.

3.2.4.3 Major volatile aroma production

Aroma compounds were extracted using a liquid-liquid extraction (Louw et al., 2009). A 5 mL sample of each treatment was used with 100 µL 4-methyl-2-pentanol as internal standard. After addition of 1 mL diethyl ether and sonicating the mixture for 5 min it was then centrifuged at 4000 rpm for 3 minutes. If separation of ether layer was not clear, sodium sulphate (Na_2SO_4) was added and the mixture was centrifuged again. The supernatant (ether layer) was then aspirated and dried on Na_2SO_4 after which it was injected into the gas chromatography– flame ionisation detector (GC-FID). Metabolites were identified and quantified by the GC-FID and a Hewlett Packard 6890 Plus gas chromatograph (Agilent, Little Falls, Wilmington, USA) fitted with a split injector. Method for quantification was conducted as stated in Smith (2013).

3.2.4.4 Statistical analysis

All univariate statistical analyses were done using the Statistica 13 analytics software package (Dell Inc., USA) to infer the effects of different treatments on yeast growth, metabolite accumulation and fermentation kinetics. Multivariate data analysis was conducted using SIMCA 13 data presentation

Stellenbosch University https://scholar.sun.ac.za

and analytics software (Umetrics, Sweden) to simultaneously investigate the treatment effect on all metabolites produced. Data in tables and graphs are presented as means ± standard error of mean.

Table 3.2 Composition of the nitrogen treatments (adapted from Smit, 2013)

Compound	Treatment A Ammonium only			Treatment B Complete amino acids			Treatment C BCAAs		
	%N	mg N/L	mg/L	%N	mg N/L	mg/L	%N	mg N/L	mg/L
NH_4Cl	21.2	300.0	1146.0	21.2	150.0	573.0	21.2	150.0	573.0
ALA	-	-	-	15.7	7.5	47.8	-	-	-
ARG	-	-	-	32.2	7.5	23.3	-	-	-
ASN	-	-	-	21.2	7.5	35.4	-	-	-
ASP	-	-	-	10.5	7.5	71.4	-	-	-
CYS	-	-	-	11.6	7.5	64.9	-	-	-
GLN	-	-	-	19.2	7.5	39.1	-	-	-
GLU	-	-	-	9.5	7.5	78.9	-	-	-
GLY	-	-	-	18.6	7.5	40.3	-	-	-
HIS	-	-	-	27.1	7.5	27.7	-	-	-
ILE	-	-	-	10.7	7.5	70.1	10.7	30.0	280.4
LEU	-	-	-	10.7	7.5	70.1	10.7	30.0	280.4
LYS	-	-	-	19.2	7.5	39.1	-	-	-
MET	-	-	-	9.4	7.5	79.8	-	-	-
PHE	-	-	-	8.5	7.5	88.2	8.5	30.0	352.9
PRO	-	-	-	12.2	7.5	61.5	-	-	-
SER	-	-	-	13.3	7.5	56.4	-	-	-
THR	-	-	-	11.8	7.5	63.6	-	-	-
TRP	-	-	-	13.7	7.5	54.7	-	-	-
TYR	-	-	-	7.7	7.5	97.4	7.7	30.0	389.6
VAL	-	-	-	12.0	7.5	62.5	12.0	30.0	250.0

3.3 Results

3.3.1 Phenotypic characterisation – plate assays

The non-*Saccharomyces* yeasts, *K. aerobia* and *W. anomalus*, were exposed to different stress assays to characterise yeast and differentiate between isolates of the same species. All the *K. aerobia* isolates showed low tolerance to oxidation in comparison to *S. cerevisiae* (VIN13). The

Stellenbosch University https://scholar.sun.ac.za

isolates Y837-A and Y895-A showed better growth on plates supplemented with 3 mM H_2O_2 compared to the other *K. aerobia* isolates (Figure 3.1 – A). Concentration of 4mM H_20_2 resulted in no growth for the *K. aerobia* isolates. With regards to osmotic and salt tolerance, *K. aerobia* isolates did not show distinctive phenotypes (Figure 3.1 – B,C). Although isolate Y895-A and the CBS strain exhibited the least growth on 1 M NaCl media compared to the rest of the isolates and VIN13. Heat stress induced by exposing the yeast for 15 and 30 minutes at 55°C did not have an effect on growth, whereas heat stress for 45 minutes showed that the *K. aerobia* isolate Y895-B and CBS strain had a slightly higher resistance to heat (Figure 3.3).

Wickerhamomyces anomalus isolates proved to have higher tolerance to oxygen, osmotic and hypersaline stress than *S. cerevisiae* (Figure 3.2). Between isolates, LO633, Y934-1 and Y934-2 had the lowest tolerance to oxygen (Figure 3.2 - A). No differences were observed between the ARC isolates or Y934-A, Y934-B, Y934-C. Furthermore, the *W. anomalus* isolates Y934-A, Y934-B, Y934-C were the most resistant to osmotic stress (Figure 3.2 - B). In comparison, the isolates Y934-1 and Y934-2 and ARC isolates 25/12, 19/17 and 19/22 were the most sensitive to osmotic stress. No differences were observed amongst the isolates LO632 and LO633 as well as ARC 40/8, 40/10, 40/20. The hypersaline stress assays showed no differences between the Y934 isolates and they were all more resistant to high salt conditions when compared to the other isolates, followed by LO633 and ARC 40/20 (Figure 3.2 - C). ARC 25/12, 19/17 and 19/22 were the least resistant to hypersaline stress, followed by ARC 40/8 and 40/10. When assessing the resistance of *W. anomalus* to heat stress, the isolates Y934-A and ARC 25/12 had the least resistance (Figure 3.3 – B). There were no differences between the remaining ARC isolates.

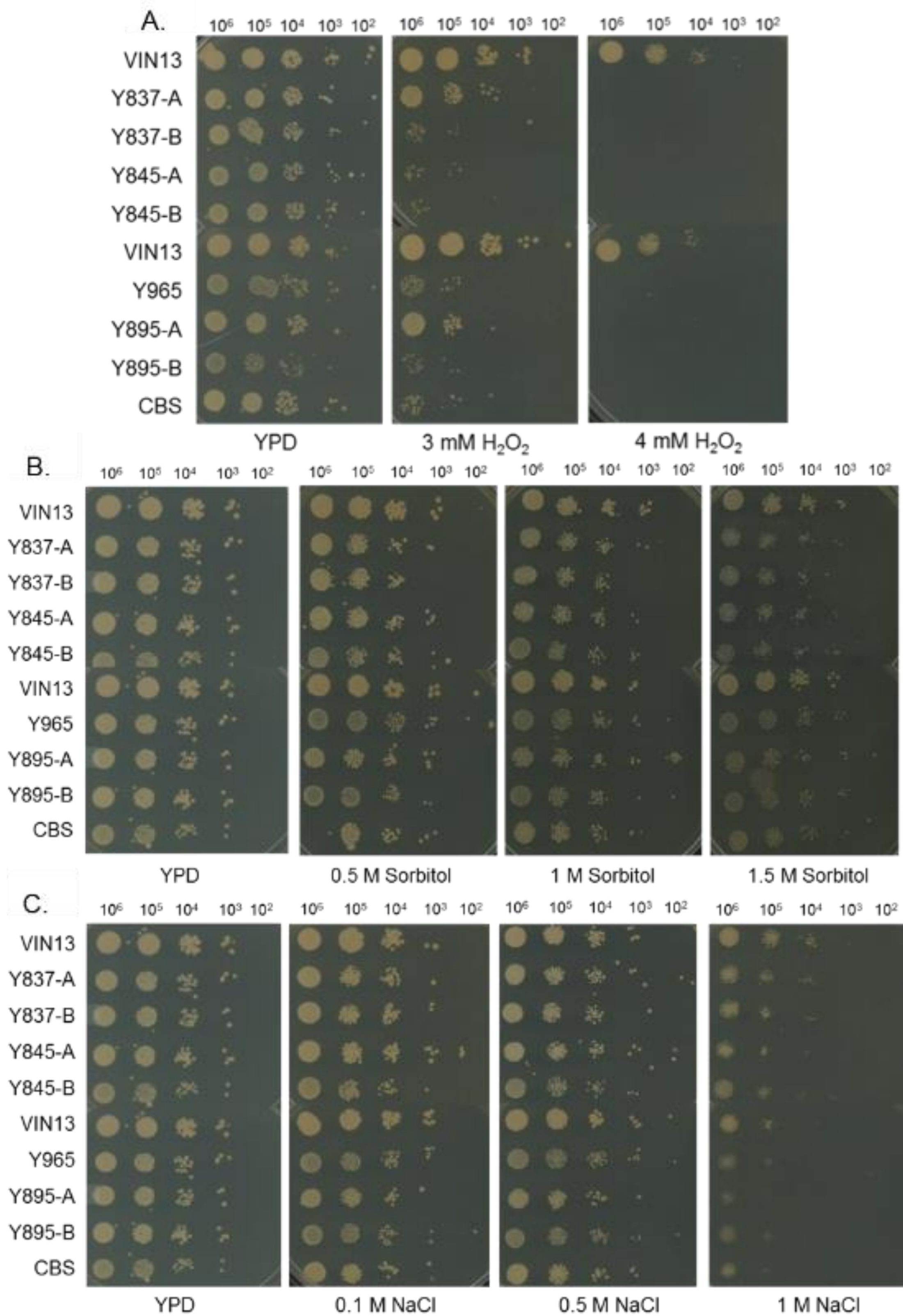


Figure 3.1 Effect of oxygen (A), osmotic (B), and hypersaline (C) stresses on *K. aerobia* isolates (Y837-A, Y837-B; Y845-A, Y845-B; Y965, Y895-A, Y895-B; CBS) at 10^6 cfu/mL to 10^2 cfu/mL. *S. cerevisiae* (VIN13) was used as control.

Stellenbosch University https://scholar.sun.ac.za

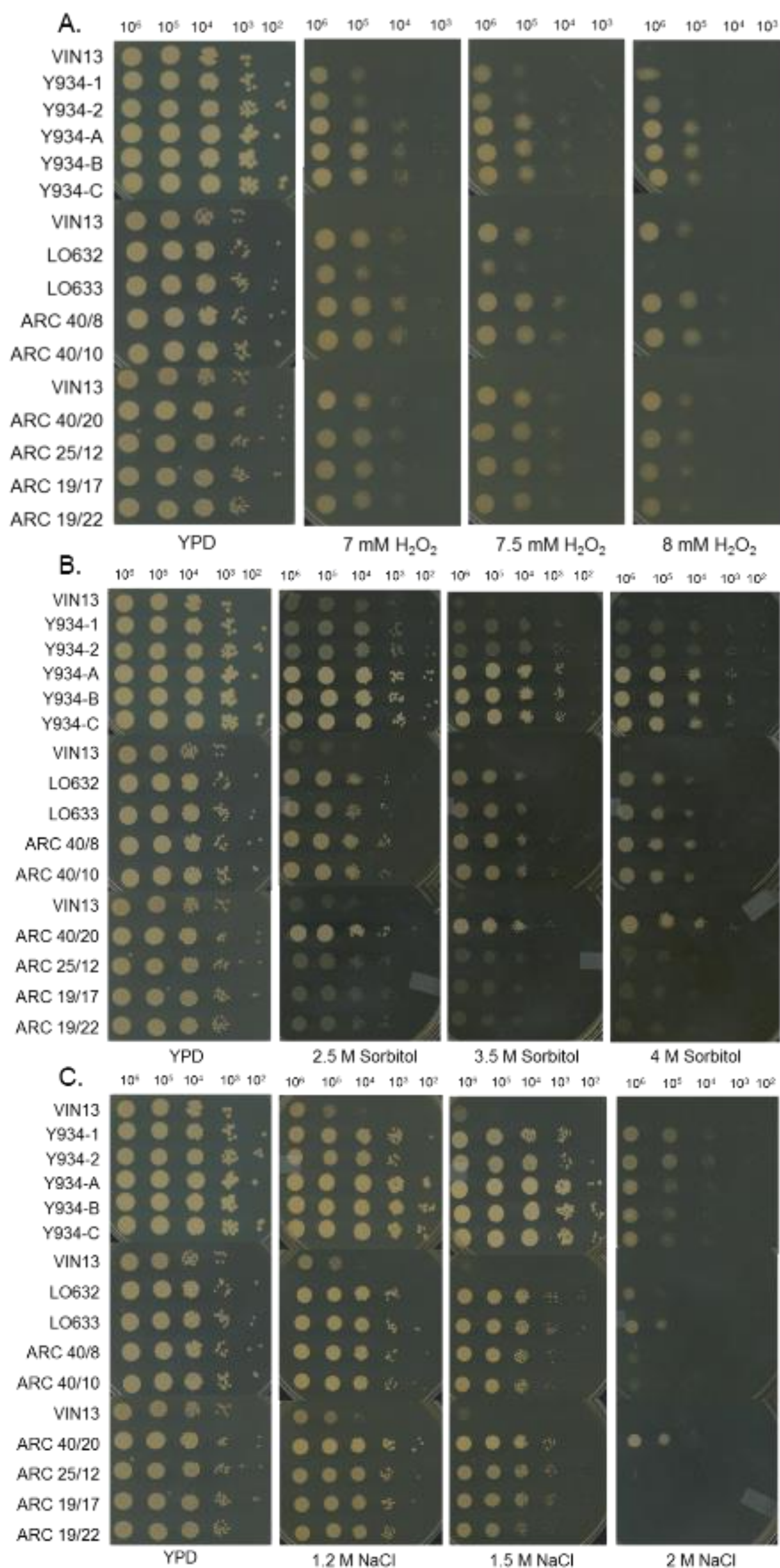


Figure 3.2 Effect of oxygen (A), osmotic (B) and hypersaline (C) stresses on *W. anomalus* isolates (Y934-A, Y934-B, Y934-C, Y934-1, Y934-2; LO632, LO633, ARC 40/8, ARC 40/10, ARC 40/20, ARC 25/12, ARC 19/17, ARC 19/22) at 10^6 cfu/mL to 10^2 cfu/mL. *S. cerevisiae* (VIN13) was used as control.

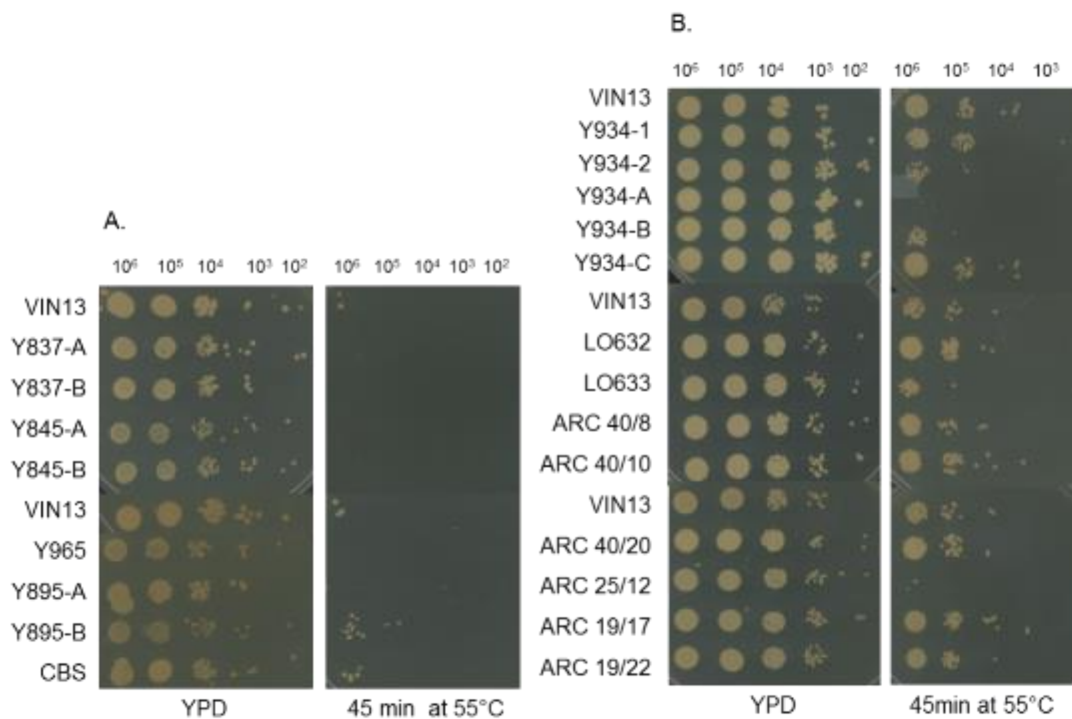


Figure 3.3 Effect of heat shock on A - *K. aerobia* isolates (Y837-A, Y837-B; Y845-A, Y845-B; Y965, Y895-A, Y895-B; CBS) and B - *W. anomalus* isolates (Y934-A, Y934-B, Y934-C, Y934-1, Y934-2; LO632, LO633, ARC 40/8, ARC 40/10, ARC 40/20, ARC 25/12, ARC 19/17, ARC 19/22) at 10^6 cfu/mL to 10^2 cfu/mL. *S. cerevisiae* (VIN13) was used as control.

3.3.2 Genotypic characterisation – RAPD

The DNA based taxonomic differentiation between isolates of *K. aerobia* and *W. anomalus* were conducted using RAPD analysis. Isolates of *K. aerobia* showed no clear genetic difference, with the exception of the CBS strain when amplified with primer OPA-01 (Figure 3.4 - A). The *K. aerobia* isolates were distinctly different from the *S. cerevisiae* VIN13 control yeast.

The primers OPA-01, OPA-05, OPA-09 showed that the *W. anomalus* isolates ARC 25/12, ARC 19/17 and ARC 19/22 were similar. Primer OPA-01 showed that the remaining isolates had the same banding pattern (Figure 3.4 - B). Using primer OPA-05 distinguished LO632 and LO633 from Y934 isolates by an extra band. ARC 40/20 had the same extra band as LO632 and LO633 whereas Y934 isolates, ARC 40/8 and 40/10 all had the same region amplified. These groupings were confirmed by amplification with primer OPA-09. Amplification with primer OPA-09 suggested that the ARC isolates 40/8, 40/10 and 40/20 were different from each other.

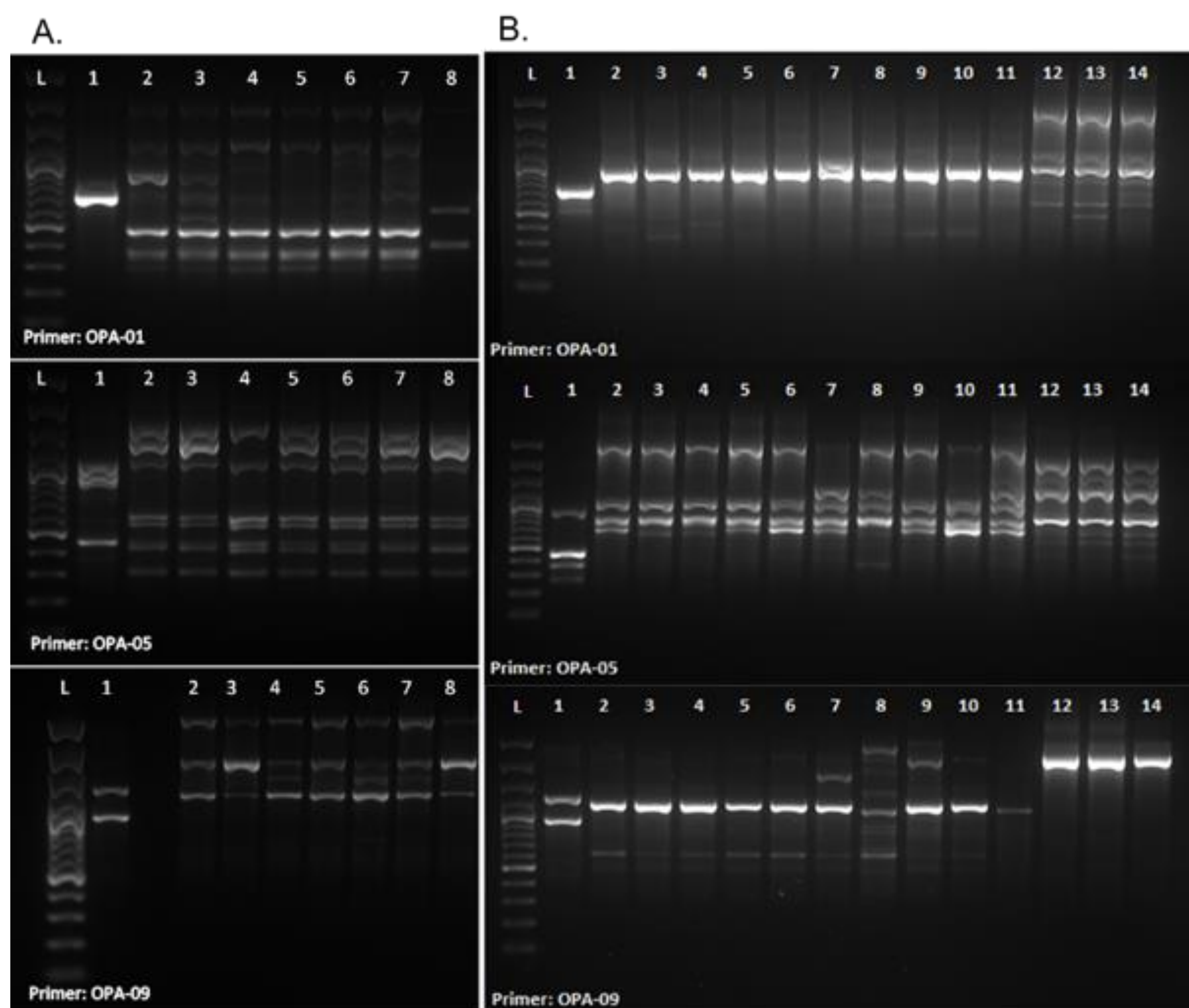


Figure 3.4 Strain characterisation of (A) *K. aerobia* and (B) *W. anomalus* isolates using RAPD. Three primers OPA-1, OPA-5 and OPA-9 were used for the PCR amplification. In A, lanes 1-8 represent *S. cerevisiae* VIN13, *K. aerobia* Y845-A, Y837-B, Y965, Y895-A, Y895-B, Y845-B and the CBS strain in that order; in B, lanes 1-14 represent *S. cerevisiae* VIN13, *W. anomalus* Y934-1, Y934-2, Y934-A, Y934-B, Y934-C, LO632, LO633, ARC 40/8, ARC 40/10, ARC 40/20, ARC 25/12, ARC 19/17, and ARC 19/22 after PCR amplification of genomic DNA. Lane L contain 0.25µg GeneRuler 100 bp Plus DNA ladder as reference.

3.3.3 *K. aerobia* and *W. anomalus* in single culture fermentations

3.3.3.1 Fermentation kinetics

Single culture fermentations were conducted with three and five phenotypically diverse *K. aerobia* and *W. anomalus* isolates. Fermentation with *S. cerevisiae* VIN13 served for comparative purposes. All fermentations were conducted in synthetic grape must supplemented with the same amount of total yeast available nitrogen, but with different nitrogen source combinations (referred to as Treatments A. B and C): no amino acids (only ammonium), all of the amino acids (with ammonium) and BCAA’s (with ammonium). All fermentations were conducted for 21 days. Fermentation rate and biomass production of yeast cultures were determined by monitoring CO_2 production and sugar consumption and change in optical density (OD) over time.

Stellenbosch University https://scholar.sun.ac.za

As expected, *S. cerevisiae* VIN13 showed the fastest fermentation rate as measured CO_2 release and sugar consumption, independently of the nitrogen treatments (Figure 3.5; Figure 3.6). Fermentation rate of *K. aerobia* isolates (with the exception of *K. aerobia* CBS) were slightly increased in the treatment with BCAA's (Figure 3.5 – A; Figure 3.6). At the start of fermentation, *K. aerobia* Y837-B displayed a higher CO_2 production independent of nitrogen treatment, although as fermentation progressed Y837-B and Y965 had similar sugar utilisation and CO_2 production. Overall, the CBS strain showed the lowest fermentation rate (specific end point values are documented in the appendix, Table 1). For the *W. anomalus* yeasts, Treatment B with all of the amino acids had the fastest fermentation rate (CO_2 production and sugar consumption) and Treatment C with BCAA's as nitrogen source, resulted in the slowest fermentation rate (Figure 3.5 - B; Figure 3.7). Between isolates of *W. anomalus,* LO632 had the fastest fermentation rate throughout the different treatments (Figure 3.5 – B Figure 3.7, specific end point values are documented in the appendix, Table 2). In contrast, the isolates ARC 40/20, 19/22 and Y934-C had the slowest fermentation rate. All of the yeast showed a preference for glucose, which was consumed at a faster rate than fructose. *Wickerhamomyces anomalus* yeasts consumed minimal amounts of fructose.

In terms of growth rate, *S. cerevisiae* exhibited the fastest biomass production, entering exponential phase after 24 hours, compared to the *K. aerobia* and *W. anomalus* isolates that had a three and two day long lag phase (Figure 3.8). After 9 days *S. cerevisiae* was in stationary phase, compared to the non-*Saccharomyces* yeast that had not yet reached stationary phase at the time that the fermentations were terminated. Nitrogen composition displayed a significant impact on biomass formation. For all *K. aerobia* yeasts, Treatment B resulted in higher biomass production and Treatment A resulted in the lowest biomass production (Figure 3.8 - A). Amongst *K. aerobia* isolates, Y965 had the highest biomass production and the CBS strain the lowest production (Figure 3.8 - A; Table 1 in appendix). In *W. anomalus* fermentations, similar to *K. aerobia* fermentations, Treatment A resulted in the lowest growth, although, in contrast, Treatment C, had the highest biomass production (specific end point values are documented in the appendix, Table 1 and Table 2). Amongst *W. anomalus* isolates, ARC 19/22 had the lowest growth and LO632 the highest, although not significant at end point.

The doubling time of yeast growth was shortest for *S. cerevisiae*, followed by *W. anomalus* and then *K. aerobia* (Table 3.3). Between treatments the doubling time differed depending on species. Doubling time for *S. cerevisiae* was the longest for Treatment C with the BCAA's. For the *K. aerobia* fermentations, doubling time was the fastest for Treatment A with no amino acids, with the exception of *K. aerobia* Y837-B that had a shorter doubling time in Treatment B with all of the amino acids. Interestingly, for *W. anomalus* fermentations, Treatment C with the BCAA's had the fastest doubling time, with the exception of *W. anomalus* ARC 40/20. The fastest doubling time was observed for either Treatment A with no amino acids or Treatment B with all of the amino acids. Amongst isolates

Stellenbosch University https://scholar.sun.ac.za

of *K. aerobia*, differences between Y837-B and Y965 were treatment dependant, with strain CBS exhibiting the longest doubling time. Amongst *W. anomalus*, LO632 had the shortest and ARC 19/22 the longest doubling time.

Table 3.3 Doubling time (Td) indicated in hours for yeast species, *S. cerevisiae* (SC), *K. aerobia* (KA), *W. anomalus* (WA), in single culture fermentation. Values calculated from three OD_{600} measurements during the exponential growth phase

Yeast species	Treatment	Td (h)	Treatment	Td (h)	Treatment	Td (h)
SC VIN13	A	1.23	B	1.22	C	1.40
KA Y837-B	A	8.76	B	4.56	C	5.85
KA Y965	A	4.95	B	7.06	C	5.68
KA CBS	A	5.35	B	16.58	C	7.49
WA Y934-C	A	3.12	B	4.67	C	2.89
WA LO632	A	3.02	B	3.60	C	2.44
WA LO633	A	4.05	B	3.88	C	2.85
WA ARC 40/20	A	3.20	B	2.46	C	3.29
WA ARC 19/22	A	5.43	B	4.38	C	4.35

Stellenbosch University https://scholar.sun.ac.za

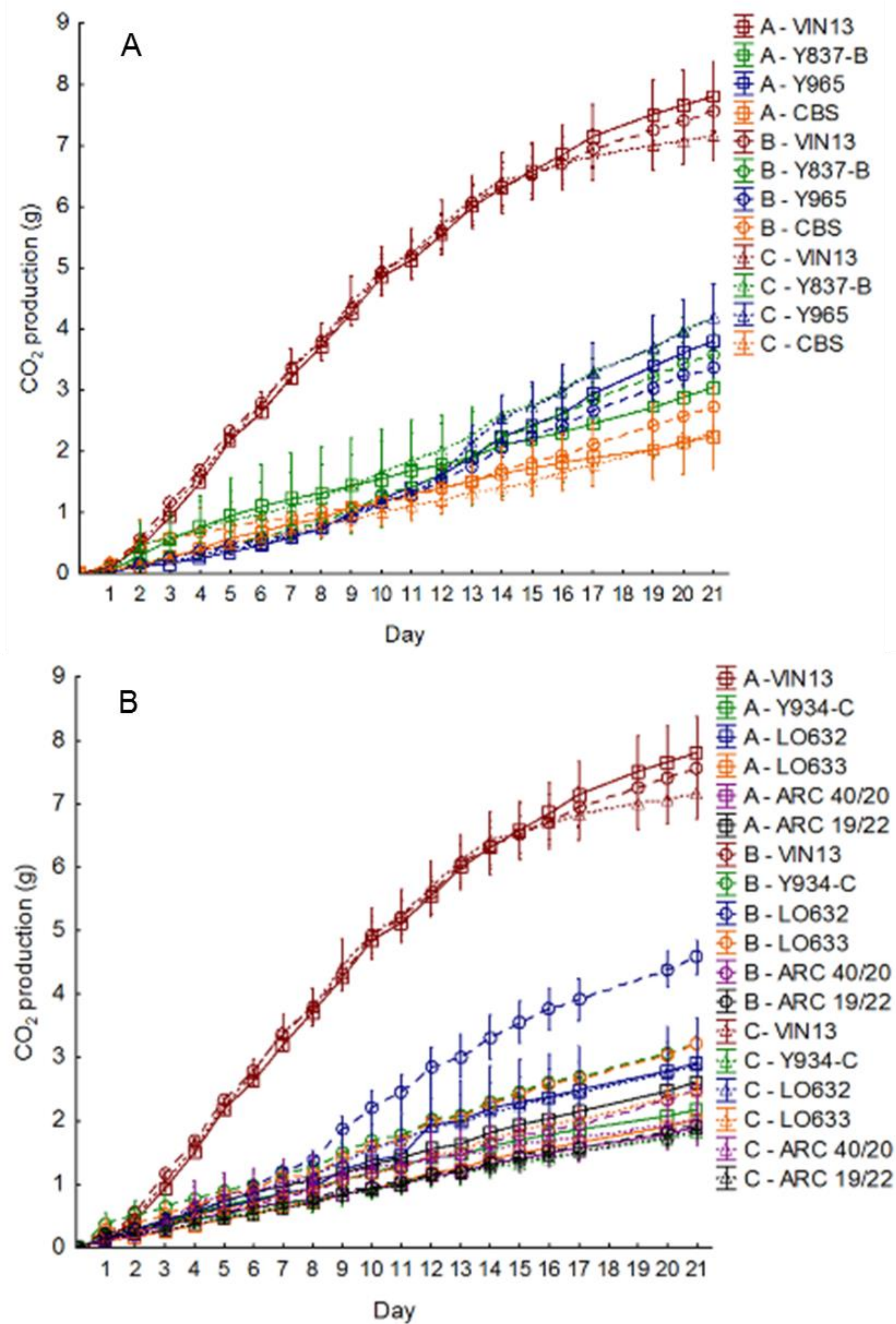


Figure 3.5 Mean CO_2 production for the duration of *K. aerobia* and *W. anomalus* fermentations displayed in graph A and B. Nitrogen treatments are indicated as A (square) - only ammonium, B (circle) - all of the amino acids and ammonia, and C (triangle) - BCAA's and ammonia. VIN13 is *S. cerevisiae* the control and the non-*Saccharomyces* yeast is indicated as their respective isolate number. All means values are indicated with standard error bars.

