

Multispecies interactions in a simplified wine yeast consortium

by

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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 authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2018

Summary

Over the last two decades various studies have evaluated the impact of non-*Saccharomyces* (NS) yeasts on alcoholic fermentation of wine, the chemical imprint they leave in the wine and how this affects wine quality. These NS yeasts are either present naturally in the grape must or inoculated together with *Saccharomyces cerevisiae*, but little is known about how these NS interact with each other and with *S. cerevisiae* and how these interactions might influence the presences of other yeast during fermentation and ultimately affect the contribution of each yeast to the wine. In recent years, several strains of the species, *Torulaspora delbrueckii*, *Pichia kluyveri*, *Metschnikowia pulcherrima* and *Lachancea thermotolerans* have been commercialized. The availability of such commercial preparations allows winemakers to perform mixed-culture fermentations through different inoculation strategies. Research has evaluated co-inoculations and sequential inoculations between NS and *S. cerevisiae*, but grape must is a complex ecosystem with a large variety of indigenous yeasts that partake during fermentation. Understanding how various yeasts interact within such a larger matrix is challenging, but will make an essential contribution to sound decision making in wineries. This study was designed to better understand how NS yeasts perform individually and how their behaviour might differ in the presence of one or more other yeasts and what effect this might have on the final wine.

Three commercial NS yeasts strains and one *S. cerevisiae* yeast strain were used to determine how these yeasts interact and how these interactions might alter the chemical composition of wine. Fifteen inoculations scenarios, including mono-culture, co-culture and combinations of three and ultimately a consortium containing all four yeasts were performed. Fermentations were carried out in synthetic grape juice at both 15°C and 25°C. The data showed significant variations in the cell densities of all species through-out fermentation depending on the nature of the co-inoculated species and the environmental conditions. These changes in population dynamics also had a clear impact on the concentration of and types of aromatic compounds produced.

Chenin blanc wines made with the consortium of all four yeasts, *S. cerevisiae* and spontaneous fermentations, showed distinct chemical profiles. However, no correlation was found, regarding population dynamics or aroma profiles of the wines, between the synthetic wine and the Chenin blanc wines both derived from the consortium inoculation. This study provides the foundation for future work on understanding how multiple species (more than two yeasts) interact within fermentations and how this will affect wine quality. It also provides a better understanding of how one yeast can suppress the presence of other yeasts and how different temperatures might affect the presence of each yeasts and how this might influence the interactions between the yeasts.

Opsomming

Gedurende die afgelope twee dekades was daar menigde studies gedoen om die impak van Nie-*Saccharomyces* (NS) giste te evalueer gedurende alkoholiese gisting van wyn, die chemiese impak wat hulle het op die wyn en hoe dit die wynkwaliteit beïnvloed. Hierdie NS giste kom of natuurlik voor op die druiwe, of word geïnkuleer tesame met *Saccharomyces cerevisiae*, maar daar is egter min informasie betreffende hoe hierdie NS giste in mekaar se teenwoordigheid reageer, asook in die teenwoordigheid van *S. cerevisiae*, en hoe hierdie interaksies die teenwoordigheid van mekaar sal beïnvloed en watter invloed dit op die wyn sal hê. In die afgelope paar jaar was daar menigde stamme van die spesies, *Torulaspora delbrueckii*, *Pichia kluyveri*, *Metschnikowia pulcherrima* en *Lachancea thermotolerans* ontwikkel vir kommersiële gebruik. Die beskikbaarheid van hierdie NS giste laat wynmakers toe om gemengde-kultuur fermentasies te doen met verskillende inokulasie strategieë. Daar is egter baie navorsing beskikbaar rakende mede-inokulasie en gesaamtlike-inokulasie van NS met *S. cerevisiae*, maar druiwe mos is 'n komplekse medium met 'n groot verskeidenheid natuurlike giste teenwoordig wat ook deelneem aan die fermentasie van wyn. Dit is egter belangrik om te verstaan hoe verskeie hoeveelhede giste interaksie voer in mekaar se teenwoordigheid en kan veral moeilik wees in sulke groot matrikse, maar sal 'n baie groot bydrae lewer tot wynmaak strategieë. Hierdie studie is dus ontwikkel om beter te verstaan hoe NS individueel fermenteer en hoe hulle moontlik anders kan fermenteer in die teenwoordigheid van meer giste en watter effek dit op die finale wyn mag hê.

Drie kommersieel beskikbare giste en een *S. cerevisiae* gis was gebruik in hierdie studie om die interaksies te bepaal tussen die giste en te bepaal watter impak die interaksies moontlik kan hê op die chemiese samestelling van die finale wyn. Vyftien inokulasie strategieë, insluitende enkel- en dubbel inokulasies, kombinasies van drie asook al vier tesame, was bestudeer. Die fermentasies was gedoen by beide 15°C en 25°C. Die data het 'n groot verskil in sel-konsentrasies van al die giste gewys asook in al die fermentasies en was afhanklik van die kombinasie gebruik en die omgewingsomstandighede teenwoordig. Die verskille in sel konsentrasies het ook tot duidelike verskille gelei in die konsentrasies van aromatiese komponente wat geproduseer was.

Chenin blanc wyne was gemaak met die gebruik van die kombinasie wat al vier giste ingesluit het, *S. cerevisiae* en spontane gisting. Die wyne het almal verskillende chemiese samestellings tot gevolg gehad. Daar was egter geen verhouding gevind tussen die resultate van die populasie samestellings en chemiese profile van die sintetiese wyn medium en die regte wyn medium nie. Die studie verskaf dus die basis vir toekomstige werk rakende die kennis van hoe verskeie giste (meer as twee) reageer in die teenwoordigheid van mekaar in fermentasies en hoe dit die wynkwaliteit sal beïnvloed. Dit verskaf ook informasie om beter te verstaan hoe die teenwoordigheid van een gis die

teenwoordigheid van ander giste kan beïnvloed en hoe verskillende temperature die interaksies moontlik kan beïnvloed tuseen die giste.

This thesis is dedicated to my mom, dad, family and friends for supporting me throughout the years
of my studies

Biographical sketch

John James Nutt was born in Bellville on April 3rd in 1992. He spent 21 years of his life in Knysna in which time he also attended Knysna Primary School and Knysna High School. In 2011 he started his studies at the University of Stellenbosch where he initially enrolled in Industrial Engineering, but later changed to BSc Viticulture and Oenology which he completed it in 2015. In 2016 he enrolled for his postgraduate studies at the Institute Wine Biotechnology to further his career in wine making.

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Preface

This thesis is presented as a compilation of 5 chapters. Referencing is done to the style of the journal South African Journal of Enology and Viticulture.

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Chapter 1

Introduction and project aims

Chapter 1 - Introduction

1.1. Introduction

The most commonly used yeast species in the wine industry is *Saccharomyces cerevisiae* which can complete fermentations to dryness. However, in both spontaneously fermented and inoculated wines, *S. cerevisiae* will ferment in the presence of a consortium of other yeasts and bacterial species. Yeasts can be defined as unicellular fungi, either ascomycetous or basidiomycetous (Jolly *et al.*, 2006). In the wine industry, such yeasts are generally referred to as non-*Saccharomyces* (NS) or *Saccharomyces* yeast. In most cases, *S. cerevisiae* tends to dominate the final stages of fermentation, primarily due to its ability to ferment in the presence of high alcohol and in anaerobic environments. However, research has shown that NS yeast strains can be detected throughout wine fermentation (Jolly *et al.*, 2003; Combina *et al.*, 2005; Fleet, 2008) and their dominance during the early stages of fermentation can leave an imprint on the final composition of the wine (Romano *et al.*, 1997; Ciani *et al.*, 2010).

Interactions between *S. cerevisiae* and NS and the effect of these interactions on the wine aroma have been researched by several groups. In addition to ethanol and carbon dioxide, NS yeasts have been found to release secondary products such as esters, higher alcohols, carbonyl compounds, acids which is of importance to the sensory characteristics of wines (Ciani and Maccarelli 1998; Egli *et al.*, 1998; Romano *et al.*, 2003; Mateo and Maicas, 2016). Consequently, the combined use of *S. cerevisiae* and NS yeasts are often sought to increase or decrease a specific wine compound such as glycerol (Ciani *et al.*, 2002), acetic acid (Bely *et al.*, 2008) or ethanol (Contreras *et al.*, 2014). Furthermore, wine aroma can be improved by modulating compounds such as varietal thiols (Anfang *et al.*, 2009), geraniol (Garcia *et al.*, 2002), and acetate esters (Rojas *et al.*, 2003; Moreira *et al.*, 2008). This can also be achieved through the use of selected mixed cultures (Viana *et al.*, 2011). A study on mixed fermentations with *Lachancea thermotolerans* and *S. cerevisiae* showed interesting oenological properties and provided favourable combinations to improve the glycerol, total acidity, 2-phenylethanol and polysaccharides content, while reducing the volatile character of the wine (Gobbi *et al.*, 2013).

Considering the abovementioned trends and the tremendous innovation potential associated with the large number of NS yeast species and strains, there is an urgent need to better describe and understand the impact of multiple yeast species and of their interactions on wine character. Furthermore, and in order to improve the usefulness of natural yeast communities, models need to be developed to predict the impact of a given yeast community on fermentation progress, wine aroma and mouthfeel. Most of the research available has been focusing on interactions between a NS and a *S. cerevisiae* species using either sequential or co-inoculation strategies. Limited research is

available on the impact of larger yeast consortia and how the interactions within such complex ecosystem will affect the population dynamics and aroma formation.

1.2. Problem statement

Some research on how inoculation with individual NS yeasts impact on wine character, and how some of these yeasts interact with *S. cerevisiae* when used individually has been carried out. The data show that these practices can significantly change the population dynamics during fermentation and the aroma profile of wine either positively or negatively.

However, there is very little research on the interactions between two or more NS yeasts together with *S. cerevisiae* and on what impact these interactions might have on the population dynamics of multiple species. Therefore, this study aims to start building a base where multiple species interactions will be determined and how these interactions could affect populations dynamics and wine aroma.

1.3. Aims

The aim of this project was to:

- 1.3.1. Determine how three NS strains of, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Pichia kluyveri* interact with each other and with *S. cerevisiae*. This was determined in synthetic grape must (AWRI, based on Henschke and Jiranek 1993) and in real grape must.
- 1.3.2. Determine how the population dynamics of these yeasts are affected when used in all possible combinations in synthetic and grape must, and how population changes influence the aroma of wine.
- 1.3.3. Determine how temperature differences will influence the population dynamics and wine aroma.

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Chapter 2

Literature Review

Yeast interaction and their influence on population dynamics
and wine aroma

Chapter 2 - Yeast interaction and their influence on population dynamics and wine aroma

2.1. Introduction

Grape must is a variable matrix and chemically complex environment in which yeast growth and fermentative metabolism lead to the production of wine (Ribéreau-Gayon *et al.* 2006; Viana *et al.* 2014). Wines can ferment spontaneously due to the presence of naturally occurring yeasts present on grapes, in the vineyards, and on cellar equipment. The initial stages of spontaneous fermenting grape must it is not dominated by the presence of *S. cerevisiae*, but rather due to the presence non-*Saccharomyces* (NS) yeasts species of the genera *Candida*, *Cryptococcus*, *Hanseniaspora* (*Kloeckera*), *Metschnikowia*, *Pichia* and *Rhodotorula* (Jolly *et al.*, 2014). During this biochemical transformation, the yeast converts grape sugar into ethanol, CO₂ and a myriad of volatile and non-volatile compounds. *S. cerevisiae* is usually present in low numbers, but will in most cases ensure complete alcoholic fermentation due to its ability to outcompete other organisms as the alcohol in the environment increases. Although *S. cerevisiae* dominates fermentation from mid-stage onwards, many of the other species of yeasts contribute to the initial stages of fermentation and in turn affects the organoleptic characteristics of the final wine (Fleet, 2008), but cannot complete fermentation and are eventually suppressed by *S. cerevisiae*.

In recent years, there has been an increasing interest regarding the use of NS yeasts to enhance complexity in wine aroma or to better control the levels of certain desirable or undesirable metabolites such as acetic acid and glycerol. Other advantages of using NS yeasts for fermentation may include the reduction of alcohol in wines. In a study by, Contreras *et al.* (2014), Chardonnay and Shiraz wines made by using *Metschnikowia pulcherrima* produced 0.9% and 1.6% (v/v) less ethanol, respectively, than the control wines produced by *S. cerevisiae*.

In general, the commercialization of some NS has given winemakers the ability to achieve more diversified aroma profiles. Current commercialised yeasts species include *Torulaspora delbrueckii*, *M. pulcherrima*, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) and *Pichia kluyveri*, the latter being sold as a frozen liquid culture made to directly inoculate wine without having to rehydrate like with most products.

Until recently, it was believed that the inoculation, of wine, with *S. cerevisiae* would overpower the growth of NS yeasts. However, numerous quantitative ecological studies have shown that not all species were completely suppressed in either inoculated or spontaneous (Romano *et al.*, 1997; Jolly *et al.*, 2003; Jolly *et al.*, 2006b). Therefore, yeast producing companies started producing more NS cultures.

This chapter will thus primarily focus on reviewing different NS and *Saccharomyces* yeasts, both lab cultures and commercial yeast products, used in wine fermentations. It will also focus on what interactions take place between different yeasts species during fermentation and how temperature affects the results. These interactions will influence the contributions made by each yeast and ultimately could affect the wine aroma and palate.

2.2. NS and *Saccharomyces* yeasts in wine

There are approximately twenty NS yeast genera that have been defined in fermenting grape must, these include: *Lachancea*, *Hanseniaspora*, *Candida*, *Pichia* and *Metschnikowia*, and less frequently those from the genera *Dekkera*, *SchizoSaccharomyces*, *ZygoSaccharomyces*, *Torulaspora* and *Saccharomycodes* (Fleet, 2003; Jolly *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006; Jolly *et al.*, 2014).

Table 2.1 shows only the most frequently encountered species of NS yeasts from grape juice, and include the genera *Hanseniaspora*, *Metschnikowia* and *Candida*, and more occasionally *Torulaspora* and *Pichia*.

Table 2.1 Some of the more frequently occurring NS yeast genera commonly isolated from wine fermentations (Jolly *et al.*, 2014)

Teleomorphic form	Anamorphic Form	Former name
<i>Citeromyces matritensis</i>	<i>Candida globose</i>	
<i>Debaryomyces hansenii</i>	<i>Candida famata</i>	<i>Pichia hansenii</i>
<i>Dekkera bruxellensis</i>	<i>Bettanomyces bruxellensis</i>	
<i>Hanseniaspora guilliermondii</i>	<i>Kloeckera apis</i>	
<i>Hanseniaspora occidentalis</i>	<i>Kloeckera javanica</i>	
<i>Hanseniaspora uvarum</i>	<i>Kloeckera apiculata</i>	
<i>Hanseniaspora vineae</i>	<i>Kloeckera africana</i>	
<i>Issatchenkia terricola</i>	<i>Kloeckera cortices</i>	<i>Pichia terricola</i>
<i>Lachancea kluyveri</i>	∞	<i>Saccharomyces kluyveri</i>
<i>Lachancea thermotolerans</i>	∞	<i>Kluyveromyces thermotolerans</i>
<i>Metschnikowia pulcherrima</i>	<i>Candida pulcherrima</i>	<i>Torulopsis pulcherrima</i>
<i>Metschnikowia reukaufii</i>	<i>Candida reukaufii</i>	
<i>Meyerozyma guilliermondii</i>	<i>Candida guilliermondii</i>	<i>Pichia guilliermondii</i>
<i>Milleroyzyma farinosa</i>	∞	<i>Pichia farinosa</i>
<i>Pichia fermentans</i>	<i>Candida lambica</i>	
<i>Pichia kluyveri</i>	∞	<i>Hansenula kluyveri</i>
<i>Pichia membranifaciens</i>	<i>Candida valida</i>	
<i>Pichia occidentalis</i>	<i>Candida sorbose</i>	<i>Issatchenkia occidentalis</i>
<i>Saccharomycodes ludwigii</i>	∞	
<i>Starmerella bombicola</i>	<i>Candida bombicola</i>	<i>Torulopsis bombicola</i>
<i>Torulaspora delbrueckii</i>	<i>Candida colliculosa</i>	<i>Saccharomyces rosei</i>
<i>Wickerhamomyces anomalus</i>	<i>Candida pelliculosa</i>	<i>Pichia anomala</i>
<i>Zygoascus meyeræ</i>	<i>Candida hellenica</i>	
<i>ZygoSaccharomyces bailii</i>		<i>Saccharomyces bailii</i>
‡	<i>Candida zemplinina</i>	<i>Candida stellata*</i>
‡	<i>Candida stellata</i>	<i>Torulopsis stellata</i>

*Names sometimes found in older literature

⌘ No teleomorphic form

∞ No anamorphic form

The occurrence of these NS and *S. cerevisiae* yeasts during fermentation is largely affected by the composition of the grape must as well as the by-products associated with fermenting grape must. Grape must is characterized by low pH, high osmotic pressure and sugar levels and frequently by the addition of sulphur dioxide. All these characteristics of the must can influence the survival of yeasts. During fermentation in closed tanks the conditions change from aerobic to anaerobic, alcohol levels increase and the amount of assimilable nitrogen decreases, which may have a negative impact on the development many of NS yeasts. Other factors influencing the growth of yeasts are temperature and SO₂ and the combined effect of the two. Studies have however shown that even strains that only persist during the beginning stages of fermentation may leave a detectable metabolic footprint that contributes to the final wine aroma composition (Pretorius, 2000; Fleet, 2003).

2.3. Influence of yeasts on secondary aroma

Of all distinguishable characteristics of wine, flavour is the most important. Two aspects that contribute to wine flavour are, taste, which primarily depends on non-volatile compounds and aroma, which is defined by volatile compounds. The compounds can vary in origin, and include grape derived elements, some of which may be qualified as varietal flavours, pre-fermentative flavours, fermentative flavours and post-fermentative flavours (Lambrechts & Pretorius, 2000).

Table 2.2. shows the main groups of compounds formed during fermentation, which include higher alcohols, esters, organic acids and to a lesser degree, aldehydes. Some compounds formed during fermentation could be undesirable when present in excess concentrations. These compounds include, acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl (Carrau *et al.*, 2008).

In the following sections, individual compounds and the impact of NS yeast on such compounds will be discussed.

Table 2.2 The main volatile fatty acids, higher alcohols, esters and carbonyl compounds found in wine fermentations

Esters	Higher Alcohols	Volatile Fatty acids	Carbonyl compounds
Ethyl Hexanoate	Propanol	Acetic Acid	Acetoin
Ethyl Caprylate	Butanol	Propionic Acid	Acetaldehyde
2-Phenylethyl Acetate	Isobutyl alcohol	Isobutyric acid	Benzaldehyde
Ethyl Caprate	Amyl alcohol	Hexanoic Acid	Butanal
Diethyl Succinate	Hexanol	Octanoic Acid	Diacetyl
Ethyl phenylacetate	Isoamyl alcohol	Decanoic Acid	Propanal
Isoamyl acetate	Phenylethanol	Butyric Acid	Isobutanal

Isobutyl acetate	2-Phenylethanol	Isovaleric Acid	Pentanal
Hexyl acetate	3-Ethoxy-1-propanol	Valeric Acid	Isovaleraldehyde
	4-Methyl-2-Pentanol	Formic acid	2-Acetyl tetrahydropyridine
		Heptanoic acid	
		Nonanoic acid	
		Tridecanoic acid	

Most abundant compounds found in wine are shown in bold

2.3.1. Volatile fatty acids

Volatile fatty acids are divided into short chain (less than 6 carbon molecules) and medium-chain fatty acids with between 6 – 12 carbon molecules. The short-chain fatty acid, acetic acid is the main acid responsible for 90% of the volatile acidity (VA) of wine. The remaining short-chain fatty acids including butanoic and propanoic acid, are present in lower concentrations, and are frequently associated with intervention of bacteria (Ribéreau-Gayon *et al.*, 2006). Acetic acid is usually acceptable between the levels of 0.2 and 0.7 g.L⁻¹, but between 0.7 and 1.1 g.L⁻¹ becomes unpleasant (Lambrechts *et al.*, 2000). During barrel maturation, winemakers expect an increase of volatile acidity by about 60 – 120 mg.L⁻¹ for each year in barrel. This is not necessarily due to microbial spoilage, but could also be due to the degradation of the hemicellulose of the oak barrel itself. Phenolic compounds could also oxidise over time to form peroxide, which can oxidise to acetaldehyde, and then to acetic acid (Zoecklein *et al.*, 1999). A direct connection has been established regarding glycerol and acetic acid production during *S. cerevisiae* fermentation (Remize *et al.*, 1999; Erasmus *et al.*, 2004; Silas, 2016). *S. cerevisiae* has to continuously equilibrate redox imbalances, which are the result of alcoholic fermentations (Silas, 2016). Anabolic responses identified with biomass formation occupy glycolytic intermediates from ethanol formation, requiring different pathways for the recovery of NAD⁺ which is required to maintain flux through glycolysis (Silas, 2016). However, acetic acid will be produced from acetaldehyde due to an excess production of NAD⁺, this reaction works as a redox sink to convert NAD⁺ to NADH (Michnick *et al.*, 1997; Remize *et al.*, 1999; Silas, 2016).

The impact of NS on acetic acid in wine has been evaluated. Sequentially inoculated fermentations with *M. pulcherrima* and various strains of *Saccharomyces cerevisiae* it was found that the acetic acid production was less than when only *Saccharomyces cerevisiae* strains were used (Contreras *et al.*, 2014). Other NS yeasts of the genera *Hanseniaspora* and *ZygoSaccharomyces* can produce excessive amount of acetic acid which led to them being considered as spoilage yeasts for many years (Du Toit *et al.*, 2000; Loureiro, 2003; Romano *et al.*, 2003). The amount of acetic acid produced however varies greatly from strain to strain. For instance, it has been found that some strains of *H. uvarum* produced levels ranging between 0.6 g.L⁻¹ – 3.4 g.L⁻¹ (Romano *et al.*, 2003). Other yeast may also help reduce acetic acid levels. Another study with *S. pombe* V2, showed acetic acid production

was less than 0.4 g.L⁻¹ and with sequential inoculation with *L. thermotolerans* CONCERTO™ reported less than 0.3 g.L⁻¹ (Benito *et al.*, 2017). *S. bacillaris* and its ability to produce low quantities of ethanol and acetic acid has been confirmed (Englezos *et al.*, 2015).

2.3.2. Higher alcohols

Higher alcohols are molecules that possess more than two carbon atoms with a higher molecular and boiling point than that of ethanol (Lambrechts & Pretorius, 2000). All the higher alcohols present in wine are given the term “fusel oil”. Individual higher alcohols generally have little impact on wine aroma but combined they may have a major impact. Their impact is generally higher in distilled wines. (Ribéreau-Gayon *et al.*, 2006).

Straight-chain higher alcohols [e.g. 1-propanol, 2-methyl-1propanol, 2-methyl-1-butanol and 3-methyl-1-butanol] have a low threshold of detection and thus has the greatest impact on wine aroma. At low concentration (<0.3 g.L⁻¹) they can contribute to the complexity of wine aroma (Lambrechts and Pretorius, 2000). However, at higher concentration, they can be overpowering and could thus impact wine aroma negatively. One of the most important function of higher alcohols in wine is their function in the formation of esters.

Data shows that some yeasts produce higher amount of fusel alcohols in wine when compared to wines made from only *S. cerevisiae*. Such yeasts species include yeasts that carry out a portion of the fermentation (e.g. *Lachancea thermotolerans*) before *S. cerevisiae* finishes fermentation, have been proven to increase fusel alcohols production in wines. The data suggests that spontaneous fermented wines would have higher concentration of fusel alcohols. Yeasts species including *L. thermotolerans*, *H. uvarum*, *C. zemplinina*, *Saccharomyces ludwigii*, and *W. anomalus* have been described as high fusel alcohol producers when used in single fermentations and mixed fermentations (Belda *et al.*, 2017). It has also been found that, depending on the strain, *T. delbrueckii* also has been proven to either decrease or increase the higher alcohol concentration in wine depending on strain differences and inoculation methods. In a study done by Azzolini *et al.*, (2014) on commercial strains, Zymaflora® Alpha (Laffort, Bordeaux, France) and BIODIVA (Lallemand, Montreal, Canada) and by Belda *et al.*, (2015) on Viniferm NS-TD® (Agrovin Alcázar de San Juan, Spain) it was found that only Viniferm was able to reduce the total amount of higher alcohols produced, in sequential fermentations with *S. cerevisiae*. It is still unclear whether these changes comes from modification of the metabolic regulation of *S. cerevisiae* (because of their coexistence), NS metabolism or presumably as a sum of both the above mentioned factors (Belda *et al.*, 2017). On the other hand, it has been found that fermentations carried out with the use of *C. stellata* and *Lachancea fermentati* (formerly *Zygosaccharomyces fermentati*) showed low levels of higher alcohols both when fermented individually or in combination with *S. cerevisiae* (Jolly *et al.*, 2014).

2.3.3. Esters

When a carboxylic acid and alcohol react, an ester is formed, and a water molecule is eliminated. Esters can be grouped into two groups, those formed enzymatically and those formed during ageing

of wine (Ribéreau-Gayon *et al.*, 2006). The two main classes of esters formed in wine during fermentation acetate esters from higher alcohols and ethyl esters from fatty acids. Esters are some of the most abundant compounds found in wine and have a significant effect on the floral and fruity aromas of wine. For this reason, the presence of several different esters often has a synergistic effect, impacting on the individual flavours well below their individual threshold levels (Table 2.3. presents ester thresholds).

Most esters occur in wine under their threshold levels and for this reason a modest change in their concentration could have a great impact on the wine aroma (Sumbly *et al.*, 2010).

Table 2.3 Threshold values for esters and their concentration in wine (Swiegers *et al.*, 2005)

Compound	Threshold level (ppm)	Concentration range (ppm)	Flavour description
Ethyl acetate	7.5 ^a	22.5–63.5	Nail polish, fruity
Ethyl butanoate	0.02 ^a	0.01–1.8	Floral, fruity
Ethyl decanoate	0.2 ^d	0–2.1	Floral, soap
Ethyl hexanoate	0.05 ^a	0.03–3.4	Green apple
Ethyl octanoate	0.02 ^a	0.05–3.8	Sweet soap, apple
Hexyl acetate	0.7 ^c	0–4.8	Sweet, perfume
Isoamyl acetate	0.03 ^a	0.1–3.4	Banana, pear
Isobutyl acetate	1.6 ^b	0.01–1.6	Banana, fruity
Phenyl ethyl acetate	0.25 ^a	0–18.5	Roses, flowery

^a10% ethanol ^bBeer ^cWine ^dSynthetic wine

Several studies have evaluated or reported on the impact of NS species on esters, with sometimes contradictory results. Such contradictions might be due to differences in wine making conditions differences in strains, and the general insights presented below are therefore amenable to being revised in future.

In general studies have shown that *Saccharomyces* species tend to produce more esters than other wine yeasts, but that the amount produced will be dependent on the strain used (Rossouw *et al.*, 2008). However, some co-inoculation strategies resulted in an overall higher ester level in the final product. This has been shown in the case of wine produced from a co-inoculation with *M. pulcherrima* together with *S. cerevisiae*, which resulted in high amounts of esters (Rodriguez *et al.*, 2010). Viana *et al.*, (2008) reported that *P. membranifaciens* specifically produced higher amount of acetate esters, and Whitener (2016) reported higher levels of esters in co-inoculations with *K. gamospora*. Furthermore, Renault *et al.*, (2009) found a couple of esters, including ethyl isobutanoate, ethyl dihydroxycinnamate, ethyl propanoate, and ethyl isobutyrate, that might be due to the presence of *T. delbrueckii*.

However, a study evaluating Sauvignon blanc and Syrah wines made with the NS species; *L. thermotolerans*, *M. pulcherrima*, *T. delbrueckii* and *Zygosaccharomyces kombuchaensis*, showed generally lower concentrations of esters produced apart from *K. gamospora*, produced more esters than the *S. cerevisiae* control (Whitener, 2016).

A study by Andorra *et al.*, (2012) found that in the presence of NS, acetate esters were more affected compared to *S. cerevisiae* wines. The study showed *C. zemplinina* and a mixture of *C. zemplinina*; *H. uvarum*; *S. cerevisiae* increased the hexyl acetate. Higher production of isoamyl acetate was linked to the presence of *H. uvarum* in mixed fermentation. 2-Phenylethyl acetate was the only acetate ester highly produced by *S. cerevisiae*, but in the presence of the NS used in the study the concentration was significantly reduced. From the NS yeasts used in the study it was found that only *C. zemplinina* increased the overall amount of acetate esters. A study done by, Mendoza and Farías (2010), showed that when *Kloeckera apiculata* and *S. cerevisiae* were co-fermented, the concentration of ester acetate in the sequentially inoculated fermentations was 15.14 mg.L⁻¹, where individually it was 33.13 mg.L⁻¹ for single *K. apiculata* fermentations, and 1.87mg.L⁻¹ for *S. cerevisiae* fermentations. This sequentially inoculated result was an increase from wine made from *S. cerevisiae* alone. Viana *et al.*, (2008) found a 10-fold increase in the concentration of 2-Phenylethyl acetate in wines fermented with *Hanseniaspora osmophila* 1471, than wines fermented with *S. cerevisiae*. Wines fermented with two different strains of *H. guilliermondii* (strain 11027 and 11102) showed different levels of esters. *H. guilliermondii* 11102 produced 2-fold more, 2-phenylethyl acetate, ethyl acetate, hexyl acetate and isobutyl acetate than *H. guilliermondii* 11027. *H. guilliermondii* 11027, produced higher levels of ethyl caproate, ethyl caprylate and diethyl succinate. Differences between *H. guilliermondii* 11102 and *H. osmophila* 1471. The latter produced non-detectable amount of isobutyl acetate, but four times more 2-phenylethyl acetate and three times more ethyl caproate and diethyl succinate (Viana *et al.*, 2008). Comitini *et al.*, (2011) found that mixed fermentations with *T. delbrueckii* resulted in the reduction of some esters such as isoamyl acetate and phenyl ethyl acetate.

The data regarding the impact of various NS yeast are summarised in Table 2.4. The table shows examples of most widely encountered NS yeasts and the main effect of these NS yeasts on wine when fermented in monoculture and how these attributes carry over into fermentations co-inoculated wines with *S. cerevisiae*. The table includes some compounds and other compounds not discussed in the section above.

Table 2.4 Fermentation behaviour of some NS and *S. cerevisiae* yeasts in pure or co-culture fermentations (Adapted from Ciani and Comitini, 2011; Lombard, 2016)

NS yeast species	Characteristic behaviour of pure culture	Effect produced by mixed fermentation with <i>S. cerevisiae</i> , compared to pure <i>S. cerevisiae</i>	Reference

<i>Candida cantarellii</i>		Enhancement of glycerol content	Toro & Vazquez (2002)
<i>Candida pulcherrima</i>		Improve wine aroma profile	Jolly <i>et al.</i> (2003)
<i>Debaryomyces variiji</i>	High level of β -glucosidase activity	Increase in terpenols	Ciani & Comitini, (2011)
		Increase in geraniol concentration	Garcia <i>et al.</i> (2002)
<i>Hanseniaspora guilliermondi</i>	High ethyl acetate producer		Moreira <i>et al.</i> (2008); Rojas <i>et al.</i> (2003); Viana <i>et al.</i> (2008)
<i>Hanseniaspora osmophila</i>	High 2-phenyl ethyl acetate producer	Increase in 2-phenyl ethyl acetate	Ciani & Comitini, (2011)
<i>Hanseniaspora uvarum</i>	High acetic acid producer	No increase in acetic acid production	Ciani & Comitini, (2011)
	High ethyl acetate producer	Slight increase in ethyl acetate production (strong reduction in comparison to pure culture)	Ciani & Comitini, (2011)
<i>Issatchenkia orientalis</i>	Utilise malic acid		Seo <i>et al.</i> (2007)
	Low ethyl acetate producer		Clemente-Jimenez (2004)
<i>Issatchenkia terricola</i>	High ethyl acetate producer		Clemente-Jimenez <i>et al.</i> (2004)
<i>Lachancea thermotolerans (Kluyveromyces thermotolerans)</i>	Low acetaldehyde producer		Ciani <i>et al.</i> (2006)
	High acid producer		Gobbi <i>et al.</i> (2013)
	Lactic acid producer (some strains)		Kapsopoulou <i>et al.</i> (2005)
<i>Kluyveromyces thermotolerans</i>	Low acetaldehyde producer	Reduction in final acetaldehyde production	Ciani & Comitini, (2011)
	Lactic acid producer (some strains)	Increase in titratable acidity	Ciani & Comitini, (2011)
<i>M. pulcherrima</i>		Synergistic effect on aroma profiles of mixed fermentations	Sadoudi <i>et al.</i> (2012)
		Lowering of ethanol	Contreras <i>et al.</i> (2014)
	High producer of 2-Methoxy-4-vinylphenol		Whitener (2016)
	High glycerol producer		Clemente-Jimenez <i>et al.</i> (2004)

<i>Pichia anomala</i> (known now as <i>Wickerhamomyces anomala</i>)	High producer of isoamyl acetate (EAHase)	Increase in isoamyl acetate production	Ciani & Comitini, (2011); Rojas <i>et al.</i> (2003)
	High producer of acetic acid		Rojas <i>et al.</i> (2003)
	High producer of ethyl acetate		Rojas <i>et al.</i> (2003)
<i>Pichia fermentans</i>	High glycerol production		Clemente-Jimenez <i>et al.</i> (2004)
		Increased polysaccharides	Domizio <i>et al.</i> (2011)
	High acetoin production or no production – fermentation condition dependant		Clemente-Jimenez <i>et al.</i> (2005, 2004)
<i>Pichia kluyveri</i>	High producer of 3-mercaptohexyl acetate	Increase in thiol production (3MHA)	Ciani & Comitini, (2011)
<i>Pichia membranifaciens</i>	High ethyl acetate		Viana <i>et al.</i> (2008)
<i>Saccharomyces ludwigii</i>	High acetoin		Ciani & Maccarelli (1998)
	High ethyl acetate		Ciani & Maccarelli (1998)
		Increased polysaccharides	Domizio <i>et al.</i> (2011)
<i>SchizoSaccharomyces spp.</i>	High rate of malic acid degradation	Reduction in titratable acidity	Ciani & Comitini, (2011)
<i>Starmerella bacillaris</i> (<i>Candida zemplinina</i>)	High producer of 3-mercaptohexan-1-ol (3MH)	Increased 3MH	Anfang <i>et al.</i> (2009)
	Low acetic acid producer	Lower acetic acid production	Rantsiou <i>et al.</i> (2012); Tofalo <i>et al.</i> (2012)
	Fructophilic yeast		Tofalo <i>et al.</i> (2012)
<i>Starmerella bombicola</i> (<i>Candida stellata</i>)	Fructophilic yeast	Combined consumption of reduced sugars/ improved consumption	Ciani & Comitini, (2011)
	High glycerol producer	Increased glycerol production	Ciani & Comitini, (2011)
	High succinic acid producer	Increase in succinic acid production	Ciani & Comitini, (2011)
	High acetaldehyde producer	No increase (combined consumption)	Ciani & Comitini, (2011)

	High acetoin producer	No increase (combined consumption)	Ciani & Comitini, (2011)
	Low ethanol producer	Reduction in final ethanol concentration	Ciani & Comitini, (2011)
<i>Torulaspora delbrueckii</i>	Low acetic acid producer	Reduction in acetic acid production	Ciani & Comitini, (2011)
		Reduction of acetaldehyde and VA	Ciani <i>et al.</i> (2006)
		Increased aromatic complexity	Azzolini <i>et al.</i> (2012); Loira <i>et al.</i> (2014)
		Increased polysaccharides	Ciani & Comitini (2011)

2.4. Yeast-yeast growth interactions in wine

The way in which yeasts interact with each other will influence the final wine composition and these interactions are categorized as competitive, neutral and mutualistic (Liu *et al.*, 2017; Mains, 2014). These interactions can be either metabolic interactions or growth interactions (figure 2.1). The interactions between various yeasts will influence the population dynamics of the yeasts during fermentation and depending on which yeasts dominate fermentation for a longer period, the aroma will be affected by these yeasts and their organoleptic attributes.