Stellenbosch University https://scholar.sun.ac.za

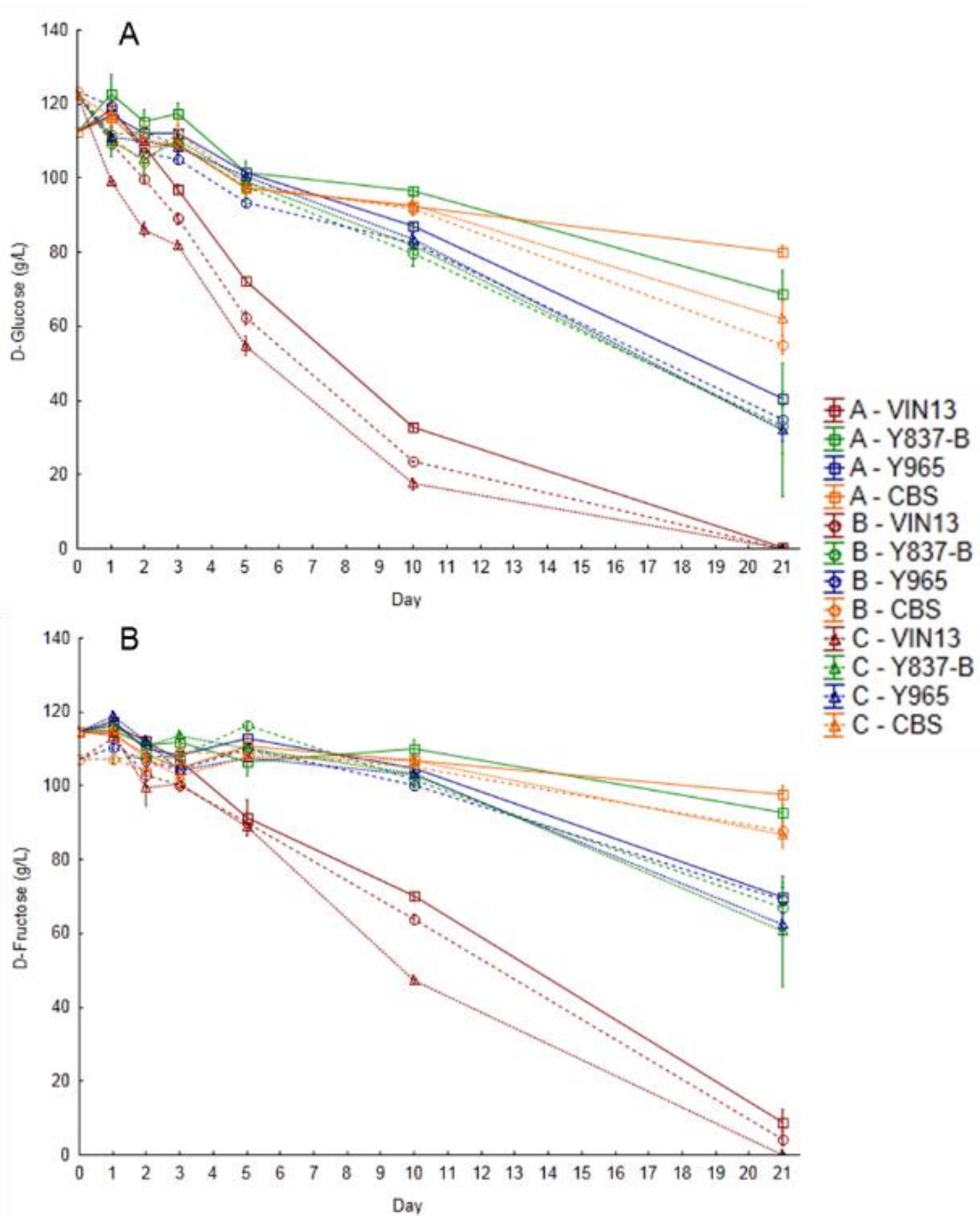


Figure 3.6 Consumption of glucose (A) and fructose (B), indicated as mean ± standard error, in single culture fermentations of *S. cerevisiae* VIN 13 (red) and *K. aerobia* Y837-B (green), Y965 (blue), CBS (orange). Nitrogen treatments are indicated as A (square) - only ammonium, B (circle) - all of the amino acids and ammonia, and C (triangle) - BCAA's and ammonia.

Stellenbosch University https://scholar.sun.ac.za

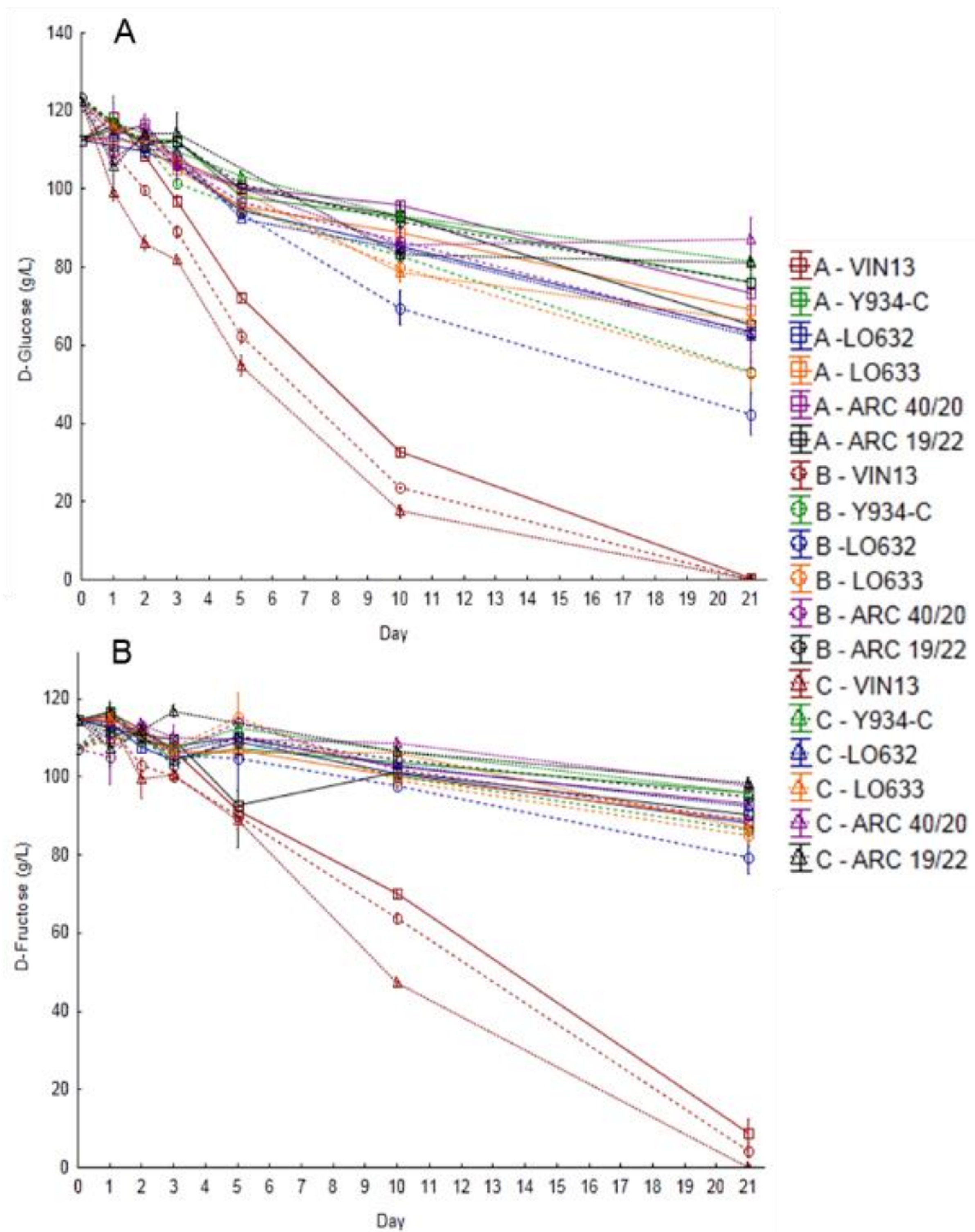


Figure 3.7 Consumption of glucose (A) and fructose (B), indicated as mean ± standard error, for single culture fermentations of *S. cerevisiae* VIN 13 (red) and *W. anomalus* Y934-C (green), LO632 (blue), LO633 (orange), ARC 40/20 (purple), ARC 19/22 (black). Nitrogen treatments are indicated as A (square) - only ammonium, B (circle) - all of the amino acids and ammonia, and C (triangle) - BCAA's and ammonia.

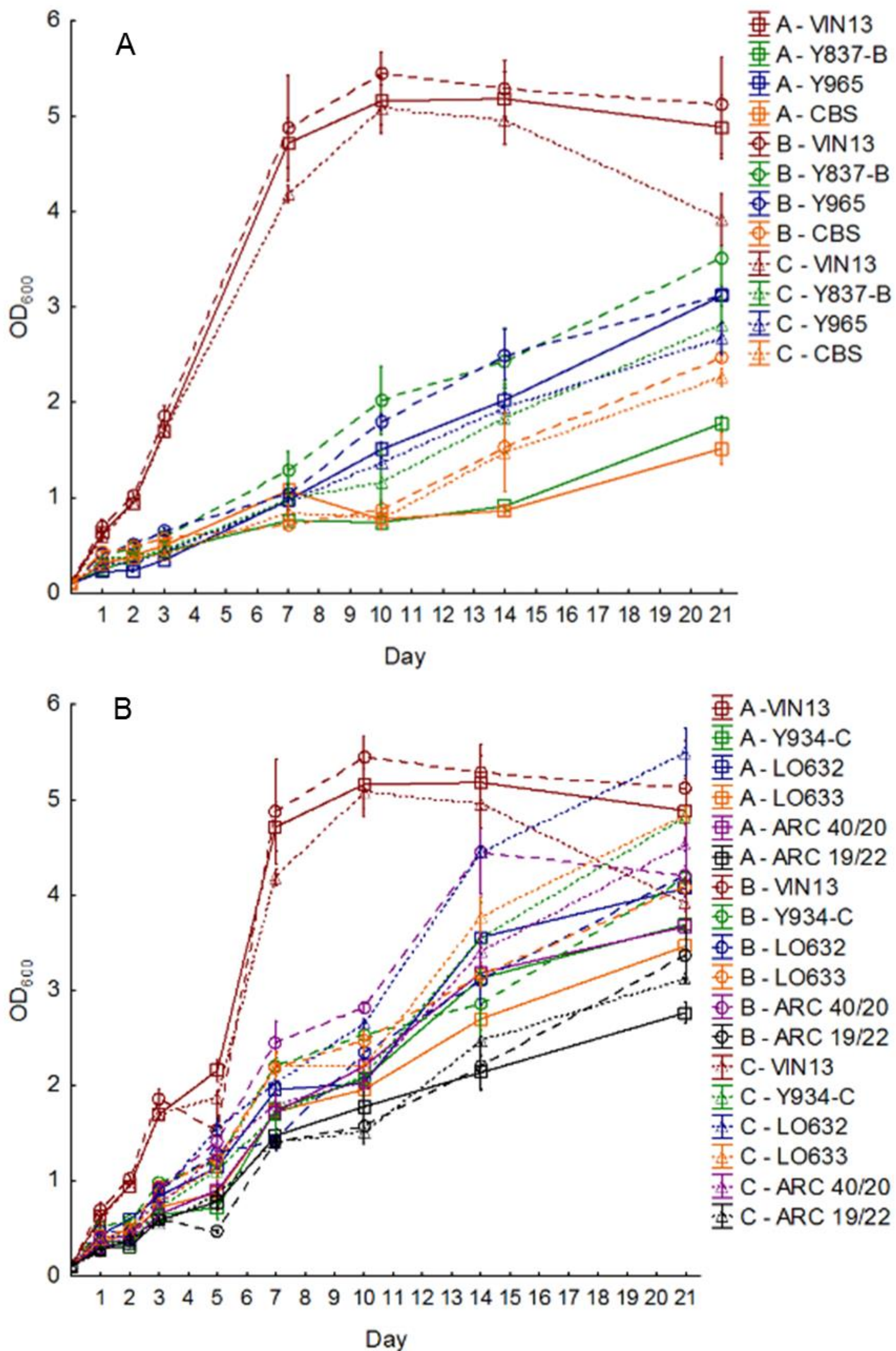


Figure 3.8 Mean OD_{600} for the duration of *K. aerobia* and *W. anomalus* fermentations displayed in graphs A and B. Nitrogen treatments are indicated as A (square) - only ammonium, B (circle) - all of the amino acids and ammonia, and C (triangle) - BCAA's and ammonia. VIN13 is *S. cerevisiae* the control and the non-*Saccharomyces* yeast is indicated as their respective isolate number. All mean values are indicated with standard error bars.

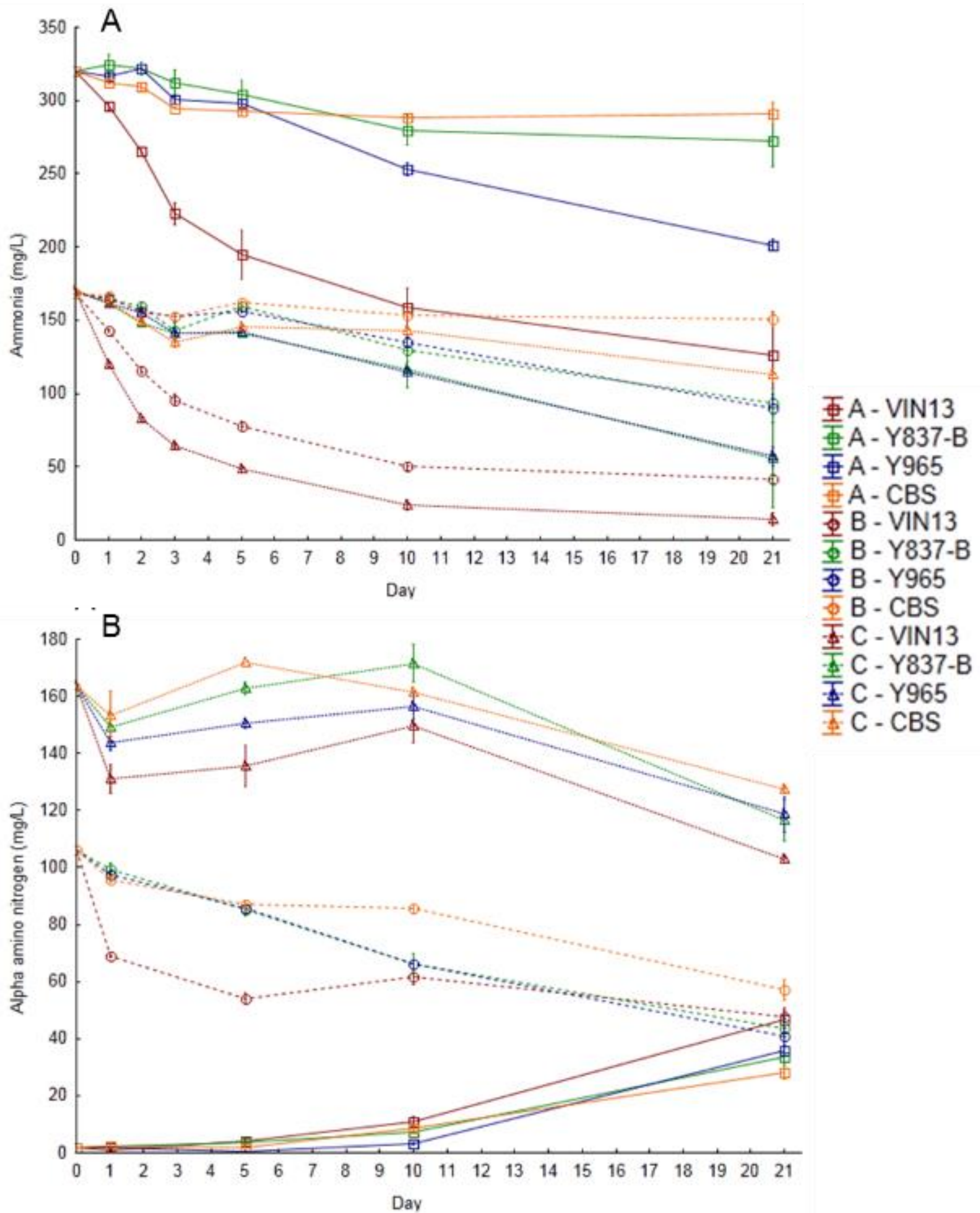


Figure 3.9 Ammonia (A) and alpha amino nitrogen (B) concentrations, indicated as mean ± standard, error in single culture fermentations for *S. cerevisiae* VIN 13 (red) and *K. aerobia* Y837-B (green), Y965 (blue), CBS (orange). Nitrogen treatments are indicated as A (square) - only ammonium, B (circle) - all of the amino acids and ammonia, and C (triangle) - BCAA's and ammonia.

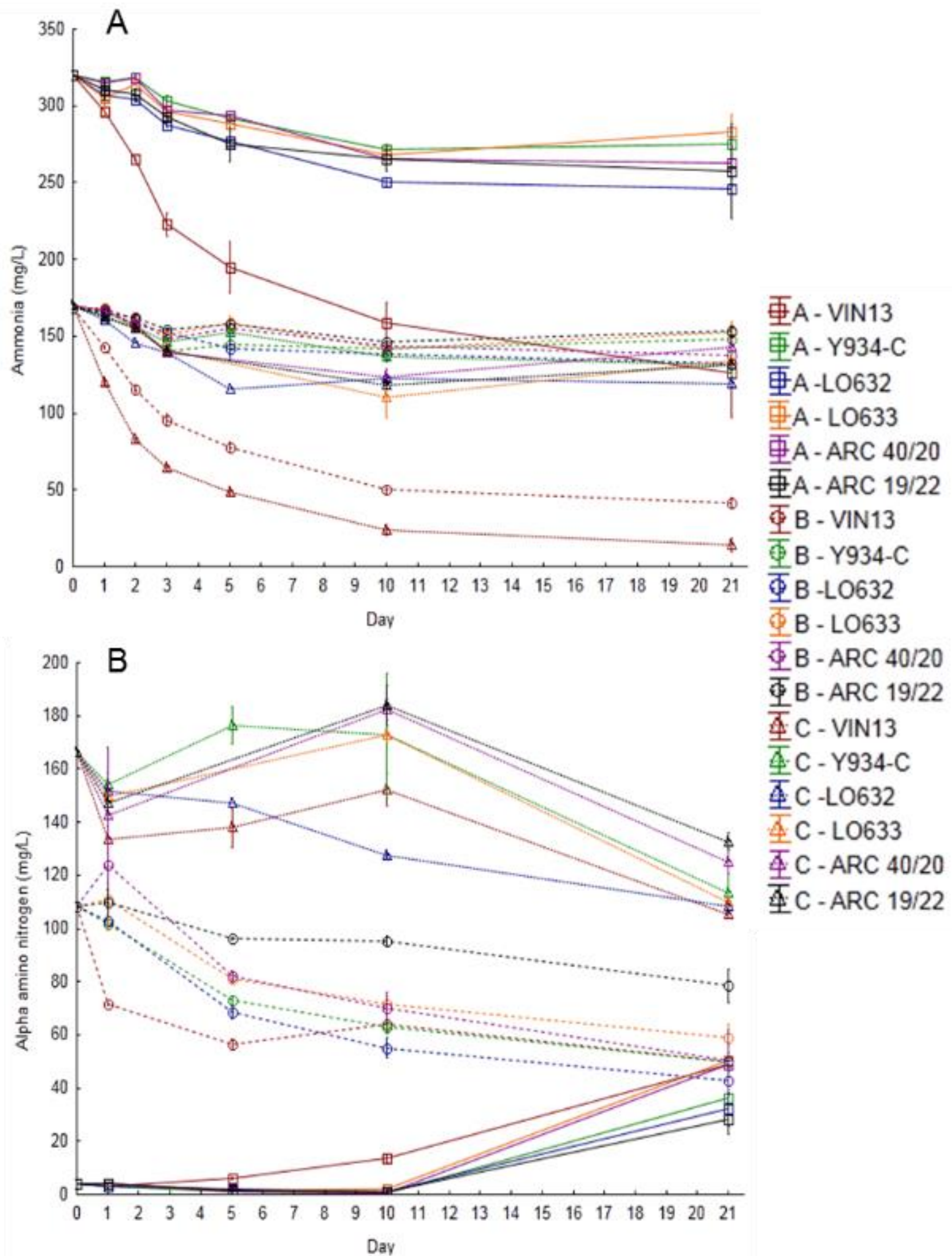


Figure 3.10 Ammonia (A) and alpha amino nitrogen (B), indicated as mean ± standard error, in single culture fermentations of *S. cerevisiae* VIN 13 (red) and *W. anomalus* Y934-C (green), LO632 (blue), LO633 (orange), ARC 40/20 (purple), ARC 19/22 (black). Nitrogen treatments are indicated as A (square) - only ammonium, B (circle) - all of the amino acids and ammonia, and C (triangle) - BCAA's and ammonia.

Chemical analysis of the single culture fermentations revealed that *S. cerevisiae* (VIN 13) had the fastest consumption rate of ammonia, although consumption of amino acids did not differ between the yeast species (Figure 3.9; Figure 3.10). In addition, amino acids increased in Treatment A where no amino acids were present initially, as yeasts synthesise amino acids. Amongst *K. aerobia* isolates, Y965 had the fastest consumption of ammonia and amino acids and the CBS isolate the slowest consumption (Figure 3.9). In terms of nitrogen treatment effect, *K. aerobia* isolates in Treatment C with the BCAA's consumed more ammonia and amino acids compared to the other treatments.

Amongst the isolates of *W. anomalus*, LO632 utilised the most ammonia and amino acids, whilst isolates Y934-C and LO633 utilised the least ammonia (Figure 3.10). Between treatments, *W. anomalus* consumed the most ammonia in Treatment A, and no differences was seen in consumption for Treatment B and C that had added amino acids. An increase in amino acid utilisation was observed when all of the amino acids were present in the must.

3.3.3.2 Major volatile aroma production

The overall data set of measured aroma compounds was analysed with PLS-DA, and suggests that the nitrogen treatment used had the largest impact on the aroma profile, as yeast separated and grouped according to treatment (Figure 3.11 - A). Overall, volatile compounds (isoamyl acetate, isobutanol, 2-phenyl ethanol, isoamyl alcohol, isobutyric acid, iso-valeric acid) increased when the relative amino acid precursors were added, as indicated in the biplot (Figure 3.11 - B).

For all of the yeast species, production of 2-phenyl ethanol, isoamyl alcohol and isovaleric acid was doubled when all of the amino acids were added to the must and tripled when the BCAA's were added (Figure 3.12). In addition, isobutanol similarly increased in Treatment C, but no difference was seen between Treatments A and B. Isobutyric acid production was the same for all yeasts irrespective of nitrogen treatment, with the exception of the production by *W. anomalus* that tripled production of this compound in the treatment with added BCAA's. In general, the addition of BCAA's had a more significant effect on compound production, *e.g.* isoamyl acetate, isoamyl alcohol, isobutanol, isobutyric acid, isovaleric acid, in *W. anomalus* yeasts compared to *S. cerevisiae* and *K. aerobia*.

Ethyl acetate and acetoin production by all yeast species were not affected by nitrogen treatment. The non-*Saccharomyces* yeasts all produced more ethyl acetate than *S. cerevisiae*, with important isolate differences. Interestingly, acetic acid production was lower in Treatment C for *S. cerevisiae* and *K. aerobia*, although no differences were observed for the other treatments or fermentations with *W. anomalus*. Acetic acid and acetoin production was highest for *K. aerobia* isolates compared to the other species. Propanol and butanol production by all yeast species were highest in Treatment A with no added amino acids and lowest in Treatment C.

Amongst *W. anomalus* isolates, the trend for compound production as consistent. The *W. anomalus* isolate, LO632 produced the most volatile compounds, followed by LO633, while ARC 19/22 produced the least of these compounds. Amongst *K. aerobia* isolates, isolate Y965 produced higher amounts of propanol, butanol, acetic acid and acetoin.

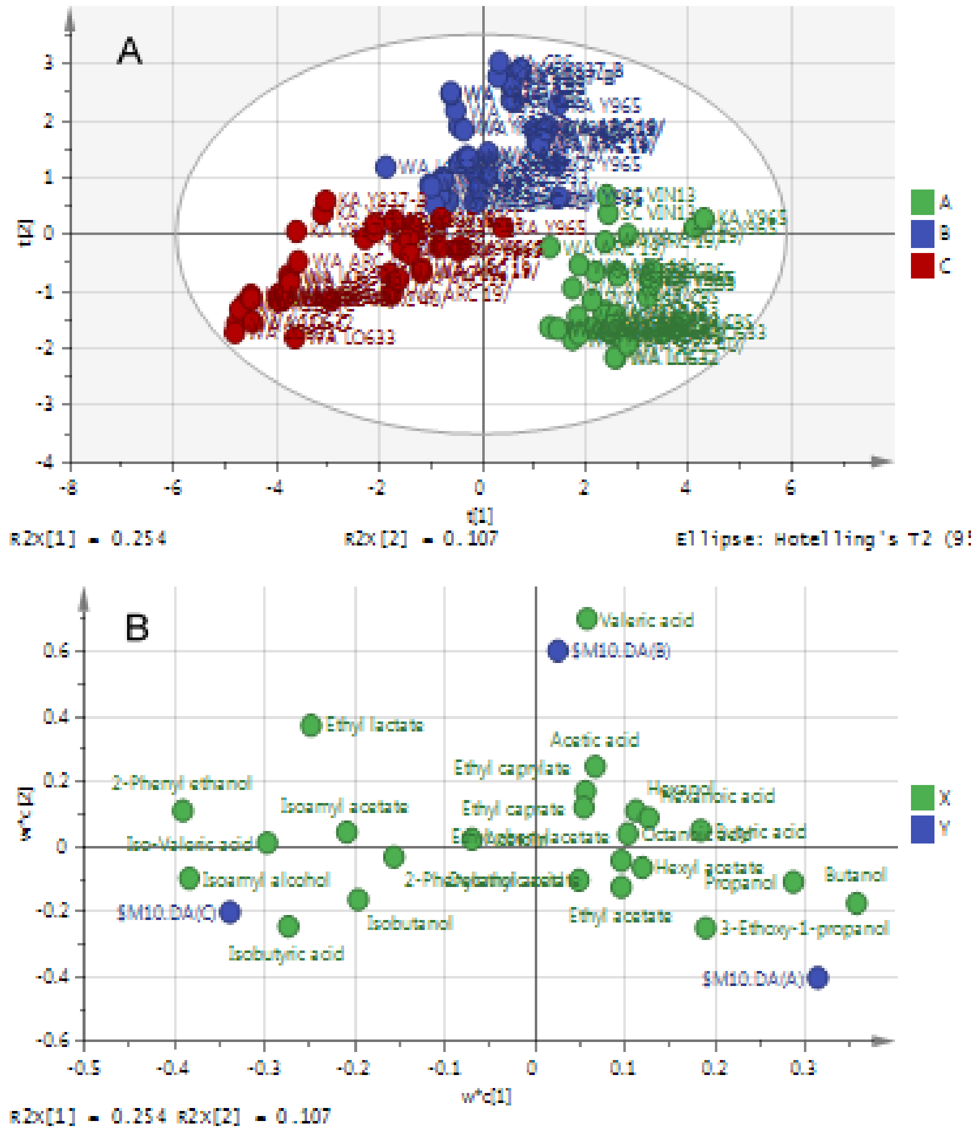
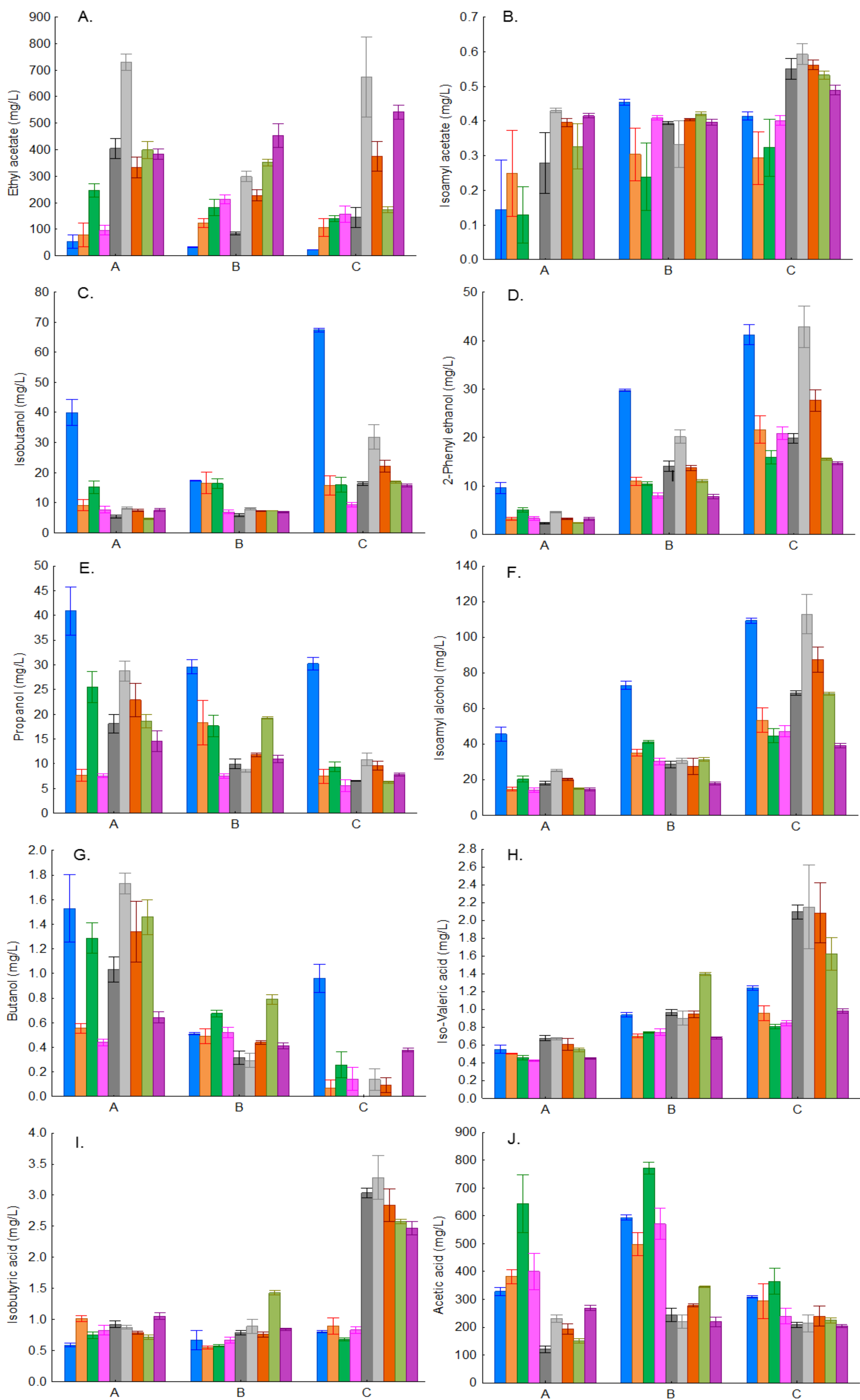


Figure 3.11 PLS-DA scores plot (A) showing the effect of treatments (A, B & C) on the global volatile aroma of wines fermented with different yeasts (the plots). Compounds driving differentiation is indicated in the biplot (B).


A.
Ethyl acetate (mg/L)
B.
Isoamyl acetate (mg/L)
C.
Isobutanol (mg/L)
D.
2-Phenyl ethanol (mg/L)
E.
Propanol (mg/L)
F.
Isoamyl alcohol (mg/L)
G.
Butanol (mg/L)
H.
Iso-Valeric acid (mg/L)
I.
Isobutyric acid (mg/L)
J.
Acetic acid (mg/L)
A
B
C

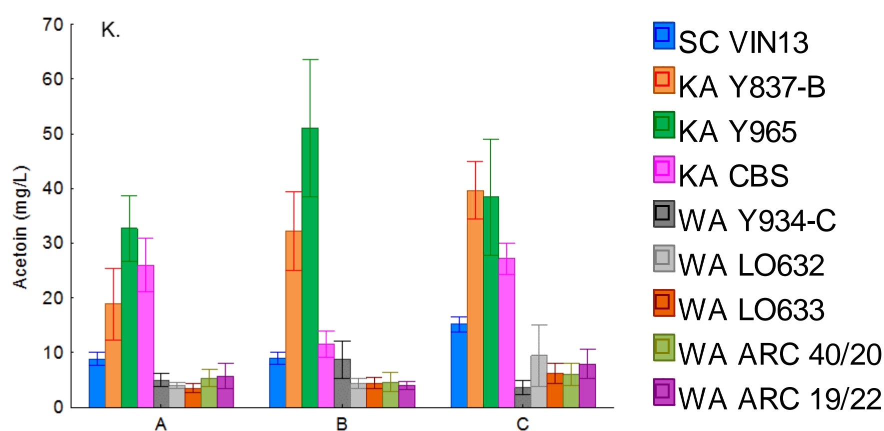


Figure 3.12 Major volatile compounds produced by single culture fermentations of *S. cerevisiae* (SC), *K. aerobia* (KA) and *W. anomalus* (WA) on day 14 in synthetic grape must fermentations with different nitrogen additions indicated as A (only ammonium), B (all of the amino acids) and C (BCAA's) on the horizontal axis. Compounds measured are A – ethyl acetate; B - isoamyl acetate; C – isobutanol; D – 2-phenyl ethanol; E – propanol; F – isoamyl alcohol; G – butanol; H – isovaleric acid; I –isobutyric acid; J – acetic acid; K – acetoin indicated in mg/L as the average of three biological repeats (each with one or two technical repeats) with standard error bars.