The metabolic interactions can be either additive, in the case of simple metabolite production or reduction and the amount of increase or decrease of these metabolites are influenced by the persistence of the yeast. It can also be synergistic where there is an exchange of metabolites between yeasts or the enhancement of metabolites. Lastly interactions can also be negative and leads to the reduction of metabolites (Ciani and Comitini, 2015a). The main growth interactions between yeasts are the competition for nutrients (oxygen, vitamins, nitrogen) and the toxic effect of certain metabolites (ethanol, killer proteins, short peptides, fatty acids) (Ciani and Comitini, 2015a).

2.4.1. Abiotic factors affecting *S. cerevisiae* / NS interactions

Competition between yeast is influenced by several abiotic factors such as osmotic pressure, pH, nitrogen, temperature and molecular sulfur dioxide. The main abiotic factor in wine fermentation is usually considered to be the increase in ethanol concentration. Ethanol supports *S. cerevisiae* domination from mid stage of fermentation since this yeast is more ethanol tolerant than most other species (Pretorius, 2000). Oxygen availability also plays an important role on the yeast's performance. Yeasts such as *L. thermotolerans* and *T. delbrueckii* require higher levels of oxygen. In low oxygen fermentation, these yeasts died off quicker when co-fermented with *S. cerevisiae* (Hansen *et al.*, 2001). In the presence of low oxygen conditions, *S. cerevisiae* and NS wine yeasts display different behavioural activities. *S. cerevisiae* is able to grow particularly well in anaerobic conditions (Hansen *et al.*, 2001) however, yeasts belonging to genera *Hanseniaspora* and *Torulasporea* was reported to grow poorly under the same conditions. Another study by Shekhawat *et al.*, (2017) showed how oxygen availability influenced the population dynamics of three NS, *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima*. Their data showed that *L. thermotolerans* required the least oxygen, followed by *T. delbrueckii* and *M. pulcherrima*. A study by Englezos *et al.*, (2018) showed that oxygen availability increased the survival time of *S. bacillaris* and decreased the growth rate of *S. cerevisiae* strains in mixed culture fermentations, whereas it did not affect the growth of the latter in pure culture fermentations.

Temperature is part of the most important factors that could influence the yeast interactions. High temperatures together with increasing levels of ethanol affects the membrane permeability and integrity. A study by Gobbi *et al.*, (2013) regarding the interaction between co-inoculated *S. cerevisiae* and *L. thermotolerans* fermentation, presented that the antagonistic effect between these

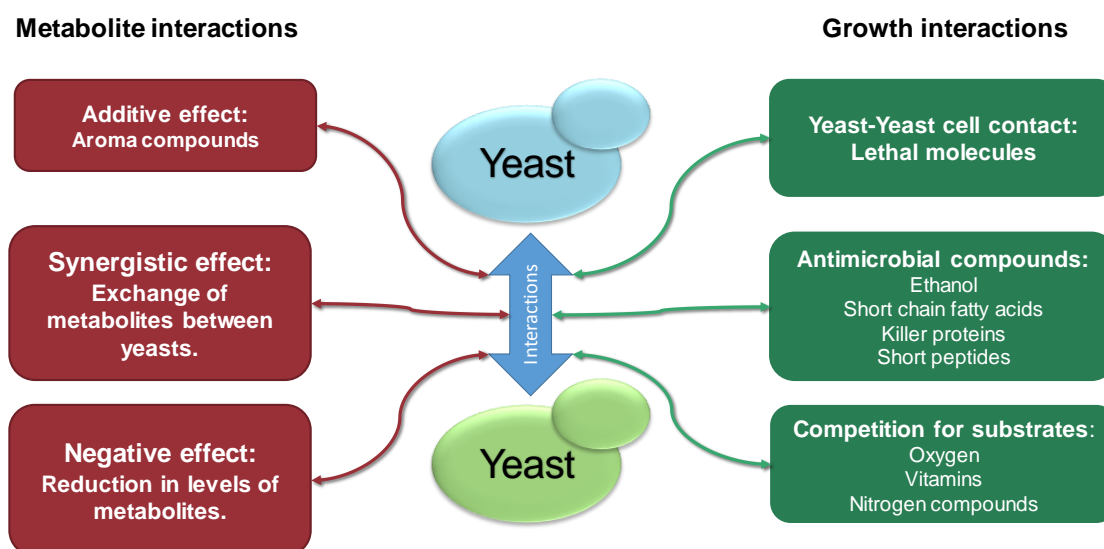


Figure 2.1 Different interactions between yeasts, both metabolic and growth relevant (Ciani and Comitini, 2015b).

two yeasts were temperature dependent. A recent study by Williams *et al.*, 2015 confirmed that the

temperature at which fermentation is carried out at, plays an important role on the ability of *S. cerevisiae* to dominate in high-sugar environments.

Another factor that might influence the interaction is the nitrogen sources available to the yeast that will help influence the secondary aroma attributes of the final wine. Kemsawasd *et al.*, (2015) reported that the growth and fermentation behaviour of *S. cerevisiae* and other NS yeasts was influenced differently by various nitrogen sources. The data suggested that alanine, arginine, asparagine, aspartic acid, glutamine, isoleucine, ammonium sulphates, serine, valine and mixtures of 19 amino acids and mixtures of 19 amino acids with ammonium sulphate had a positive impact of the growth and performance of *S. cerevisiae*. Furthermore, they observed beneficial effects of nitrogen sources on all performance parameters for three NS, *L. thermotolerans* (affected by serine), *H. uvarum* (affected by alanine), *M. pulcherrima* (affected by alanine and asparagine) and *T. delbrueckii* (arginine, asparagine, glutamine, isoleucine and mixtures of 19 amino acids). Yeasts from the genera *Hanseniaspora* and *Metschnikowia* have been proven to contribute to the enrichment of nitrogen within a medium due to their proteolytic activity (Dizy and Bisson, 2000; Ciani *et al.*, 2016).

2.4.2. Biotic factors affecting *S. cerevisiae* / NS interactions

Biotic factors that impact population dynamics in wine includes the production of antimicrobial compounds such as, killer proteins, short peptides, ethanol, medium fatty acids, acetaldehyde and acetic acid (Bisson *et al.*, 2005; Ciani *et al.*, 2009). Biological antimicrobial compounds such as, killer toxins (Table 2.5) secreted by certain NS yeast, including *K. wickerhamii*, *P. anomala*, *P. membranifaciens* have been defined as possibly being able to control the growth of *B. bruxellensis* (Comitini *et al.*, 2004; Santos *et al.*, 2009; 2011). Killer toxins secreted by NS display a wider range of activity, affecting the performance of species both within NS and *Saccharomyces* genera (Ciani and Comitini, 2011). Nissen *et al.*, (2003) proposed that *S. cerevisiae* S101 adopted a contact-dependent mechanism resulting in the culturability loss of some NS strains (*L. thermotolerans* and *T. delbrueckii*). Toxic compounds excreted by *S. cerevisiae* CCMI 885 inhibited the growth of *H. guilliermondii* and *H. uvarum*, indicating the interaction between these species through the excretion of antimicrobial compounds (Perez-Nevado *et al.*, 2006; Wang *et al.*, 2016).

Cell-to-cell contact is another interaction which seems to play a role between *S. cerevisiae* and other NS species such as, *T. delbrueckii*, *Hanseniaspora uvarum* and *L. thermotolerans* (Arneborg *et al.*, 2005). Nissen *et al.*, (2003) showed how cell-to-cell contact, between two NS (*T. delbrueckii* and *L. thermotolerans*) and *S. cerevisiae*, influenced the performance of the NS. The two NS yeasts were inoculated into a container with a dialysis membrane to keep species physically apart but allow metabolic exchanges to occur. From this the authors found that *S. cerevisiae* reached similar cell densities in the compartmentalized and non-compartmentalized fermentations while *T. delbrueckii* and *L. thermotolerans* were significantly inhibited when being in physical contact with *S. cerevisiae*. The molecular nature of the impact asserted through physical contact is however unknown.

Table 2.5 Main killer toxins involved in wine making (Ciani *et al.*, 2016)

Killer Yeast	Killer toxin	Sensitive strain	Applicative indication	Reference
<i>S. cerevisiae</i> “Prise de mousse”	K2 type	<i>S. cerevisiae</i>	Control of <i>S. cerevisiae</i> wild strains	Shimizu, 1993
<i>S. cerevisiae</i>	K2 type	<i>S. cerevisiae</i>	Enhanced autolysis in sparkling wine	(Todd <i>et al.</i> , 2000)
<i>Tetrapisispora phaffi</i>	Kpkt	<i>Hanseniaspora/Kloeckera</i>	Control of “apiculate” yeast	(Ciani and Comitini, 2011)
<i>Kluyveromyces wickerhamii</i>	Kwkt	<i>Dekkera/Brettanomyces</i>	Anti-Brett activity	(Comitini <i>et al.</i> , 2004)
<i>Wickerhanomyces anomalus</i>	Pikt	<i>Dekkera/Brettanomyces</i>	Anti-Brett activity	(Comitini <i>et al.</i> , 2004)
<i>P. kluyveri</i>	Zymocins	Certain <i>S. cerevisiae</i> strains	Control of <i>S. cerevisiae</i> wild strains	(Jolly <i>et al.</i> , 2014)
<i>Pichia membranifaciens</i>	PMKT2	<i>Dekkera/Brettanomyces</i>	Anti-Brett activity	(Santos <i>et al.</i> , 2009)
<i>T. delbrueckii</i>	Kbarr-1	<i>S. cerevisiae</i> strain killer	Broad anti-wine yeast activity	(Ramírez <i>et al.</i> , 2015)
<i>T. delbrueckii</i>	TdKT	<i>Pichia</i> and <i>Brettanomyces/Dekkera</i>	Spoilage wine yeast	(Villalba <i>et al.</i> , 2016)

2.5. A focus on commercial species widely used in wine fermentation

2.5.1. *Saccharomyces cerevisiae*

S. cerevisiae is the most commonly used yeast in the wine industry due to its fermentative properties and ability to ferment wine to dryness. It is also more tolerant to higher levels of ethanol and SO₂ than most other yeast species (Ludovico *et al.*, 2001; Fleet, 2003; Arroyo *et al.*, 2010), and also tolerant to temperature fluctuations (Goddard, 2008; Salvadó *et al.*, 2011). Swiegers and Pretorius (2005), reported *S. cerevisiae* to produce many aromatic secondary metabolites which mostly positively impacts the sensory profile of wine. Molina *et al.*, (2009) evaluated the aroma production by two strains of *S. cerevisiae*. They found with EC1118, chemical defined grape juice media showed higher solvent, fatty and pineapple aroma attributes and VIN13 exhibited aromas of banana, fruity, yeasty and green attributes.

S. cerevisiae is found at lower CFU.mL⁻¹ in grape must than most other NS. Thus, during the early stages of fermentation NS yeasts dominate during the first two to three days where after *S. cerevisiae* population increases and starts to dominate and complete the rest of the fermentation. *S. cerevisiae* have an inhibitory effect on the other yeasts since it outcompetes the other yeasts for nutrients such as, glucose and amino acids (Fleet, 2008) and it more tolerant with an increase in ethanol. For this reason, *S. cerevisiae* has been the preferred yeast for inoculating wines for many years.

2.5.2. *Metschnikowia pulcherrima*

The ecological distribution of *M. pulcherrima* is broad and includes flowers, fruits and other plants (Oro *et al.*, 2014). Among the various fruits, the grape berry surface represents an optimal and nutrient-rich habitat for *M. pulcherrima*, which is considered common wine yeast on mature grape berries, overripe grape berries, grape used to produce ice wine and botrytized grapes. Moreover, *M. pulcherrima* is generally present during the first stages of grape juice fermentation (Prakitchaiwattana *et al.*, 2004; Combina *et al.*, 2005). The presence of *M. pulcherrima*, in co-inoculated wine, with *S. cerevisiae* was limited to the first days of fermentation in a study by Oro *et al.*, (2014) and was confirmed by results obtained by Sadoudi *et al.*, (2012) and Milanovic *et al.*, (2013).

M. pulcherrima reacts antagonistic towards some other yeasts. According to, Oro *et al.* 2014, The yeast genera *Hanseniaspora*, *Candida* and *Pichia* showed very high sensitivity towards all the *M. pulcherrima* strains tested. The main exceptions here were *Hanseniaspora valbyensis* strain Hva28 and *P. membranaefaciens* strain Pm17, which showed no sensitivity to *M. pulcherrima* strains. Different behaviours were seen among yeasts in the genera *Torulaspora*, *ZygoSaccharomyces* and *Kluyveromyces*, which were generally sensitive to the antimicrobial effects of *M. pulcherrima* strains. In particular, *M. pulcherrima* strains #46 and #48 exhibited an effective antimicrobial activity against all strains of *Torulaspora*, *ZygoSaccharomyces* and *Kluyveromyces* that were investigated. In contrast, none of the eighteen *S. cerevisiae* starter strains were sensitive to the antimicrobial actions of either of *M. pulcherrima* strains. It is found that this antimicrobial effect of *M. pulcherrima* is due to the formation of pulcherriminic acid in the medium, which immobilizes the iron in the growth medium (Oro *et al.*, 2014). With this information, *M. pulcherrima* could be proposed in a control mixed fermentation with *S. cerevisiae* to not only improve the aromatic profile of the wine but also counteract the spoilage microbiota. *M. pulcherrima* has also been used in co-inoculation with *S. cerevisiae* to produce wines with lower alcohol content. Contreras *et al.*, (2014) demonstrated that *M. pulcherrima* AWRI1149 could produce wine with reduced alcohol concentration when used in sequential inoculation with a wine strain of *S. cerevisiae*.

Whitener (2016), performed fermentations where *M. pulcherrima* was sequentially inoculated with *S. cerevisiae* in Sauvignon blanc and Syrah must. The findings showed that 15 out of 76 compounds were at significantly higher concentrations in the co-inoculated Sauvignon blanc wine such as a 53-fold increase in phenol 2-methoxy-4-vinylphenol. For the co-inoculated Syrah wines, 24 out of the 66 compounds were significantly higher in concentrations, such as a 80-fold increase in phenol 2-methoxy-4-vinylphenol.

2.5.3. *Torulaspora delbrueckii*

T. delbrueckii is widely recommended for improving the complexity and for enhancing certain specific characteristics of wine (Bely *et al.*, 2008; Renault *et al.*, 2009; Azzolini *et al.*, 2014; Jolly *et al.*, 2014). This yeast has been found to increase glycerol (Contreras *et al.*, 2015) and mannoproteins (Belda *et al.*, 2015) or to reduce ethanol (Contreras *et al.*, 2015) in wine. Although there are commercial

products available that only contains *T. delbrueckii* its impact on wine quality is still far from satisfactory due to the difficulty of determining how much of the inoculated *T. delbrueckii* participates in the fermentation due to the influences of other natural occurring yeasts in the must. Renault *et al.*, (2015) found that mixed inoculations of *T. delbrueckii* with *S. cerevisiae* allowed for the increase, in comparison to pure *S. cerevisiae* culture, of some esters specifically produced by *T. delbrueckii* and significantly correlated to the maximal *T. delbrueckii* population reached in mixed culture. Ethyl propanoate, ethyl isobutanoate and ethyl dihydrocinnamate were considered as activity markers of *T. delbrueckii*. Renault *et al.*, (2015) also proved how both the sequential and simultaneous inoculation of *S. cerevisiae* and *T. delbrueckii*, influenced the maximal population compared to the pure culture fermentations. The sequential inoculation of the two yeasts, showed to have decrease the population of *S. cerevisiae* more than *T. delbrueckii* compared to their pure culture fermentations, but the simultaneous fermentations showed to have similar impact on the population densities of both yeasts (Table 2.6).

Table 2.6 Ethanol and residual sugar concentration, kinetic parameters and maximal cell populations in pure and mixed *T. delbrueckii* and *S. cerevisiae* cultures (laboratory scale conditions) (Renault *et al.*, 2015)

	<i>T. delbrueckii</i> Pure culture	Sequential Mixed culture	Simultaneous Mixed culture	<i>S. cerevisiae</i> Pure culture
Ethanol (% vol.)	6.2 ± 0.3 ^a	11.9 ± 0.8 ^b	12.4 ± 0.4 ^b	11.8 ± 0.4 ^b
Sugar (g/L)	107 ± 4 ^b	0.60 ± 0.10 ^a	0.60 ± 0.10 ^a	0.90 ± 0.20 ^a
Vmax (g/L/h)	0.39 ± 0.01 ^a	0.56 ± 0.03 ^b	0.84 ± 0.01 ^c	1.00 ± 0.01 ^d
Fermentation duration (h)	350 ± 7 ^b	473 ± 3 ^d	390 ± 2 ^c	334 ± 3 ^a
Maximal population (viable cells/ mL)				
<i>T. delbrueckii</i>	8.1 x 10 ⁷ ± 2.8 x 10 ⁶ ^c	6.1 x 10 ⁷ ± 7.1 x 10 ⁶ ^b	4.3 x 10 ⁷ ± 3.5 x 10 ⁶ ^a	-
<i>S. cerevisiae</i>	-	2.4 x 10 ⁷ ± 7.1 x 10 ⁵ ^a	4.4 x 10 ⁷ ± 2.3 x 10 ⁶ ^b	7.6 x 10 ⁷ ± 1.8 x 10 ⁶ ^c
^{a, b, c, d} represents significantly different statistical groups (p < 0.05)				
Vmax: Maximum CO2 production rate				

Whitener (2016), noted that *T. delbrueckii* fermented in Sauvignon blanc resulted in 69 significant differences of which only 10 differences were positive. One such difference was the 56 times higher level of phenethyl propionate. For Syrah wines, 62 significant differences were observed of which only 14 differences were positive such as the 53 times higher level of phenethyl propionate. The fermentation contained notably lower amounts of esters. With the exception for isobornyl acetate, isoeugenyl phenylacetate and phenethyl propionate, all the other esters demonstrated a negative fold change. Ethyl 2-hydroxy-4-methyl pentanoate showed a negative fold change in Sauvignon blanc, but was found to be a positive fold change in Syrah.

2.5.4. *Pichia kluyveri*

There is not a lot of information regarding how *P. kluyveri* reacts to interactions from other yeasts in a consortium. According to certain companies that sell the yeast (exp. CHR Hansen – Frootzen) the yeast can only tolerate a medium with an alcohol percentage of 4-5% ethanol. For this reason, the yeast is used as a co-inoculant together with *S. cerevisiae* to assure fermentation will complete. Research done by Whitener (2016), showed that 23 compounds were significantly higher in the *P. kluyveri* wines than in the other fermentations. Eight of these compounds were esters that have significant fruity aroma, three of which were 3-methylbutyl of three different organic acids. Two possibly fault inducing compounds found in *P. kluyveri* fermentations were 3-methyl-butanoic acid and phenethylamine. The first compound is associated with off-putting sour, sweaty, and cheesy aromas, and in to high concentration could be considered as a faulty wine. When looking at the 3MH and 3MHA production by *P. kluyveri*, Anfang *et al.*, (2009) found a significant increase in 3MHA levels in co-fermentation with *P. kluyveri* and VL3 compared to the single fermentation of VL3.

2.6. Conclusion

Many studies conducted, focused on the effects NS yeasts might have on wine aroma and how these effects different according to different inoculation strategies. Since research found benefits of using certain NS yeasts, an ever-increasing market developed for isolating and commercializing NS yeasts. Table 2.7 shows some NS yeasts available to the wine industry. Many studies available looked at co-inoculation strategies with NS yeasts and *S. cerevisiae* with good results. One such study by, Shekhawat *et al.*, (2017) showed promising results regarding co-inoculation with two NS yeasts, *L. thermotolerans* and *T. delbrueckii*, and the effect of oxygen availability on the persistence of these yeasts. Under anaerobic conditions these yeasts could grow and persist albeit at low levels. The growth of both yeasts was significantly enhanced under oxygenation, resulting in cell numbers reaching up to 10^9 and 10^{10} in *T. delbrueckii* and *L. thermotolerans* fermentations. There are however few studies where the effect of multiple species (consortium), on wine aroma and population dynamics, was monitored.

Table 2.7 Commercially available NS wine dry yeast products (Adapted from, Mains, 2014)

Yeast Company	Product	NS yeast strain(s)
Lallemand	Level ² Td™	<i>T. delbrueckii</i> + <i>S. cerevisiae</i>
	Flavia™ Mp346	<i>M. pulcherrima</i>
	Biodiva™ TD291	<i>T. delbrueckii</i>
	Promalic®	<i>S. pombe</i>
Chr. Hansen	Prelude™	<i>T. delbrueckii</i>
	Viniflora® Concerto™	<i>L. thermotolerans</i>
	Frootzen™	<i>P. kluyveri</i>

	Melody™	<i>L. thermotolerans</i> + <i>T. delbrueckii</i> + <i>S. cerevisiae</i>
Laffort	Zymaflore®Alpha n. Sacch	<i>T. delbrueckii</i>
	Zymaflore® ÉGIDE™DMP	<i>T. delbrueckii</i> + <i>M. pulcherrima</i>

The research shows the benefits from using NS yeasts together with *S. cerevisiae* in the strategy for enhancing the organoleptic properties of wine. Some of the positive effects include the suppression of negatively perceived volatile compounds (e.g. acetic acid), the production of certain killer toxin to suppress the growth of other undesirable yeasts (e.g. *B. bruxellensis*) and many other abiotic and biotic factors.

To gain further insight into the potential benefits and possible negative effects of using multiple yeasts in consortium, more investigation need to be conducted to determine which NS yeasts are to be used with *S. cerevisiae* in wine, to improve wine quality. Moreover, the effect of interactions between the yeasts should be investigated and how this might impact the population dynamics and ultimately the imprint it could have on wine. Also, the effect temperature might have on these interactions and on the population dynamics is also still to be investigated in a consortium. In this manner, a more holistic interpretation could be made on how population dynamics of yeasts are affected within a consortium and how this might influence wine aroma.

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Chapter 3

Research results

Evaluating population dynamics of a simplified wine yeast consortium and the effect of temperature on the interactions and aroma production in synthetic grape juice

Chapter 3 - Evaluating population dynamics of a simplified wine yeast consortium and the effect of temperature on the interactions and aroma production in synthetic grape juice

3.1. Introduction

The impact of non-*Saccharomyces* yeasts on the final wine composition is significant, and is in many cases perceived as positive (Jolly *et al.*, 2006; Benito *et al.*, 2015; Garcia *et al.*, 2016; Padilla *et al.*, 2016). Data show that in both inoculated and spontaneous fermentations, NS yeasts tend to dominate during the earlier stages of fermentation, and that some species can persist throughout the process. The later stages of the process are however almost always dominated by *Saccharomyces cerevisiae*. Data also show that many of these species can impact the chemical composition of the final wine (Fleet, 2008; Sadoudi *et al.*, 2012; Bagheri *et al.*, 2017; Benito *et al.*, 2017).

These findings have led to increased interest and research on the influence of non-*Saccharomyces* (NS) yeasts on wine chemical composition, and has in the past few years spurred the production of several commercial starter cultures which are now used as co-inoculants with *S. cerevisiae* (Jolly *et al.*, 2014; Wang *et al.*, 2016; Lu *et al.*, 2017). Each of these commercialized species and strains has been selected for one or several specific purposes, allowing wine makers to modulate or diversify their product range or to reduce the risk of certain off-flavours. Species that have been commercialized are described Table 2.6, chapter 2. The specific suggested application of these strains are the following: (i) *P. kluyveri* (Frootzen, Chr. Hansen) for the increased production of 3MH in Sauvignon blanc wine and has been shown to increase the levels of 3MH in Sauvignon blanc wines (Anfang *et al.*, 2009). Whitener (2016) also found that Shiraz wines initially inoculated with Frootzen, had higher levels of acetaldehyde and lower levels of esters; (ii) *T. delbrueckii* (Biodiva, Lallemand, France) according to the manufacturer are said to improve wine character and mouthfeel and this has been proven by Whitener, (2016), who found that it had a 10 and 14 positive changes to Sauvignon blanc and Shiraz wines. The wines were however notably lacking in significant number of ester which could be undesirable. Whitener (2016) also noted a 66-fold increase in the levels of 5-methylfurfural of Sauvignon blanc wines. Lastly, (iii) *M. pulcherrima* (Flavia, Lallemand, France) is suggested to improve varietal character, thiols and esters in wine when co-inoculated with *S. cerevisiae*, which was confirmed by Whitener, (2016).

There are many parameters in winemaking that influence the performance and effects of NS yeasts on the final wine. Some of these parameters include oxygen availability, nutrient availability, metabolic interactions and fermentation temperature. A study by Shekhawat *et al.*, (2017), on the effect of oxygen on *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* showed that oxygen

availability strongly influenced the population dynamics, but clear species-dependent differences were observed. *L. thermotolerans* required the least amount of oxygen, followed by *T. delbrueckii* and *M. pulcherrima*. Data showed, *M. pulcherrima* displayed the strongest dependence on the amount of oxygen supplied during fermentation. In anaerobic conditions it declined below detection within the first 24 to 48 h, but its contribution to the final aroma compound levels was significant. However, it was found that with an increase in dissolved oxygen (DO), this yeast displayed protracted persistence with viable cell count reaching up to 10^{10} CFU.mL⁻¹ (Shekhawat *et al.*, 2017). Furthermore, oxygen availability also affects the growth and fermentation performance of yeast species during fermentation (Ciani *et al.*, 2006; Brandam *et al.*, 2013; Jolly *et al.*, 2014; Taillandier *et al.*, 2014). Fermentations carried out, at two different conditions of oxygen availability, with *Stam. bacillaris* and *S. cerevisiae* showed that strain combination and oxygen availability influenced the population dynamics throughout the fermentations. Oxygen concentration increased the survival time of *Stam. bacillaris* and decreased the growth rate of *S. cerevisiae* strains in mixed culture fermentations, whereas it did not affect the growth of the latter in pure culture fermentations (Englezos *et al.*, 2018).

Another way in which the impact on the final wine is influenced is due to the interaction between yeast species and the subsequent changes of the population dynamics. Liu *et al.*, (2017) showed that competition for available nutrients, such as vitamins and nitrogen compounds, contributes to modulate the presence and dominance of yeast species within a fermentation. The population dynamics and thus the biomass produced by each yeast within a medium, will also be influenced by the available nitrogen. The yeast assimilable nitrogen (YAN), comprising of ammonium and amino acids is essential for biomass production, and in turn the aroma profile of the final wine (Mains, 2014).

There are two different types of interactions: (i) direct, which implies physical interaction and (ii) indirect. Each of these interactions have different interactions which includes either predation, parasitism, neutralism, commensalism, mutualism, antagonism and competition (Liu *et al.*, 2017; Mains, 2014). Synergistic (commensalism) interaction between yeasts can be the result of the exchange of metabolites between yeasts and may lead to increased production in wine. There are also effects which could cause a reduction in certain metabolites. The production of antimicrobial compounds such as ethanol, short-chain fatty acids, killer proteins and short peptides can also impact growth (Ciani & Comitini, 2015a; Ciani *et al.*, 2016; Wang *et al.*, 2016; Liu *et al.*, 2017).

Temperature has also been shown to impact yeast interactions and relative performance during fermentation. In general, available data suggest that lower temperature favour the persistence of NS yeast on *S. cerevisiae*. Using cooler temperatures to ferment co-cultures of *S. cerevisiae* and NS could therefore be used to better exploit the aroma contribution from NS whilst still ensuring fermentation will complete due to *S. cerevisiae* (Ciani & Ferraro, 1998; Jolly, Augustyn & Pretorius, 2003). An increase in the concentration of ethanol during the fermentations process is another of the

main factors that determines the dominance of *S. cerevisiae* toward NS yeasts (Pretorius, 2000; Ciani *et al.*, 2016). The effect of temperature may indeed be at least in part linked to the fact that NS yeasts are better able to tolerate high ethanol concentrations at lower temperatures.

In this study, we evaluated the interactions between three commercial strains of NS yeast, *T. delbrueckii*, *M. pulcherrima* and *P. kluyveri* between each other and with *S. cerevisiae* in synthetic grape juice. To better characterise interactions between these yeasts we fermented the four yeasts in every possible combination at 15°C and 25°C and monitored fermentation kinetics and population dynamics throughout each fermentation. All fermentations containing *S. cerevisiae* was further analysed on their metabolic profiles. The main aim is to better understand how yeasts interact within a consortium and to understand how combinations of certain yeasts result in different attributes in the final wine. We would like to see if we can use the results from the single fermentations to determine the outcome of multispecies fermentations and if the results differ determine what caused the change and how this change affects the imprint left on the final wine.

3.2. Material and methods

3.2.1. Yeast strains and culture conditions

Table 3.1 lists the four different yeasts strains used in this study along with their distributor and origin. All the cultures are industrial, freeze-dried cultures kept in 4°C fridge, exception for Frootzen which was kept at -80°C as it is used for direct inoculation. *S. cerevisiae* EC1118 was inoculated, according to package instructions, but at a dosage of 0.05 g.L⁻¹. All inoculation combinations with abbreviations used from now on are described in table 3.2.

Table 3.1 Selected wine yeasts strains used in this study

Yeast Species	Strains	Origin	Collection/Reference
<i>Saccharomyces cerevisiae bayanus</i>	Lalvin EC1118 (Prise de mousse)	Champagne region, France	Lallemand
<i>Torulaspota delbrueckii TD291</i>	Level 2 Solution Biodiva		Lallemand
<i>Metschnikowia pulcherrima</i>	Level 2 Solution Flavia	University de Santiago, Chile (USACH)	Lallemand
<i>Pichia kluyveri</i>	Viniflora® Frootzen™	Auckland University, New Zealand	Cnr. Hansen

T. delbrueckii and *M. pulcherrima* was inoculated at 0.25 g.L⁻¹ for all fermentations. *P. kluyveri* comes packaged as a 500 g solid frozen culture used to directly inoculate 5000L of grape must. Thus, to add the same concentration to each fermentation flask, a 20g piece was dissolved in 50 ml synthetic grape juice and cell densities were established through optical density measurements. Synthetic

grape juice was prepared according to Henschke and Jiranek (1993) and Bely *et al.* (1990) and is described in Table 3.3. The fermentations were carried out in triplicate at both 15°C and 25°C. The carbon sources, acids and salts, for 1.4 L of synthetic grape must, were added to 700 mL dH₂O in a 2 L Schott bottle, with stirrer bar, and allowed to dissolve. Afterwards the pH was adjusted to 3.4 using 5M KOH. The content of the Schott bottle was poured into a 2 L measuring cylinder and filled to 1.4 L with dH₂O. The content was then autoclaved and thereafter stored at 4°C until needed.

Table 3.2 All fermentations combinations done in this chapter with abbreviations used where _15 is for 15°C and _25 for 25°C. All yeasts were inoculated simultaneously at time 0.

Mono-cultures	One-on-one	3 Yeasts	Consortium
<i>S. cerevisiae</i> (Sc) (Sc_15 / SC_25)	<i>S. cerevisiae</i> + <i>M. pulcherrima</i> (MS_15 / MS_25)	<i>S. cerevisiae</i> + <i>M. pulcherrima</i> + <i>T. delbrueckii</i> (TMS_15 / TMS_25)	<i>S. cerevisiae</i> + <i>M. pulcherrima</i> + <i>T. delbrueckii</i> + <i>P. kluyveri</i> (TPMS_15 / TPMS_25)
<i>M. pulcherrima</i> (Mp) (Mp_15 / MP_25)	<i>S. cerevisiae</i> + <i>T. delbrueckii</i> (ST_15 / ST_25)	<i>S. cerevisiae</i> + <i>T. delbrueckii</i> + <i>P. kluyveri</i> (TPS_15 / TPS_25)	
<i>T. delbrueckii</i> (TdB) (TdB_15 / TdB_25)	<i>S. cerevisiae</i> + <i>P. kluyveri</i> (SP_15 / SP_25)	<i>S. cerevisiae</i> + <i>P. kluyveri</i> + <i>M. pulcherrima</i> (MPS_15 / MPS_25)	
<i>P. kluyveri</i> (Pk) (Pk_15 / PK_25)	<i>M. pulcherrima</i> + <i>T. delbrueckii</i> (TM_15 / TM_25)	<i>M. pulcherrima</i> + <i>T. delbrueckii</i> + <i>P. kluyveri</i> (TPM_15 / TPM_25)	
	<i>T. delbrueckii</i> + <i>P. kluyveri</i> (TP_15 / TP_25)		
	<i>P. kluyveri</i> + <i>M. pulcherrima</i> (PM_15 / PM_25)		

Table 3.3 Synthetic grape must (AWRI) as amended from Henschke and Jiranek (1993) Bely *et al.* (1990) adjusted pH of 3.4 using 5M KOH

Nitrogen sources (Made in 1L stock solution)		Lipids/Oxygen	
Tyrosine	1.4g	Ergosterol	10mg
Tryptophan	13.7g	Tween 80	0.5ml
Isoleucine		Trace Elements (Made in 1L DH ₂ O, x1000 stock solution)	
Aspartic acid	3.4g	Manganese chloride	0.2g
Glutamic acid	9.2g	Zink chloride	0.135g
Arginine	28.6g	Ferri chloride	0.03g
Leucine	3.7g	Cupri chloride	0.015g
Threonine	5.8g	Boric acid	0.005g
Glycine	1.4g	Cobalt nitrate	0.03g
Glutamine	38.6g	Sodium molybdate	0.025g

Alanine	11.1g	Potassium iodate	0.01g
Valine	3.4g	Vitamins (Made in 1L DH ₂ O, x100 stock solution)	
Methionine	2.4g	Myo-Inositol	10g
Phenylalanine	2.9g	Pyridoxine.HCL	0.2g
Serine	6.0g	Nicotinic acid	0.2g
Histidine	2.5g	Calcium Pantothenate	0.1g
Lysine	1.3g	Thiamine.HCl	0.05g
Cysteine	1.0g	PABA.K	0.02g
Proline	46.8g	Riboflavin	0.02g
Carbon sources (Made in 1L DH₂O)		Biotin	0.0125g
Glucose	100g	Folic acid	0.02g
Fructose	100g		
Acids			
KH-tartrate	2.5g		
L-Malic acid	3.0g		
Citric acid	0.2g		
Salts			
K₂HPO₄	1.14g		
MgSO₄.7H₂O	1.23g		
CaCl₂.2H₂O	0.44g		

3.2.2. Monitoring population dynamics of fermentation

The fermentation flasks were weighed after inoculation at time point 0 and then everyday thereafter at the same time as inoculation, until no more than 0.1g weight loss for three consecutive days was reported. Population dynamics were measured at t₀, t₁₂, t₂₄, t₄₈ and then every second day after t₄₈ until fermentation was complete. At each time point a 1.5 mL sample was taken, from each flask, of which a 100µL sample was used immediately to perform serial dilutions for determination of yeast population. The rest of the sample was centrifuged at 13000 rpm for 10 minutes, the supernatant collected, and the samples stored at -20°C until analysis.