3.4 Discussion

3.4.1 Phenotypic characterisation with stress assays

Wickerhamomyces anomalus showed to be very resilient to the different stresses compared to *S. cerevisiae* and can easily survive in the wine environment. This yeast had higher tolerance to oxygen, saline and sugar, which is characteristic of this species as it is known to survive in stressful environments (Kurtzman, 2011). *Saccharomyces cerevisiae* is not resistant to high levels of sodium chloride (Mendes et al., 2013). The high resistance of *W. anomalus* to oxygen is advantageous as yeast cells are known to synthesize reactive oxygen species (ROS) when limited oxygen is available (Mendes-Ferreira et al., 2010). In addition, prominent isolate differences could be seen and the stresses categorised the isolates into 7 possible groups. This species is known to show large physiological variation (Kurtzman, 2011), similar to *S. cerevisiae*, for which large variations in phenotypes have been reported (Barbosa et al., 2014; Cubillos et al., 2011; Kvitek et al., 2008; Liti et al., 2009; Warringer et al., 2011). However, the impact of the stresses was not so marked for the *K. aerobia* isolates. This yeast was less resistant to the stresses than *S. cerevisiae*. These yeasts were all sourced from the same environment and it is likely that they are all the same strain.

This is the first attempt to phenotypically characterise *K. aerobia* and *W. anomalus* by means of stress assays. These results not only show the stress level of the isolates but enabled differentiation between isolates. Other studies also found the stress assays to be effective in discriminating between strains (Barbosa et al., 2014; Kvitek et al., 2008; Mendes et al., 2013).

3.4.2 Genotypic characterisation with RAPD analysis

The RAPD analysis confirmed that there were no differences between the *K. aerobia* yeasts isolated from Stellenbosch. Elsewhere, Lin et al. (1996) also struggled to detect strain differences in isolates from the same source suggesting that geographical separation could be the major driver of strain development. The *K. aerobia* strain CBS 9918, was isolated from aerobically decomposing maize silage in Japan (Lu et al., 2004). As expected, it appeared genetically different, although it was phenotypically similar to the Stellenbosch isolates further asserting the hypothesis that for *K. aerobia,* location is the determinant of yeast genetic variability.

Genetic characterisation of the *W. anomalus* isolates confirmed the findings from the phenotypic stress assay, although no genetic differences were evident between the Y934 isolates. The use of different primers could improve the accuracy of the RAPD analysis since it is documented that not all primers are capable of identifying DNA polymorphisms (Lin et al., 1996; Zahavi et al., 2002). However, some studies have reported that the use of only two primers are sufficient for strain characterisation and no further knowledge is gained by increasing the number of primers (Hopkins, 2001). In this study, using the OPA-01 primer only could not differentiate between the *W. anomalus* isolates Y934, LO632, LO633 and the ARC isolates 40/8, 40/10, 40/20, but adding primers OPA-05 and OPA-09 showed the differences in strains.

3.4.3 Impact of different nitrogen compositions on single culture fermentations of *K. aerobia* and *W. anomalus*

3.4.3.1 Fermentation kinetics

In order to reduce variability and create a constant environment to investigate the physiological reaction and metabolite production of yeast it is the best to use synthetic grape must to optimise the results (Barrajón-Simancas et al., 2011; Carrau et al., 2008). Single culture fermentations with different isolates of the *K. aerobia* and *W. anomalus* yeast showed that these yeasts do not ferment wines to dryness, echoing findings by Jolly et al. (2003) after fermenting SGM with *Hanseniaspora uvarum*, *Starmarella bombicola*, *Candida pulcherrima* and *C. colliculosa.* The slower fermentation rate is also consistent with previous data and is a general trait of non-*Saccharomyces* yeasts (Ciani et al., 2010; Jolly et al., 2003a). These yeasts are glucophilic like *S. cerevisiae* (Mains, 2014) and most other non-*Saccharomyces* yeasts (De Koker, 2015). Fructose utilisation had a significant impact on the duration of fermentation. The bigger the ratio between glucose and fructose, the weaker the fermentative performance of the isolates were (Barbosa et al., 2014; Berthels et al., 2004). Low consumption of sugars of the non-*Saccharomyces* yeasts could be attributed to the low ammonia consumption by these yeasts. Studies have found that when nitrogen is utilised in higher amounts, fermentation is conducted at a faster rate (Barbosa et al., 2014). Although, data suggests

Stellenbosch University https://scholar.sun.ac.za

that *W. anomalus* yeasts used nitrogen more for biomass formation and less for fermentation performance (Berthels et al., 2004).

Amino acid concentration in musts possibly do not affect the rate of fermentation (Arias-Gil et al., 2007). Indeed, a previous study, conducted under the same nitrogen conditions as the current study, showed no significant differences in terms of sugar consumption (Smit, 2013); similar to the current findings. However, others found more rapid CO_2 production in wine with added ammonia compared to the addition of only amino acids (Miller et al., 2007). Moreover, similar amino acid utilisation was observed for *S. cerevisiae* and the non-*Saccharomyces* yeasts, although indigenous yeasts have been found to consume less amino acids compared to commercial *S. cerevisiae* strains (Barrajón-Simancas et al., 2011). However, these values do not account for possible differences in consumption of specific amino acids (Jiranek et al., 1995; Mckinnon, 2013).

Most amino acids can be synthesised by *S. cerevisiae*, although it is strain dependant as to which specific amino acids are synthesised (Barrajón-Simancas et al., 2011). In addition, secretion of amino acids, clearly observed in the fermentation with no added amino acids, is possibly a function of autolysis (Hernawan and Fleet, 1995; Martinez-Rodriquez and Polo, 2000). Others observed nitrogen secretion by yeast during the later stages of fermentation due to an increase in ethanol concentration which increased membrane permeability while solute active transport is decreased (Monteiro and Bisson, 1992; Ough et al., 1991). Furthermore, amino acids (proline, methionine, leucine, tryptophan and cysteine) can be secreted in fermentation possibly for the reoxidation of NAD(P)H to restore the redox balance in wine (Valero et al., 2003).

Moreover, it has been noted that ammonium is not fully consumed without shaking of the fermentation vessels (De Koker, 2015; Vilanova et al., 2007), possibly causing the high residual ammonia concentration in this study. Although, to the contrary, others found ammonium to be completely consumed in a spontaneous fermentation, regardless of the amino acid concentration of the must (Arias-Gil et al., 2007). Uptake of ammonium is preferred by yeast compared to other nitrogen sources, impacting the nitrogen catabolite repression influencing metabolism of amino acids (Cooper and Sumrada, 1983; Valero et al., 2003). This preference can lead to reduced utilisation of amino acids when ammonia is added to must (Miller et al., 2007; Smit, 2013). Although, when amino acids were present in the grape must, less ammonia was utilised by *W. anomalus*, suggesting that *W. anomalus* prefer amino acids to ammonia.

Slight differences between isolates, although genetically the same strain, is expected as individual phenotypic variation, not only due to the genotype, but also to environmental pressures, impact how individuals respond to stress and developmental deviations (Vogt et al., 2008).

3.4.3.2 Major volatile aroma production

Major volatile aroma compounds were affected by the addition of amino acids, confirming the work of previous authors (Arias-Gil et al., 2007; Mckinnon, 2013; Smit, 2013). Many have studied the effect of BCAA's on higher alcohols and acids (García et al., 1994; Hazelwood et al., 2008; Mendes-Ferreira et al., 2011) and the corresponding esters (Hernández-Orte et al., 2002; Herraiz and Ough, 1993; Saerens et al., 2010). Indeed, the increase in isobutyric acid and isobutanol can be attributed to the increased presence of valine (Barrajón-Simancas et al., 2011; Mendes-Ferreira et al., 2011). The BCAA's, phenylalanine and leucine, lead to an increase in 2-phenyl ethanol, and isovaleric acid and isoamyl alcohol (including its esterified acetate – isoamyl acetate) respectively (Boulton et al., 1996; Mendes-Ferreira et al., 2011). The *W. anomalus* yeasts were able to convert amino acids more effectively into aroma compounds, possibly due to an increase in branched-chain amino acid transaminases (BCAAT) (Lilly et al., 2006). Additionally, *W. anomalus* produced significantly more biomass than *K. aerobia* which added to the increase in aroma compounds (Bell and Henschke, 2005). In addition, high production of isoamyl acetate by *W. anomalus* was previously reported (Rojas et al., 2003). The increased amino acid contribution in the treatment with BCAA's, possibly lead to a greater consumption of these compounds, further increasing the aroma profile (Arias-Gil et al., 2007). Although nitrogen in the must was sufficient, amino acids were utilised for secondary metabolite production (Miller et al., 2007). These findings possibly indicate the similarities in the metabolisms of *S. cerevisiae, K. aerobia* and *W. anomalus.*

The reason for the decreased production of propanol and butanol is uncertain, although a decrease in propanol production have been found in a setup with all amino acids compared to only ammonium (Smit, 2013). However, it was not similarly decreased when the BCAA's were present. Increase of amino acids in the treatment with only ammonium as nitrogen, attributed to the aromatic profile of yeast. Tyrosine, phenylalanine, isoleucine and leucine are secreted by yeast during fermentations and these amino acids possibly led to the increase in aromatic compounds (*e.g.* 2-phenyl ethanol, and isovaleric acid and isoamyl alcohol) in the fermentation treatments with no added amino acids (Arias-Gil et al., 2007). Furthermore, tyrosine and phenylalanine are only secreted when small quantities of amino acids are present in the grape musts, leading to an increase in amino acid secretion in the absence of amino acids (Arias-Gil et al., 2007).

Many non-*Saccharomyces* yeasts are known to produce high amounts of acetic acid, similarly found for *K. aerobia* (Ciani et al., 2010). Interestingly, the presence of BCAA's resulted in lower acetic acid production, as observed previously for *S. cerevisiae* (Mckinnon, 2013). It has been reported that *W. anomalus* produce high amounts of acetic acid (Rojas et al., 2003), although it was not observed in this study, showing the importance of strain variation. The high amount of ammonia left in the wines fermented with *K. aerobia* could possibly add to the increased concentrations of acetoin and acetic acid present in these wines (Bell and Henschke, 2005; Carrau, 2006; Vilanova et al., 2007).

Bell & Henschke (2005) showed that branched-chain fatty acid and ester concentrations are higher at lower nitrogen levels, and acetic acid and medium-chain fatty esters increased at higher nitrogen levels in the must.

Non-*Saccharomyces* yeasts from the genera *Candida, Hansenula* and *Pichia,* have been found to produce high amounts of ethyl acetate (Plata et al., 2003; Rojas et al., 2003; Romano et al., 1997). The levels of ethyl acetate produced in this study (especially by *W. anomalus*) is undesirable in wine fermentations and contributes to a nail polish remover, glue, varnish aroma (Ribéreau-Gayon, 1978). High nitrogen concentrations can lead to an increase in ethyl acetate, as seen in the fermentations with *K. aerobia* (Bell and Henschke, 2005).

In general, the non-*Saccharomyces* yeasts used were not as aromatic as *S. cerevisiae*, although this could be attributed to relative biomass production. Although, this specific *S. cerevisiae* strain, VIN13, is commercialised to produce aromatic wines (Anchor Yeast, South Africa). The compounds ethyl acetate, isoamyl acetate, 2-phenyl ethanol and isoamyl alcohol increased with increased biomass production of isolates. Differences in aroma production by different strains in studies focussing on nitrogen additions have been observed previously (Hernández-Orte et al., 2005; Vilanova et al., 2007).

3.5 Conclusion

Kazachstania aerobia isolates could not be phenotypically classified into different strains, but were genetically different from the CBS reference strain. *Wickerhamomyces anomalus* isolates were categorised into seven phenotypic groupings based on environmental stress factors and five strains using RAPD analysis.

In single culture fermentations, both non-*Saccharomyces* yeasts were found to be weak fermenters, although *W. anomalus* produced a biomass similar to *S. cerevisiae*. The chemical profile of wine was indeed altered by these yeasts, although they are not as aromatic as *S. cerevisiae*. This study showed the impact of amino acids on the aroma profile of wines and is the first to report on the use of nitrogen by these two non-*Saccharomyces* yeasts. The yeasts response to amino acids is similar to that of *S. cerevisiae*, although *W. anomalus* showed a significantly higher production of certain compounds. High production of acetic acid and ethyl acetate for respectively *K. aerobia* and *W. anomalus* is a cause of concern when these yeasts are present in must.

This study gives insight into the phenotypic space in terms of fermentative performance and aroma production of *K. aerobia* and *W. anomalus* yeasts. It was found that isolates differed between geographical locations, and identified as possible different strains. Additional stress assays could show supplementary differences between isolates, in addition to using a greater database of strains, especially when characterising *K. aerobia*.

Stellenbosch University https://scholar.sun.ac.za

3.6 References

Agustini, B.C., Silva, L.P., Bloch, C., Bonfim, T.M.B., Da Silva, G.A., 2014. Evaluation of MALDI-TOF mass spectrometry for identification of environmental yeasts and development of supplementary database. Appl. Microbiol. Biotechnol. 98, 5645–5654.

Ali, M.N., Khan, M.M., 2014. Screening, identification and characterization of alcohol tolerant potential bioethanol producing yeasts. Curr. Res. Microbiol. Biotechnol. 2, 316–324.

Arias-Gil, M., Garde-Cerdán, T., Ancín-Azpilicueta, C., 2007. Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. Food Chem. 103, 1312–1318.

Bagheri, B., 2014. Comparative analysis of fermentative yeasts during spontaneous fermentation of grapes from different management systems. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Barbosa, C., Lage, P., Vilela, A., Mendes-Faia, A., Mendes-Ferreira, A., 2014. Phenotypic and metabolic traits of commercial *Saccharomyces cerevisiae* yeasts. AMB Express 4, 1–14.

Barrajón-Simancas, N., Giese, E., Arévalo-Villena, M., Úbeda, J., Briones, A., 2011. Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. Food Chem. 127, 441–446.

Beckner Whitener, M.E., 2016. Metabolomic profiling of non-*Saccharomyces* yeasts in wine. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Bell, S.J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242–295.

Berthels, N.J., Cordero Otero, R.R., Bauer, F.F., Thevelein, J.M., Pretorius, I.S., 2004. Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Res. 4, 683–689.

Boulton, R.B., Singleton, V.L., Bisson, L.F., Kunkee, R.E., 1996. Principles and practices of winemaking, 1st ed. Springer US, New York.

Camarasa, C., Sanchez, I., Brial, P., Bigey, F., Dequin, S., 2011. Phenotypic landscape of *Saccharomyces cerevisiae* during wine fermentation: Evidence for origin-dependent metabolic traits. PLoS One 6, e25147.

Carrau, F., 2006. Native yeasts for low input winemaking: Searching for wine diversity and increased complexity. Int. Wine Microbiol. Symp. 33–39.

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. FEMS Yeast Res. 8, 1196–1207.

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

Stellenbosch University https://scholar.sun.ac.za

Cooper, T.G., Sumrada, R.A., 1983. What is the function of nitrogen catabolite repression in *Saccharomyces cerevisiae*? J. Bacteriol. 155, 623–627.

Cubillos, F.A., Billi, E., Zörgö, E., Parts, L., Fargier, P., Omholt, S., Blomberg, A., Warringer, J., Louis, E.J., Liti, G., 2011. Assessing the complex architecture of polygenic traits in diverged yeast populations. Mol. Ecol. 20, 1401–1413.

De Koker, S., 2015. Nitrogen utilisation of selected non-*Saccharomyces* yeasts and the impact on volatile compound production. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Dickinson, J.R., Harrison, S.J., Dickinson, J.A., Hewlins, M.J.E., 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 275, 10937–10942.

Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 273, 25751–25756.

Dickinson, J.R., Lanterman, M.M., Danner, D.J., Pearson, B.M., Sanz, P., Harrison, S.J., Hewlins, M.J., 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 272, 26871–26878.

Dunn, B., Richter, C., Kvitek, D.J., Pugh, T., Sherlock, G., 2012. Analysis of the *Saccharomyces cerevisiae* pan-genome reveals a pool of copy number variants distributed in diverse yeast strains from differing industrial environments. Genome Res. 22, 908–924.

García, A.I., García, L.A., Díaz, M., 1994. Fusel alcohols production in beer fermentation processes. Process Biochem. 29, 303–309.

Hazelwood, L.A., Daran, J., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl. Environ. Microbiol. 74, 2259–3920.

Hernández-Orte, P., Cacho, J.F., Ferreira, V., 2002. Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. J. Agric. Food Chem. 50, 2891–2899.

Hernández-Orte, P., Ibarz, M.J., Cacho, J., Ferreira, V., 2005. Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. Food Chem. 89, 163–174.

Hernawan, T., Fleet, G., 1995. Chemical and cytological changes during the autolysis of yeasts. J. Ind. Microbiol. 14, 440–450.

Herraiz, T., Ough, C.S., 1993. Formation of ethyl esters of amino acids by yeasts during the alcoholic fermentation of grape juice. Am. J. Enol. Vitic. 44, 41–48.

Hopkins, K.L., 2001. Use of multiple primers in RAPD analysis of clonal organisms provides limited improvement in discrimination. Biotechniques 30, 1262–1267.

Stellenbosch University https://scholar.sun.ac.za

Izquierdo Cañas, P.M., Esteban, C., Romero, G., Heras, J.M., Mónica, M., González, F., 2014. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. Eur. Food Res. Technol. 239, 279–286.

Izquierdo Cañas, P.M., Palacios Garcia, A.T., Garcia Romero, E., 2011. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starter. Vitis 50, 177–182.

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Amino acid and ammonium utilization by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. Am. J. Enol. Vitic. 46, 75–83.

Jolly, N.P., 2004. Characterisation, evaluation and use of non-*Saccharomyces* yeast strains isolated from vineyards and must. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Jolly, N.P., Augustyn, O.H.P., Pretorius, I.S., 2003. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55–62.

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

Kurtzman, C.P., 2011. *Wickerhamomyces*; Kurtzman, Robnett & Basehoar-Powers (2008), in: Kurtzman, C.P., Fell, J.W., Boekhout, T. (Eds.), The Yeasts, A Taxonomic Study. Elsevier B.V., London, UK, pp. 902–903.

Kvitek, D.J., Will, J.L., Gasch, A.P., 2008. Variations in stress sensitivity and genomic expression in diverse *S. cerevisiae* isolates. PLoS Genet. 4, e1000223.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its importance to wine aroma - A review. S. Afr. J. Enol. Vitic. 21, 97–129.

Lilly, M., Bauer, F.F., Styger, G., Lambrechts, M.G., Pretorius, I.S., 2006. The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. FEMS Yeast Res. 6, 726–743.

Lin, A.W., Usera, M.A., Barrett, T.J., Goldsby, R.A., 1996. Application of random amplified polymorphic DNA analysis to differentiate strains of Salmonella enteritidis. J. Clin. Microbiol. 34, 870–876.

Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., Davey, R.P., Roberts, I.N., Burt, A., Tsai, I.J., Bergman, C.M., Bensasson, D., Michael, J.T., Kelly, O., van Oudenaarden, A., Barton, D.B.H., Bailes, E., Ba, A.N.N., Jones, M., Quail, M.A., Goodhead, I., Sims, S., Blomberg, A., Durbin, R., Louis, E.J., 2009. Population genomics of domestic and wild yeasts. Eur. PMC Funders Gr. 458, 337–341.

Llungdahl, P.O., Daignan-Fornier, B., 2012. Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. Genetics 190, 885–929.

Louw, L., Roux, K., Tredoux, A., Tomic, O., Naes, T., Nieuwoudt, H.H., Van Rensburg, P., 2009. Characterization of selected South African young cultivar wines using FTMIR Spectroscopy, Gas chromatography, and multivariate data analysis. J. Agric. Food Chem. 57, 2623–2632.

Stellenbosch University https://scholar.sun.ac.za

Lu, H.Z., Cai, Y., Wu, Z.W., Jia, J.H., Bai, F.Y., 2004. *Kazachstania aerobia* sp. nov., an ascomycetous yeast species from aerobically deteriorating corn silage. Int. J. Syst. Evol. Microbiol. 54, 2431–2435.

Mains, A.O., 2014. Evaluating the impact of yeast co-inoculation on individual yeast metabolism and wine composition. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Martinez-Rodriquez, A.J., Polo, M.C., 2000. Enological aspects of yeast autolysis. Recent Res. Dev. Microbiol. 285–301.

Mckinnon, A., 2013. The impact of amino acids on growth performance and major volatile compound formation by industrial wine yeast. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Mendes, I., Franco-Duarte, R., Umek, L., Fonseca, E., Drumonde-Neves, J., Dequin, S., Zupan, B., Schuller, D., 2013. Computational models for prediction of yeast strain potential for winemaking from phenotypic profiles. PLoS One 8, e66523.

Mendes-Ferreira, A., Barbosa, C., Lage, P., Mendes-Faia, A., 2011. The impact of nitrogen on yeast fermentation and wine quality. Ciência Téc. Vitiv. 26, 17–32.

Mendes-Ferreira, A., Sampaio-Marques, B., Barbosa, C., Rodrigues, F., Costa, V., Mendes-Faia, A., Ludovico, P., Leao, C., 2010. Accumulation of non-superoxide anion reactive oxygen species mediates nitrogen-limited alcoholic fermentation by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 76, 7918–7924.

Miller, A.C., Wolff, S.R., Bisson, L.F., Ebeler, S.E., 2007. Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in chardonnay juice fermentations. Am. J. Enol. Vitic. 58, 470–483.

Monteiro, F.F., Bisson, L.F., 1992. Nitrogen supplementation of grape juice. 1. Effect in amino acid utilization during fermentation. Am. J. Enol. Vitic. 43, 1–10.

Nikolaou, E., Soufleros, E.H., Bouloumpasi, E., Tzanetakis, N., 2006. Selection of indigenous *Saccharomyces cerevisiae* strains according to their oenological characteristics and vinification results. Food Microbiol. 23, 205–211.

Ough, C.S., Huang, Z., An, D., Stevens, D., 1991. Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: effects on urea excretion and reabsorption. Am. J. Enol. Vitic. 42, 26–40.

Plata, C., Millán, C., Mauricio, J.C., Ortega, J.M., 2003. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. Food Microbiol. 20, 217–224.

Ribéreau-Gayon, P., 1978. Wine flavour, in: Charalambous, G., Inglett, G.E. (Eds.), Flavor of Foods and Beverages. Academic Press, New York, pp. 362–371.

Rojas, V., Gil, J. V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int. J. Food Microbiol. 86, 181–188.

Romano, P., Suzzi, G., Domizio, P., Fatichenti, F., 1997. Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. Antonie Van Leeuwenhoek 71, 239–242.

Stellenbosch University https://scholar.sun.ac.za

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 165–177.

Smit, A.Y., 2013. The impact of nutrients on aroma and flavour production during wine fermentation. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Ugliano, M., Henschke, P.A., Herderich, M.J., Pretorius, I.S., 2007. Nitrogen management is critical for wine flavour and style. Wine Ind. J. 22, 24–30.

Valero, E., Millán, C., Ortega, J.M., Mauricio, J.C., 2003. Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains. J. Sci. Food Agric. 83, 830–835.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Appl. Microbiol. Biotechnol. 77, 145–157.

Vogt, G., Huber, M., Thiemann, M., van den Boogaart, G., Schmitz, O.J., Schubart, C.D., 2008. Production of different phenotypes from the same genotype in the same environment by developmental variation. J. Exp. Biol. 211, 510–23.

Warringer, J., Zörgö, E., Cubillos, F.A., Zia, A., Gjuvsland, A., Simpson, J.T., Forsmark, A., Durbin, R., Omholt, S.W., Louis, E.J., Liti, G., Moses, A., Blomberg, A., 2011. Trait variation in yeast is defined by population history. PLoS Genet. 7, e1002111.

Zahavi, T., Droby, S., Cohen, L., Wiss, B., Ben-Arie, R., 2002. Characterization of the yeast flora on the surface of grape berries in Israel. Vitis 41, 203–208.

Zuzuarregui, A., del Olmo, M., 2004. Analyses of stress resistance under laboratory conditions constitute a suitable criterion for wine yeast selection. Antonie Van Leeuwenhoek 85, 271–280.

Stellenbosch University https://scholar.sun.ac.za

3.7 Appendix

Table 1. Mean end point CO_2 and OD_{600} with one way ANOVA post hoc analysis for single culture fermentations of *S. cerevisiae* VIN13 and *K. aerobia* isolates conducted in three different nitrogen treatments – A) with only ammonia, B) with amino acids and ammonia, C) with BCAA's and ammonia.

Treatment	Yeast species	CO_2 production	OD_{600}
A	VIN13	7.81 ± 0.57^{a}	4.89 ± 0.33^{a}
A	Y837-B	3.04 ± 0.83^{b}	1.78 ± 0.08^{d}
A	Y965	3.80 ± 0.03^{b}	3.12 ± 0.10^{bc}
A	CBS	2.25 ± 0.55^{b}	1.51 ± 0.16^{d}
B	VIN13	7.57 ± 0.32^{a}	5.11 ± 0.51^{a}
B	Y837-B	3.59 ± 0.35^{b}	3.52 ± 0.45^{bc}
B	Y965	3.38 ± 0.18^{b}	3.12 ± 0.05^{bc}
B	CBS	2.73 ± 0.34^{b}	2.46 ± 0.03^{cd}
C	VIN13	7.15 ± 0.39^{a}	3.92 ± 0.27^{ab}
C	Y837-B	4.20 ± 0.23^{a}	2.82 ± 0.29^{bcd}
C	Y965	4.19 ± 0.54^{a}	2.67 ± 0.16^{bcd}
C	CBS	2.30 ± 0.13^{b}	2.26 ± 0.09^{cd}

Values with the same letter in the same column are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

Stellenbosch University https://scholar.sun.ac.za

Table 2. Mean end point CO_2 and OD_{600} with one way ANOVA post hoc analysis for single culture fermentations of *S. cerevisiae* VIN13 and *W. anomalus* conducted in three different nitrogen treatments – A) with only ammonia, B) with amino acids and ammonia, C) with BCAA's and ammonia.

Treatment	Yeast species	CO_2 production (g)	OD_{600}
A	VIN13	7.81 ± 0.57^{a}	4.89 ± 0.33^{abc}
A	Y934-C	2.18 ± 0.50^{c}	3.69 ± 0.06^{defg}
A	LO632	2.91 ± 0.70^{bc}	4.07 ± 0.08^{bcdef}
A	LO633	2.03 ± 0.07^{c}	3.46 ± 0.08^{efg}
A	ARC 40/20	1.91 ± 0.12^{c}	3.67 ± 0.07^{defg}
A	ARC 19/22	2.62 ± 0.28^{c}	2.76 ± 0.11^{g}
B	VIN13	7.57 ± 0.32^{a}	5.11 ± 0.51^{ab}
B	Y934-C	3.22 ± 0.11b^{c}	4.18 ± 0.07^{bcdef}
B	LO632	4.58 ± 0.27^{b}	4.20 ± 0.27^{bcdef}
B	LO633	3.21 ± 0.13b^{c}	4.09 ± 0.07^{bcdef}
B	ARC 40/20	2.49 ± 0.37^{c}	4.20 ± 0.37^{bcdef}
B	ARC 19/22	1.93 ± 0.10^{c}	3.36 ± 0.26^{efg}
C	VIN13	7.15 ± 0.39^{a}	3.92 ± 0.27^{cdefg}
C	Y934-C	1.82 ± 0.11^{c}	4.82 ± 0.08^{abcd}
C	LO632	2.88 ± 0.46^{bc}	5.50 ± 0.25^{a}
C	LO633	2.47 ± 0.18^{c}	4.83 ± 0.10^{abcd}
C	ARC 40/20	2.01 ± 0.40^{c}	4.53 ± 0.22^{abcde}
C	ARC 19/22	1.86 ± 0.14^{c}	3.13 ± 0.04^{fg}

Values with the same letter in the same column are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

Stellenbosch University https://scholar.sun.ac.za

Chapter 4

Research results

Determining the fermentation potential and aroma production of non-*Saccharomyces* yeast in mixed culture fermentations with *Saccharomyces cerevisiae*

Chapter 4 – Determining the fermentation potential and aroma production of non-*Saccharomyces* yeast in mixed culture fermentations with *Saccharomyces cerevisiae*

4.1 Introduction

The impact of non-*Saccharomyces* yeasts on the aroma bouquet and the development of unique and complex wines have been investigated by several research groups (Anfang et al., 2009; Ciani et al., 2010; Gobbi et al., 2013; Izquierdo Cañas et al., 2011; Jolly et al., 2014; Lambrechts and Pretorius, 2000; Lema et al., 1996; Rossouw and Bauer, 2016; Sadoudi et al., 2012; Soden et al., 2000; Swiegers et al., 2005). With the use of inoculated *Saccharomyces* starter cultures, a rapid and reliable fermentation is usually ensured, although indigenous yeast tend to be suppressed (Fleet and Heard, 1993b; Mas et al., 2016). However, it is the general observation that the indigenous microflora contributes to the aromatic complexity of wine, and it has been hypothesised that typical terroir specific characters of wine may in part be the result of the impact of the regional microflora (Bokulich et al., 2013). Spontaneous fermentations are thus also perceived to counteract the perceived uniformity of *S. cerevisiae* fermentations (Mas et al., 2016).

More than 40 yeast species have been isolated from grape must and these can be further divided into numerous different strains (Jolly et al., 2014). The impact on fermentation for many of these species and strains is still relatively unknown. In spontaneous fermentations, sequential dominance of yeast populations have been reported (Jackson, 2008). In order to more accurately evaluate the impact of yeast in a natural fermentation, the contribution of each yeast species needs to be fully characterised and compared to the traditional wine yeast *S. cerevisiae.* Furthermore, in most spontaneous fermentations, it is known that *S. cerevisiae* eventually dominates the microbial biomass and completes the fermentation (Fleet and Heard, 1993b). Thus, the relationship of any non-*Saccharomyces* yeast of oenological importance with *S. cerevisiae* is worthy of investigation. Two inoculation strategies are usually followed with these mixed culture fermentations: *S. cerevisiae* is either inoculated simultaneously to the non-*Saccharomyces* yeasts (known as a co-inoculation) or sequentially 1 hour to 15 days later (known as a sequential inoculation) (Herraiz et al., 1990; Jolly et al., 2003a; Soden et al., 2000).

Studies in South Africa have indicated that the non-*Saccharomyces* yeasts *Kazachstania aerobia* and *Wickerhamomyces anomalus* may be present in uncommonly high numbers in South African grape must when compared to similar data from other wine growing regions (Bagheri et al., 2015; Setati et al., 2012). Dataset on these non-*Saccharomyces* yeasts indigenous to South African grape musts and their effect on aroma and fermentation is limited. Previous studies have been conducted using either metagenomics or culture based methods and showed that the presence of indigenous yeast species during wine-making significantly impacted the character of South African wine (Jolly

Stellenbosch University https://scholar.sun.ac.za

et al., 2003b; Setati et al., 2012). Consequently, it is paramount that the impact of individual non-*Saccharomyces* yeasts and their contributions to fermentation be further evaluated. *Kazachstania aerobia* has only recently been used in alcoholic fermentation with *S. cerevisiae*, and data suggest an increase in esters, ethyl acetate and terpenes, although, sensorially, wines were characterised as bitter and as presenting a solvent-like character (Beckner Whitener et al., 2016). *Wickerhamomyces anomalus* (formerly *Hansenula anomala* and *Pichia anomala*) on the other hand has been used successfully in sequential inoculation with *S. cerevisiae* and products have been reportedly favoured by tasters (Izquierdo Cañas et al., 2014). These wines showed an increase in lineal alcohols and ethyl and acetate esters.