Yeast viability was monitored by surface plating on Wallerstein Laboratory (WL) nutrient agar (Fluka Analytical, Sigma-Aldrich), using appropriate serial dilutions. The plates were incubated at 30°C for 5 days or until colonies were big enough to count or easily distinguishable. Afterwards the plates were counted, and mixed culture fermentations were differentiated on basis of colony morphology. After fermentations had ceased (lost less than 0.1 g per day for three consecutive days) 50 mL of synthetic wine was centrifuged and the supernatant kept for HPLC and GC-FID analysis.

3.2.3. Gas chromatographic analysis

For the extractions of volatile aroma from the samples, the protocol described by Louw *et al.* (2010) was followed with a few minor adjustments. 5 mL of sample was aliquoted into a 15 mL culture tube. The internal standard 4-methyl-2-pentanol (100 μL) and 1 mL diethyl ether were added to each sample. A small magnetic stirrer bar was added to each culture tube and the tubes were placed on a magnetic stirrer, stirred for 20 min and inverted every 5 min. Afterwards the tubes were centrifuged at 2000 rpm for 3 min, Na_2SO_4 was added to the mixture and centrifuged again.

The ethyl layer was removed and dried on additional Na_2SO_4 in vial and then placed into the vial insert and vial capped. Only 12 samples were prepared per day. The volatile higher alcohols, esters, fatty acids and carbonyl compounds (Table 2) were quantified in duplicate using a Hewlett Packard 6890 Plus gas chromatograph (Little Falls, USA) with a split/splitless injector and a flame ionization detector. The protocol described by Malherbe (2011) was followed with a few modifications. The separation of compounds was achieved using a DB- FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) with dimensions 20 m length x 0.1 mm inside diameter x 0.2 μm film thickness. The initial oven temperature was maintained at 33°C for 8 minutes after which the temperature was increased by 12°C /minute until 240 was reached. This temperature was held for 5 minutes. A 1 μL sample was injected when the oven temperature reached 250°C.

The split ratio 10:1 and the split flow rate was 36.7 cm/s. The column flow rate was 6.6 mL/min using hydrogen as the carrier gas. The detector temperature was 230°C. After each sample, oven temperature was maintained at 250°C, with a column flow rate of 30 mL/min to clean the column of all contaminants with high boiling points.

After 48 injections, the column was cleaned both thermally and chemically by a hexane injection at an oven temperature of 250°C (Louw *et al.*, 2010).

3.3. Results

3.3.1. Fermentations performance

All fermentations were carried out in synthetic grape juice at two different temperatures, 15°C and 25°C. Fermentations were monitored until weight loss ceased. Figure 3.1 and 3.2 below show the population count for all species in all single and mixed species fermentations. For clarity, standard deviations were not included in the graphs, but are shown in Table 1, Appendix A. For total colony counts at the end of fermentation and total days to end of fermentations, please also consult Table 2 and Figure 2, appendix A. Please note that not all yeasts have population counts at some time points. This is due to the dilution factor at those time points being too high. Some instances the dilution factor had to be higher in order to get countable plates for the dominating yeasts. An example of this is the fermentation of *T. delbrueckii* and *S. cerevisiae*. *T. delbrueckii* was inoculated at 25g.hL⁻¹ and *S. cerevisiae* at 1g.hL⁻¹, thus in order to dilute enough to dilute *T. delbrueckii*, to get to countable

range, *S. cerevisiae* were undetected at time 0, until it reached high enough cell densities to be detected at the dilutions made.

Of all the yeasts used, *S. cerevisiae* showed the numerically biggest variance with regards to population growth in the different fermentations. In all co-cultures *S. cerevisiae* generally reached higher cell densities in the presence of *M. pulcherrima* as can be seen from Fig. 3.1 (a) and (b) where for MS_15, MPS_15 and MS_25 it had a higher growth rate initially, but at 120 hours had similar cell densities to the mono-culture. In all cases *S. cerevisiae* performed significantly less well in the presence of *T. delbrueckii* at both temperatures. It seems like *P. kluyveri* also had an inhibitory effect on the cell density of *S. cerevisiae*. In the case of TPS_15 the combined effect of *P. kluyveri* and *T. delbrueckii* had a substantial effect on the cell densities of *S. cerevisiae* resulting in a decline in population density from 48 h to 120 h.

For both temperatures, *M. pulcherrima* reached the highest cell density in mono-culture (Fig. 3.1 (c) and (d)). It seems that at 15°C *S. cerevisiae* has a substantially lesser effect on *M. pulcherrima*'s performance compared to the same fermentation as 25°C. With the exception of TPMS_15 it is clear that at 15°C the presence of *T. delbrueckii* had a significant influence on the performance of *M. pulcherrima*, which was no longer present after 24 h. On the other hand, the presence of *P. kluyveri* at 15°C did not have as great an influence compared to *T. delbrueckii*, but still resulted in much lower cell density of *M. pulcherrima* compared to the mono-culture. *M. pulcherrima* grows well in the MS_25 fermentation until 48 hours, but then the cell numbers drop rapidly.

In all fermentations at 15°C the effect of the other yeasts seemed to have had little impact on the performance of *T. delbrueckii*. In TMS_15 and ST_15 combinations, the strain showed slightly higher cell densities (Fig. 3.2 (a)). It does however appear that the strongest inhibition, regarding a decrease in cell densities, at 15°C appeared in the presence of *P. kluyveri*. The combined presence of *P. kluyveri* and *S. cerevisiae*, seemed to have affected the growth rate of *T. delbrueckii*, in TPMS_25 and TPS_25, more so than in the presences of these yeasts individually (Fig. 3.2 (b)).

In mono-culture a delay in onset of fermentation was observed for *P. kluyveri* at 15°C with higher cell densities observed for all other fermentations at 24 h (Fig. 3.2 (c)). At 48 hours the strain reached the highest cell density when grown in mono-culture, remaining so until the end. It is clear that *T. delbrueckii* had the greatest influence on *P. kluyveri*, at both temperatures and this effect was more apparent at 25°C (Fig. 3.2 (d)), where *P. kluyveri* became undetectable after 48 h. The presence of *M. pulcherrima*, at 25°C, seemed to have less of an effect for PM_25 and MPS_25, but *P. kluyveri* still disappeared after 48 h. Although it reached lower cell densities compared to mono-culture, when in the presence of only *S. cerevisiae*, *P. kluyveri* was still at a high density at 120 h.

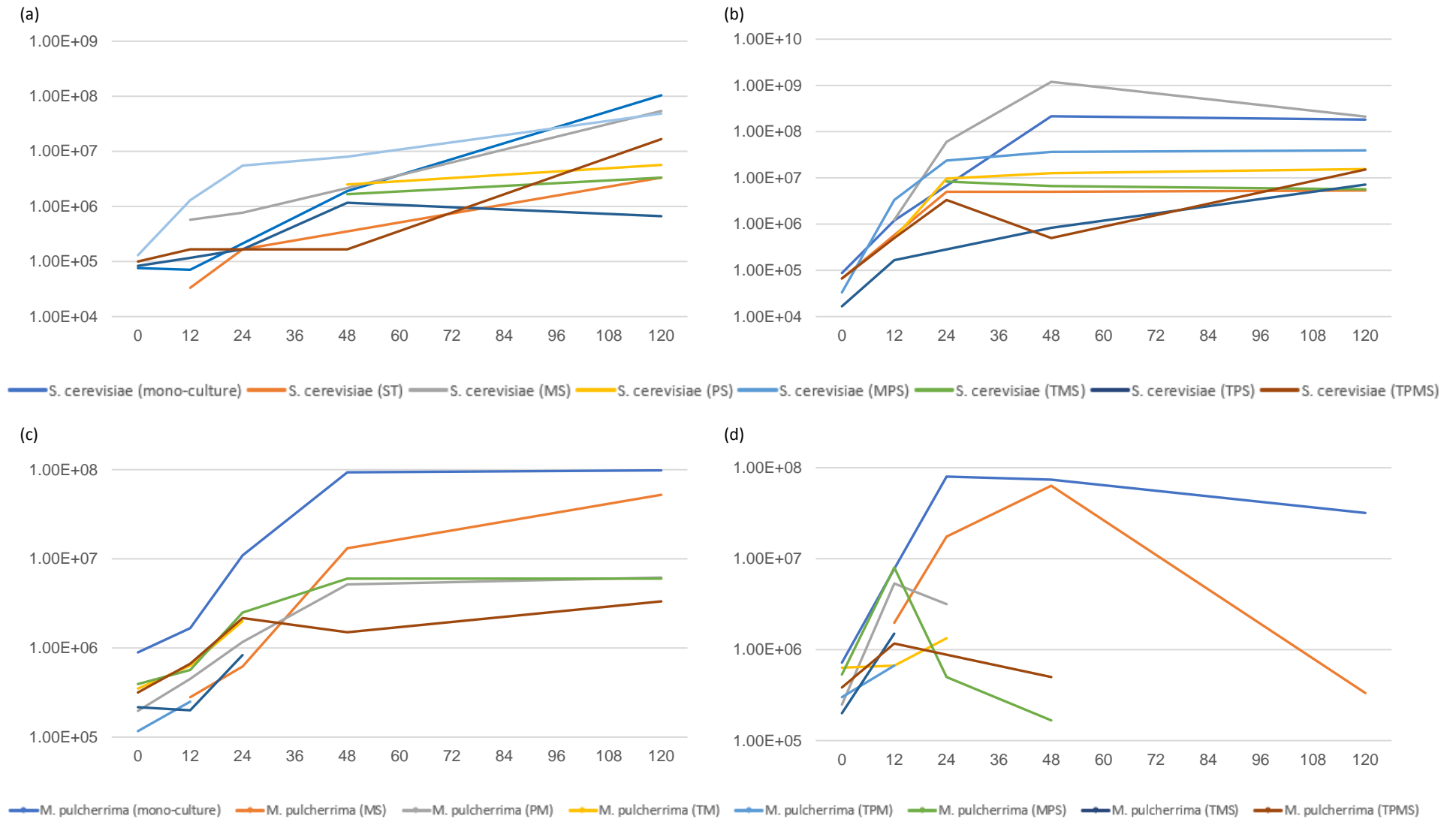


Figure 3.1 *S. cerevisiae* cell growth rate for the first 120 hours at, (a) 15°C and (b) 25°C. *M. pulcherrima* cell growth rate for the first 120 hours at, (c) 15°C and (d) 25°C.

Note: All y-axis display CFU.mL⁻¹ and x-axis display hours

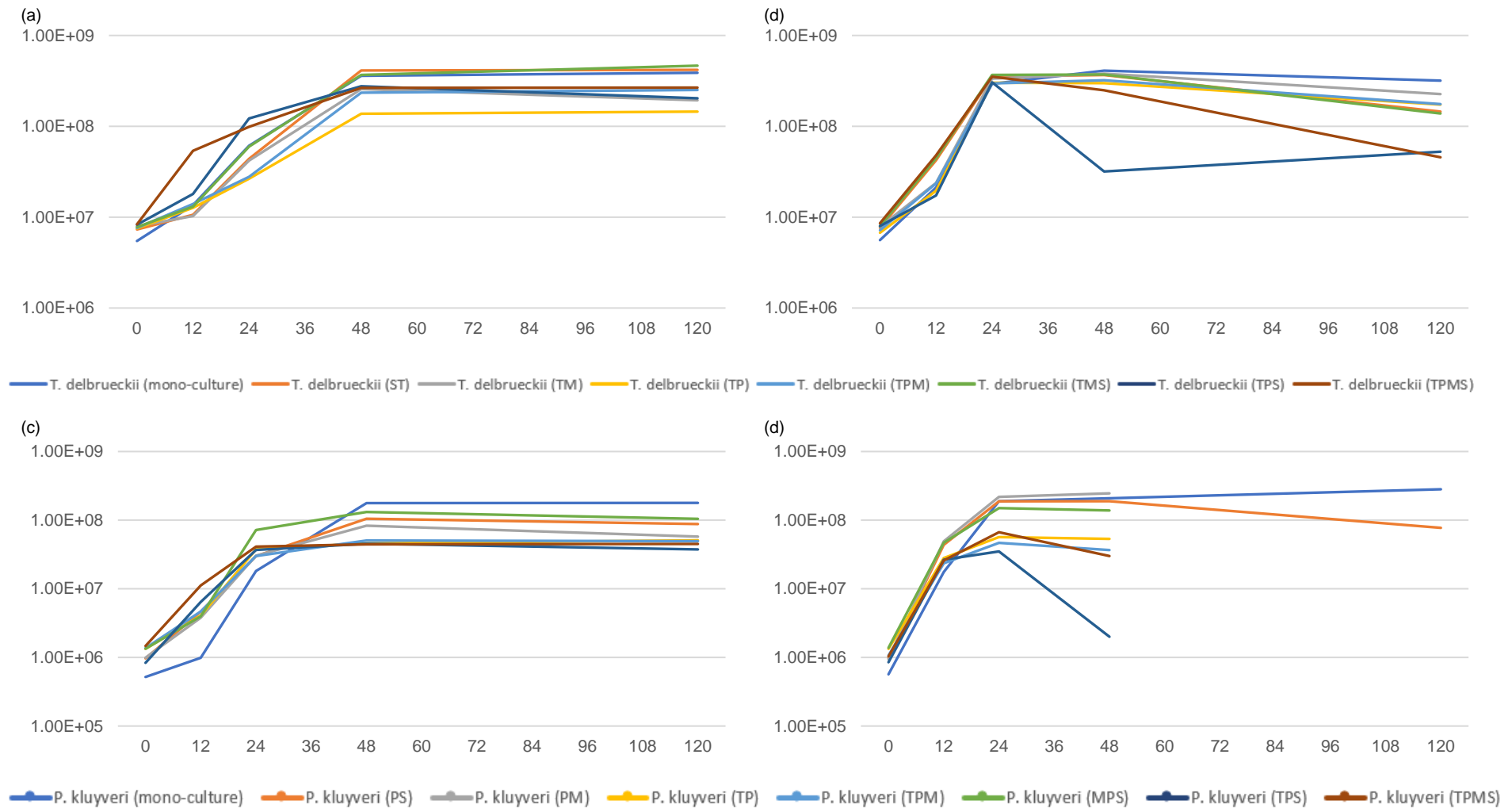


Figure 3.2 *T. delbrueckii* cell growth rate for the first 120 hours at, (a) 15°C and (b) 25°C. *P. kluyveri* cell growth rate for the first 120 hours at, (c) 15°C and (d) 25°C.

Note: All y-axis display CFU.mL⁻¹ and x-axis display hours

Figure 3.3 shows the weight loss for all synthetic grape juice fermentations, for the first 120 hours after inoculation. The values on the graphs are the average weight loss per triplicate with standard deviation. For all fermentations, good reproducibility was achieved.

It is clear from the results that both *S. cerevisiae* and *T. delbrueckii*, at 25°C, showed the similar amount regarding weight loss at 120 h, but when paired together in co-culture the weight loss at the same time point is lower. This is reflected in the lower cell density of *S. cerevisiae* compared to the mono-culture. *T. delbrueckii* showed significantly higher weight loss compared to any other mono-culture fermentations and the most weight loss compared to all other fermentations at 15°C. The weight loss of PM is more compared to that of PS and MS at 15°C, but this does not hold true when at 25C where it lost less weight compared to PS and MS. It is seen from the table that the presence of three of four yeasts had similar weight loss results at 25°C, but at 15°C MPS lost less weight.

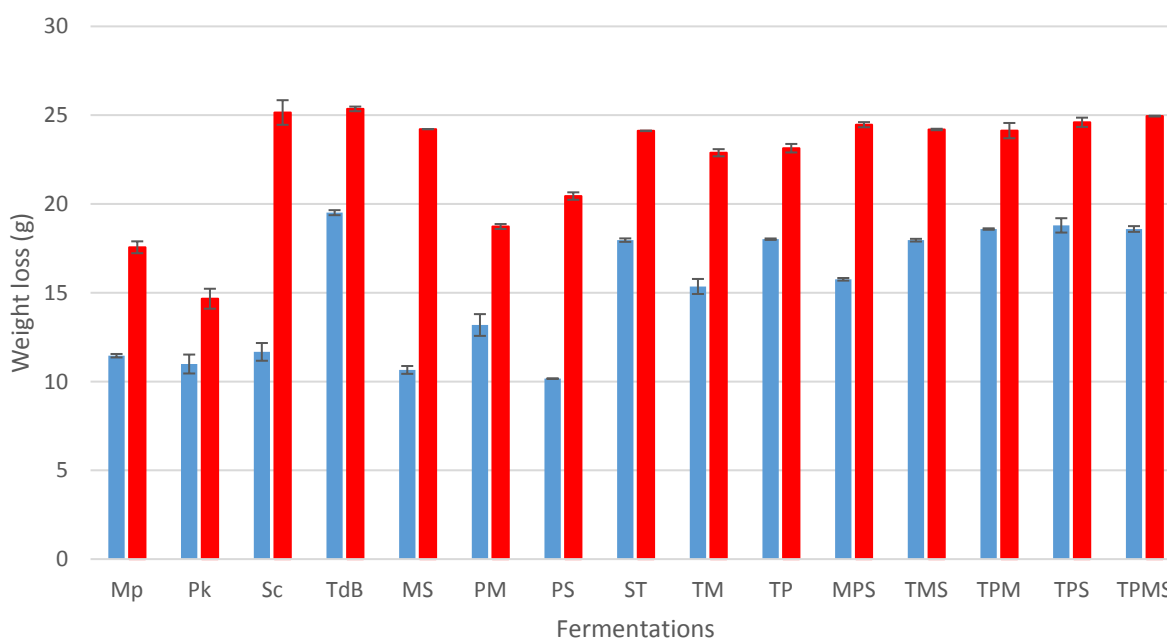


Figure 3.3 Total accumulative CO₂ weight loss for all fermentations at both temperatures with standard deviation for the first 120 hours. Blue representing 15°C and red, 25°C. Error bars show +/- standard deviation of three biological repeats.