The aim of this study is to investigate the impact of mainly South African isolates of *K. aerobia* and *W. anomalus* on fermentations when fermented as single and mixed cultures with *S. cerevisiae* in synthetic grape must and Sauvignon blanc grape must. Sauvignon blanc is one of the most commonly cultivated varieties in South Africa and was thus chosen for this study to give a more realistic view on the possible effects of these yeasts on fermentation. This study is a further stepping stone to understanding the yeast microbiome and its impact on fermentations in a South African and possibly global context, shedding light on possible strain differences within species.

4.2 Materials and Methods

4.2.1 Mixed culture fermentations: *K. aerobia* and *W. anomalus* with *S. cerevisiae* in synthetic grape must

4.2.1.1 Inoculation strategy

Fermentations were conducted in synthetic grape must (SGM) composed as described earlier in section 3.2.4.1 for the treatment with a yeast assimilable nitrogen (YAN) component consisting of all amino acids contributing in equal amounts. All fermentations were performed in triplicate in 100 mL spice bottles fitted with fermentation locks containing 80mL SGM, with the exception of the *W. anomalus* sequential culture fermentations that contained 60mL SGM.

All strains were cultured as described previously in section 3.2.1 and grown overnight in at 30°C in Yeast Peptone Dextrose (YPD) broth (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar), purchased from Biolab, SA.

Mixed culture fermentations with *K. aerobia* were conducted with the *K. aerobia* Y837-B and Y965 isolates and *K. aerobia* CBS 9918 strain, while *S. cerevisiae* VIN13 (Anchor, SA) served as the control wine yeast. Inoculation rates are shown in Table 4.1. An OD_{600} of 0.01 equates to a cell number of 10^5 colony forming units per mL (cfu/mL), OD_{600} of 0.1 equates to a cell number of 10^6 cfu/mL and OD_{600} of 1 equates to a cell number of 10^7 cfu/mL

Table 4.1 Treatment outline for fermentations in SGM with *K. aerobia*

Treatment	Yeast species	Inoculation density (OD_{600})	Time (h) *	Ratio (*K. aerobia*: VIN13)
A. Monoculture	*K. aerobia*	1	0	-
	VIN13	0.1	0	-
	VIN13	0.01	0	-
B. Co-inoculation	*K. aerobia*	1	0	10:1
	VIN13	0.1	48	
C. Co-inoculation	*K. aerobia*	1	0	100:1
	VIN13	0.01	48	
D. Sequential inoculation	*K. aerobia*	1	0	10:1
	VIN13	0.1	48	

*hours after start of fermentation

Co-culture fermentations with *W. anomalus* were conducted with the *W. anomalus* strains Y934-C, LO632, LO633, ARC 40/20, ARC 19/22, including *S. cerevisiae* VIN13 and EC1118 (Lallemand, SA). Each *W. anomalus* strain was inoculated with VIN13 and EC1118 respectively. For the sequential culture fermentations only *W. anomalus* Y934-C and LO632 were used. Here also monocultures of *W. anomalus* Y934-C and LO632 and *S. cerevisiae* VIN13 and EC1118 was fermented. Inoculation of the yeasts occurred simultaneously, except for the sequential culture fermentations where *S. cerevisiae* strains were inoculated after 48 hours. For the fermentation setup with *W. anomalus*, all strains were inoculated at an OD_{600} of 0.1 (10^6 cfu/mL).

All fermentations were incubated at 30°C and conducted under static conditions with the exception of being shaken once a day during weighing.

4.2.1.2 Fermentation kinetics

Carbon dioxide production and sugar consumption were used to establish the fermentation potential of the isolates. This was determined by daily weighing of fermentation flasks before and after sampling and measuring sugar (glucose and fructose) using the Arena 20XT Photometric Analyzer (Thermo Electron Oy, Finland). Fermentations were conducted for three and two weeks for *K. aerobia* and *W. anomalus* treatments respectively.

For the *K. aerobia* fermentations, samples were taken every day for the first 3 days and thereafter every second or third day. Sugar concentrations were determined for days 3, 7, 14 and 21. For the *W. anomalus* fermentations, samples were taken at day 0-3, 5, 7 and 14 as well as day 4 for the sequential culture fermentations. Sugar concentrations were determined for days 4, 7 and 14.

Stellenbosch University https://scholar.sun.ac.za

4.2.1.3 Yeast enumeration

Change in optical density (OD) at 600nm wavelength in order to determine growth and biomass formation was monitored with every sampling point as stipulated above in section 4.2.1.2. Cell viability was determined by plating out 0.1 mL aliquots at every sampling point on Wallerstein Laboratory Nutrient (WLN) agar (BioLab, Merck, South Africa). Each sample was plated out in duplicate after dilution to concentrations of 10^2 and 10^3 cfu/mL. Plates were incubated for 2 to 3 days at 30°C after which colony forming units (cfu's) were counted. The yeast was identified based on colony morphology and colour and only plates with less than 300 colonies were counted.

4.2.2 Mixed culture fermentations in Sauvignon blanc grape must

4.2.2.1 Microvinification procedure

Sauvignon blanc grapes were sourced from Welgevallen farm, Stellenbosch, South Africa in February 2016. Grapes were destemmed, crushed and pressed at the Department of Viticulture and Oenology (DVO) experimental cellar according to the standard winemaking procedures. To prevent spoilage and to aid must clarification, respectively 30 ppm SO_2 and 4mL/hL pectinase (Rapidase® Clear, Anchor Yeast, SA) were added to the juice and the juice was then allowed to settle overnight at 15°C. Thereafter, the juice was racked from the sediment and the sugar content, acidity and yeast assimilable nitrogen (YAN) were determined. Acidity and YAN were adjusted to 6.46 g/L and 352 mg N/L with tartaric acid and 50 g/hLThiazote® (Laffort, France) respectively. Initial residual sugar was 229.5 g/L and after the acidity adjustment, the must had a pH of 3.34. The chemical parameters were measured using a Winescan FT120 instrument (FOSS Analytical A/S, Hillerød, Denmark). The juice was then aliquoted into 100 mL fermentation vessels prior to inoculation.

4.2.2.2 Yeast species, isolates and strains

Grape must was fermented with two *W. anomalus* (Y934-C and LO632) and two *K. aerobia* strains (Y965 and CBS). *Saccharomyces cerevisiae* EC1118 was used as a control fermentation in monoculture and to conduct the sequential culture fermentations.

4.2.2.3 Inoculation strategy and culture conditions

All strains were cultured as described previously in section 3.2.1 and grown overnight at 30°C in Yeast Peptone Dextrose (YPD) broth (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar) purchased from Biolab, SA. The non-*Saccharomyces* yeasts were inoculated at an OD_{600} of 0.3 and *S. cerevisiae* EC1118 at an OD_{600} of 0.1. Sequential culture fermentations was conducted by inoculating EC1118 48 hours after the inoculation of the non-*Saccharomyces* yeast. All yeast species (non-*Saccharomyces* and *S. cerevisiae*) were also fermented as monocultures.

Spontaneous fermentations were conducted to determine population dynamics and fermentation potential of native yeast species. All fermentations were conducted in triplicate in 100mL spice bottles containing 60 mL juice fitted with fermentation locks. After inoculation, fermentations were incubated at 15°C under static conditions with the exception of being shaken once a day when weighing the flasks. Grape must was fermented until dryness was achieved (sugar level less than 2g/L).

4.2.2.4 Fermentation kinetics

Carbon dioxide production and sugar consumption were used to determine the fermentation potential of the strains. The fermentations were weighed daily, before and after sampling, and samples were taken during the lag phase (day 0, 2, 4), exponential phase (day 7, 10 and 14) and stationary phase (day 17, 21, 25 and 28).

Glucose and fructose was analysed for days 0, 10, 21, 28 using the Arena 20XT Photometric Analyzer (Thermo Electron Oy, Finland). Organic acids (malic, lactic, citric acid, tartaric acid and total acidity), saccharose, ethanol, pH and glycerol was analysed on day 21 using FT-IR ATR mid infrared spectrometry (Bruker). One mL sample was injected directly onto the diamond surface.

4.2.2.5 Yeast enumeration

Biomass was determined using optical density (OD) measurements at 600nm wavelength. These measurements were taken with every sampling point, as stipulated above in section 4.2.2.4. Cell viability was determined by plating out 0.1 mL aliquots at every sampling point on Wallerstein Laboratory Nutrient (WLN) agar (BioLab, Merck, South Africa) in the same manner as in section 4.2.1.3. The agar was supplemented with 34 mg/L chloramphenicol and 150 mg/L biphenyl for total yeast enumeration. Chloramphenicol inhibits the growth of bacteria whereas biphenyl inhibits the growth of filamentous fungi. Differentiation between yeasts were based on colour and morphology.

4.2.3 Major volatile aroma production

The major volatile aroma production was measured at end point for all fermentations using GC-FID as stated in Chapter 3. Thirty three compounds were measured, but only those within the calibration range are reported on.

4.2.4 Statistical analysis

All univariate statistical analysis were done using Statistica 13 (Dell Inc.) to infer the effects of different treatments on yeast growth, metabolite accumulation and fermentation kinetics. Multivariate data analysis was conducted using SIMCA 13 (Umetrics) to simultaneously investigate the treatment

effect on all metabolites produced. Unless stated otherwise data in tables and graphs are presented as means ± standard error of mean.

4.3 Results

4.3.1 Mixed culture fermentations: *K. aerobia* and *S. cerevisiae*

4.3.1.1 Fermentation kinetics

Kazachstania aerobia was inoculated in co-culture fermentations with *S. cerevisiae* VIN13 at inoculation ratios of 10:1 and 100:1 (non-*Saccharomyces*: *S. cerevisiae*) in synthetic grape must (SGM). In addition, *S. cerevisiae* was inoculated 48 hours after introducing *K. aerobia,* at an inoculation ratio of 10:1, in a sequential fermentation setup. Control fermentations were conducted with *S. cerevisiae* VIN13 as monocultures inoculated at OD_{600}=0.1 and OD_{600}=0.01 respectively, with additional monocultures of the *K. aerobia* isolates inoculated at OD_{600}=1. Fermentations were terminated after 21 days, at which point the total residual sugar was less than 2 g/L with the exception of the monoculture fermentations that were suspended on day 12.

The control *S. cerevisiae* fermentations had the fastest fermentation rate in terms of CO_2 production, with the exception of the co-inoculation with *K. aerobia* Y965 (ratio 10:1) (Figure 4.1). Overall the co-inoculation treatments (inoculation rate 10:1), showed the fastest sugar consumption followed by *S. cerevisiae* monocultures (Figure 4.2). Co-inoculation with *S. cerevisiae* at a higher OD (OD_{600}=0.1) resulted in a slightly faster fermentation rate compared to the co-inoculation treatments where *S. cerevisiae* was inoculated at a lower OD (OD_{600}=0.01) (Figure 4.1; Figure 4.2). Fermentation rate, in terms of CO_2 production, increased in sequential culture fermentations after addition of *S. cerevisiae* on day 2; slowing down on day 8. Irrespective of treatment, *K. aerobia* isolate Y965 in mixed culture fermentations had the fastest fermentation rate (CO_2 production and sugar consumption) whereas no significant differences were found between isolates Y837-B and CBS (Figure 4.1; Figure 4.2 – A, C). The non-*Saccharomyces* monocultures demonstrated a significantly slower fermentation rate than the mixed culture fermentations with *K. aerobia* Y965 as monoculture having the slowest fermentation rate (Figure 4.1; Figure 4.2 – B, D).

Fermentation rate slowed down after 7 days and fermentations were dry (total sugar <2 g/L) on day 21 for the mixed culture treatments and controls, with no statistical difference between treatments (Table 1, appendix). Glucose was consumed at a faster rate compared to fructose.

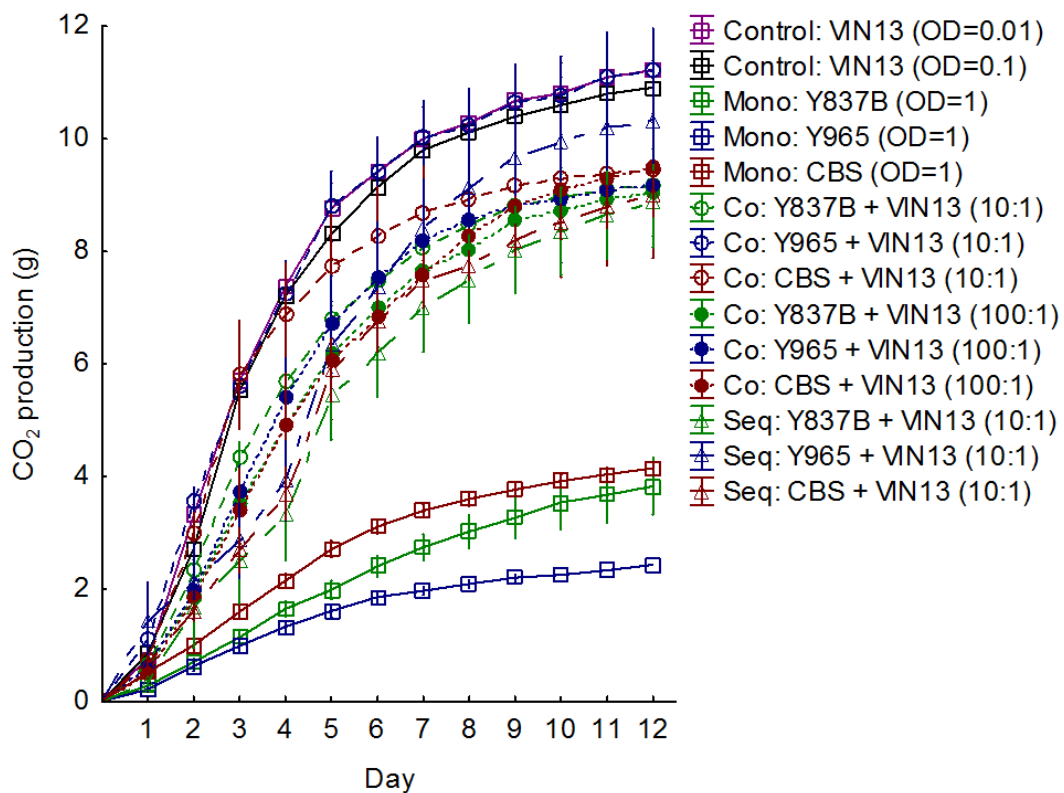


Figure 4.1 CO_2 production of monoculture fermentations (indicated with □); co-inoculation, 10:1 (indicated with O), co-inoculation, 100:1 (indicated with ●) and sequential culture fermentations (indicated with ∆) of *K. aerobia* (Y937B, Y965, CBS) and *S. cerevisiae* (VIN13). Values are the average of 3 biological repeats ± standard error of the mean.

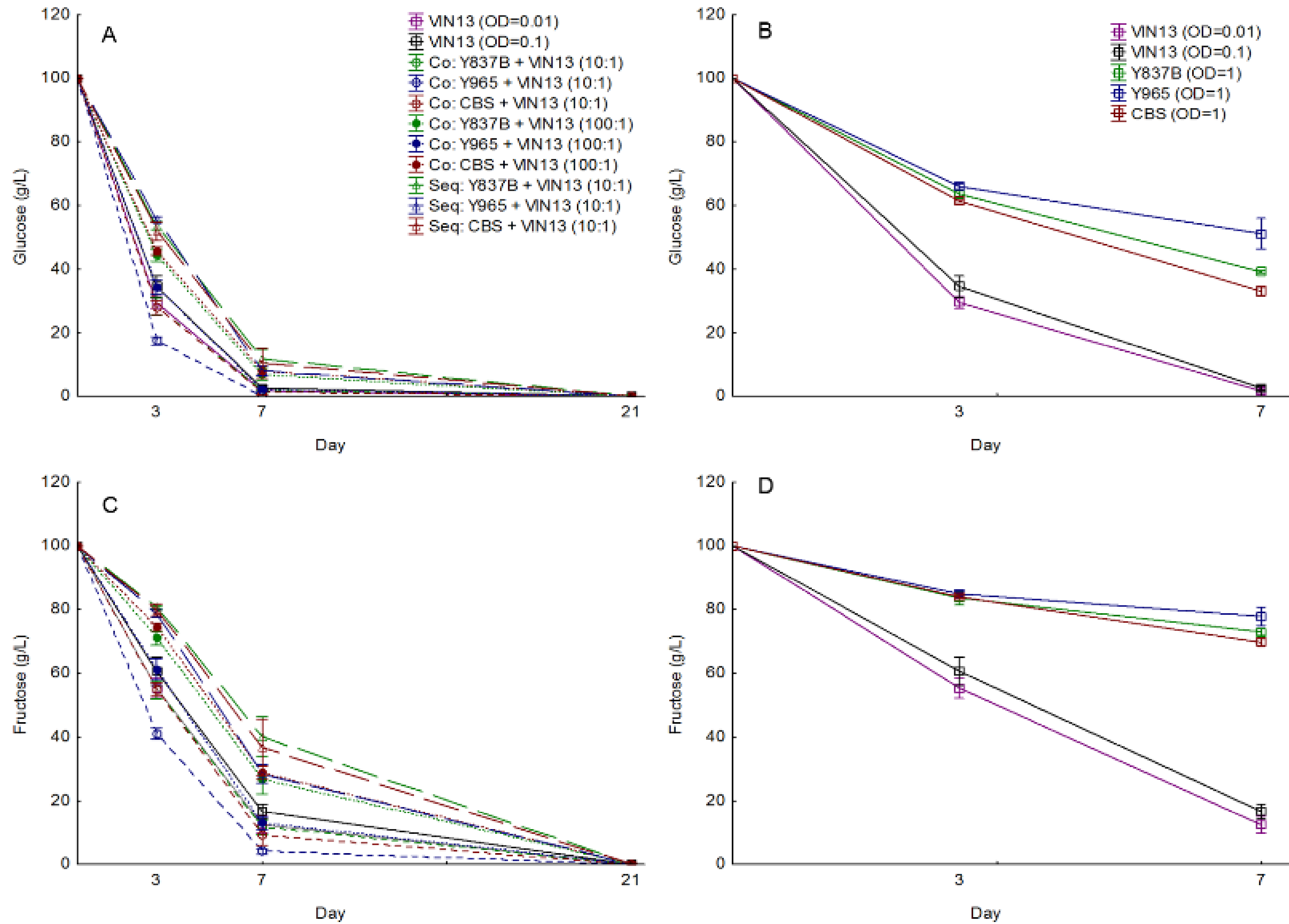


Figure 4.2 Sugar utilisation, glucose (A, B) and fructose (C,D), of *K. aerobia* (Y837B, Y965, CBS) and *S. cerevisiae* VIN13 in co- and sequential culture fermentations (graph A and C) and monocultures (graph B and D). Values are indicated as the mean ± standard error of the mean.

4.3.1.2 Yeast enumeration

All of the yeasts entered exponential phase within one day of fermentation. The *S. cerevisiae* monocultures reached a similar or lower biomass production (expressed as OD_{600}) compared to the co-inoculation treatments (Figure 4.3). Between *K. aerobia* isolates, no difference was observed amongst the monoculture fermentations, although isolate Y965 had a higher OD in the co- and sequential inoculation treatments compared to the other *K. aerobia* isolates.

When comparing yeast growth for the individual species, it is clear that the non-*Saccharomyces* yeasts impacted the growth of *S. cerevisiae*, with the slowest growth rate of this yeast observed in the sequential culture fermentations (Figure 4.4 - A). The *S. cerevisiae* population was highest for all the treatments when fermented in conjunction with *K. aerobia* Y965.

In contrast to *S. cerevisiae*, the *K. aerobia* population declined rapidly after a few days (Figure 4.4 - B). The *K. aerobia* monoculture fermentations reached the highest population of *K. aerobia* between treatments. At higher inoculations of *S. cerevisiae*, the *K. aerobia* yeasts demonstrated the fastest decline in population. By day 7 all of the non-*Saccharomyces* yeast had died off in the co-inoculation treatments, although it survived until day 9 in the sequential treatments. In all of the treatments *K. aerobia* Y965 obtained the lowest population density throughout fermentation.

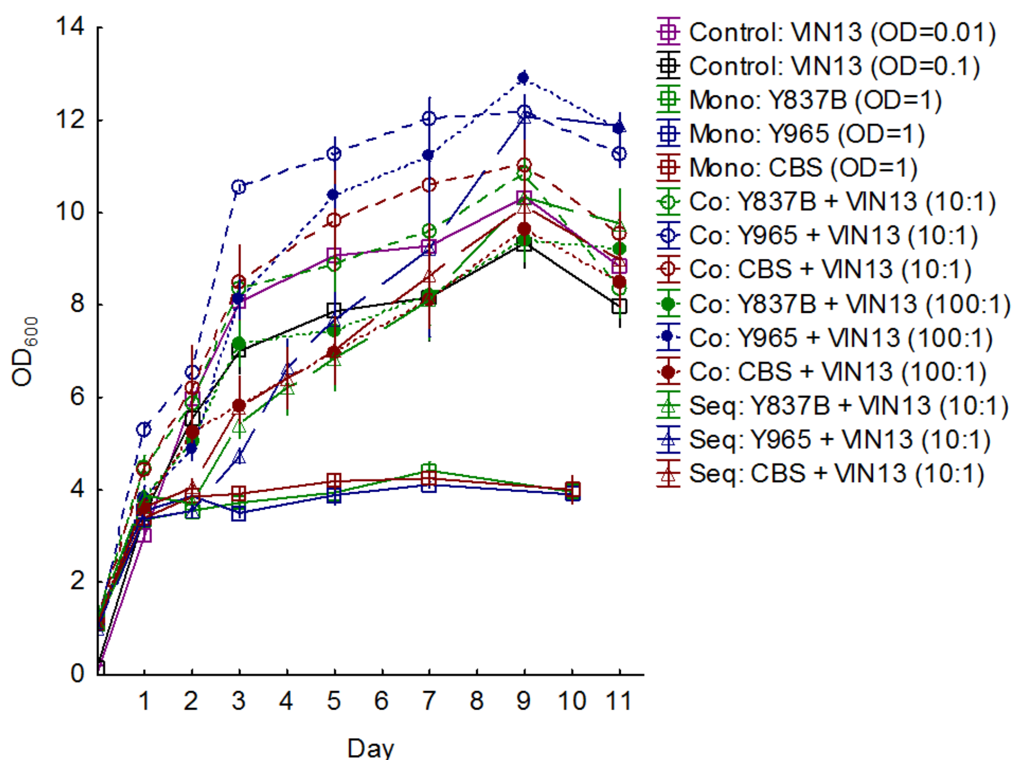


Figure 4.3 Growth rate (expressed as OD_{600}) of *K. aerobia* (Y837B, Y965, CBS) and *S. cerevisiae* (VIN13) in monoculture fermentations (indicated with □) and co-inoculations at ratios 10:1 (indicated with O) and 100:1 (indicated with ●) and sequential inoculations (indicated with Δ). Values are indicated as the mean ± standard error of the mean.

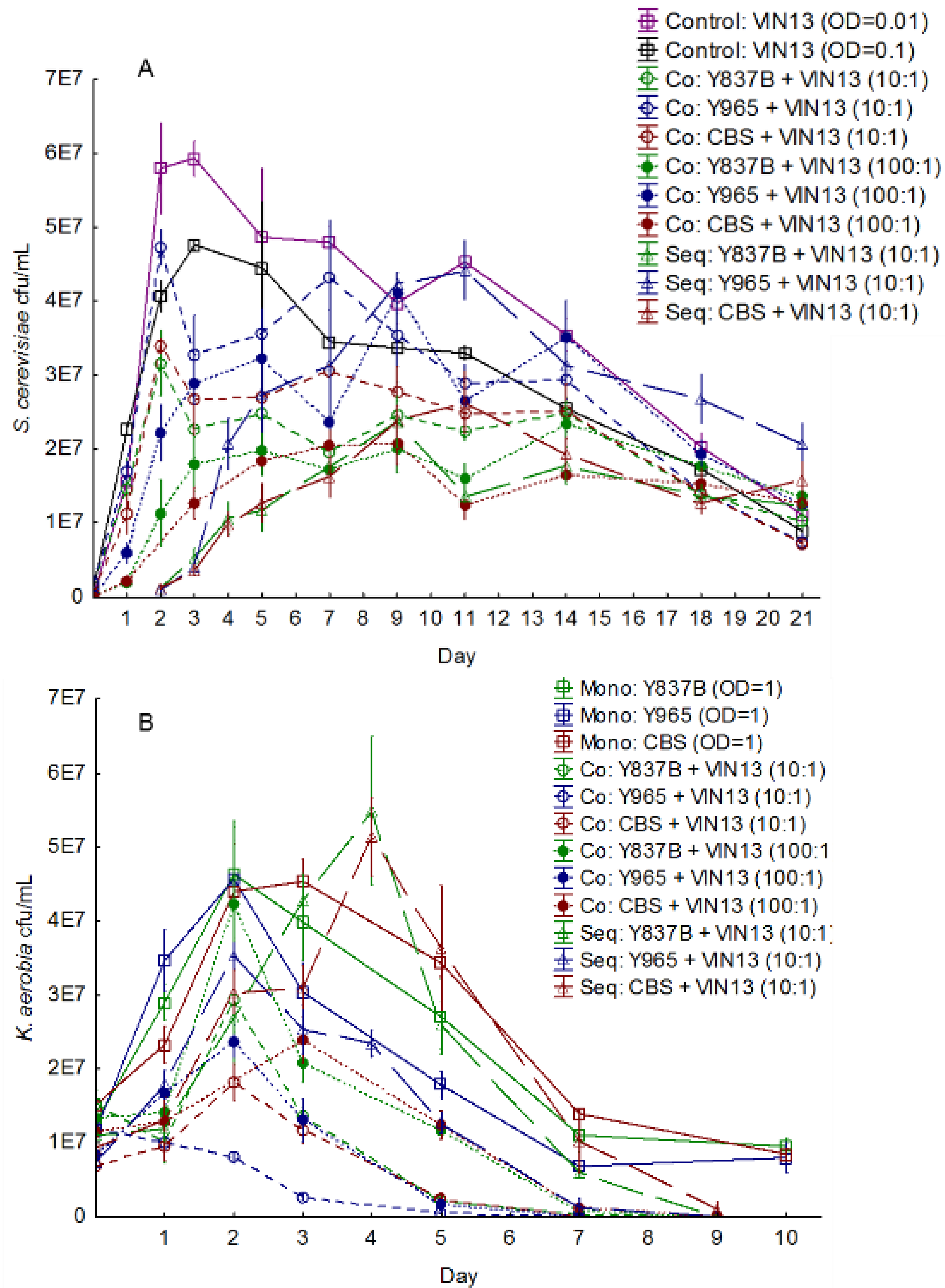


Figure 4.4 Cell growth rate indicated as cfu/mL for *S. cerevisiae* (VIN13) (graph A) and *K. aerobia* (Y837B, Y965, CBS) (graph B) monoculture fermentations (indicated with □), co-inoculation fermentations at ratios 10:1 (indicated with O) and 100:1 (indicated with ●) and sequential inoculations (indicated with Δ). Values are indicated as the mean ± standard error of the mean.

Stellenbosch University https://scholar.sun.ac.za

4.3.1.3 Major volatile aroma production

Mixed culture fermentations (co- and sequential inoculation) resulted in wines with higher concentrations of most of the analysed aromatic compounds compared to the control *S. cerevisiae* fermentations with the exception of isobutyric acid and ethyl caprylate (Table 4.2). The overall data set was analysed with PCA, and suggests that all treatments produced somewhat distinct aroma profiles (Figure 4.5). The *S. cerevisiae* monoculture clearly separated from all other treatments, and sequential culture fermentations produced different PCA scores when compared to the co-inoculations (Figure 4.5 - A). Sequential culture fermentations showed a distinct aroma profile due to higher concentrations of propanol, isobutanol, butanol, isoamyl alcohol and acetic acid (Figure 4.5 – C; Table 4.2). In addition, the differences in aroma compounds between the co-inoculation treatments were not pronounced.

Amongst the *K. aerobia* isolates, Y965 consistently produced higher concentrations of ethyl acetate, propanol, butanol, isoamyl alcohol, ethyl caprylate and 2-phenyl ethanol, but lower concentrations of acetic acid, acetoin and isobutyric acid than the other isolates. The PCA scores plot confirms that *K. aerobia* Y965 produced a distinct aromatic profile irrespective of the treatment (Figure 4.5 - B). The isolates Y837-B and CBS produced more similar concentrations of these compounds and grouped closer to each other in the PCA scores plot. Nevertheless, the CBS strain produced higher acetic acid concentrations in all of the treatments. There were no noteworthy differences between the two inoculation strategies for the monocultures of *S. cerevisiae*.

Table 4.2 Aroma compounds detected and within limit of quantification (LOQ) in SGM produced by *S. cerevisiae* (VIN13) and *K. aerobia* (Y837B, Y965, CBS) in mixed culture fermentations, compared using a one-way ANOVA between different yeast combinations. Differences between means were inferred using Unequal N HSD test and values in the table represents mean ± standard error of mean.

Compound (mg/L)	**OD=0.01**	**OD=0.1**	**Co inoculation (10:1)**			**Co inoculation (100:1)**			**Sequential inoculation**		
	VIN13		**Y837B**	**Y965**	**CBS**	**Y837B**	**Y965**	**CBS**	**Y837B**	**Y965**	**CBS**
Ethyl acetate	40.03 ± 1.55^{ab}	32.53 ± 5.81^{b}	36.65 ± 0.78^{ab}	35.25 ± 0.44^{ab}	36.70 ± 3.00^{ab}	37.96 ± 1.02^{ab}	47.21 ± 1.45^{a}	38.99 ± 0.68^{ab}	35.94 ± 1.78^{ab}	40.78 ± 0.43^{ab}	35.88 ± 2.23^{ab}
Ethyl caprylate	0.21 ± 0.01^{b}	0.36 ± 0.05^{a}	0.16 ± 0.00^{bc}	0.17 ± 0.00^{bc}	0.14 ± 0.01^{bc}	0.17 ± 0.01^{bc}	0.21 ± 0.01^{b}	0.18 ± 0.01^{bc}	0.11 ± 0.00^{c}	0.10 ± 0.01^{c}	0.11 ± 0.00^{c}
Ethyl caproate(*)	0.11 ± 0.00^{ab}	0.24 ± 0.04^{ab}	0.11 ± 0.00^{ab}	0.12 ± 0.01^{ab}	0.05 ± 0.02^{b}	0.15 ± 0.01^{ab}	0.13 ± 0.01^{ab}	0.13 ± 0.01^{ab}	0.11 ±0.01^{ab}	0.04 ± 0.04^{ab}	0.30 ± 0.10^{a}
2-Phenylethyl acetate	0.47 ± 0.01^{e}	0.50 ± 0.01^{e}	0.93 ± 0.01^{cd}	0.69 ± 0.03^{de}	0.91 ± 0.02^{d}	1.33 ± 0.05^{b}	0.67 ± 0.02^{de}	1.25 ± 0.08^{bc}	1.54 ± 0.09^{ab}	0.62 ± 0.02^{de}	1.62 ± 0.10^{a}
Propanol	30.76 ± 1.73^{d}	28.13 ± 1.66^{d}	38.08 ± 2.11^{cd}	43.00 ± 0.94^{abcd}	46.72 ± 4.00^{abc}	40.06 ± 2.24^{abcd}	52.96 ± 3.39^{ab}	37.16 ± 2.02^{bcd}	48.82 ± 3.23^{abc}	57.87 ± 0.71^{a}	48.82 ± 4.00^{abc}
Isobutanol	18.54 ± 0.16^{bc}	16.05 ± 0.63^{c}	22.86 ± 0.38^{a}	21.54 ± 0.45^{ab}	24.12 ± 1.01^{a}	22.07 ± 0.52^{a}	22.01 ± 0.56^{a}	22.32 ± 0.27^{ab}	24.79 ± 0.70^{a}	23.42 ± 0.24^{a}	24.87 ± 1.10^{a}
Butanol(*)	0.54 ± 0.01^{de}	0.44 ± 0.01^{e}	0.67 ± 0.01^{cd}	0.66 ± 0.02^{cd}	0.76 ± 0.01^{c}	0.73 ± 0.01^{c}	0.94 ± 0.09^{ab}	0.75 ± 0.03^{bc}	1.00 ± 0.03^{a}	0.99 ± 0.02^{a}	1.02 ± 0.03^{a}
Isoamyl alcohol	85.50 ± 2.86^{c}	80.25 ± 1.99^{c}	110.47 ± 2.17^{ab}	119.14 ± 3.32^{ab}	118.81 ± 4.03^{ab}	110.40 ± 2.51^{ab}	124.19 ± 2.73^{ab}	108.39 ± 1.10^{b}	124.25 ± 2.94^{ab}	131.43 ± 1.96^{a}	121.35 ± 4.44^{ab}
2-Phenyl ethanol	34.43 ± 0.47^{de}	33.71 ± 0.90^{e}	35.76 ± 0.42^{cde}	38.23 ± 0.71^{abc}	35.88 ± 0.29^{cde}	36.61 ± 0.44^{bcd}	40.92 ± 0.11^{a}	37.18 ± 0.54^{bcd}	36.34 ± 0.60^{bcde}	39.78 ± 0.10^{ab}	34.84 ± 0.71^{de}
Acetic acid	620.38 ± 7.08^{e}	565.20 ± 13.73^{e}	880.62 ± 15.69^{d}	862.34 ± 15.54^{d}	972.00 ± 19.72^{c}	1017.19 ± 11.39^{c}	869.62 ± 4.83^{d}	1142.97 ± 17.46^{b}	1228.04 ± 19.56^{b}	1155.00 ± 25.93^{b}	1313.26 ± 24.69^{a}
Isobutyric acid(*)	1.09 ± 0.04^{a}	0.94 ± 0.03^{b}	0.82 ± 0.04^{bcd}	0.87 ± 0.01^{bc}	0.87 ± 0.05^{bc}	0.71 ± 0.02^{de}	0.64 ± 0.01^{e}	0.68 ± 0.01^{de}	0.71 ± 0.02^{de}	0.63 ± 0.02^{de}	0.73 ± 0.02^{cde}
Acetoin**	7.91 ± 1.14	7.32 ± 0.66	7.59 ± 0.63	6.43 ± 0.44	8.34 ± 0.49	6.25 ± 0.45	7.02 ± 0.90	6.87 ± 0.48	6.62 ± 0.34	6.90 ± 0.35	7.70 ± 0.25

** indicates no significant difference between treatments. (*) indicates when only one treatment is within the LOQ

Values with the same letter in the same column are statistically similar when compared with Unequal N HSD post-hoc test at 95 % confidence level.