Note: For abbreviations please refer to Table 3.2

3.3.2. Influence of mixed starter culture on volatile aroma formation

GC-FID was utilized for aromatic compounds analysis on all fermentations containing *S. cerevisiae* as these were the only fermentations in which most of the sugar was consumed as high sugar content samples did not show accurate results. A number of 20 major volatile compounds were quantified within the limitations of the machine. The subsequent subsections will discuss these compounds according to their chemical class and Table 3.4 which shows the concentration at the end of fermentation, will be referred to throughout.

3.3.2.1. Esters

In general, Table 3.4 shows that the total amount of esters produced were higher in fermentations containing *P. kluyveri*. Figure 3.4 (a) shows that all fermentations containing *P. kluyveri* produced a substantial amount of ethyl acetate and fermentations at 15°C produced more ethyl acetate than at 25°C. TPS_15 produced four times more than TPS_25, MPS_15 produced twice as much as at 25°C and SP_15 produced more than SP_25, but not as significantly as the other fermentations. Fermentation SP at both temperatures produced the highest amounts of ethyl acetate. With the consortium at 15°C it is also clear that the presence of *P. kluyveri* increased the ethyl acetate production twice as much as at 25°C, but this is the combination where this compound was the lowest of all fermentations.

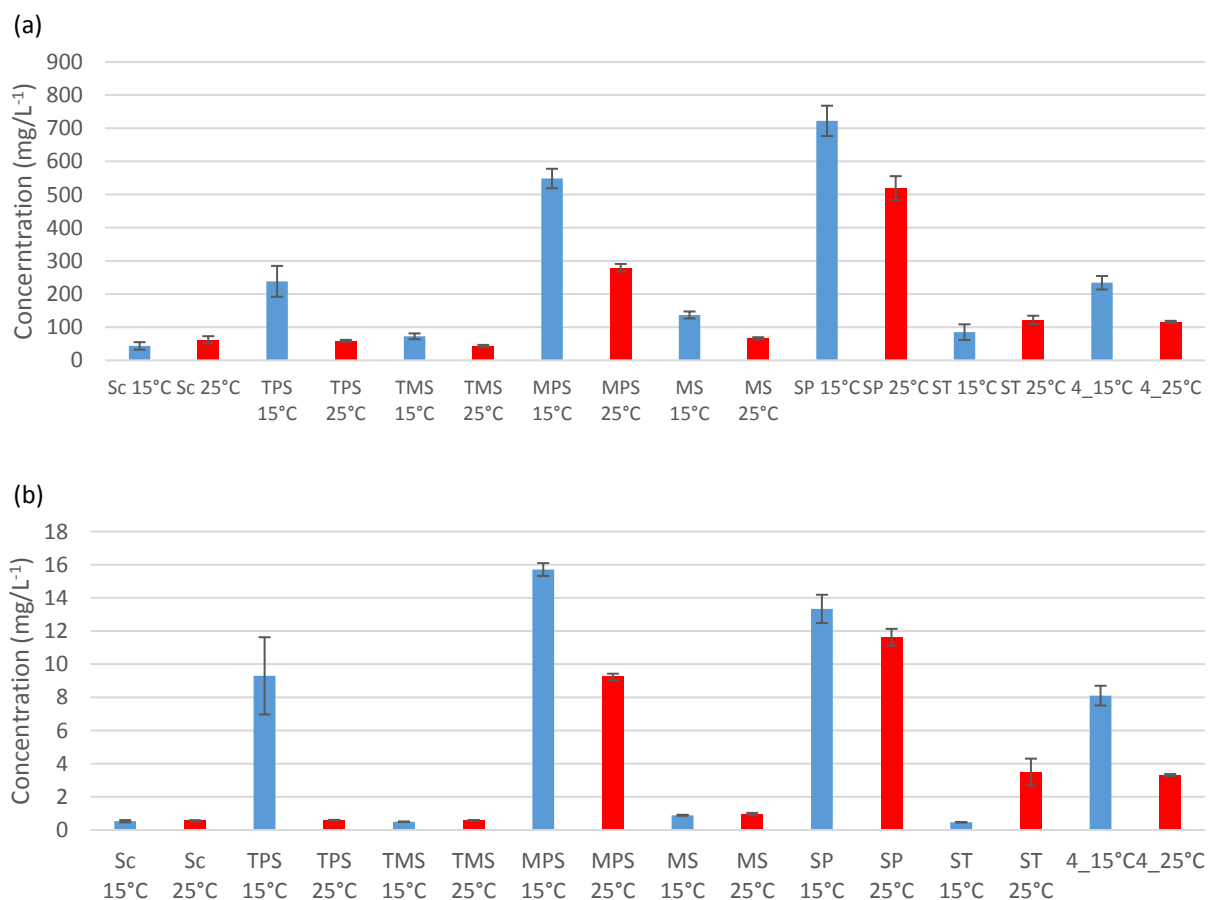


Figure 3.4 (a) The amount of ethyl acetate produced and (b) showing the amount of isoamyl acetate produced. Error bars show standard deviation of three biological repeats.

Figure 3.4 (b) shows that isoamyl acetate production was also substantially higher in fermentations containing *P. kluyveri*. All fermentations containing *P. kluyveri*, with the exception to the consortium and TPS at 25°C, showed substantial higher levels of isoamyl acetate. Interestingly fermentation TPS_25 produced the same levels of isoamyl acetate than fermentation not containing *P. kluyveri*. TPS_25 produced +/- 1 mg.L⁻¹ of isoamyl acetate compared to TPS_15 that produced +/- 15 mg.L⁻¹ (Fig. 3.4 (b)). Ethyl caprylate was higher for all 15°C fermentations with the exception to ST_15, with more than 0.35 g.L⁻¹ produced by, Sc_15, MPS_15, MS_15 and SP_15.

3.3.2.2. Higher alcohols

The total amount of higher alcohols seems to have been influenced by the presence of *P. kluyveri* in most cases having less higher alcohols compared to the fermentations excluding *P. kluyveri* (Table 3.4). The amount of propanol and isoamyl alcohol production from all fermentations differed except for mono-culture fermentations of *S. cerevisiae*. With all the fermentations the amount of butanol (Fig. 3.5) was less for all fermentations at 15°C. For isobutanol production (Table 3.4), Sc_15 (23.9 mg.L⁻¹) produced almost have the amount compared to that of Sc_25 (41.32 mg.L⁻¹). The Isoamyl alcohol production for fermentations TMS, MPS, ST and TPMS at 15°C where more than for the fermentations at 25°C. The amount of 3-ethoxy-1-propanol for fermentations Sc_15, MPS_15 and MS_15 resulted in roughly twice as much as for 25°C (Table 3.4). For all other fermentations, 25°C produced more 3-ethoxy-1-propanol with TMS_25 and ST_25 producing roughly double the amount of compared to the fermentations at 15°C. Fermentations TMS_15 and ST_15 produced by far more 2-Phenyl ethanol than any other fermentations with 145.76 mg.L⁻¹ and 116.42 mg.L⁻¹ respectively.

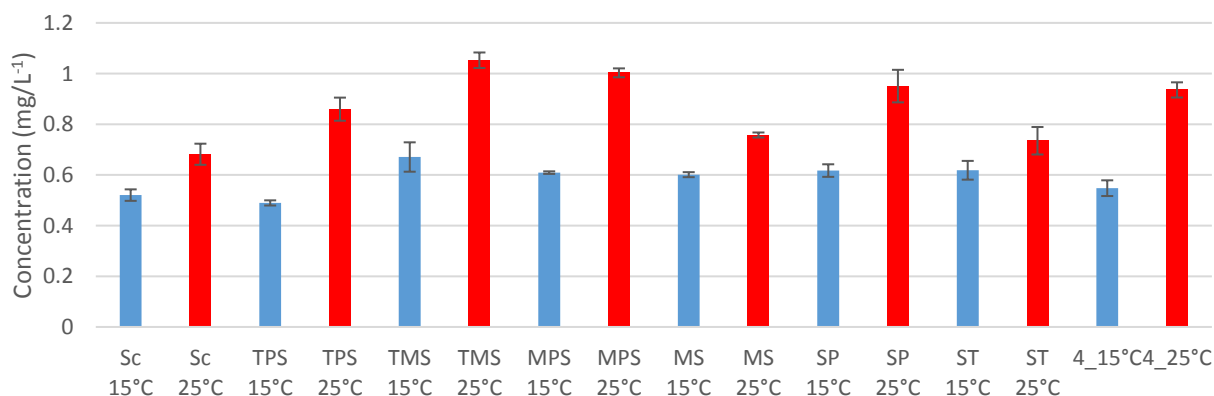


Figure 3.5 The amount of butanol produced at the end of fermentation. Error bars show standard deviation of three biological repeats.

3.3.2.3. Volatile fatty acids

Fermentation SP at both temperatures produced the highest amount of acetic acid and the most total volatile fatty acids of all the fermentations. The butyric acid production (Figure 3.6 (a)) for all fermentations at 15°C where higher than at 25°C. The octanoic and decanoic acid production for all fermentations, excluding TMS, ST and TPMS were higher at 15°C.

It is clear from the total volatile fatty acids (Figure 3.6 (b)) that all fermentation combinations where *T. delbrueckii* and *S. cerevisiae* were paired, the total amount of volatile fatty acids produced, were higher at 15°C than at 25°C.

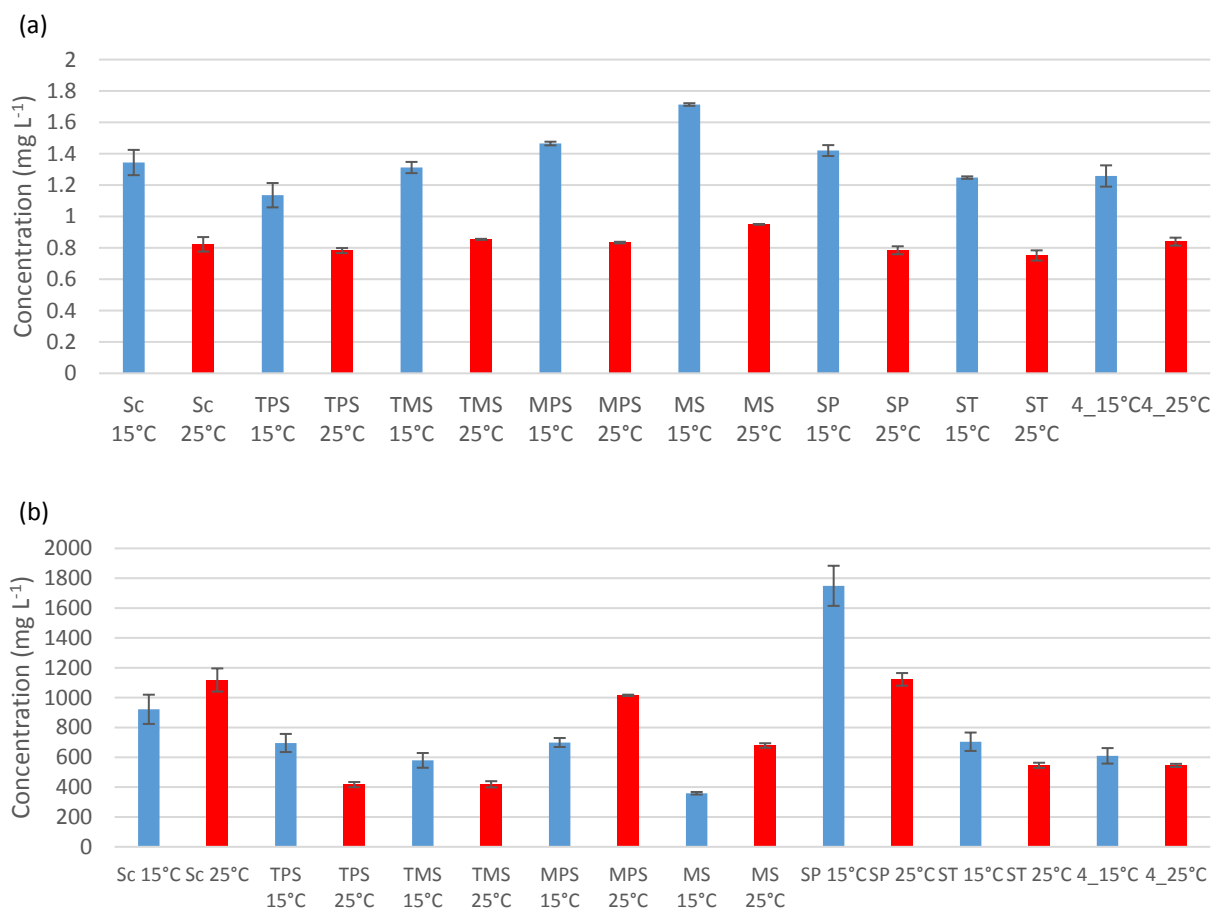


Figure 3.6 (a) Amount of butyric acid and (b) total amount of volatile fatty acids produced at the end of fermentation. Error bars show standard deviation of three biological repeats.

3.3.3. Multivariate data analysis (GC-FID)

All quantifiable data was used to create the PCA plot. The PCA plot (Figure 3.7), described by the first and the second principle component, accounts for 60.68% of the total variance. In most cases the three biological repeats grouped well together, indicating a high reproducibility of the experimental procedure, and suggesting the significant and consistent impacts of the treatments. In some cases, only two of the biological repeats grouped together, with one outlier.

It is clear from the results that PC1 (explains 15.97%) separates due to the difference in temperature. PC1 also separates between chemical compounds where the 15°C fermentations were associated with volatile fatty acids and higher alcohols and 25°C fermentations associated with volatile fatty acids and esters. PC2 (explains 44.71%) separated all fermentations containing *T. delbrueckii* with fermentations excluding *T. delbrueckii*. From the data it is clear that the effect of *M. pulcherrima* to produce volatile fatty acids and esters were suppressed in the presence of *T. delbrueckii*.

PC2 mainly separates between a combination of higher alcohols and volatile fatty acid or esters with higher alcohols. Regarding 15°C fermentations it seems that in the presence of *T. delbrueckii* is resulted in wines associated with higher alcohol formation, whereas the absences of *T. delbrueckii* with the presence of *P. kluyveri* and *M. pulcherrima* resulted in wines driven by higher alcohol and ester formation. The same holds true at 25°C, but fermentations containing *T. delbrueckii* showed to be associated with acetoin and isobutanol formation.

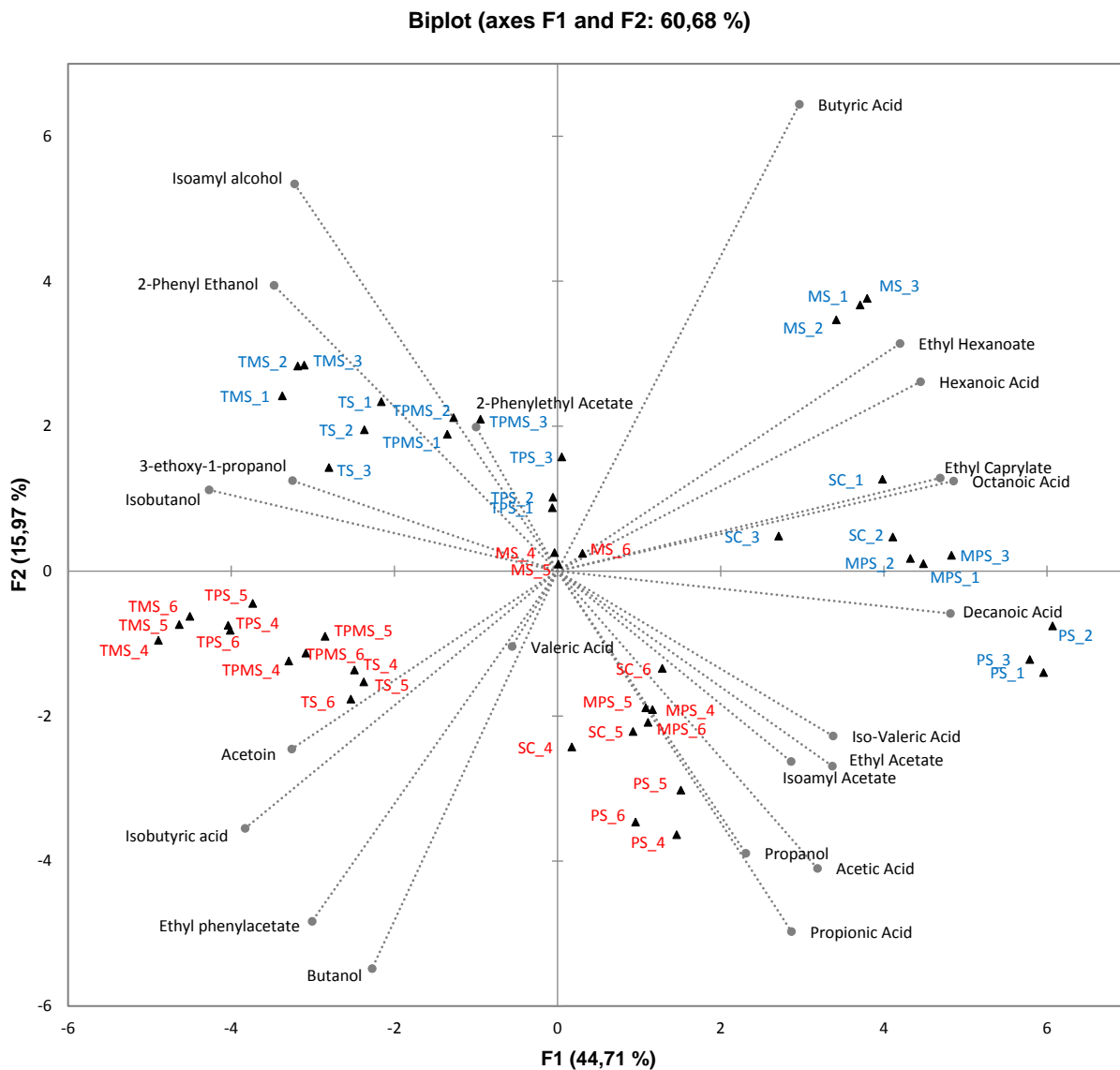


Figure 3.7 Biplot of the principle component analysis (PC1 vs PC2) for the synthetic grape must fermentation at both 15°C (in blue) and 25°C (in red).