Stellenbosch University https://scholar.sun.ac.za

Figure 4.5 The PCA scores plot (A) indicates the influence of inoculation timing on aroma profiles of *K. aerobia* and *S. cerevisiae* mixed culture and *S. cerevisiae* (VIN13) control fermentations. Scores labels denote the isolates used. Green and blue scores represents inoculation of *S. cerevisiae* at 0 or 48 hours after inoculation of *K. aerobia*. PCA scores plot (B) indicates the effect of the yeast isolate used with the inoculation strategy indicated next to the scores. Groupings of treatments suggest similar aroma profiles. The loadings plot (C) indicate the compounds driving the variations in the aroma profile of the different treatments.

Stellenbosch University https://scholar.sun.ac.za

4.3.2 Mixed culture fermentations: *W. anomalus* and *S. cerevisiae*

4.3.2.1 Fermentation kinetics

Sequential culture fermentations were conducted by inoculating the *S. cerevisiae* strains VIN13 and EC1118 48 hours after introducing *W. anomalus* to SGM at equal concentrations (OD_{600}=0.1). Monocultures of *W. anomalus* and *S. cerevisiae* served as control fermentations. Similar to findings in *K. aerobia* fermentations, *S. cerevisiae* demonstrated a faster fermentation rate in terms of CO_2 production and sugar consumption compared to the *W. anomalus* mono- and sequential cultures (Figure 4.6; Figure 4.7). Fermentation rate increased after addition of *S. cerevisiae* in sequential culture fermentations on day 2. The *S. cerevisiae* strain EC1118 had a slightly faster fermentation rate than VIN13 as monocultures. Amongst strains of *W. anomalus,* LO632 fermented at a faster rate than Y934-C in the mono- and sequential culture fermentations.

All of the yeasts had a preference for glucose and this was consumed at a faster rate compared to fructose (Figure 4.7). After two weeks the *S. cerevisiae* control and sequential culture fermentations were completed (sugar < 2 g/L), but the *W. anomalus* monocultures had not yet fermented to dryness and had stopped fermenting.

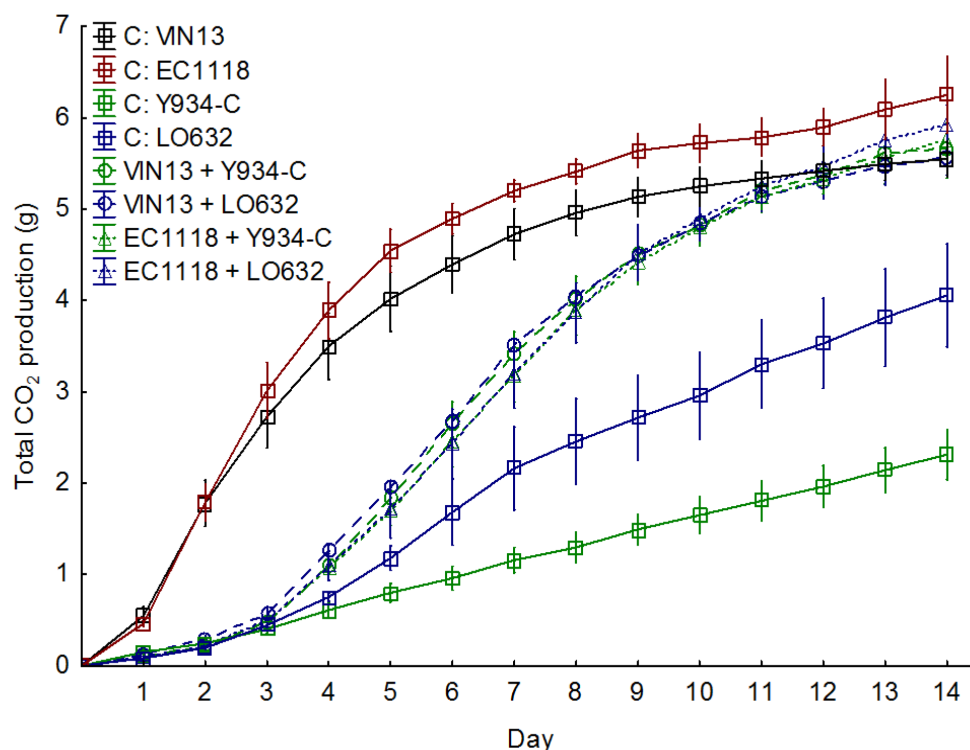


Figure 4.6 CO_2 production of *S. cerevisiae* (VIN13, EC1118) and *W. anomalus* (Y934-C, LO632) in monoculture fermentations (indicated with □) and sequential culture fermentations with either VIN13 (indicated with O) or EC1118 (indicated with Δ). Values are indicated as the mean ± standard error of the mean.

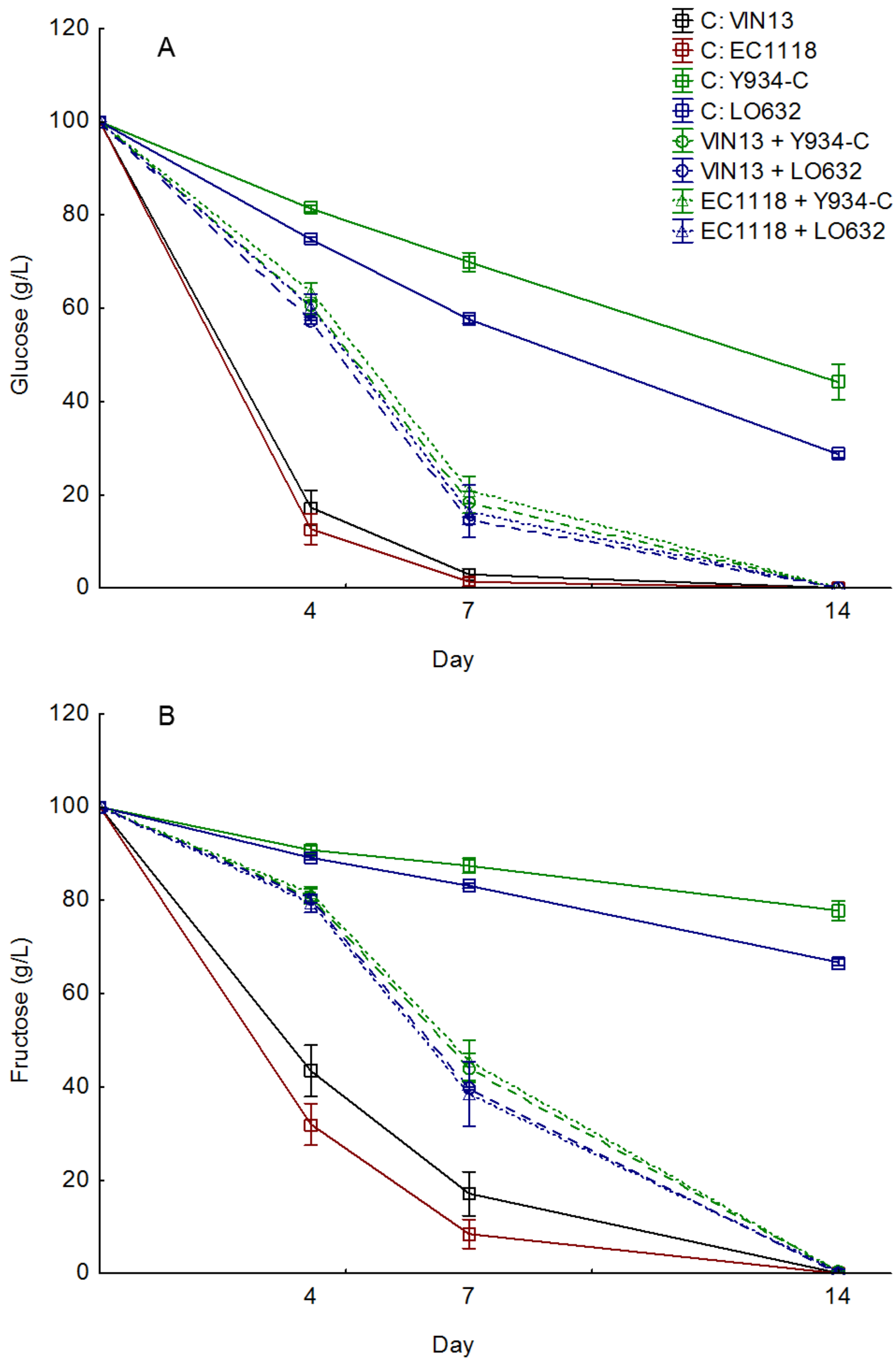


Figure 4.7 Glucose (A) and fructose (B) consumption by *W. anomalus* (Y934-C, LO632) and *S. cerevisiae* (VIN13, EC1118) monoculture fermentations (indicated with □) and sequential culture fermentations with either VIN13 (indicated with O) or EC1118 (indicated with ∆). Values are indicated as the mean ± standard error of the mean.

4.3.2.2 Yeast enumeration

In addition to sequential inoculations with *W. anomalus*, co-inoculation fermentations were conducted with five *W. anomalus* strains and *S. cerevisiae* strains EC1118 and VIN13, to determine the effect of different *S. cerevisiae* strains on the performance of *W. anomalus*. All yeasts were inoculated at an equal OD_{600} of 0.1 in SGM. After one day of fermentation, the *S. cerevisiae* population was 10 times the initial inoculated density, compared to the declining population of *W. anomalus* yeasts (Figure 4.8). By day 5 all *W. anomalus* yeast had died off. Amongst strains of *W. anomalus*, Y934-C reached the highest cell density. There were no clear differences in the population of *W. anomalus* strains when fermenting with different *S. cerevisiae* strains, although cell population of *S. cerevisiae* VIN13 was almost twice as high as compared to EC1118. In co-inoculations, when the *W. anomalus* population was lower (*e.g.* for ARC 19/22 and LO632), the *S. cerevisiae* population (EC1118 and VIN13) was slightly higher.

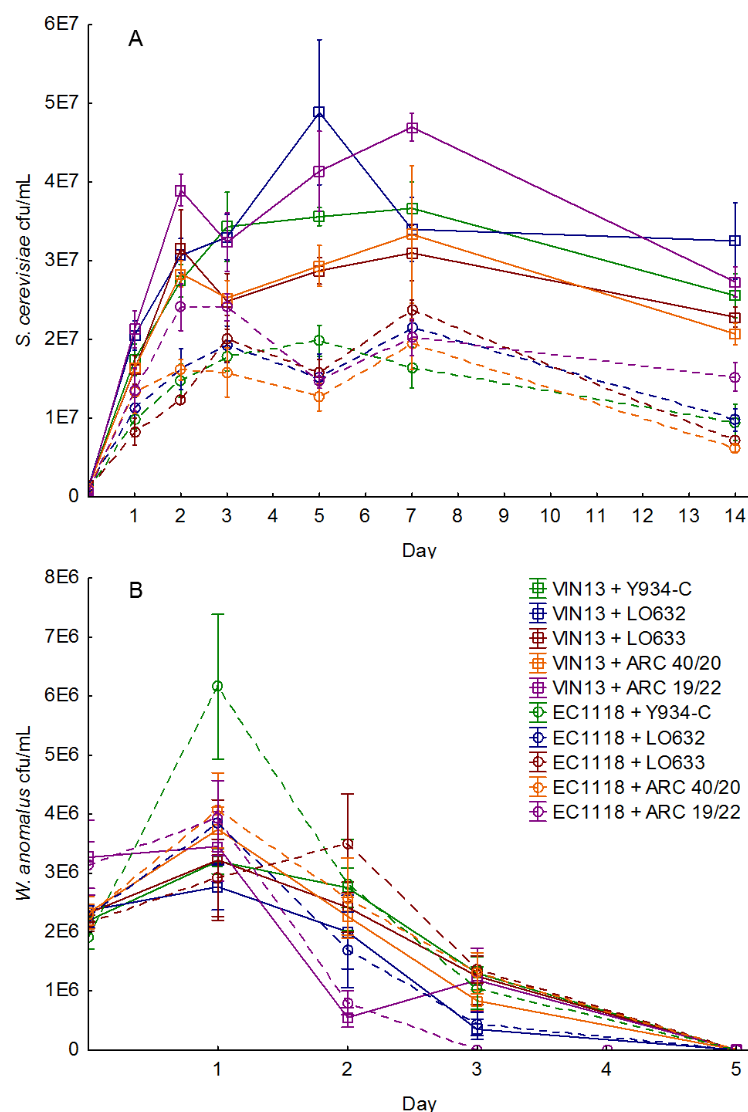


Figure 4.8 Yeast cell populations of *S. cerevisiae* (graph A) and *W. anomalus* (graph B). Co-inoculation fermentations were conducted with *S. cerevisiae* VIN 13 (indicated with □) and EC1118 (indicated with O) Values are indicated as the mean ± standard error of the mean.

In addition, control fermentations of *S. cerevisiae* showed the highest growth rate in terms of biomass production (OD_{600}), rapidly entering exponential phase and reaching stationary phase after 3 days (Figure 4.9). After inoculation of *S. cerevisiae* on day 2 in the sequential culture fermentations, biomass increased. Between strains, for either *S. cerevisiae* or *W. anomalus*, differences were not significant at endpoint (data not shown). With regards to individual yeast population dynamics, the *S. cerevisiae* yeasts were present in higher densities compared to *W. anomalus* (Figure 4.10). As seen with the co-inoculations, strain VIN13 reached a higher yeast population compared to EC1118 (Figure 4.10 - A). Amongst *W. anomalus* strains, Y934-C obtained the highest cell growth irrespective of treatment (Figure 4.10 - B). Furthermore, the *W. anomalus* yeast populations did not change with the use of different *S. cerevisiae* strains.

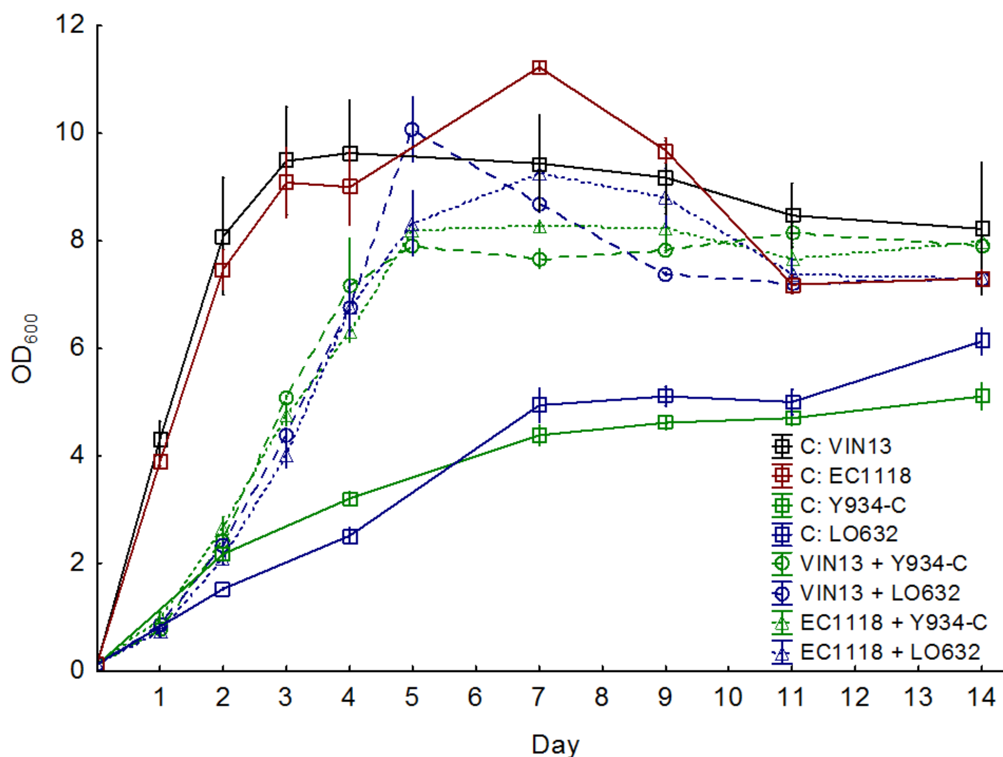


Figure 4.9 Growth rate expressed as optical density (OD_{600}) for control and monoculture fermentations (indicated with □) of *S. cerevisiae* and *W. anomalus* (Y934-C, LO632) and sequential culture fermentations with either VIN13 (indicated with O) or EC1118 (indicated with Δ). Values are indicated as the mean ± standard error of the mean.

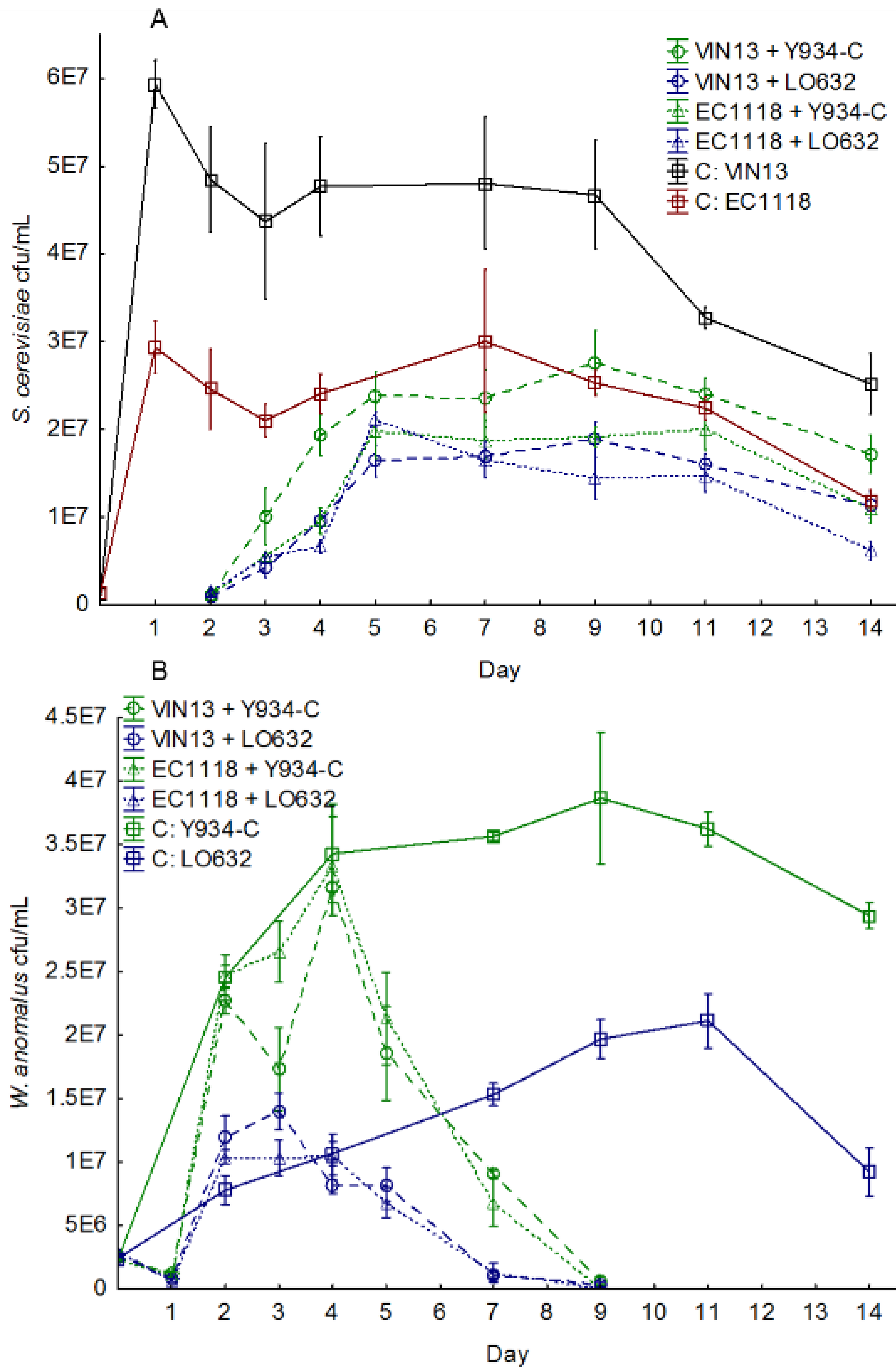


Figure 4.10 *S. cerevisiae* (VIN13, EC1118) (graph A) and *W. anomalus* (Y934-C, LO632) (graph B) population growth expressed as colony forming units per mL (cfu/mL) for monocultures (C) (indicated with □) and sequential culture fermentations with either VIN13 (indicated with O) or EC1118 (indicated with Δ). Values are indicated as the mean ± standard error of the mean.

Stellenbosch University https://scholar.sun.ac.za

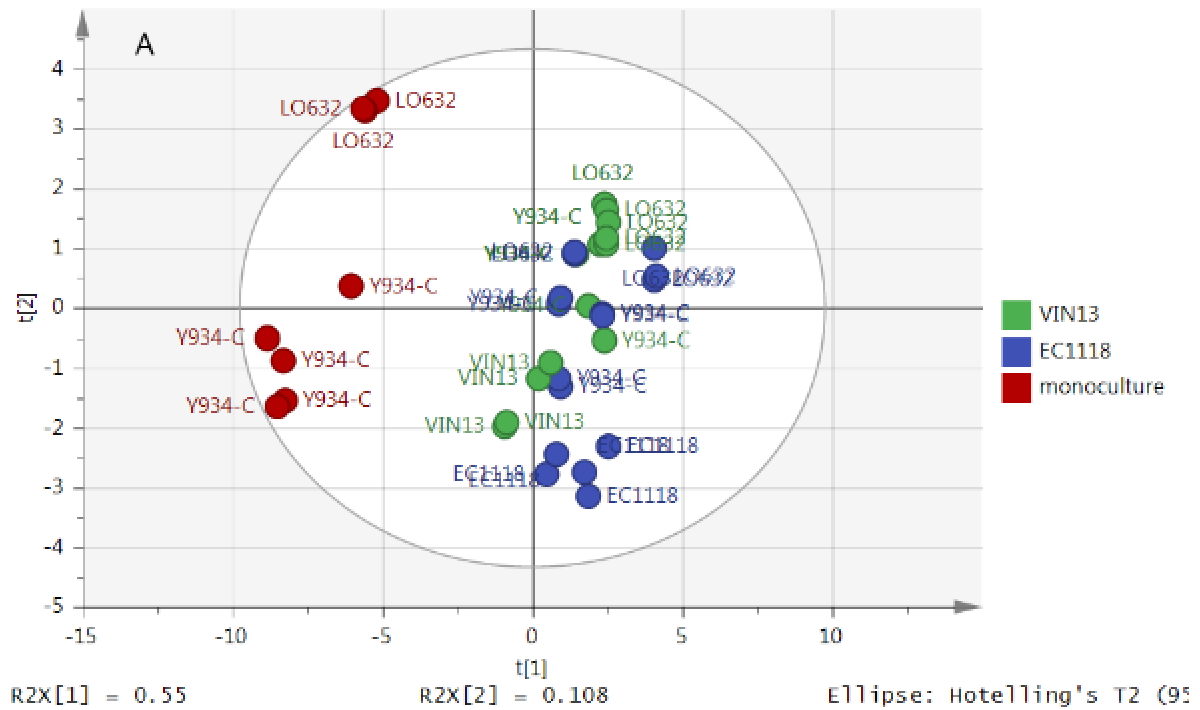
4.3.2.3 Major volatile aroma production

Aroma compounds produced by *W. anomalus* and *S. cerevisiae* in mono- and sequential culture fermentations were measured at termination of fermentations (day 14). As with *K. aerobia*, analysis showed that sequential culture fermentations with *W. anomalus* resulted in a higher concentration of aroma compounds measured, in terms of esters, higher alcohols and fatty acids, compared to the monoculture fermentations of either *W. anomalus* or *S. cerevisiae* (Table 4.3).

The overall data set was analysed with PCA, and suggests that the *W. anomalus* monocultures had a distinct metabolite profile compared to the sequential cultures and *S. cerevisiae* controls (Figure 4.11 - A). However, differences in the sequential setup between strains were less prominent and more similar to the *S. cerevisiae* monocultures.

The *W. anomalus* strains, LO632 and Y934-C in mono- and sequential culture fermentation, produced six and three times the amount of ethyl acetate, in comparison with the *S. cerevisiae* monocultures (Table 4.3). However, ethyl caproate, ethyl caprylate, 3- ethoxy-1-propanol was not produced by *W. anomalus* in monocultures. In sequential cultures, production of higher alcohols (propanol, isobutanol and isoamyl alcohol) was greater, compared to when the yeast species were fermented as monocultures. In addition, *W. anomalus* produced significantly lower concentrations of acetic acid compared to *S. cerevisiae,* especially *W. anomalus* Y934-C. Acetoin production was reduced when *W. anomalus* was fermented in combination with *S. cerevisiae* VIN13.

Amongst the *W. anomalus* strains, isobutyric acid and isovaleric acid were drivers for the differentiation between the monoculture fermentations (Figure 4.11 - B). Furthermore, the specific *S. cerevisiae* strain used impacted certain compounds (Table 4.3). Strain VIN13 showed the biggest impact on production of valeric acid and the higher alcohols isoamyl alcohol and propanol, whereas EC1118 contributed to the production of isobutanol and the acids hexanoic and octanoic acid in sequential culture fermentations.



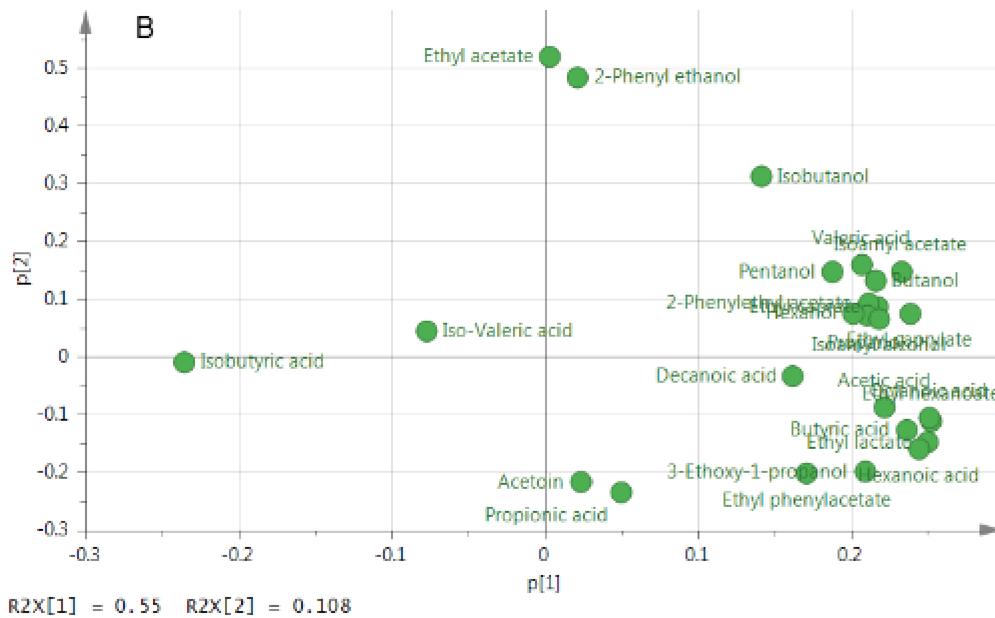


Figure 4.11 PCA scores plot (A) indicating influence of *W. anomalus* and *S. cerevisiae* co-culturing on aroma profiles. Scores labels denote the strains used. Blue and green scores represents EC1118 and VIN13 scores respectively as monoculture and sequentially cultured with *W. anomalus* strains shown on the label. Scores for the monocultures of *W. anomalus* Y934-C and LO632 is indicated in red. Loadings plot (B) suggesting the metabolite responsible for the volatile aroma profile variations.

Table 4.3 Aroma compounds detected and within limit of quantification (LOQ) in *W. anomalus* (Y934-C, LO632) and *S. cerevisiae* (VIN13, EC1118) mono- and sequential inoculation fermentations compared using a one-way ANOVA between different yeast combinations. Differences between means were inferred using Unequal N HSD test and value in the table represents mean ± standard error of mean.