Table 3.4: GC-FID values for all fermentations containing *S. cerevisiae* at both fermentation temperatures with standard deviation. All values displayed in the table are in mg.L⁻¹.

	SC_15	SC_25	TPS_15	TPS_25	TMS_15	TMS_25	MPS_15	MPS_25	MS_15	MS_25	SP_15	SP_25	ST_15	ST_25	TPMS_15	TPMS_25
Esters																
2-Phenylethyl Acetate	0.42 ±0.05	0.40 ±0.01	39.25 ±5.74	0.47 ±0.01	0.42 ±0.01	0.44 ±0.03	0.56 ±0.02	0.61 ±0.04	0.88 ±0.03	0.55 ±0.10	0.46 ±0.03	0.46 ±0.03	0.45 ±0.00	16.26 ±3.74	48.50 ±1.53	16.01 ±0.82
Ethyl Acetate	43.40 ±11.33	61.89 ±10.90	238.25 ±46.51	58.94 ±2.87	72.47 ±8.71	42.98 ±3.59	548.38 ±29.36	279.41 ±11.27	136.84 ±10.54	66.29 ±3.37	722.22 ±45.71	519.30 ±36.13	84.98 ±23.64	122.01 ±12.50	233.95 ±20.40	116.07 ±3.08
Ethyl Caprylate	0.42 ±0.08	0.28 ±0.06	0.16 ±0.00	0.12 ±0.00	0.11 ±0.01	0.10 ±0.00	0.43 ±0.02	0.24 ±0.01	0.45 ±0.01	0.19 ±0.03	0.38 ±0.03	0.19 ±0.01	0.11 ±0.01	0.14 ±0.01	0.17 ±0.01	0.14 ±0.01
Ethyl Hexanoate	0.74 ±0.06	0.64 ±0.02	0.65 ±0.00	0.60 ±0.00	0.61 ±0.01	0.59 ±0.00	0.81 ±0.01	0.67 ±0.01	0.94 ±0.01	0.68 ±0.01	0.78 ±0.02	0.64 ±0.01	0.60 ±0.00	0.60 ±0.01	0.64 ±0.01	0.62 ±0.02
Ethyl phenylacetate	0.45 ±0.08	0.73 ±0.11	0.50 ±0.01	1.01 ±0.05	0.40 ±0.03	1.12 ±0.06	0.54 ±0.01	0.62 ±0.01	0.46 ±0.01	0.81 ±0.02	0.47 ±0.03	0.67 ±0.01	0.46 ±0.03	1.03 ±0.06	0.47 ±0.04	1.00 ±0.05
Isoamyl Acetate	0.53 ±0.07	0.55 ±0.04	9.30 ±2.33	0.59 ±0.02	0.50 ±0.02	0.58 ±0.01	15.71 ±0.39	9.23 ±0.19	0.89 ±0.03	0.96 ±0.07	13.33 ±0.85	11.63 ±0.50	0.46 ±0.01	3.49 ±0.81	8.10 ±0.60	3.31 ±0.06
Total Esters	45.97	64.49	288.11	61.71	74.51	45.81	566.42	290.78	140.47	69.48	737.64	532.89	87.07	143.54	291.83	137.15
Higher Alcohols																
2-Phenyl Ethanol	18.54 ±3.49	21.56 ±3.26	25.48 ±0.60	62.26 ±2.38	145.76 ±0.38	86.09 ±5.50	20.52 ±0.18	17.04 ±0.17	25.47 ±0.62	37.22 ±1.17	13.92 ±0.30	18.25 ±0.60	116.42 ±3.12	28.74 ±2.53	55.33 ±3.89	42.65 ±2.15
3-ethoxy-1-propanol	12.73 ±2.43	5.87 ±0.64	20.33 ±2.08	25.35 ±1.44	12.11 ±0.70	22.44 ±0.52	10.88 ±0.16	5.05 ±0.07	15.30 ±0.12	7.97 ±0.24	4.91 ±0.13	3.97 ±0.26	12.16 ±0.96	27.05 ±1.28	15.63 ±0.87	25.36 ±0.58
Butanol	0.52 ±0.02	0.68 ±0.04	0.49 ±0.01	0.86 ±0.05	0.67 ±0.06	1.05 ±0.03	0.61 ±0.01	1.00 ±0.02	0.60 ±0.01	0.76 ±0.01	0.62 ±0.02	0.95 ±0.06	0.62 ±0.04	0.73 ±0.05	0.55 ±0.03	0.94 ±0.03
Isoamyl alcohol	135.39 ±9.35	135.48 ±6.99	121.75 ±5.57	160.83 ±3.97	242.86 ±8.41	195.26 ±2.94	133.72 ±1.20	119.07 ±1.57	161.51 ±2.75	176.75 ±2.18	89.93 ±2.22	99.81 ±2.74	194.46 ±2.01	128.59 ±5.39	199.99 ±11.90	157.60 ±3.52
Isobutanol	23.90 ±2.80	41.32 ±1.95	43.60 ±2.26	70.94 ±1.99	77.28 ±5.37	80.10 ±3.68	45.26 ±2.10	54.29 ±0.52	56.48 ±3.09	51.00 ±1.16	36.96 ±1.07	48.11 ±1.87	61.79 ±1.03	62.98 ±0.91	64.30 ±5.74	76.04 ±2.95
Propanol	75.13 ±8.91	75.24 ±5.99	39.50 ±1.01	50.21 ±1.38	38.79 ±2.70	41.46 ±1.63	65.55 ±3.79	81.02 ±1.29	81.60 ±3.65	55.08 ±1.25	51.14 ±2.24	85.50 ±5.14	41.79 ±1.70	80.24 ±9.44	35.16 ±2.31	59.15 ±0.58
Total Higher Alcohols	266.21	280.16	251.15	370.44	517.46	426.40	276.53	277.48	340.96	328.78	197.48	256.59	427.24	328.34	370.96	361.75

Volatile Fatty Acids																
Acetic Acid	907.30 ±96.52	1103.52 ±76.46	684.19 ±60.24	402.31 ±16.98	567.60 ±48.82	401.97 ±20.19	683.58 ±29.79	1002.79 ±4.25	346.98 ±8.18	667.53 ±14.58	1732.97 ±133.86	1107.64 ±41.93	694.02 ±61.44	532.52 ±16.43	596.63 ±51.47	530.12 ±9.88
Butyric Acid	1.34 ±0.08	0.82 ±0.05	1.14 ±0.08	0.78 ±0.02	1.31 ±0.04	0.85 ±0.00	1.46 ±0.01	0.83 ±0.01	1.71 ±0.01	0.95 ±0.00	1.42 ±0.03	0.78 ±0.03	1.25 ±0.01	0.75 ±0.03	1.26 ±0.07	0.84 ±0.03
Decanoic Acid	1.64 ±0.25	1.27 ±0.08	0.84 ±0.03	0.75 ±0.01	0.63 ±0.01	0.64 ±0.01	2.20 ±0.09	1.40 ±0.16	2.23 ±0.06	1.33 ±0.10	2.91 ±0.10	1.58 ±0.07	0.66 ±0.03	0.73 ±0.02	0.78 ±0.01	0.84 ±0.13
Isobutyric acid	3.23 ±0.28	4.86 ±0.27	4.45 ±0.05	8.25 ±0.30	5.08 ±0.33	10.62 ±0.54	3.60 ±0.02	3.81 ±0.05	1.40 ±0.02	2.70 ±0.01	3.70 ±0.06	5.77 ±0.20	3.75 ±0.06	7.16 ±0.29	5.71 ±0.21	8.37 ±0.45
Iso-Valeric Acid	2.25 ±0.11	1.93 ±0.13	1.22 ±0.01	1.25 ±0.03	1.08 ±0.05	1.45 ±0.02	1.91 ±0.02	1.39 ±0.02	1.47 ±0.00	1.85 ±0.02	1.76 ±0.05	1.62 ±0.02	0.97 ±0.01	1.29 ±0.02	1.50 ±0.04	1.41 ±0.02
Octanoic Acid	2.44 ±0.36	1.62 ±0.36	1.20 ±0.11	0.73 ±0.03	0.81 ±0.02	0.73 ±0.02	2.60 ±0.06	1.54 ±0.05	2.78 ±0.14	1.64 ±0.26	2.58 ±0.06	1.31 ±0.04	0.74 ±0.02	0.88 ±0.09	1.11 ±0.02	0.91 ±0.04
Propionic Acid	2.93 ±0.32	3.36 ±0.28	2.50 ±0.15	2.25 ±0.07	2.44 ±0.16	2.31 ±0.02	3.27 ±0.02	2.82 ±0.01	1.86 ±0.05	2.40 ±0.05	3.64 ±0.21	3.15 ±0.10	2.54 ±0.12	2.61 ±0.04	2.41 ±0.15	2.45 ±0.08
Valeric Acid	0.52 ±0.07	0.60 ±0.06	0.39 ±0.01	0.56 ±0.03	0.37 ±0.01	0.58 ±0.02	0.58 ±0.01	0.51 ±0.01	0.70 ±0.02	0.88 ±0.03	0.27 ±0.01	0.45 ±0.02	0.37 ±0.00	0.67 ±0.06	0.38 ±0.02	0.68 ±0.00
Total Volatile Fatty Acids	921.65	1117.98	695.93	416.88	579.32	419.16	699.20	1015.10	359.14	679.27	1749.25	1122.29	704.28	546.61	609.78	545.62

3.4. Discussion

In this study the fermentations kinetics and metabolic compounds by different combinations of yeast species was evaluated. To study such complex multispecies system, we decided to use synthetic grape must to achieve a good reproducibility of results (Carrau *et al.*, 2008).

The data overall confirms the significant impact of individual yeast species and of different combinations of such yeast on fermentations and aroma production (Wang *et al.*, 2016). The study also reveals a number of interactions, including that *S. cerevisiae* benefits from the presence of *M. pulcherrima*. This does hold true even when other, numerically more dominant species such *T. delbrueckii* are present. *T. delbrueckii* in particular had the most negative effect on the growth and fermentative performance of *S. cerevisiae*. The data also show the negative effect of *P. kluyveri*, although not as strong as *T. delbrueckii*, also had an influence on the performance of *S. cerevisiae*. This effect was somewhat additive, since *S. cerevisiae* performed the least well in combination with both of these yeasts (Figure 3.1 (a) and (b)).

It seems the difference in temperature caused the other yeasts to influence *T. delbrueckii* differently when comparing the cell densities (Figure 3.2 (a) and (b)). At 15°C it was clear that it reached higher cell densities in the presence of only *S. cerevisiae* and this holds true when *M. pulcherrima* is added but this is not the case at 25°C. When fermented in only the presence of *M. pulcherrima*, *T. delbrueckii* did not display the same cell densities compared cell densities of combined *T. delbrueckii*, *S. cerevisiae* and *M. pulcherrima*. *P. kluyveri* had the biggest inhibitory effect on the performance of *T. delbrueckii*. This inhibitory effect was enhanced when in the combined presence *S. cerevisiae* and *P. kluyveri*, at 25°C.

It was clear from the data (Figure 3.1 (c) and (d); Appendix A, Table 1)) that all yeasts negatively affected the performance of *M. pulcherrima* and these effects were more apparent at higher temperature. At 15°C these effects were more limited in the presence of only *S. cerevisiae* and when in the presence of *P. kluyveri* or the combination of both. Although all inoculation combinations resulted in *P. kluyveri* growth retardation, it was evident that co-fermentation with *T. delbrueckii* at 25°C resulted in the most considerable inhibition of *P. kluyveri*. Conversely *S. cerevisiae* had the least negative effect against *P. kluyveri*.

In most fermentations the addition of NS increased the chemical compound structure of the wine (Table 3.4). The exception being for fermentations that contained *P. kluyveri* where the amount of ethyl acetate produced was substantially higher than the other fermentations, which could produce wines with undesirable aroma such as nail polish remover, glue-like or varnish. The addition of *T. delbrueckii* and/or *M. pulcherrima* however caused a slight decrease in ethyl acetate production compared to the SP co-fermentation. The total amount of volatile fatty acid production for fermentations that included NS was also substantially lower than the pure *S. cerevisiae* fermentations, apart from SP_15, SP_25 and MPS_25. Higher alcohols were elevated in fermentations containing *T. delbrueckii*.

This study gives an insight into the phenotypic space in terms of fermentative performance and aroma production of *S. cerevisiae*, *P. kluyveri*, *M. pulcherrima* and *T. delbrueckii* in mixed fermentations at two different temperatures. This study sheds light onto how various NS interact with each other and in the presence of *S. cerevisiae*, in a controlled environment, on aroma production and how these outcomes might differ at different temperatures. It also shows how interactions between various yeasts influence the performance of each yeast within the consortium and how this might affect the fermentation kinetics and population dynamics.

3.5. References

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3.6. Appendix A

Table 1 The cell growth rate for Sc_15, Sc_25, Mp_15 and Mp_25 for the first 120 h.

Note: SD are shown below each value and represents three biological repeats

15°C	<i>S. cerevisiae</i> (mono-culture)	<i>S. cerevisiae</i> (ST)	<i>S. cerevisiae</i> (MS)	<i>S. cerevisiae</i> (PS)	<i>S. cerevisiae</i> (MPS)	<i>S. cerevisiae</i> (TMS)	<i>S. cerevisiae</i> (TPS)	<i>S. cerevisiae</i> (TPMS)
0	7.62E+04	0.00E+00	0.00E+00	0.00E+00	1.30E+05	0.00E+00	8.33E+04	1.00E+05
	7.91E+03	0.00E+00	0.00E+00	0.00E+00	1.32E+04	0.00E+00	2.89E+04	8.66E+04
12	7.08E+04	3.33E+04	5.73E+05	0.00E+00	1.30E+06	0.00E+00	1.17E+05	1.67E+05
	3.18E+03	5.77E+04	2.52E+04	0.00E+00	1.32E+05	0.00E+00	7.64E+04	2.89E+05
24	0.00E+00	1.67E+05	7.70E+05	0.00E+00	5.50E+06	0.00E+00	1.67E+05	1.67E+05
	0.00E+00	2.89E+05	3.12E+04	0.00E+00	1.80E+06	0.00E+00	2.89E+05	2.89E+05
48	1.91E+06	0.00E+00	2.15E+06	2.50E+06	8.00E+06	1.67E+06	1.17E+06	1.67E+05
	9.26E+04	0.00E+00	2.65E+05	8.66E+05	2.65E+06	2.89E+06	2.89E+05	2.89E+05
120	1.04E+08	3.33E+06	5.40E+07	5.67E+06	4.85E+07	3.33E+06	6.67E+05	1.67E+07
	1.26E+06	2.89E+06	1.80E+06	1.53E+06	7.94E+06	2.89E+06	2.89E+05	5.77E+06
25°C	<i>S. cerevisiae</i> (mono-culture)	<i>S. cerevisiae</i> (ST)	<i>S. cerevisiae</i> (MS)	<i>S. cerevisiae</i> (PS)	<i>S. cerevisiae</i> (MPS)	<i>S. cerevisiae</i> (TMS)	<i>S. cerevisiae</i> (TPS)	<i>S. cerevisiae</i> (TPMS)
0	8.73E+04	6.67E+04	0.00E+00	0.00E+00	3.33E+04	0.00E+00	1.67E+04	6.67E+04
	6.37E+03	5.77E+04	0.00E+00	0.00E+00	1.44E+04	0.00E+00	2.89E+04	7.64E+04
12	1.20E+06	0.00E+00	1.23E+06	5.00E+05	3.33E+06	0.00E+00	1.67E+05	5.00E+05
	8.66E+04	0.00E+00	1.76E+05	5.00E+05	1.44E+06	0.00E+00	2.89E+05	5.00E+05
24	0.00E+00	5.00E+06	6.02E+07	9.67E+06	2.37E+07	8.33E+06	0.00E+00	3.33E+06
		5.00E+06	7.64E+05	2.75E+06	1.53E+06	2.89E+06	0.00E+00	2.89E+06
48	2.15E+08	5.00E+06	1.20E+09	1.27E+07	3.65E+07	6.67E+06	8.33E+05	5.00E+05
	4.04E+06	5.00E+06	7.70E+07	1.04E+06	2.18E+06	2.89E+06	1.04E+06	0.00E+00
120	1.83E+08	5.33E+06	2.12E+08	1.55E+07	3.93E+07	5.67E+06	7.17E+06	1.52E+07
	8.55E+06	7.64E+05	7.58E+07	4.44E+06	2.93E+06	1.44E+06	3.62E+06	2.93E+06