Compound (mg/L)	VIN13		EC1118		Monoculture		Control	
	Y934-C	LO632	Y934-C	LO632	Y934-C	LO632	VIN13	EC1118
Ethyl acetate	100.05 ± 1.53^{c}	287.91 ± 12.10^{a}	78.94 ± 2.88^{d}	223.61 ± 15.88^{b}	88.21 ± 9.59^{cd}	331.39 ± 24.11^{a}	25.26 ± 0.91^{e}	30.05 ± 1.55^{e}
Ethyl caprylate	0.30 ± 0.02^{a}	0.27 ± 0.01^{ab}	0.21 ± 0.03^{bc}	0.28 ± 0.02^{ab}	nd	ns	0.13 ± 0.00^{c}	0.18 ± 0.02^{c}
Ethyl caproate	0.30 ± 0.04^{a}	0.21 ± 0.01^{ab}	0.21 ± 0.03^{ab}	0.33 ± 0.05^{a}	nd	ns	0.09 ± 0.00^{b}	0.16 ± 0.02^{b}
Propanol	33.85 ± 0.71^{a}	37.92 ± 1.29^{a}	25.59 ± 0.90^{bc}	24.19 ± 1.43^{c}	9.03 ± 0.65^{d}	11.31 ± 0.37^{d}	31.58 ± 1.72^{ab}	22.13 ± 2.13^{c}
Isobutanol	22.70 ± 0.83^{ab}	26.04 ± 0.95^{a}	24.51 ± 0.38^{ab}	26.95 ± 1.15^{a}	16.22 ± 1.67^{c}	28.27 ± 2.19^{a}	19.00 ± 1.38^{bc}	23.83 ± 1.94^{ab}
Isoamyl alcohol	106.06 ± 1.93^{a}	89.64 ± 1.61^{abc}	101.96 ± 3.11^{ab}	81.25 ± 2.10^{c}	35.21 ± 8.14^{d}	55.21 ± 2.15^{d}	84.06 ± 8.64^{bc}	78.41 ± 3.14^{c}
3-ethoxy-1-propanol	2.08 ± 0.15^{b}	3.24 ± 0.06^{ab}	3.49 ± 0.23^{ab}	4.95 ± 0.57^{a}	nd	nd	3.02 ± 0.72^{ab}	5.18 ± 0.83^{a}
2-Phenyl ethanol	32.12 ± 0.75^{bc}	31.39 ± 0.30^{bcd}	33.15 ± 0.39^{b}	34.43 ± 0.76^{b}	27.41 ± 1.97^{d}	40.25 ± 1.52^{a}	27.91 ± 1.88^{cd}	27.08 ± 0.25^{d}
Acetic acid	556.48 ± 16.48^{b}	654.41 ±16.18^{ab}	570.35 ±12.72^{ab}	784.15 ± 21.03^{a}	311.26 ± 34.35^{c}	541.45±143.00^{bc}	593.36 ±29.32^{ab}	751.95 ±56.69^{ab}
Isobutyric acid	1.19 ± 0.07^{cd}	0.86 ± 0.03^{e}	1.42 ± 0.05^{bc}	1.18 ± 0.09^{cd}	2.35 ± 0.06^{a}	1.64 ± 0.11^{b}	1.11 ± 0.01^{dce}	1.03 ± 0.03^{de}
Valeric acid	1.81 ± 0.05^{a}	1.97 ± 0.00^{a}	1.11 ± 0.23^{bc}	1.83 ± 0.04^{a}	0.34 ± 0.03^{d}	0.68 ± 0.02^{cd}	1.39 ± 0.24^{ab}	1.15 ± 0.15^{bc}
Hexanoic acid	1.36 ± 0.03^{b}	1.26 ± 0.00^{bc}	1.54 ± 0.04^{a}	1.57 ± 0.04^{a}	0.33 ± 0.02^{d}	0.33 ± 0.00^{d}	1.18 ± 0.03^{c}	1.55 ± 0.05^{a}
Octanoic acid	1.95 ± 0.08^{b}	1.95 ± 0.03^{b}	2.13 ± 0.08^{ab}	2.45 ± 0.14^{a}	0.46 ± 0.02^{d}	0.44 ± 0.02^{d}	1.48 ± 0.03^{c}	2.16 ± 0.08^{ab}
Acetoin	2.00 ± 0.10^{b}	1.86 ± 0.39^{b}	4.88 ± 0.62^{ab}	3.40 ± 0.47^{ab}	2.50 ± 1.56^{ab}	4.41 ± 1.08^{ab}	3.26 ± 0.06^{ab}	5.78 ± 0.52^{a}

Values with the same letter in the same column are statistically similar when compared with Unequal N HSD post-hoc test at 95 % confidence level.

"nd" = not detected; "ns" = not significant

Stellenbosch University https://scholar.sun.ac.za

4.3.3 Mixed culture fermentations in Sauvignon blanc grape must

4.3.3.1 Fermentation kinetics

Sequential culture fermentations were conducted in Sauvignon blanc grape must by inoculating with *W. anomalus* and *K. aerobia* strains respectively and introducing *S. cerevisiae* EC1118 after 48 hours. In addition, all yeasts were also inoculated separately as single strains and referred to as monoculture fermentations. In addition, a spontaneous fermentation was also conducted.

All fermentations proceeded in the normal sigmoidal pattern (Figure 4.12). After 21 days of fermentation, the total amount of residual sugar was reduced to 2 g/L or less, with the exception of the monoculture fermentations and the spontaneous fermentation that took 28 days to reach the same sugar concentrations (Figure 4.13). For all treatments, glucose was the preferred carbon source and was completely consumed after 21 days in all fermentations except for the monoculture fermentation of *K. aerobia* CBS and the spontaneous fermentation (Figure 4.13).

Saccharomyces cerevisiae exhibited the fastest fermentation rate, in terms of CO_2 production and sugar consumption, followed by the sequential culture fermentations (Figure 4.12; Figure 4.13). However, the total CO_2 production between treatments showed no significant differences (Figure 4.12, Table 2 in appendix). Sequential culture fermentations proceeded in a similar manner between yeast strains and after 10 days there were no statistical differences in the metabolic activities in terms of glucose and fructose consumption (Figure 4.13; Table 3 in appendix). Amongst strains, *W. anomalus* LO632 showed the highest production of CO_2 compared to the other single strain fermentations, although initially the *W. anomalus* strains had the lowest fermentation rate (in terms of sugar consumption and CO_2 release). Overall, the spontaneous fermentations had the lowest consumption of sugars and CO_2 release, followed by the *K. aerobia* CBS strain (Figure 4.12; Figure 4.13). Factorial ANOVA analysis for accumulative CO_2 production between day 21 and 28 for the monoculture fermentations and spontaneous fermentations showed no significant interaction between day and treatment (Table 4.4).

Furthermore, in terms of ethanol production, the spontaneous fermentation and the monoculture of *K. aerobia* CBS displayed the lowest production after 21 days (Table 4.5). However, there was no difference in ethanol yield between treatments. In addition, the *S. cerevisiae* control fermentation produced the lowest amount of glycerol. The glycerol yield in the spontaneous fermentation and *K. aerobia* monoculture fermentations were significantly higher than the other fermentations. Other chemical analysis did not show noteworthy results – no significant difference were found in saccharose, tartaric acid and lactic acid (Table 4 in appendix). The pH of the monoculture fermentations and the spontaneous fermentation was slightly lower than the sequential culture fermentations and control. In addition, lower malic and total acidity was observed in the spontaneous

fermentations compared to the other treatments (that did not show a significant difference between one another).

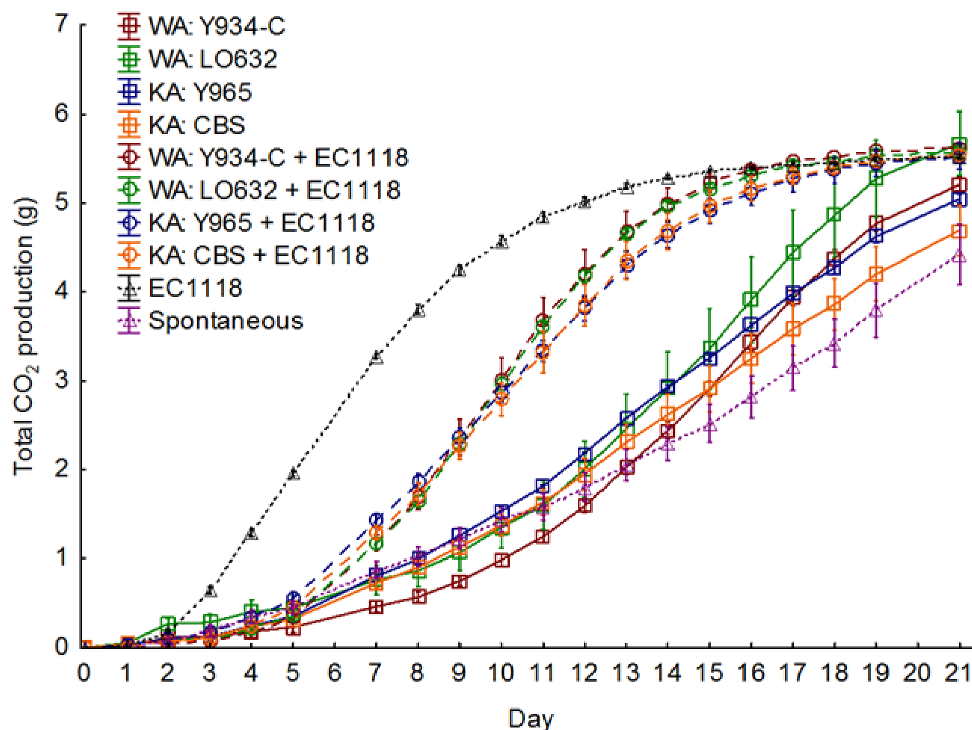


Figure 4.12 Total CO_2 production of *W. anomalus* (WA) and *K. aerobia* (KA) monoculture fermentations (indicated with ☐), sequential culture fermentations (indicated with O) and *S. cerevisiae* EC1118 monoculture fermentations (indicated with Δ) and spontaneous fermentation (indicated with Δ). Values plotted as mean ± standard error of mean.

Table 4.4 Univariate analysis for total CO_2 production of the spontaneous and monoculture fermentations on day 21 and 28. Significant differences indicated in boldface.

Effect	Degr. of freedom	CO_2 production SS	CO_2 production MS	CO_2 production F	CO_2 production p
Intercept	1	**742.14**	**742.14**	**3216.10**	**0.00**
Day	1	**1.17**	**1.17**	**5.07**	**0.04**
Treatment	4	**3.25**	**0.81**	**3.52**	**0.03**
Day*Treatment	4	0.69	0.17	0.75	0.57
Error	18	4.15	0.23		
Total	27	9.38			

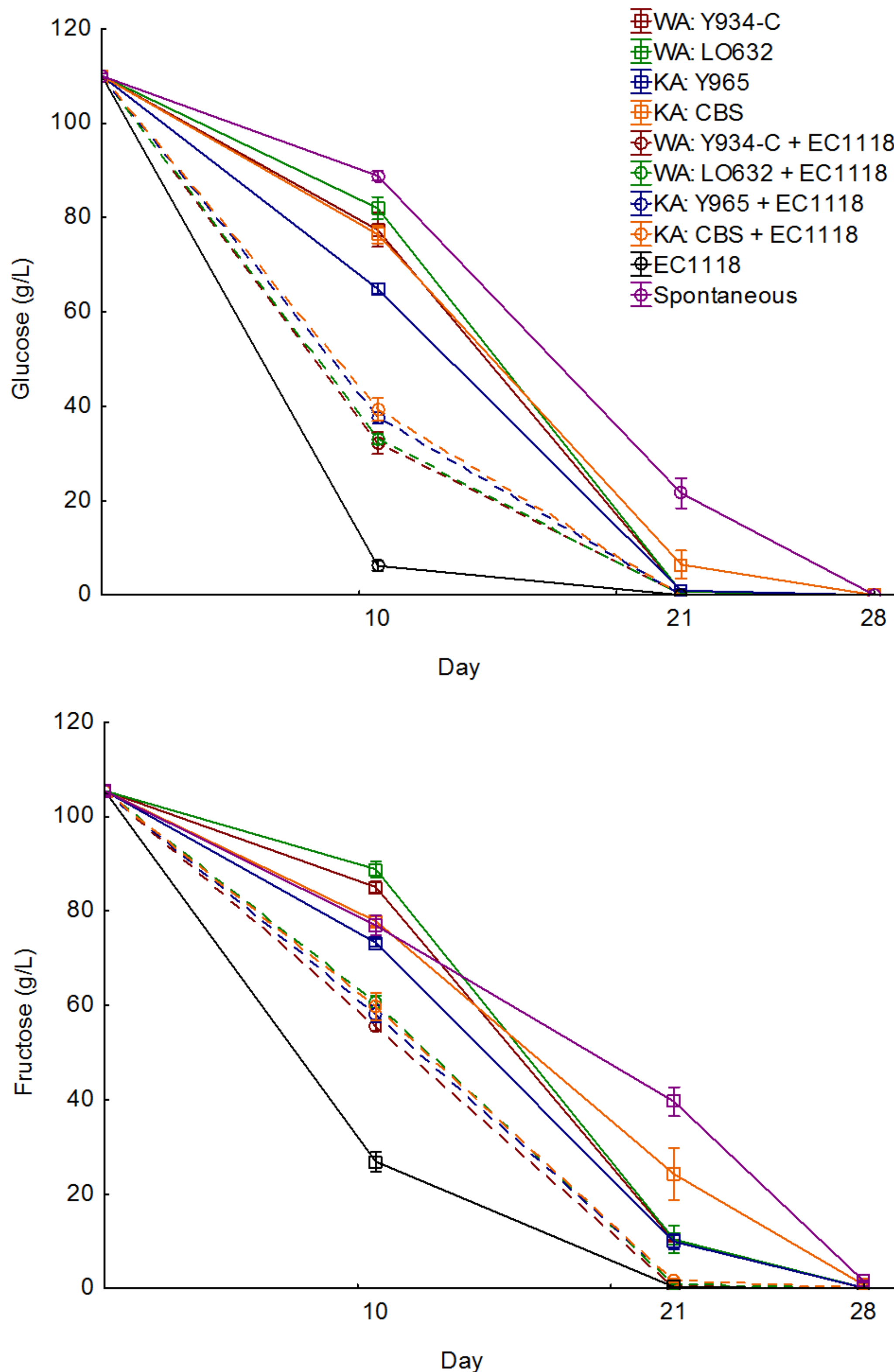


Figure 4.13 Glucose (A) and fructose (B) consumption of *W. anomalus* (WA) and *K. aerobia* (KA) as monoculture fermentations (indicated with □), and sequential culture fermentations with EC1118 (indicated with O) as well as *S. cerevisiae* EC1118 monoculture fermentation and spontaneous fermentation (indicated with ∆). The data points represents mean ± standard error of mean ($n = 3$). Fermentations proceeded until the media was considered dry (total sugar less than 2 g/L).

Stellenbosch University https://scholar.sun.ac.za

Table 4.5 Chemical analysis of ethanol (%v/v), glycerol (g/L) further expressed as ethanol and glycerol yield (g/g sugars utilised) of fermentations conducted with *S. cerevisiae* (EC1118) and *W. anomalus* (WA) and *K. aerobia* (KA) in mono- and sequential cultures as well as the spontaneous fermentation at day 21. Values indicated as mean ± standard error of the mean.

Treatment	Yeast strain	Ethanol (%v/v)	Ethanol yield	Glycerol (g/L)	Glycerol yield
Control	EC1118	13.13 ± 0.03^{a}	0.48 ± 0.00	7.00 ± 0.06^{d}	0.03 ± 0.00^{c}
Monocultures	WA: Y934-C	12.20 ± 0.15^{ab}	0.47 ± 0.01	8.30 ± 0.26^{bcd}	0.04 ± 0.00^{bc}
	WA: LO632	12.33 ± 0.23^{ab}	0.48 ± 0.00	8.37 ± 0.03^{bcd}	0.04 ± 0.00^{bc}
	KA: Y965	11.97 ± 0.03^{ab}	0.46 ± 0.00	10.27 ± 0.18^{a}	0.05 ± 0.00^{ab}
	KA: CBS	11.17 ± 0.49^{bc}	0.48 ± 0.00	9.63 ± 0.18^{ab}	0.05 ± 0.00^{a}
Sequential cultures	WA: Y934-C	12.80 ± 0.15^{a}	0.47 ± 0.01	7.37 ± 0.15^{d}	0.03 ± 0.00^{c}
	WA: LO632	12.67 ± 0.19^{ab}	0.47 ± 0.01	7.37 ± 0.09^{d}	0.03 ± 0.00^{c}
	KA: Y965	12.27 ± 0.03^{ab}	0.45 ± 0.00	9.00 ± 0.25^{abc}	0.04 ± 0.00^{bc}
	KA: CBS	12.60 ± 0.06^{ab}	0.47 ± 0.00	8.23 ± 0.23^{bcd}	0.04 ± 0.00^{c}
	Spontaneous	9.90 ± 0.75^{c}	0.51 ± 0.04	7.70 ± 0.76^{cd}	0.05 ± 0.01^{ab}

Values with the same letter in the same column are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

4.3.3.2 Yeast enumeration

Yeast growth during fermentation was determined by measuring total biomass formation (OD_{600}) and individual yeast growth on differentiation plates. Similar to fermentations in SGM, the *S. cerevisiae* EC1118 fermentation had the shortest lag phase and entered exponential phase after two days of fermenting (Figure 4.14). It reached stationary phase after 10 days, at which point it exhibited the highest biomass production (OD_{600}=11). The sequential culture fermentations entered exponential phase after 5 days, with those inoculated with *W. anomalus* reaching stationary phase at day 16, compared to the *K. aerobia* fermentations that only reached stationary phase on day 18. The *W. anomalus* yeasts in mono- and sequential culture fermentations reached a higher biomass than *K. aerobia* in the corresponding fermentations. The monoculture fermentations and spontaneous fermentation had a long lag phase and slow exponential growth phase.

Individual population growth was in accordance with total biomass production, as *S. cerevisiae* yeasts displayed the highest cell counts as monoculture, followed by fermentation in sequential culture with *W. anomalus* yeasts (Figure 15 – A). The spontaneous fermentation had the lowest *S. cerevisiae* counts, followed by that in the *K. aerobia* fermentations. Overall, *S. cerevisiae* displayed the most dominant presence in must, contributing the most to total biomass. Furthermore, cell counts showed that the inoculated yeasts were dominant at the start of fermentation, although exhibiting different survival rates (Figure 4.15 - D). The *W. anomalus* yeasts initially had the highest

Stellenbosch University https://scholar.sun.ac.za

cell density, but died off fairly quickly after *S. cerevisiae* was inoculated, for the sequential culture fermentations, or when it started to take over the fermentations in the monoculture fermentations, surviving until day 10 and 17 respectively (Figure 4.15 – B). No prominent differences were observed between the *W. anomalus* strains. The *K aerobia* yeast were not affected by the presence of *S. cerevisiae* and continued to grow even in the presence of *S. cerevisiae*, although it had a lower yeast growth. *Kazachstania aerobia* yeasts were viable until just after day 17 and until day 21, for the sequential and monoculture fermentations respectively. Amongst strains, *K. aerobia* CBS was still detected after 28 days in the monoculture fermentations, although Y965 reached a higher population during fermentation.

As expected, the spontaneous fermentation had the highest density of indigenous non-*Saccharomyces* yeasts in the grape must (Figure 4.15 - C). The indigenous population was furthermore relatively higher in the fermentations with *K. aerobia* compared to the *W. anomalus* fermentations. In addition, growth of the indigenous microflora persisted for longer in the monoculture fermentations compared to the sequential culture fermentations. After 7 days the indigenous *S. cerevisiae* started to take over the monoculture fermentations, reaching a peak after 21 days and then dying off.

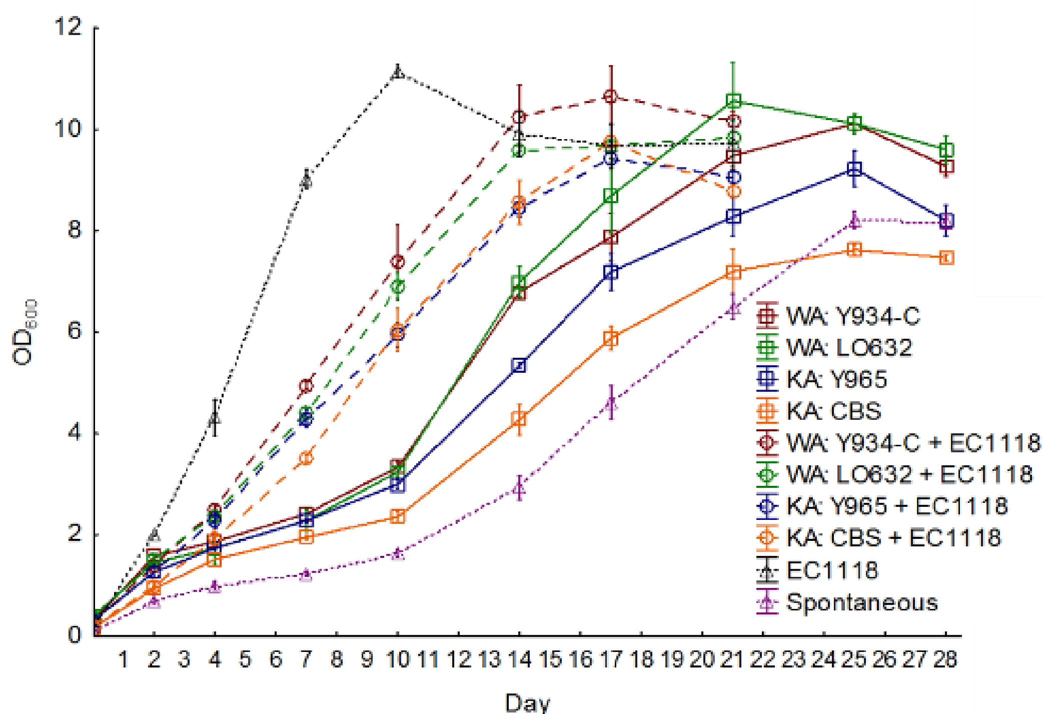


Figure 4.14 Growth kinetics, expressed as OD_{600}, indicated as mean ± standard error of mean, of *W. anomalus* (WA) and *K. aerobia* (KA) monoculture fermentations (indicated with □), sequential culture fermentations (indicated with O) and *S. cerevisiae* EC1118 monoculture fermentation and spontaneous fermentation (indicated with Δ).

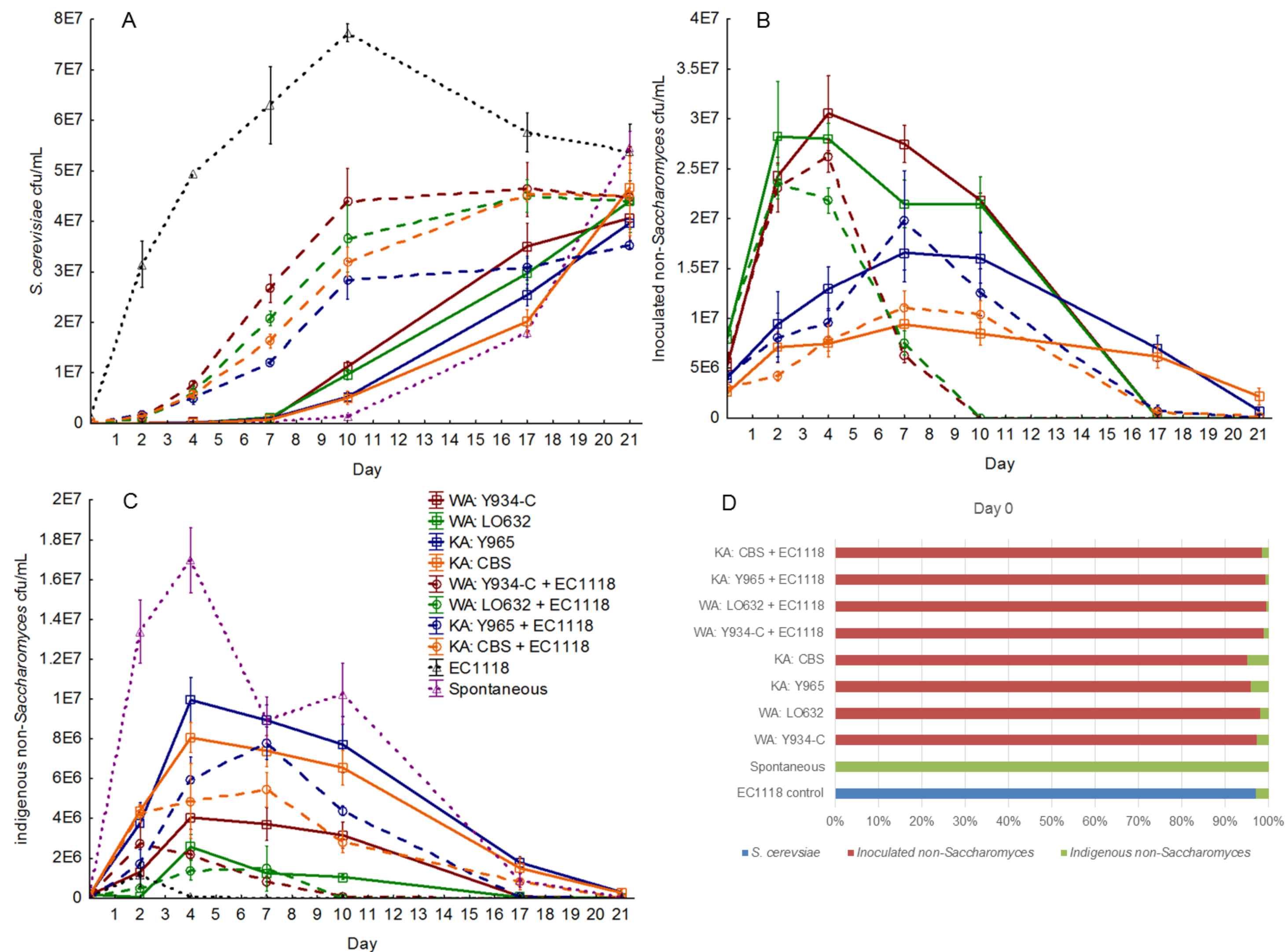


Figure 4.15 Population dynamics during fermentation in Sauvignon blanc grape must with *S. cerevisiae* (EC1118), *K. aerobia* (KA) and *W. anomalus* (WA) indicated as *S. cerevisiae* yeast (A), inoculated non-*Saccharomyces* yeast (B) and indigenous non-*Saccharomyces* population (C)in the respective monoculture fermentations (☐); sequential fermentations (O) and control and spontaneous (Δ) fermentations. Population indicated as mean cfu/mL ± standard error of mean. Directly after inoculation the inoculated yeast was dominant (D).

Stellenbosch University https://scholar.sun.ac.za

4.3.3.3 Major volatile aroma production

Aroma production in terms of some of the major volatile compounds was determined for all treatments at completion of the control and sequential culture fermentations (day 21). After completion of fermentations for the spontaneous and monoculture fermentations these treatments' aroma porduction was measured again, but differences were not noteworthy and were in line with trends observed on day 21 (Figure 3 in appendix).

The overall data set was analysed with PCA, and similar to findings in SGM, suggests that sequential culture treatments and monocultures produced divergent aroma profiles (Figure 4.16-A). The *S. cerevisiae* monoculture clearly separated from all other treatments, and was least similar to the non-*Saccharomyces* monoculture fermentations (mainly due to the latter's high production of ethyl acetate, acetoin, and acetic acid) (Figure 4.16 - B). Furthermore, although *W. anomalus* and *K. aerobia* fermentations separated from each other, within strains the differences were not as pronounced. The exception was for the *K. aerobia* sequential culture fermentations that grouped separately due to differences in acetic acid production (Figure 4.16-A; Table 4.6). The spontaneous fermentation resulted in a volatile profile similar to *K. aerobia* monoculture fermentations.

Similar to what was observed in SGM, ethyl acetate production by *W. anomalus* was high compared to the other treatments, producing five to seven times more ethyl acetate than the *S. cerevisiae* control (Table 4.6). In the sequential cultures this concentration was less, although still three to four times more than the control. Amongst the *W. anomalus* strains, LO632 produced the highest amount of ethyl acetate. Furthermore, production of ethyl acetate by *K. aerobia* monocultures was also four times more than the control, although reduced by half in the sequential cultures. In addition, the non-*Saccharomyces* yeasts, especially *K. aerobia*, had an increased 2-phenylethyl acetate production. The *W. anomalus* yeasts displayed the highest production of higher alcohols (*i.e.* propanol, isobutanol, butanol, 2-phenyl ethanol and isoamyl alcohol). In addition, isobutanol was produced in high concentrations by *K. aerobia* yeasts and the spontaneous fermentation.

Furthermore, sequential culture fermentations with *W. anomalus* showed increased fatty acid concentrations of butyric, hexanoic and octanoic acid. In contrast, hexanoic and octanoic acid was decreased in sequential culture fermentations with *K. aerobia*. However, production of acetic acid was doubled in the *K. aerobia* monocultures, similar to what was observed in SGM, compared to all the other treatments, especially *W. anomalus* fermentations. The non-*Saccharomyces* yeasts as monocultures produced excessive amounts of acetoin along with the indigenous yeast in the spontaneous fermentation. These concentrations were reduced in the sequential culture fermentations to levels similar to that produced by *S. cerevisiae*. Overall, the indigenous yeast in the spontaneous fermentation produced lower amounts of the compounds measured compared to the

inoculated treatments. In addition, the *S. cerevisiae* control fermentation produced higher amounts of ethyl caprylate, 3-ethoxy-1-propanol and propionic acid compared to the non-*Saccharomyces* inoculated fermentations.

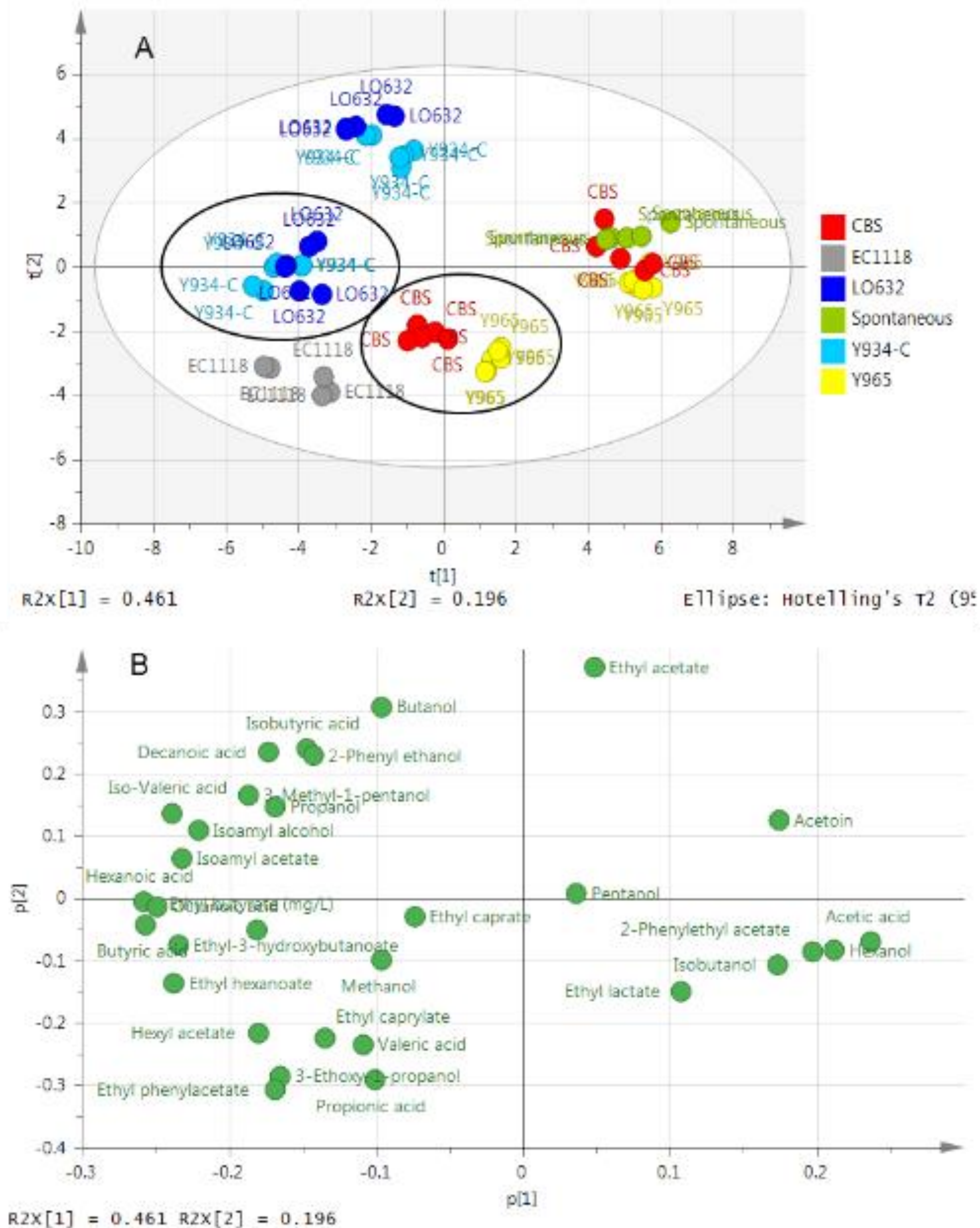


Figure 4.16 PCA scores plot (A) indicating differences between the *S. cerevisiae* and non-*Saccharomyces* mono- and sequential culture fermentations (encircled on the plot) at day 21. Yeast species are coloured according to the legend. PCA loadings plot (B) indicates drivers of differentiation between treatments. Circles indicate sequential fermentations

Stellenbosch University https://scholar.sun.ac.za

Table 4.6 Mean aroma compounds detected and within limit of quantification (LOQ), produced by *S. cerevisiae* (EC1118), *K. aerobia* (KA) and *W. anomalus* (WA) in mono- and sequential culture fermentations on day 21 of fermentation, compared using a one-way ANOVA between different yeast combinations. Differences between means were inferred using Unequal N HSD test and value in the table represents mean ± standard error of mean.

Compound (mg/L)	Monocultures				Sequential cultures				Control	Spontaneous
	WA: Y934-C	WA: LO632	KA: Y965	KA: CBS	WA: Y934-C	WA: LO632	KA: Y965	KA: CBS	EC1118	
Ethyl acetate	394.10 ± 25.53[b]	562.02 ± 13.48[a]	319.81 ±13.75[c]	312.46 ± 9.14[c]	210.78 ± 8.50[d]	305.17 ± 8.34[c]	184.20 ± 1.97[d]	176.76 ± 8.87[d]	74.56 ± 0.83[e]	359.57 ± 7.82[bc]
Isoamyl acetate	1.95 ± 0.05[bc]	2.31 ± 0.18[ab]	1.37 ± 0.07[de]	1.31 ± 0.07[de]	2.33 ± 0.09[a]	1.94 ± 0.07[bc]	1.69 ± 0.03[cd]	1.83 ± 0.02[c]	2.04 ± 0.08[abc]	1.21 ± 0.08[e]
Ethyl caprylate	0.13 ± 0.01[c]	0.12 ± 0.00[c]	0.07 ± 0.01[d]	0.12 ± 0.00[c]	0.20 ± 0.00[b]	0.21 ± 0.01[b]	0.18 ± 0.00[b]	0.19 ± 0.01[b]	0.33 ± 0.01[a]	0.21 ± 0.01[b]
2-Phenylethyl acetate	0.68 ± 0.01[ef]	0.72 ± 0.03[de]	1.25 ± 0.03[b]	1.40 ± 0.04[a]	0.61 ± 0.00[fg]	0.65 ± 0.02[ef]	1.08 ± 0.01[c]	1.17 ± 0.04[bc]	0.53 ± 0.01[g]	0.79 ± 0.00[d]
Methanol**	44.49 ± 0.94	43.90 ± 1.17	43.56 ± 1.57	42.37 ± 0.48	43.76 ± 1.26	44.25 ± 2.43	44.45 ± 0.37	45.01 ± 0.52	47.13 ± 1.65	40.63 ± 1.05
Propanol	88.96 ± 2.06[a]	87.73 ± 0.99[a]	70.96 ± 2.12[bcde]	65.01 ± 3.86[e]	80.78 ± 1.88[abcd]	81.35 ± 4.97[abc]	68.86 ± 1.90[de]	82.01 ± 1.90[ab]	78.45 ± 3.00[abcde]	68.14 ± 2.83[cde]
Isobutanol	20.07 ± 0.16[ef]	20.42 ± 0.44[def]	27.88 ± 0.46[a]	22.55 ± 0.98[cd]	20.32 ± 0.22[def]	20.85 ± 0.92[de]	26.19 ± 0.22[ab]	23.92 ± 0.25[bc]	17.96 ± 0.51[f]	22.99 ± 0.88[cd]
Hexanol	2.11 ± 0.01[de]	2.06 ± 0.02[de]	2.59 ± 0.03[bc]	2.75 ± 0.06[ab]	2.16 ± 0.02 [de]	2.02 ± 0.02 [e]	2.47 ± 0.08 [bc]	2.32 ± 0.09[cd]	2.31 ± 0.09[cde]	2.98 ± 0.11[a]
Butanol	1.21 ± 0.02[b]	1.55 ± 0.02[a]	0.87 ± 0.01[e]	1.02 ± 0.02[cd]	1.01 ± 0.02[d]	1.11 ± 0.03[c]	0.80 ± 0.02[ef]	1.04 ± 0.02[cd]	0.72 ± 0.01[f]	0.74 ± 0.02[f]
Isoamyl alcohol	168.32 ± 2.40[a]	163.53 ± 3.27[ab]	136.17 ± 2.49[d]	119.66 ± 6.49[e]	172.25 ± 1.12[a]	162.48 ± 1.62[a]	142.37 ± 0.61[cd]	149.44 ± 0.74[bc]	145.14 ± 4.87[cd]	113.05 ± 2.08[e]
2-Phenyl ethanol	18.05 ± 0.35[cd]	20.34 ± 0.30[ab]	16.46 ± 0.31[ef]	15.46 ± 0.40[f]	19.14 ± 0.25[bc]	21.29 ± 0.10[a]	15.44 ± 0.04[f]	16.51 ± 0.13[ef]	15.22 ± 0.66[f]	17.35 ± 0.15[de]

Stellenbosch University https://scholar.sun.ac.za

3-Ethoxy-1-propanol	8.29 ± 0.13^{ef}	9.84 ± 0.80^{de}	10.84 ± 0.15^{d}	9.66 ± 0.22 ^{def}	15.90 ± 0.40^{bc}	17.67 ± 0.93^{b}	14.43 ± 0.25^{c}	15.81 ± 0.12^{bc}	20.75 ± 0.60^{a}	7.53 ± 0.16^{f}
Acetic acid	478.73 ± 6.96^{fg}	584.32 ±28.10^{de}	1009.18 ± 10.97^{a}	1065.87 ± 18.35^{a}	358.72 ± 7.08^{h}	451.47 ± 34.85^{g}	763.68 ± 12.73^{b}	665.67 ±20.07^{cd}	545.05 ± 8.96^{ef}	731.54 ±11.20^{bc}
Propionic acid	1.28 ± 0.03^{e}	1.47 ± 0.02^{cd}	1.48 ± 0.01^{d}	1.49 ± 0.04^{cd}	1.47 ± 0.02^{d}	1.65 ± 0.08^{bc}	1.68 ± 0.02^{b}	1.75 ± 0.01^{b}	1.93 ± 0.05^{a}	1.20 ± 0.01^{e}
Butyric acid	1.20 ± 0.02^{d}	1.23 ± 0.01^{d}	0.67 ± 0.00^{f}	0.71 ± 0.02^{f}	1.65 ± 0.02^{a}	1.58 ± 0.01^{b}	1.10 ± 0.01^{e}	1.23 ± 0.02^{d}	1.42 ± 0.01^{c}	0.58 ± 0.01^{g}
Hexanoic acid	2.74 ± 0.03^{c}	2.82 ± 0.05^{c}	1.25 ± 0.02^{e}	1.51 ± 0.03^{e}	3.69 ± 0.05^{a}	3.34 ± 0.09^{b}	2.29 ± 0.04^{d}	2.42 ± 0.10^{d}	3.25 ±0.14^{b}	1.39 ± 0.05^{e}
Octanoic acid	2.77 ± 0.02^{c}	2.83 ± 0.04^{c}	1.55 ± 0.02^{d}	1.93 ± 0.06^{d}	3.74 ± 0.02^{a}	3.37 ± 0.17^{ab}	2.47 ± 0.04^{c}	2.57 ± 0.11^{c}	3.29 ± 0.15^{b}	1.77 ± 0.08^{d}
Acetoin	11.91 ± 0.69^{c}	12.05 ± 3.07^{c}	10.87 ± 1.29^{c}	27.72 ± 5.38^{b}	2.64 ± 0.19^{c}	3.30 ± 0.41^{c}	4.15 ± 0.30^{c}	4.32 ± 0.43^{c}	5.42 ± 0.62^{c}	42.56 ± 4.01^{a}

Values with the same letter in the same column are statistically similar when compared with Unequal N HSD post-hoc test at 95 % confidence level.

** indicates that there were no significant differences between the treatments.

Stellenbosch University https://scholar.sun.ac.za

4.4 Discussion

4.4.1. Fermentation potential of *K. aerobia* and *W. anomalus* in single and mixed culture fermentations

The overall faster fermentation rate of the control fermentation with *S. cerevisiae* (in synthetic and Sauvignon blanc grape must) compared to mixed cultures was expected, as this yeast is well known for its rapid fermentation speed (Fleet and Heard, 1993b; Moreno-Arribas and Polo, 2005). Lower fermentative performance of the non-*Saccharomyces* yeasts are typically reported (Jolly et al., 2003a), although between the species and strains differences occur. Moreover, all of the yeasts preferentially metabolised glucose compared to fructose, similar to other species (De Koker, 2015; Mains, 2014). Interestingly, in SGM, *K. aerobia* strains performed similarly in terms of sugar consumption and biomass production, although in Sauvignon blanc grape must, the CBS strain had lower fermentative performance, possibly due to it not being isolated from a wine environment (Lu et al., 2004). This study furthermore confirms the preference of *W. anomalus* yeast to produce biomass rather than metabolising sugars (Rojas et al., 2003). However, the strain differences in terms of sugar consumption have not been found before (Charoenchai et al., 1998), and most studies focused on one strain without investigating fermentative abilities (Sabel et al., 2014; Swangkeaw et al., 2009). For both *K. aerobia* and *W. anomalus*, strain differences were observed in terms of fermentation rate and growth. Studies show that non-*Saccharomyces* yeasts can display a large phenotypic space with regard to sugar utilisation (Contreras et al., 2014; Rossouw and Bauer, 2016).

Differences in yeast population due to the inoculation strategies is expected. The data demonstrated an increased *K. aerobia* population when *S. cerevisiae* was inoculated at a lower density, as seen in other studies investigating inoculation density (Bely et al., 2008; Comitini et al., 2011; Pérez-Nevado et al., 2006). In comparison, with a delay in *S. cerevisiae* inoculation (48h hours after the non-*Saccharomyces* yeast), both *K. aerobia* and *W. anomalus* yeast showed increased growth compared to simultaneous inoculation of *S. cerevisiae*, possibly due to the improved competition, *i.e.* available nitrogen, of the non-*Saccharomyces* yeasts (Ciani et al., 2006; Fleet, 2003; Kapsopoulou et al., 2007; Mendoza et al., 2007). In addition, a similar lag in sugar consumption has also been reported when delaying inoculation of *S. cerevisiae* in fermentations with *Torulaspora delbrueckii* (Bely et al., 2008; Taillandier et al., 2014) and *Metschnikowia pulcherrima* (Contreras et al., 2015).

Autolysis of *K. aerobia* yeast in SGM, releasing valuable nutrients for *S. cerevisiae*, could have led to the increase in biomass production in the mixed culture fermentations compared to the *S. cerevisiae* control fermentations, or possibly that dead non-*Saccharomyces* yeast cells increased turbidity in optical density readings. Indeed, the effect of non-*Saccharomyces* yeast on the

performance of *S. cerevisiae* in mixed culture fermentations has been reported by others (Comitini et al., 2011; Mendoza et al., 2007; Sadoudi et al., 2012).

The data showed that *W. anomalus* does not perform well in the presence of *S. cerevisiae*, similar to findings in previous studies although exhibiting a strong growth rate (Heard and Fleet, 1985; Rojas et al., 2003). Competition for nutrients is possibly one of the main reasons for lower population growth (Bagheri, 2014). However, *K. aerobia* and *W. anomalus* has a low level of ammonium and amino acids utilisation in general (Chapter 3). Numerous reasons exist for cell death of the non-*Saccharomyces* yeasts, including sensitivity to rising ethanol levels (Jackson, 2008), decreasing oxygen (Hansen et al., 2001), high temperatures (Egli et al., 1998; Gobbi et al., 2013; Zott et al., 2008), excretion of toxic molecules (killer toxins and medium chain fatty acids) by *S. cerevisiae* (Fleet, 2003; Pérez-Nevado et al., 2006) and physical interactions between yeasts, recently known as a cell-cell contact mechanism (Nissen and Arneborg, 2003). Indeed, studies have noted that *W. anomalus* is not tolerant to high ethanol concentrations (Kalathenos et al., 1995; Fredlund et al., 2002), although findings in the current study suggests otherwise (Chapter 3). Recently glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-derived antimicrobial peptides was detected as one of the reason why many non-*Saccharomyces* yeast dies off in the presence of *S. cerevisiae* (Branco et al., 2014). The exact cause of cell death for specifically *W. anomalus* and *K. aerobia* falls outside of the scope of this study. Testing the growth reaction of *W. anomalus* to different *S. cerevisiae* strains possibly indicate that the reaction is not specific to a specific strain of *S. cerevisiae*. However, some studies have found that *W. anomalus* persist to the end of fermentation, which indicates that interaction can be strain specific (Díaz et al., 2013).

However, in Sauvignon blanc must, killer toxins produced by *W. anomalus* could be the reason for the early death of the indigenous yeast population (Comitini et al., 2004; El-Banna et al., 2011; Sun et al., 2012). Interestingly, with the increased presence of indigenous yeast in the fermentations with *K. aerobia, S. cerevisiae* played a lesser role compared to the fermentations with *W. anomalus* where it completely took over the fermentations. Herraiz et al. (1990) found that *Hanseniaspora uvarum* had an inhibitory effect on *S. cerevisiae* growth, although this was not due to killer activity or any other known inhibition at that time. Antimicrobial peptides produced by non-*Saccharomyces* yeast (depleting iron in must due to production of pulcherriminic acid) could have different effects on indigenous yeasts (Oro et al., 2014) and *S. cerevisiae* (Panon, 1997), as seen with *M. pulcherrima*.

In synthetic and Sauvignon blanc grape must *K. aerobia* performed similarly in mono- and sequential culture fermentations, suggesting that *S. cerevisiae* had little interaction with this yeast. In addition, *S. cerevisiae* growth was also less affected in co-inoculations treatments, with specifically *K. aerobia* Y965. This same lack of impact was seen in mixed fermentations with *M. pulcherrima* and *S. cerevisiae* (Comitini et al., 2011).

Stellenbosch University https://scholar.sun.ac.za

Although fermentations proceeded relatively similarly in synthetic and Sauvignon blanc grape must, monocultures fermented to dryness in the latter must, due to *S. cerevisiae* growth. Wine yeast is usually present in vineyards and cellars and has also been found on grape berry surfaces (Bagheri, 2014) thus explaining how *S. cerevisiae* was introduced to non-*Saccharomyces* monoculture fermentations. *Saccharomyces cerevisiae* is generally very low in grape must (Martini et al., 1996; Mortimer et al., 1999; Combina et al., 2005; Mercado et al., 2007; Di Maro et al., 2007; Guzzon et al., 2011), although it is known to dominate from the middle of fermentation up to the end due to its high fermentative abilities (Fleet and Heard, 1993b; Fleet, 2003).

In addition to the effect of indigenous yeasts in Sauvignon blanc must, differences in population growth between synthetic and Sauvignon blanc grape must could possibly be due to differences in fermentation temperatures. It has been found that non-*Saccharomyces* yeasts compete better against *S. cerevisiae* at lower temperatures (20°C) compared to temperatures of 30°C (Gobbi et al., 2013), and persists for longer (Mills et al., 2002), as similarly found in terms of *K. aerobia* growth at 15°C compared to 30°C in SGM. Gao and Fleet (1988) found that at lower temperatures, *H. uvarum* and *Starmerella bombicola* had increased tolerance to ethanol. Certain non-*Saccharomyces* yeasts has indeed been found to survive throughout fermentation (Combina et al., 2005; Jolly et al., 2006; Mills et al., 2002; Zott et al., 2008).

This data gave insight into the metabolic activity and growth of the non-*Saccharomyces* yeasts *K. aerobia* and *W. anomalus* in synthetic and Sauvignon blanc grape must. It further demonstrated that the presence of these yeasts had no influence on the final ethanol concentration. Earlier studies have found mixed cultures to have similar or higher ethanol concentrations to single *S. cerevisiae* fermentations (Comitini et al., 2011; Toro and Vazquez, 2002; Zironi et al., 1993). In comparison, the yeasts did increase glycerol content, a common trait of non-*Saccharomyces* yeasts (Ciani and Maccarelli, 1998; Tofalo et al., 2012; Toro and Vazquez, 2002), although possibly enhanced by the production of glycerol by the indigenous yeasts.

4.4.2 Aroma production of *K. aerobia* and *W. anomalus* in single and mixed culture fermentations

The impact of the non-*Saccharomyces* yeasts on aroma biosynthesis is evident, as observed for previous fermentations with these species (Beckner Whitener, 2016; Izquierdo Cañas et al., 2014, 2011; Rojas et al., 2003). Even when the non-*Saccharomyces* yeast did not proliferate considerably in the must, it confirmed the impact of non-*Saccharomyces* yeasts present in lower numbers on aroma production (Lema et al., 1996; Romano et al., 1997; Sadoudi et al., 2012; Toro and Vazquez, 2002), as metabolic activity is not necessarily affected (Fleet and Heard, 1993b). Non-*Saccharomyces* yeasts have aromatic capabilities inherent to their metabolism, although interactions with other yeasts or an increased biomass also play a role in aroma production (Ciani et al., 2010;

Sadoudi et al., 2012). The phenotypic space of *K. aerobia* and *W. anomalus* was more readily observed in the monoculture fermentations compared to the mixed culture fermentations. The overall difference between compounds produced in monoculture fermentations compared to mixed culture fermentations is expected (Gobbi et al., 2013; Moreira et al., 2008; Toro and Vazquez, 2002).

The data demonstrated that *K. aerobia* is not a high producer of fatty acids, as similarly observed with the non-*Saccharomyces* yeast *T. delbrueckii,* suggesting that some non-*Saccharomyces* yeasts metabolism does not result in excessive fatty acid biosynthesis (Azzolini et al., 2015). This decrease in fatty acids is favourable for wine quality, as it mostly contributes fatty, rancid, unpleasant odours to wine (Azzolini et al., 2015; Lambrechts and Pretorius, 2000). In addition, increased esters could be due to the conversion of medium chain fatty acids in treatments with *K. aerobia* in SGM to ethyl esters (Saerens et al., 2010). In contrast, most of the measured fatty acids (*e.g.* butyric, hexanoic and octanoic acid) were increased in mixed fermentations with *W. anomalus* and *S. cerevisiae*, possibly due to its excretion during autolysis of *W. anomalus* yeasts (Alexandre and Guilloux-Benatier, 2006; Chen et al., 1980). Octanoic acid production by *S. cerevisiae,* which is toxic to yeast, could be one of the reasons for the decrease in *W. anomalus* population in sequential cultures with *S. cerevisiae* (Alexandre et al., 1996; Fleet and Heard, 1993b; Moreno-Arribas and Polo, 2005).

Numerous non-*Saccharomyces* yeasts are known for their high production of acetic acid (Carrau, 2006; Romano et al., 2003; Sadoudi et al., 2012; Toro and Vazquez, 2002) as characteristic for *K. aerobia* yeasts in this study. Reduction in sequential culture fermentations in Sauvignon blanc fermentations have been observed for numerous mixed culture fermentations (Ciani and Comitini, 2011; Rantsiou et al., 2012; Rojas et al., 2003; Sadoudi et al., 2012) as a result of interactions between yeasts or acetic acid co-metabolism (Dos Santos et al., 2003; Sadoudi et al., 2012). Production of acetic acid is the response of the yeast to repair the redox reaction due to an imbalance caused by increased glycerol production (Scanes et al., 1998; Vilanova et al., 2007), as observed in the *K. aerobia* fermentations. In contrast, lower levels of acetic acid, as shown in fermentations with *W. anomalus*, have been observed for strains of *T. delbrueckii* yeast as single or mixed culture (Azzolini et al., 2015) and *M. pulcherrima* (Sadoudi et al., 2012). These findings contradicts a previous study that showed *W. anomalus* to produce very high levels of acetic acid in single culture (Rojas et al., 2003).

Furthermore, increased acetoin production in monoculture fermentations with *K. aerobia* and *W. anomalus,* could be due to production by indigenous yeast present in Sauvignon blanc grape must, as these concentrations were not observed in synthetic grape must. Non-*Saccharomyces* yeasts have been shown to increase acetoin production (Toro and Vazquez, 2002). However, in sequential culture fermentations in Sauvignon blanc grape must, possible interactions between yeasts lead to a decrease in acetoin to levels lower than the *S. cerevisiae* control. Acetoin can be utilised by *S. cerevisiae* to form 2,3-butanediol or other secondary by-products or possibly increase

Stellenbosch University https://scholar.sun.ac.za

ethanol content (Herraiz et al., 1990; Romano and Suzzi, 1996; Zironi et al., 1993). This same decrease in concentration was seen in mixed culture fermentations of *S. cerevisiae* with *Candida canterellii* (Toro and Vazquez, 2002) and *Pichia fermentans* (Clemente-Jimenez et al., 2005).

Apiculate yeast are known to increase ethyl acetate (Carrau, 2006; Gobbi et al., 2013), specifically *W. anomalus* (Passoth et al., 2006; Rojas et al., 2003, 2001), as similarly observed in this study. High ethyl acetate levels are caused by an increase in alcohol acetyl transferase activity (Lilly et al., 2000). Strain differences for *W. anomalus* in terms of ethyl acetate production, confirms previous findings (Domizio et al., 2011), Although Beckner Whitener (2016) observed high ethyl acetate production in real grape must fermented with *K. aerobia,* this was not documented in the current study and increased ethyl acetate in fermentations with *K. aerobia* could be due to production by indigenous yeast. Furthermore, ethyl acetate production is favoured at lower temperatures, as seen in this study for fermentation at 15°C (Gobbi et al., 2013). However in general, *K. aerobia* has been found to produce high amounts of esters, specifically 2-phenylethyl acetate (Beckner Whitener, 2016).

The increase in production of higher alcohols (especially 2-phenyl ethanol) in sequential culture fermentations with *W. anomalus* confirms previous observations (Rojas et al., 2003) and is common in mixed culture fermentations with non-*Saccharomyces* yeasts such as *T. delbrueckii, M. pulcherrima, Lachancea thermotolerans* and *Starmarella bacillaris* (Gobbi et al., 2013; Sadoudi et al., 2012). Likewise, propanol and isoamyl alcohol production was increased in sequential culture fermentations compared to monocultures, as observed in a similar study (Rojas et al., 2003). Furthermore, lower temperatures in the Sauvignon blanc grape must fermentations, could have led to increased production of higher alcohols, as previously found in fermentations conducted at 20°C compared to 30°C (Gobbi et al., 2013). In fermentations with *K. aerobia*, an increase in higher alcohols was noted with a delay in inoculation of *S. cerevisiae* in SGM, possibly due to the additional time that *K. aerobia* had to grow and metabolise in the grape must. Increased production of higher alcohols such as isobutanol and isoamyl alcohol, and 2-phenylethyl acetate, in mixed culture fermentations could be due to an increase of amino acids caused by autolysis of dying *K. aerobia* and *W. anomalus* yeast cells (Jackson, 2008; Toro and Vazquez, 2002).

Neutral interactions have been observed between *T. delbrueckii* and *S. cerevisiae*, where aromatic profiles were similar and differences in volatile compounds was only due to a biomass effect (Gobbi et al., 2013; Sadoudi et al., 2012), as perceived for *K. aerobia* and *S. cerevisiae* yeasts in mixed culture fermentations in SGM. Furthermore, Gobbi et al. (2013) found that differences in monoculture fermentations and mixed culture fermentations were less at a higher fermentation temperature (30°C), possibly due to the increase in *S. cerevisiae* growth at the higher fermentation temperature. In contrast, interactions between *W. anomalus* and *S. cerevisiae* was more notable. Negative interactions between *W. anomalus* and *S. cerevisiae* caused a decrease in production of isobutyric

acid. These interactions, not dependant on biomass, have been observed between *Starmarella bacillaris* and *S. cerevisiae* leading to a decrease in lactone and terpene concentrations (Gobbi et al., 2013). Other negative interactions between yeasts have been reported by Sadoudi et al. (2012). Moreover, positive interactions caused an increase in production of ethyl caprylate and ethyl caproate, as both *W. anomalus* and *S. cerevisiae* produced low concentrations of these esters as monocultures in both musts, but doubled the concentration in mixed culture fermentations. Synergistic effects, not dependant on biomass, have been reported between *M. pulcherrima* and *S. cerevisiae* (Gobbi et al., 2013; Sadoudi et al., 2012).

In Sauvignon blanc grape must, changes in aroma production from days 21 until day 28 were minimal, suggesting that aromas measured in this experiment were produced during the exponential phase and not in the stationary phase; confirming data found by other researchers (Miller et al., 2007; Plata et al., 2003). This study furthermore contrasts the perception of certain markets and winemakers that favours spontaneous fermentations, as this fermentation yielded lower aromatic compounds (Pretorius, 2000).

Even though *W. anomalus* was present for a shorter time in the fermentations compared to *K. aerobia* it had a more significant impact on aroma production (higher alcohols and fatty acids), probably due to its increased biomass during the start of fermentation. In addition, *W. anomalus* is known to be more aromatic than other non-*Saccharomyces* yeasts (Rojas et al., 2001). The prominent differences between *K. aerobia* and *W. anomalus* have been noted for ethyl acetate and acetic acid, but numerous differences in terms of fatty acid and higher alcohol production have been noted. Many more metabolites can be analysed to determine the differences between these species.

This study shows the phenotypic space of the production of certain compounds by *W. anomalus* and *K. aerobia* strains. Significant differences was observed for ethyl acetate and acetic acid production as well as many higher alcohols such as isobutanol, butanol, and isoamyl alcohol. Differences are more pronounced in monoculture fermentations. Other yeasts from *Candida*, *Hanseniaspor*a and *Pichia* species have also shown large intra-strain variability (Viana et al., 2008).

4.5 Conclusion

This study showed the potential of *K. aerobia* and *W. anomalus* to alter wine character and quality in synthetic and Sauvignon blanc grape must fermentations. In addition, these findings show the potential growth and fermentative abilities of these yeasts in combination with *S. cerevisiae*. However, it is paramount to monitor accumulation of undesirable compounds as it became evident that *W. anomalus* produced high ethyl acetate, with *K. aerobia* identified as a higher acetic acid producer. Furthermore, *W. anomalus* had an increased impact on aroma production compared to *K. aerobia*. These yeast also contributed to desirable aromatic compounds, for instance 2-phenylethyl

acetate known to give a rose aroma to wine. It is essential to take into consideration the observed phenotypic variation between the strains in terms of fermentative performance, growth and aroma production.

The probable cause for cell death of especially *W. anomalus* still needs to be investigated. If these yeasts were to be considered for possible use in mixed culture fermentations, many more metabolites will need to be measured, including sensory analysis, to make an informed decision. In addition, increasing the number of strains could further enhance our understanding of the phenotypic space of these yeasts. Although methods for detection of yeasts are not yet readily available in wineries, this study gives insight into the possible effect of these yeasts in fermentations. These finding contribute to understanding the impact of the possible typical *terroir* of South African musts, although further work still need to be conducted to determine the effect of different non-*Saccharomyces* and *S. cerevisiae* strains to fully characterise their impact on winemaking.

4.6 References

Alexandre, H., Guilloux-Benatier, M., 2006. Yeast autolysis in sparkling wine - A review. Aust. J. Grape Wine Res. 12, 119–127.

Alexandre, H., Mathieu, B., Charpentier, C., 1996. Alteration in membrane fluidity and lipid composition, and modulation of H+-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid. Microbiology 142, 469–475.

Anfang, N., Brajkovich, M., Goddard, M.R., 2009. Co-fermentation with *Pichia kluyveri* increases varietal thiol concentrations in Sauvignon blanc. Aust. J. Grape Wine Res. 15, 1–8.

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., Zapparoli, G., 2015. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 31, 277–293.

Bagheri, B., 2014. Comparative analysis of fermentative yeasts during spontaneous fermentation of grapes from different management systems. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Bagheri, B., Bauer, F.F., Setati, M.E., 2015. The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S. Afr. J. Enol. Vitic. 36, 243–251.

Beckner Whitener, M.E., 2016. Metabolomic profiling of non-*Saccharomyces* yeasts in wine. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Beckner Whitener, M.E., Stanstrup, J., Panzeri, V., Carlin, S., Divol, B., Du Toit, M., Vrhovsek, U., 2016. Untangling the wine metabolome by combining untargeted SPME – GCxGC-TOF-MS and sensory analysis to profile Sauvignon blanc co-fermented with seven different yeasts. Metabolomics 12. doi:10.1007/s11306-016-0962-4

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122, 312–320.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2013. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. U. S. A. 111, E139–48.

Branco, P., Francisco, D., Chambon, C., Hébraud, M., Arneborg, N., Almeida, M.G., Caldeira, J., Albergaria, H., 2014. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl. Microbiol. Biotechnol. 98, 843–853.

Carrau, F., 2006. Native yeasts for low input winemaking: Searching for wine diversity and increased complexity. Int. Wine Microbiol. Symp. 33–39.

Charoenchai, C., Fleet, G.H., Henschke, P.A., 1998. Effects of temperature, pH, and sugar concentration on the growth rates and cell biomass of wine yeasts. Am. J. Enol. Vitic. 49, 283–288.

Chen, E.C.-H., Jamieson, A.M., Van Gheluwe, G., 1980. The release of fatty acids as a consequence of yeast autolysis. J. Am. Soc. Brew. Chem. 38, 13–18.

Ciani, M., Beco, L., Comitini, F., 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int. J. Food Microbiol. 108, 239–245.

Ciani, M., Comitini, F., 2011. Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. Ann. Microbiol. 61, 25–32.

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

Ciani, M., Maccarelli, F., 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World J. Microbiol. Biotechnol. 14, 199–204.

Clemente-Jimenez, J.M., Mingorance-Cazorla, L., Martínez-Rodríguez, S., Las Heras-Vázquez, F.J., Rodríguez-Vico, F., 2005. Influence of sequential yeast mixtures on wine fermentation. Int. J. Food Microbiol. 98, 301–308.

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

Comitini, F., De, J.I., Pepe, L., Mannazzu, I., Ciani, M., 2004. *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/ Brettanomyces* spoilage yeasts. FEMS Mircobiology Lett. 238, 235–240.

Comitini, F., Gobbi, M., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae.* Food Microbiol. 28, 873–882.

Contreras, A., Curtin, C., Varela, C., 2015. Yeast population dynamics reveal a potential “collaboration” between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. Appl. Microbiol. Biotechnol. 99, 1885–1895.

Stellenbosch University https://scholar.sun.ac.za

Contreras, A., Hidalgo, C., Henschke, P.A., Chambers, P.J., Curtin, C., Varela, C., 2014. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. Appl. Environ. Microbiol. 80, 1670–1678.

De Koker, S., 2015. Nitrogen utilisation of selected non-*Saccharomyces* yeasts and the impact on volatile compound production. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Díaz, C., Molina, A.M., Nähring, J., Fischer, R., 2013. Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. Biomed Res. Int. Article ID 540465

Domizio, P., Romani, C., Comitini, F., Gobbi, M., Lencioni, L., Mannazzu, I., Ciani, M., 2011. Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Ann. Microbiol. 61, 137–144.

Dos Santos, M.M., Gombert, A.K., Christensen, B., Olsson, L., Nielsen, J., 2003. Identification of in vivo enzyme activities in the co-metabolism of glucose and acetate by *Saccharomyces cerevisiae* by using 13 C-labeled substrates. Society 2, 599–608.

Egli, C.M., Edinger, W.D., Mitrakul, C.M., Henick-Kling, T., 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. J. Appl. Microbiol. 85, 779–789.

El-Banna, A.A., El-Sahn, M.A., Shehata, M.G., 2011. Yeasts producing killer toxins: An overview. Alex. J. Fd. Sci. Technol 8, 41–53.

Fleet, G.H., 2003. Yeast interactions and wine flavour. Int. J. Food Microbiol. 86, 11–22.

Fleet, G.H., Heard, G.M., 1993. Yeasts - Growth during fermentation, in: Fleet, G.H. (Ed.), Wine Microbiology and Biotechnology. Harwood Academic Publishers, pp. 28–47.

Gao, C., Fleet, G.H., 1988. The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. J. Appl. Bacteriol. 65, 405–409.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. Food Microbiol. 33, 271–281.

Hansen, H., Nissen, P., Sommer, P., Nielsen, J.C., Arneborg, N., 2001. The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. J. Appl. Microbiol. 91, 541–7.

Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microbiol. 50, 727–728.

Herraiz, T., Reglero, G., Herraiz, M., Martin-Alvarez, P.J., Cabezudo, M.D., 1990. The influence of the yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide. Am. J. Enol. Vitic. 41, 313–318.

Stellenbosch University https://scholar.sun.ac.za

Izquierdo Cañas, P.M., Esteban, C., Romero, G., Heras, J.M., Mónica, M., González, F., 2014. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. Eur. Food Res. Technol. 239, 279–286.

Izquierdo Cañas, P.M., Palacios Garcia, A.T., Garcia Romero, E., 2011. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starter. Vitis 50, 177–182.

Jackson, R.S., 2008. Fermentation - Biochemistry of alcoholic fermentation, in: Wine Science Principles and Applications. Elsevier, London, pp. 358–372.

Jolly, N.P., Augustyn, O.H.P., Pretorius, I.S., 2003a. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55–62.

Jolly, N.P., Augustyn, O.H.P.H., Pretorius, I.S., 2003b. The occurrence of non-*Saccharomyces* cerevisiae yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S. Afr. J. Enol. Vitic. 24, 63–69.

Jolly, N.P., Augustyn, O.P.H., Pretorius, I.S., 2006. The role and use of non-*Saccharomyces* yeasts in wine production. S. Afr. J. Enol. Vitic. 27, 15–39.

Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: Non-S*accharomyces* yeasts in wine production uncovered. FEMS Yeast Res. 14, 215–237.

Kapsopoulou, K., Mourtzini, A., Anthoulas, M., Nerantzis, E., 2007. Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 23, 735–739.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its importance to wine aroma - A review. S. Afr. J. Enol. Vitic. 21, 97–129.

Lema, C., Garcia-Jares, C., Orriols, I., Angulo, L., 1996. Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albarino wine aroma. Am. J. Enol. Vitic. 47, 206–216.

Lilly, M., Lambrechts, M.G., Pretorius, I.S., 2000. Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. Appl. Environ. Microbiol. 66, 744–753.

Lu, H.Z., Cai, Y., Wu, Z.W., Jia, J.H., Bai, F.Y., 2004. *Kazachstania aerobia* sp. nov., an ascomycetous yeast species from aerobically deteriorating corn silage. Int. J. Syst. Evol. Microbiol. 54, 2431–2435.

Mains, A.O., 2014. Evaluating the impact of yeast co-inoculation on individual yeast metabolism and wine composition. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Mas, A., Padilla, B., Esteve-Zarzoso, B., Beltran, G., Reguant, C., Bordons, A., 2016. Taking advantage of natural biodiversity for wine making: The Wildwine project. Agric. Agric. Sci. Procedia 8, 4–9.

Stellenbosch University https://scholar.sun.ac.za

Mendoza, L.M., De Nadra, M.C.M., Farías, M.E., 2007. Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. Biotechnol. Lett. 29, 1057–1063.

Miller, A.C., Wolff, S.R., Bisson, L.F., Ebeler, S.E., 2007. Yeast strain and nitrogen supplementation: Dynamics of volatile ester production in Chardonnay juice fermentations. Am. J. Enol. Vitic. 58, 470–483.

Mills, D.A., Johannsen, E.A., Cocolin, L., 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. Appl. Environ. Microbiol. 68, 4884–4893.

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., Vasconcelos, I., 2008. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. Int. J. Food Microbiol. 124, 231–238.

Moreno-Arribas, V.M., Polo, C.M., 2005. Winemaking biochemistry and microbiology: current knowledge and future trends. Crit. Rev. Food Sci. Nutr. 45, 265–286.

Nissen, P., Arneborg, N., 2003. Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. Arch. Microbiol. 180, 257–263.

Oro, L., Ciani, M., Comitini, F., 2014. Antimicrobial activity of *Metschnikowia pulcherrima* on wine yeasts. J. Appl. Microbiol. 116, 1209–1217.

Panon, G., 1997. Influence of oxygen on fermentation pattern in model media containing mixed or sequential cultures of three cider-producing yeasts: *Saccharomyces cerevisiae* var. *uvarum*, *Hanseniaspora valbyensis* and *Metschnikowia pulcherrima*. Sci. Aliments 17, 193–217.

Passoth, V., Fredlund, E., Druvefors, U.Ä., Schnürer, J., 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 6, 3–13.

Pérez-Nevado, F., Albergaria, H., Hogg, T., Girio, F., 2006. Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 108, 336–345.

Plata, C., Millán, C., Mauricio, J.C., Ortega, J.M., 2003. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. Food Microbiol. 20, 217–224.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast 16, 675–729.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolina, L., 2012. *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. Appl. Environ. Microbiol. 78, 1987–1994.

Rojas, V., Gil, J. V., Piaga, F., Manzanares, P., 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int. J. Food Microbiol. 70, 283–289.

Rojas, V., Gil, J. V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int. J. Food Microbiol. 86, 181–188.

Stellenbosch University https://scholar.sun.ac.za

Romano, P., Fiore, C., Paraggio, M., Caruso, M., Capece, A., 2003. Function of yeast species and strains in wine flavour. Int. J. Food Microbiol. 86, 169–180.

Romano, P., Suzzi, G., 1996. Origin and production of acetoin during wine yeast fermentation. Appl. Environ. Microbiol. 62, 309–315.

Romano, P., Suzzi, G., Comi, G., Zironi, R., Maifreni, M., 1997. Glycerol and other fermentation products of apiculate wine yeasts. J. Appl. Microbiol. 82, 615–618.

Rossouw, D., Bauer, F.F., 2016. Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. Food Microbiol. 55, 32–46.

Sabel, A., Martens, S., Petri, A., König, H., Claus, H., 2014. *Wickerhamomyces anomalus* AS1: A new strain with potential to improve wine aroma. Ann. Microbiol. 64, 483–491.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.-J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H., Alexandre, H., 2012. Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol. 32, 243–253.

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 165–177.

Scanes, K.T., Hohmann, S., Prior, B.A., 1998. Glycerol production by the yeast *Saccharomyces cerevisiae* and its relevance to wine: A review. S. Afr. J. Enol. Vitic. 19, 17–24.

Setati, M.E., Jacobson, D., Andong, U., Bauer, F., 2012. The vineyard yeast microbiome, a mixed model microbial map. PLoS One 7, e52609.

Soden, A., Francis, I.L., Oakey, H., Henschke, P.A., 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. Aust. J. Grape Wine Res. 6, 21–30.

Sun, H.-Y., Wang, K., Chi, Z., Xu, H.-M., Chi, Z.-M., 2012. Simultaneous production of single cell protein and killer toxin by *Wickerhamomyces anomalus* HN1-2 isolated from mangrove ecosystem. Process Biochem. 47, 251–256.

Swangkeaw, J., Vichitphan, S., Butzke, C.E., Vichitphan, K., 2009. The characterisation of a novel *Pichia anomala* β-glucosidase with potentially aroma-enhancing capabilities in wine. Ann. Microbiol. 59, 335–343.

Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139–173.

Taillandier, P., Lai, Q.P., Julien-Ortiz, A., Brandam, C., 2014. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. World J. Microbiol. Biotechnol. 30, 1959–1967.

Tofalo, R., Schirone, M., Torriani, S., Rantsiou, K., Cocolin, L., Perpetuini, G., Suzzi, G., 2012. Diversity of *Candida zemplinina* strains from grapes and Italian wines. Food Microbiol. 29, 18–26.

Stellenbosch University https://scholar.sun.ac.za

Toro, M.E., Vazquez, F., 2002. Fermentation behaviour of controlled mixed and sequential cultures of *Candida cantarellii* and *Saccharomyces cerevisiae* wine yeasts. World J. Microbiol. Biotechnol. 18, 347–354.

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

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Appl. Microbiol. Biotechnol. 77, 145–157.

Zironi, R., Romano, P., Suzzi, G., Battistutta, F., Comi, G., 1993. Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. Biotechnol. Lett. 15, 235–238.

Zott, K., Miot-Sertier, C., Claisse, O., Lonvaud-Funel, A., Masneuf-Pomarede, I., 2008. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. Int. J. Food Microbiol. 125, 197–203.

4.7 Appendix

Table 1. Test of SS Whole Model vs. SS Residual (Test of significance) for end point (dependent variable day 21) CO_2 production and OD_{600} production of *K. aerobia* mixed culture fermentations and *S. cerevisiae* control (significant differences indicated in boldface, p<0.05)

Variable	Multi-ple R	Multi-ple R²	Adjust ed R²	SS Model	df Model	MS Mode l	SS Resi-dual	df Resi-dual	MS Resi-dual	F	p
CO_2	0.50	0.25	-0.04	21.48	10	2.15	63.18	25	2.53	0.85	0.59
OD_{600}	0.90	0.81	0.74	56.28	10	5.63	13.05	25	0.52	10.78	0.00

Table 2. CO_2 production after 21 days of fermentations with *K. aerobia* and *W. anomalus* in Sauvignon blanc grape must as monocultures and sequential cultures inoculated with *S. cerevisiae*. *S. cerevisiae* EC1118 served as control. Values indicated as mean ± standard error.

Treatment	**Yeast isolate**	**CO_2 production (day 21)**	**CO_2 production (day 28)***
Control	EC1118	5.52 ± 0.03 a	-
Monocultures	WA: Y934-C	5.21 ± 0.09 ab	5.41 ± 0.11
	WA: LO632	5.67 ± 0.36 ab	5.93 ± 0.35
	KA: Y965	5.05 ± 0.05 ab	5.27 ± 0.01
	KA: CBS	4.69 ± 0.28 ab	5.08 ± 0.15
Sequential cultures	WA: Y934-C	5.62 ± 0.04 ab	-
	WA: LO632	5.57 ± 0.08 a	-
	KA: Y965	5.53 ± 0.15 a	-
	KA: CBS	5.55 ± 0.03 ab	-
	Spontaneous	4.42 ± 0.34 b	5.44 ± 0.45

Values with the same letter in the same column are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

*No statistical difference

- Fermentations terminated

Table 3. Residual sugar (glucose and fructose) of fermentations conducted by *W. anomalus* (WA), *K. Aerobia* (KA) compared to *S. cerevisiae* (EC1118). The data points represents mean ± standard error of mean (n = 3).

Treatment	Yeast isolate	Glucose (g/L)	Fructose (g/L)
Control	EC1118	6.19 + 1.07 a	26.87 + 2.11 a
Monocultures	WA: Y934-C	77.37 + 3.61 d	85.06 + 1.13 de
	WA: LO632	81.97 + 2.38 de	88.85 + 1.72 e
	KA: Y965	64.88 + 1.15 c	73.26 + 0.82 c
	KA: CBS	76.42 + 1.93 d	77.85 + 1.06 cd
Sequential cultures	WA: Y934-C	32.20 + 2.31 b	55.55 + 0.87 b
	WA: LO632	33.07 + 1.17 b	60.77 + 1.34 b
	KA: Y965	37.53 + 1.16 b	58.16 + 1.8 b
	KA: CBS	39.31 + 2.38 b	59.70 + 2.87 b
	Spontaneous	88.84 + 1.00 e	76.94 + 2.14 cd

Values with the same letter in the same column are statistically similar when compared with Tukey's HSD post-hoc test at 95 % confidence level.

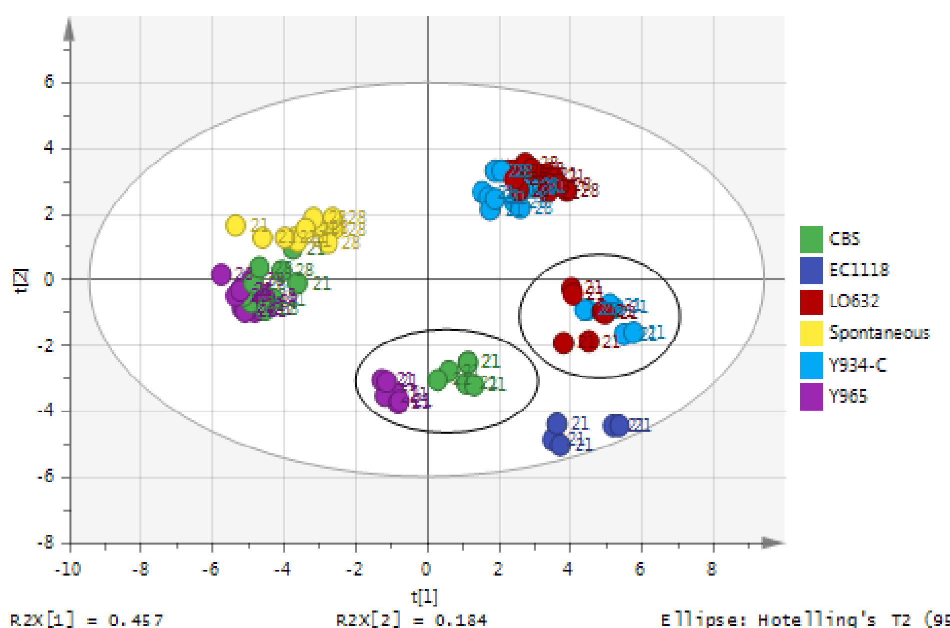


Figure 1. PCA scores plot compares aroma compounds produced in Sauvignon blanc grape must by *W. anomalus* (Y934-C, LO632) and *K. aerobia* (Y965, CBS) in mono- and sequential culture fermentations (indicated with circles on plot) on day 21 and day 28. Scores denotes yeast strains used and labels indicate day of fermentation.

Table 4. Chemical analysis conducted after 21 days of fermentation with *K. aerobia* (KA) and *W. anomalus* (WA) as monoculture fermentations and in sequential inoculations with *S. cerevisiae* EC1118. Measurements were done using FT-IR ATR mid-infrared spectrometry. All values indicated as mean ± standard error.

Treatment	Saccharose (g/L)*	pH	Tartaric acid (g/L)*	Malic acid (g/L)	Lactic acid (g/L)*	Citric acid (g/L)	Total acid (g/L)
EC1118	1.20 ± 0.15	3.12 ± 0.01^{a}	2.07 ± 0.12	1.93 ± 0.03^{ab}	0.07 ± 0.03	0.00 ± 0.00^{b}	4.67 ± 0.12^{ab}
WA: Y934-C	0.50 ± 0.25	3.03 ± 0.02^{cde}	2.00 ± 0.29	1.87 ± 0.15^{ab}	0.13 ± 0.07	0.10 ± 0.10^{ab}	5.23 ± 0.24^{ab}
WA: LO632	0.30 ± 0.25	3.01 ± 0.01^{de}	1.93 ± 0.19	2.07 ± 0.18^{a}	0.13 ± 0.13	0.07 ± 0.03^{b}	5.37 ± 0.22^{ab}
KA: Y965	0.83 ± 0.19	3.02 ± 0.01^{de}	1.93 ± 0.15	1.70 ± 0.12^{ab}	0.03 ± 0.03	0.03 ± 0.03^{b}	5.37 ± 0.03^{ab}
KA: CBS	0.43 ± 0.13	3.00 ± 0.02^{de}	1.93 ± 0.12	1.97 ± 0.19^{ab}	0.00 ± 0.00	0.07 ± 0.03^{b}	5.67 ± 0.19^{a}
WA: Y934-C + EC1118	0.70 ± 0.40	3.11 ± 0.03^{abc}	2.17 ± 0.09	1.83 ± 0.03^{ab}	0.30 ± 0.17	0.23 ± 0.12^{ab}	5.13 ± 0.12^{ab}
WA: LO632 + EC1118	1.13 ± 0.18	3.10 ± 0.01^{ab}	2.13 ± 0.03	1.90 ± 0.15^{ab}	0.13 ± 0.07	0.03 ± 0.03^{b}	4.93 ± 0.07^{ab}
KA: Y965 + EC1118	0.97 ± 0.15	3.04 ± 0.01^{bcde}	1.97 ± 0.09	1.50 ± 0.06^{ab}	0.03 ± 0.03	0.27 ± 0.03^{ab}	5.27 ± 0.09^{ab}
KA: CBS + EC1118	0.77 ± 0.15	3.07 ± 0.00^{abcd}	2.17 ± 0.07	1.63 ± 0.07^{ab}	0.20 ± 0.06	0.07 ± 0.03^{b}	5.47 ± 0.09^{a}
Spontaneous	0.73 ± 0.09	2.99 ± 0.02^{e}	1.77 ± 0.03	1.47 ± 0.03^{b}	0.10 ± 0.10	0.37 ± 0.03^{a}	5.03 ± 0.20^{b}

Values with the same letter in the same column are statistically similar when compared with Unequal N HSD post-hoc test at 95% confidence level

*No statistical difference

Stellenbosch University https://scholar.sun.ac.za

Chapter 5

General discussion and conclusions

Stellenbosch University https://scholar.sun.ac.za

General discussion and conclusions

5.1 Concluding remarks and future prospects

Much evidence exists for the impact of the natural microflora on the aroma of wine. However, it is perceived that the use of inoculated *S. cerevisiae* reduces wine complexity, producing a more uniform wine. This statement is more anecdotal than scientific, but can be taken seriously as many such claims exist. However, this claim is not yet supported by many scientific data sets. It has been documented that regional differences in microflora exist (Bokulich et al., 2013; Knight et al., 2015) and it is suggested these local microbiomes contributes to a *terroir* specific character of wine. However, there is no convincing evidence that these differences actually and consistently impact the character of wine, and in such ways as to be recognisably different from wines in other regions.

Research conducted to elucidate the local microflora of South African grape must environment is an attempt to understand these claims. The dataset of non-*Saccharomyces* yeasts indigenous to South African grape musts and its effect on aroma and fermentation is limited, with only a few studies conducted using either metabolomics or culture based methods to determine the local yeast microflora (Bagheri et al., 2015; Jolly et al., 2003b; Setati et al., 2012; Van Zyl and Du Plessis, 1961). Consequently, it is paramount that the impact of non-*Saccharomyces* yeasts on fermentation and the character of South African wine be further evaluated.

In attempt to further understand the impact of local microflora on wine, this study looked at the recently isolated yeast *K. aerobia* and *W. anomalus,* found to be dominant in certain grape musts (Bagheri et al., 2015; Setati et al., 2012). Isolates of these yeasts were characterised employing stress assays, RAPD analysis, and monitoring nitrogen usage. In addition, the fermentative and aroma production abilities of these yeasts were determined in single and mixed culture fermentations in varying conditions in synthetic as well as real (Sauvignon blanc) grape must.

After conducting monoculture fermentations with phenotypic diverse strains, it was concluded that the non-*Saccharomyces* yeasts showed a low ammonia consumption rate with a subsequently low fermentation rate compared to *S. cerevisiae*, typical of non-*Saccharomyces* yeasts (Jolly et al., 2003a). According to our knowledge, this is the first time that nitrogen consumption in these yeasts was investigated. Similar to findings of the metabolism of *S. cerevisiae*, the addition of amino acids significantly impacted aroma production (Arias-Gil et al., 2007; Mckinnon, 2013; Smit, 2013), confirming the effect of BCAA's on the production of higher alcohols, acids (García et al., 1994; Hazelwood et al., 2008; Mendes-Ferreira et al., 2011), and esters (Hernández-Orte et al., 2002; Herraiz and Ough, 1993; Saerens et al., 2010). *Wickerhamomyces anomalus* converted amino acids more effectively into aroma compounds, possibly due to increased branched-chain amino acid

transaminases (BCAAT) (Lilly et al., 2006) or its increased biomass production (Bell and Henschke, 2005).

In mixed culture fermentations with *S. cerevisiae*, these yeasts did not survive until completion of fermentation. With a delay in inoculation of *S. cerevisiae*, or inoculation at a lower density, the non-*Saccharomyces* yeasts proliferated for longer in the must, with an increased impact on the aroma profile of the wines, as observed in similar studies (Bely et al., 2008; Gobbi et al., 2013; Kapsopoulou et al., 2007; Pérez-Nevado et al., 2006). Interestingly, *K. aerobia* yeasts survived for longer in Sauvignon blanc grape must, although not as dominant as *W. anomalus* or *S. cerevisiae*. Mendoza et al. (2007) also found that even though the production of biomass is lower, certain non-*Saccharomyces* strains can have an increased persistence during fermentation when in a mixed culture fermentation. In contrast, a strong antagonistic effect was observed between *W. anomalus* and *S. cerevisiae* as the former yeast died off as soon as *S. cerevisiae* was inoculated, confirming previous results (Heard and Fleet, 1985; Rojas et al., 2003).

Typical for mixed culture fermentations, higher alcohols were increased, irrespective of the non-*Saccharomyces* yeast (Rojas et al., 2003). Production of favourable aroma compounds, such as 2-phenylethyl acetate, indicates the possible positive impact of indigenous microflora in wines. *Kazachstania aerobia* and *W. anomalus,* although only reported on in a limited amount of studies, have been shown to favourably increase certain flavour compounds (Beckner Whitener, 2016; Izquierdo Cañas et al., 2014, 2011). However, high acetic acid and ethyl acetate concentrations (common for non-*Saccharomyces* yeasts), produced by *K. aerobia* and *W. anomalus* respectively, is a cause of concern when present in must (Carrau, 2006; Gobbi et al., 2013; Passoth et al., 2006; Rojas et al., 2003, 2001; Romano et al., 1997; Sadoudi et al., 2012; Toro and Vazquez, 2002). With regards to these compounds, phenotypic differences are evident, and could lead to classification as either spoilage or favourable yeast (Azzolini et al., 2015; Romano et al., 1992).

Negative characteristics in monoculture fermentations of these yeasts can be deluding, as interaction with *S. cerevisiae* can lower the concentrations of ethyl acetate and acetic acid (Ciani and Comitini, 2011; Rantsiou et al., 2012; Rojas et al., 2003; Sadoudi et al., 2012). Although high levels of acetic acid could possibly inhibit growth of *S. cerevisiae* during mixed culture fermentations (Mortimer, 2000). Positive interaction between these yeasts and *S. cerevisiae*, documented for many other yeasts, resulted in increased esters and decreased ethyl acetate and acetic acid (Ciani and Comitini, 2015; Sadoudi et al., 2012). Increased esters have an important impact as volatile esters have an additive effect and can thus be observed at low concentrations (Meilgaard, 1975). Similar studies show the larger phenotypic space of *W. anomalus*, as previously this yeast had been found to produce high acetic acid (Rojas et al., 2003).

Overall fermentations proceeded similarly in both synthetic and Sauvignon blanc grape musts, although, indigenous yeasts does impact the aroma profile of especially monoculture inoculated

Stellenbosch University https://scholar.sun.ac.za

wines (Lema et al., 1996; Sadoudi et al., 2012; Toro and Vazquez, 2002). However, the aroma profile of sequential culture fermentations were similar in both musts.

It has yet to be determined if these yeasts are dominant in most grape must or other areas globally. Although *W. anomalus* have been detected in other areas (Cordero-Bueso et al., 2011; Díaz et al., 2013; Mora and Mulet, 1991; Regueiro et al., 1993; Rojas et al., 2003; Zagorc et al., 2001), *K. aerobia* has yet to be isolated in other wine environments. This will enable further characterisation of these yeasts and enable determination of the full phenotypic space, even between regions. Indeed, this study showed strain differences between regions, shedding light on the hypothesis of *terroir* specific microflora.

Future prospects are to elucidate the impact of other indigenous yeasts on *K. aerobia* and *W. anomalus*, as microflora between grape musts differ. There is a need to understand microbial dynamics in grape musts to either exploit or suppress natural microflora (Egli et al., 1998). Specific interactions between these yeasts in mixed culture fermentations have not yet been determined and all omics approaches (transcriptomic, proteomic and metabolomic) are necessary to elucidate the metabolic mechanisms involved (Ciani et al., 2010). In addition, mechanisms causing cell death were not investigated and could be noteworthy to clarify.

To summarise; this study was an initial attempt to determine the phenotypic space of the non-*Saccharomyces* yeasts, *K. aerobia* and *W. anomalus*. The isolates were grouped into different strains and the yeasts showed a pronounced impact on fermentation metabolites in mixed culture fermentations with *S. cerevisiae*. These findings show the possible impact of these yeasts when present in musts, and if winemakers were able to identify specific yeasts in grape must, this could affect decisions regarding winemaking practises such as extended maceration times or conducting of spontaneous fermentations. Both a positive and negative impact on wine aroma was observed. In addition, the impact of amino acids on the aroma profile of single culture non-*Saccharomyces* yeasts were shown. Many more relatively unknown non-*Saccharomyces* yeasts are present in grape must and the impact of these yeasts on spontaneous fermentations or those inoculated with a commercial starter culture is yet unknown. Ultimately the question still arise whether these yeasts can impart a typical characteristic to wine. To further prove such a claim, we would need multiyear studies of vineyard and winery microbiomes, and we would have to be able to consistently link the aromatic feature to the resulting wines to these microbiomes.

5.2 References

Arias-Gil, M., Garde-Cerdán, T., Ancín-Azpilicueta, C., 2007. Influence of addition of ammonium and different amino acid concentrations on nitrogen metabolism in spontaneous must fermentation. Food Chem. 103, 1312–1318.

Stellenbosch University https://scholar.sun.ac.za

Azzolini, M., Tosi, E., Lorenzini, M., Finato, F., Zapparoli, G., 2015. Contribution to the aroma of white wines by controlled *Torulaspora delbrueckii* cultures in association with *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 31, 277–293.

Bagheri, B., Bauer, F.F., Setati, M.E., 2015. The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S. Afr. J. Enol. Vitic. 36, 243–251.

Beckner Whitener, M.E., 2016. Metabolomic profiling of non-*Saccharomyces* yeasts in wine. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Bell, S.J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242–295.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., Dubourdieu, D., 2008. Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. Int. J. Food Microbiol. 122, 312–320.

Bokulich, N.A., Thorngate, J.H., Richardson, P.M., Mills, D.A., 2013. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proc. Natl. Acad. Sci. U. S. A. 111, 139-148

Carrau, F., 2006. Native yeasts for low input winemaking: Searching for wine diversity and increased complexity. Int. Wine Microbiol. Symp. 33–39.

Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation. Curr. Opin. Food Sci. 1, 1–6.

Ciani, M., Comitini, F., 2011. Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. Ann. Microbiol. 61, 25–32.

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

Cordero-Bueso, G., Arroyo, T., Serrano, A., Tello, J., Aporta, I., Vélez, M.D., Valero, E., 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. Int. J. Food Microbiol. 145, 132–139.

Díaz, C., Molina, A.M., Nähring, J., Fischer, R., 2013. Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. Biomed Res. Int. Article ID 540465

Egli, C.M., Edinger, W.D., Mitrakul, C.M., Henick-Kling, T., 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. J. Appl. Microbiol. 85, 779–789.

García, A.I., García, L.A., Díaz, M., 1994. Fusel alcohols production in beer fermentation processes. Process Biochem. 29, 303–309.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M., 2013. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. Food Microbiol. 33, 271–281.

Stellenbosch University https://scholar.sun.ac.za

Hazelwood, L.A., Daran, J., van Maris, A.J.A., Pronk, J.T., Dickinson, J.R., 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl. Environ. Microbiol. 74, 2259–3920.

Heard, G.M., Fleet, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. Appl. Environ. Microbiol. 50, 727–728.

Hernández-Orte, P., Cacho, J.F., Ferreira, V., 2002. Relationship between varietal amino acid profile of grapes and wine aromatic composition. Experiments with model solutions and chemometric study. J. Agric. Food Chem. 50, 2891–2899.

Herraiz, T., Ough, C.S., 1993. Formation of ethyl esters of amino acids by yeasts during the alcoholic fermentation of grape juice. Am. J. Enol. Vitic. 44, 41–48.

Izquierdo Cañas, P.M., Esteban, C., Romero, G., Heras, J.M., Mónica, M., González, F., 2014. Influence of sequential inoculation of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* in the quality of red wines. Eur. Food Res. Technol. 239, 279–286.

Izquierdo Cañas, P.M., Palacios Garcia, A.T., Garcia Romero, E., 2011. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starter. Vitis 50, 177–182.

Jolly, N.P., Augustyn, O.H.P., Pretorius, I.S., 2003a. The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. S. Afr. J. Enol. Vitic. 24, 55–62.

Jolly, N.P., Augustyn, O.H.P.H., Pretorius, I.S., 2003b. The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa. S. Afr. J. Enol. Vitic. 24, 63–69.

Kapsopoulou, K., Mourtzini, A., Anthoulas, M., Nerantzis, E., 2007. Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. World J. Microbiol. Biotechnol. 23, 735–739.

Knight, S., Klaere, S., Fedrizzi, B., Goddard, M.R., 2015. Regional microbial signatures positively correlate with differential wine phenotypes: evidence for a microbial aspect to terroir. Nature, Sci. Reports 5.

Lema, C., Garcia-Jares, C., Orriols, I., Angulo, L., 1996. Contribution of *Saccharomyces* and non-*Saccharomyces* populations to the production of some components of Albarino wine aroma. Am. J. Enol. Vitic. 47, 206–216.

Lilly, M., Bauer, F.F., Styger, G., Lambrechts, M.G., Pretorius, I.S., 2006. The effect of increased branched-chain amino acid transaminase activity in yeast on the production of higher alcohols and on the flavour profiles of wine and distillates. FEMS Yeast Res. 6, 726–743.

Mckinnon, A., 2013. The impact of amino acids on growth performance and major volatile compound formation by industrial wine yeast. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Stellenbosch University https://scholar.sun.ac.za

Meilgaard, M.C., 1975. Flavor chemistry of beer. II. Flavor and threshold of 239 aroma volatiles. MBAA TQ 12, 151–168.

Mendes-Ferreira, A., Barbosa, C., Lage, P., Mendes-Faia, A., 2011. The impact of nitrogen on yeast fermentation and wine quality. Ciência Téc. Vitiv. 26, 17–32.

Mendoza, L.M., De Nadra, M.C.M., Farías, M.E., 2007. Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. Biotechnol. Lett. 29, 1057–1063.

Mora, J., Mulet, A., 1991. Effects of some treatments of grape juice on the population of growth of yeast species during fermentation. Am. J. Enol. Vitic. 42, 133–136.

Mortimer, R.K., 2000. Evolution and variation of the yeast (*Saccharomyces*) genome. Genome Res. 10, 403–409.

Passoth, V., Fredlund, E., Druvefors, U.Ä., Schnürer, J., 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 6, 3–13.

Pérez-Nevado, F., Albergaria, H., Hogg, T., Girio, F., 2006. Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 108, 336–345.

Rantsiou, K., Dolci, P., Giacosa, S., Torchio, F., Tofalo, R., Torriani, S., Suzzi, G., Rolle, L., Cocolina, L., 2012. *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. Appl. Environ. Microbiol. 78, 1987–1994.

Regueiro, L.A., Costas, C.L., Rubio, J.E.L., 1993. Influence of viticultural and enological practices on the development of yeast populations during winemaking. Am. J. Enol. Vitic. 44, 405–408.

Rojas, V., Gil, J. V., Piaga, F., Manzanares, P., 2001. Studies on acetate ester production by non-*Saccharomyces* wine yeasts. Int. J. Food Microbiol. 70, 283–289.

Rojas, V., Gil, J. V., Piñaga, F., Manzanares, P., 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. Int. J. Food Microbiol. 86, 181–188.

Romano, P., Suzzi, G., Comi, G., Zironi, R., 1992. Higher alcohol and acetic acid production by apiculate wine yeasts. J. Appl. Bacteriol. 73, 126–130.

Romano, P., Suzzi, G., Domizio, P., Fatichenti, F., 1997. Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. Antonie Van Leeuwenhoek 71, 239–242.

Sadoudi, M., Tourdot-Maréchal, R., Rousseaux, S., Steyer, D., Gallardo-Chacón, J.-J., Ballester, J., Vichi, S., Guérin-Schneider, R., Caixach, J., Alexandre, H., Alexandre, H., 2012. Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. Food Microbiol. 32, 243–253.

Saerens, S.M.G., Delvaux, F.R., Verstrepen, K.J., Thevelein, J.M., 2010. Production and biological function of volatile esters in *Saccharomyces cerevisiae*. Microb. Biotechnol. 3, 165–177.

Stellenbosch University https://scholar.sun.ac.za

Setati, M.E., Jacobson, D., Andong, U., Bauer, F., 2012. The vineyard yeast microbiome, a mixed model microbial map. PLoS One 7, e52609.

Smit, A.Y., 2013. The impact of nutrients on aroma and flavour production during wine fermentation. Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Toro, M.E., Vazquez, F., 2002. Fermentation behaviour of controlled mixed and sequential cultures of Candida cantarellii and *Saccharomyces cerevisiae* wine yeasts. World J. Microbiol. Biotechnol. 18, 347–354.

Van Zyl, J.A., Du Plessis, L. de W., 1961. The microbiology of South African winemaking. I. The yeasts occurring in vineyards, must and wines. S. Afr. J. Agric. Sci. 4, 393–403.

Zagorc, T., Maráz, A., Cadez, N., Povhe Jemec, K., Péter, G., Resnik, M., Nemanič, J., Raspor, P., 2001. Indigenous wine killer yeasts and their application as a starter culture in wine fermentation. Food Microbiol. 18, 441–451.