15°C	<i>M. pulcherrima</i> (mono-culture)	<i>M. pulcherrima</i> (MS)	<i>M. pulcherrima</i> (PM)	<i>M. pulcherrima</i> (TM)	<i>M. pulcherrima</i> (TPM)	<i>M. pulcherrima</i> (MPS)	<i>M. pulcherrima</i> (TMS)	<i>M. pulcherrima</i> (TPMS)
0	8.92E+05 7.02E+04	0.00E+00 0.00E+00	1.97E+05 4.86E+04	3.50E+05 5.00E+04	1.17E+05 7.64E+04	3.93E+05 4.86E+04	2.17E+05 5.77E+04	3.17E+05 1.61E+05
12	1.67E+06 5.97E+04	2.80E+05 2.60E+04	4.50E+05 8.66E+04	6.33E+05 1.61E+05	2.50E+05 2.29E+05	5.67E+05 2.47E+05	2.00E+05 1.32E+05	6.67E+05 7.64E+05
24	1.09E+07 3.75E+05	6.20E+05 1.80E+04	1.17E+06 2.89E+05	2.00E+06 1.73E+06	0.00E+00 0.00E+00	2.50E+06 8.66E+05	8.33E+05 1.04E+06	2.17E+06 1.26E+06
48	9.33E+07 6.93E+06	1.31E+07 2.93E+05	5.17E+06 2.36E+06	0.00E+00 0.00E+00	0.00E+00 0.00E+00	6.00E+06 8.66E+05	0.00E+00 0.00E+00	1.50E+06 1.32E+06
120	9.83E+07 9.25E+06	5.23E+07 2.75E+06	6.17E+06 2.57E+06	0.00E+00 0.00E+00	0.00E+00 0.00E+00	6.00E+06 2.50E+06	0.00E+00 0.00E+00	3.33E+06 2.89E+06
25°C	<i>M. pulcherrima</i> (mono-culture)	<i>M. pulcherrima</i> (MS)	<i>M. pulcherrima</i> (PM)	<i>M. pulcherrima</i> (TM)	<i>M. pulcherrima</i> (TPM)	<i>M. pulcherrima</i> (MPS)	<i>M. pulcherrima</i> (TMS)	<i>M. pulcherrima</i> (TPMS)
0	7.18E+05 4.65E+04	0.00E+00 0.00E+00	2.48E+05 2.75E+04	6.33E+05 1.53E+05	3.00E+05 8.66E+04	5.32E+05 5.84E+04	2.00E+05 1.00E+05	3.83E+05 7.64E+04
12	7.73E+06 7.52E+05	1.97E+06 2.75E+05	5.33E+06 2.89E+05	6.67E+05 1.04E+05	6.67E+05 7.64E+05	8.00E+06 1.50E+06	1.50E+06 1.00E+06	1.17E+06 7.64E+05
24	7.98E+07 2.75E+06	1.75E+07 3.28E+06	3.17E+06 2.89E+05	1.33E+06 1.26E+06	0.00E+00 0.00E+00	5.00E+05 8.66E+05	0.00E+00 0.00E+00	0.00E+00 0.00E+00
48	7.38E+07 8.22E+06	6.33E+07 2.89E+06	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.67E+05 2.89E+05	0.00E+00 0.00E+00	5.00E+05 5.00E+05
120	3.18E+07 2.57E+06	3.33E+05 5.77E+05	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00
15°C	<i>T. delbrueckii</i> (mono-culture)	<i>T. delbrueckii</i> (ST)	<i>T. delbrueckii</i> (TM)	<i>T. delbrueckii</i> (TP)	<i>T. delbrueckii</i> (TPM)	<i>T. delbrueckii</i> (TMS)	<i>T. delbrueckii</i> (TPS)	<i>T. delbrueckii</i> (TPMS)
0	5.47E+06 4.04E+05	7.32E+06 4.04E+05	7.93E+06 8.81E+05	7.52E+06 4.51E+05	7.62E+06 7.75E+05	7.78E+06 3.25E+05	8.27E+06 2.52E+05	8.35E+06 3.28E+05

12	1.36E+07	1.06E+07	1.03E+07	1.27E+07	1.40E+07	1.30E+07	1.80E+07	5.38E+07	
	4.25E+05	5.03E+05	9.64E+05	1.61E+05	5.30E+05	2.02E+05	8.75E+05	6.60E+06	
24	6.12E+07	4.38E+07	4.20E+07	2.65E+07	2.78E+07	5.98E+07	1.22E+08	9.87E+07	
	3.33E+06	2.02E+06	5.29E+06	1.80E+06	1.04E+06	2.52E+06	5.77E+06	9.61E+06	
48	3.60E+08	4.12E+08	2.57E+08	1.38E+08	2.34E+08	3.68E+08	2.76E+08	2.65E+08	
	3.04E+07	3.82E+07	2.42E+07	7.47E+06	1.70E+07	2.75E+07	4.58E+06	8.66E+06	
120	3.88E+08	4.18E+08	1.93E+08	1.45E+08	2.51E+08	4.65E+08	2.04E+08	2.67E+08	
	5.35E+07	6.29E+07	6.00E+06	1.24E+07	2.11E+07	4.44E+07	1.32E+07	1.53E+07	
<hr/>									
25°C	<i>T. delbrueckii</i> (mono-culture)	<i>T. delbrueckii</i> (ST)	<i>T. delbrueckii</i> (TM)	<i>T. delbrueckii</i> (TP)	<i>T. delbrueckii</i> (TPM)	<i>T. delbrueckii</i> (TMS)	<i>T. delbrueckii</i> (TPS)	<i>T. delbrueckii</i> (TPMS)	
0	5.58E+06	7.62E+06	7.83E+06	6.73E+06	7.18E+06	7.98E+06	7.98E+06	8.57E+06	
	3.88E+05	5.75E+05	3.40E+05	3.06E+05	1.61E+05	2.52E+05	6.53E+05	2.89E+05	
12	2.12E+07	4.22E+07	2.38E+07	1.95E+07	2.35E+07	4.45E+07	1.73E+07	4.78E+07	
	6.51E+05	2.75E+06	7.29E+05	3.50E+06	2.18E+06	4.36E+06	3.40E+06	3.75E+06	
24	2.94E+08	3.53E+08	3.36E+08	3.02E+08	2.97E+08	3.68E+08	3.05E+08	3.52E+08	
	8.32E+06	5.77E+06	9.71E+06	4.19E+07	4.04E+07	1.44E+07	5.00E+06	1.26E+07	
48	4.10E+08	3.67E+08	3.80E+08	2.98E+08	3.21E+08	3.72E+08	3.18E+07	2.50E+08	
	2.18E+07	2.08E+07	1.50E+07	2.02E+07	1.56E+07	3.21E+07	3.25E+06	1.34E+07	
120	3.18E+08	1.45E+08	2.26E+08	1.73E+08	1.76E+08	1.39E+08	5.25E+07	4.57E+07	
	4.07E+07	7.86E+06	1.05E+07	1.89E+06	1.10E+07	1.50E+06	8.32E+06	3.79E+06	
<hr/>									
15°C	<i>P. kluyveri</i> (mono-culture)	<i>P. kluyveri</i> (PS)	<i>P. kluyveri</i> (PM)	<i>P. kluyveri</i> (TP)	<i>P. kluyveri</i> (TPM)	<i>P. kluyveri</i> (MPS)	<i>P. kluyveri</i> (TPS)	<i>P. kluyveri</i> (TPMS)	
0	5.18E+05	9.67E+05	9.90E+05	1.33E+06	1.37E+06	1.34E+06	8.33E+05	1.47E+06	
	6.29E+04	2.02E+04	1.07E+05	7.64E+04	2.02E+05	8.02E+04	2.52E+05	1.61E+05	
12	9.88E+05	4.63E+06	3.78E+06	4.05E+06	4.67E+06	3.97E+06	6.42E+06	1.12E+07	
	6.51E+04	6.79E+05	7.64E+04	1.08E+06	4.54E+05	5.01E+05	7.75E+05	7.64E+05	
24	1.82E+07	2.98E+07	3.03E+07	3.77E+07	3.00E+07	7.18E+07	3.68E+07	4.12E+07	
	1.97E+06	1.04E+06	3.01E+06	5.11E+06	8.66E+05	7.64E+06	4.04E+06	4.65E+06	

48	1.77E+08	1.05E+08	8.30E+07	4.60E+07	5.05E+07	1.32E+08	4.53E+07	4.43E+07
	1.24E+07	1.61E+06	3.61E+06	8.32E+06	3.50E+06	1.05E+07	4.80E+06	5.77E+05
120	1.78E+08	8.77E+07	5.75E+07	5.08E+07	4.93E+07	1.04E+08	3.75E+07	4.50E+07
	1.17E+07	7.65E+06	5.07E+06	2.36E+06	5.62E+06	8.25E+06	1.00E+06	1.32E+07
25°C	<i>P. kluyveri</i> (mono-culture)	<i>P. kluyveri</i> (PS)	<i>P. kluyveri</i> (PM)	<i>P. kluyveri</i> (TP)	<i>P. kluyveri</i> (TPM)	<i>P. kluyveri</i> (MPS)	<i>P. kluyveri</i> (TPS)	<i>P. kluyveri</i> (TPMS)
0	5.67E+05	9.83E+05	9.43E+05	1.33E+06	1.07E+06	1.38E+06	8.50E+05	1.03E+06
	4.54E+04	1.76E+04	1.00E+05	1.61E+05	3.25E+05	1.61E+05	1.32E+05	5.77E+04
12	1.75E+07	4.37E+07	4.92E+07	2.80E+07	2.35E+07	4.70E+07	2.65E+07	2.58E+07
	1.05E+06	4.86E+06	7.64E+05	1.80E+06	2.65E+06	2.00E+06	4.82E+06	5.77E+05
24	1.89E+08	1.88E+08	2.19E+08	5.67E+07	4.67E+07	1.50E+08	3.50E+07	6.67E+07
	1.13E+07	8.96E+06	8.50E+06	1.26E+07	1.04E+07	4.93E+06	1.32E+07	1.89E+07
48	2.08E+08	1.89E+08	2.46E+08	5.33E+07	3.67E+07	1.39E+08	2.00E+06	3.00E+07
	1.38E+07	5.03E+06	3.25E+06	1.53E+07	3.75E+06	4.27E+06	5.00E+05	6.95E+06
120	2.82E+08	7.73E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
	5.77E+06	3.18E+06	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00

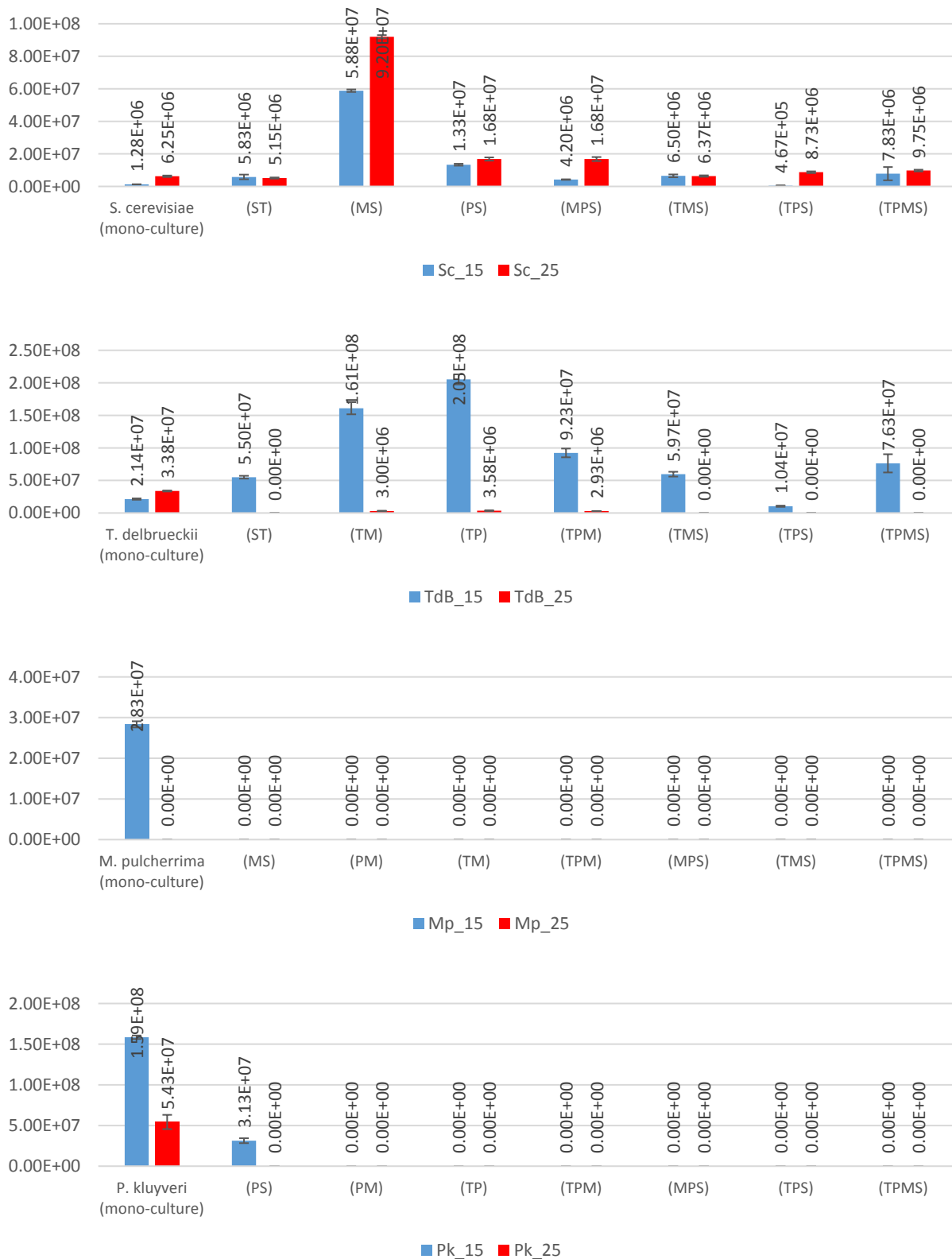


Figure 2 End population count per yeast for each treatment (x-axis), at both temperatures (15°C in blue and 25°C in red)

Note: x-axis Fermentation combinations; y-axis display colony count in CFU.mL⁻¹

Table 2 The amount of days it took each fermentation to cease at both temperatures

Yeast combinations	Days until fermentation ceased 15C	Days until fermentation ceased 25C
TdB	20	20
Mp	20	15
Pk	19	15
Sc	17	14
TM	19	19
PM	24	18
TP	15	15
ST	15	12
MS	19	13
PS	22	13
TPM	15	15
TMS	15	12
MPS	12	11
TPS	12	11
TPMS	12	11

Chapter 4

Research results

Evaluation of population dynamics and the effect of a consortium of yeast on
aroma production in real grape juice

Chapter 4 - Evaluation population dynamics and the effect of a consortium of yeast on aroma production in real grape must

4.1. Introduction

For the second part of this project, we evaluated the consortium of all four yeasts in real grape must. As controls, fermentations were inoculated with *S. cerevisiae* or left to ferment spontaneously. Real grape juice is significantly different from synthetic grape juice. Differences include nutrient composition, the presence of macromolecules such as protein and complex sugars or phenolic polymers, the presence of indigenous microorganisms, (Ciani *et al.*, 2010) and the difference in scale, which might in particular impact on oxygen availability (Brandam *et al.*, 2013; Shekhawat *et al.*, 2017; Englezos *et al.*, 2018).

Grape must is a complex chemical medium that has other compounds in the matrix which cannot be introduced into synthetic grape must (example, thiols) and it is believed that these compounds could influence the performance of yeasts and ultimately their metabolite production.

The purpose of the experiment was to evaluate to what degree the patterns observed in the synthetic grape juice would be apparent in this very different environment.

4.2. Material and Methods

4.2.2. Wine making practice and inoculation strategy

Chenin blanc grapes were used for this project. The grapes were harvested by hand at Nietvoorbij Research Institute, Stellenbosch and transported to the cellar using baskets. The grapes (1.9 tons) arrived at the cellar and were crushed and destemmed, where after they was pressed using a hydraulic basket-press. Juice recovery with this system was 50%. In total 830 Litres of juice was pumped into a master tank and the juice was sulphured to 15ppm. Settling enzyme, Rapidase Clear (Oenobrand, France), was added to the juice at 1 mL.hL⁻¹ to ensure settling overnight. The following day the clear juice was racked off the lees into another tank, sparged with CO₂, to minimize oxidation. The tank was then homogenised buy using a tank mixer and 80 litres of juice was pumped into nine 90 litre tanks. Three tanks where used per treatment (Fig. 4.1.). Three tanks each were inoculated with the combination of EC1118 (*S. cerevisiae* at 0.05 g.L⁻¹), Biodiva (*T. delbrueckii* 0.25 g.L⁻¹) and Flavia (*M. pulcherrima* 0.25 g.L⁻¹) (Lallemand, France) and Frootzen (*P. kluyveri*, because it is a frozen liquid it was diluted to an OD_{600nm} of 0.17 and inoculated) (Chr Hansen) (Treatment 1). The second three (Treatment 2) was inoculated with EC1118 (Lallemand, France) at a concentration of 0.05 g.L⁻¹ and the remaining three were left to ferment spontaneously (Treatment 3).

After inoculation a 50 mL sample was taken from each tank for culture plating on WL agar plates to determine inoculation dosages and the remainder of the 50 mL was stored for later analysis. For every day after inoculation the balling and temperature was measured using a density meter (Anton Paar, Portable Density meter: DMA 35,). A 50 mL sample was also taken for five days for cell counts (used 1 mL from the 50 mL) by plating and the remaining sample was frozen at -20°C for later analysis. After a 120 hour, the balling and temperature continued to be monitored, but the 50 mL sample (for plating and storage) was only taken every second day. The sampling strategy is shown in Fig. 4.1. For logistical reasons only two tanks per treatment could be sampled every single day. One tank per treatment was therefore sampled daily, whereas the other two tanks were sampled every alternative day.

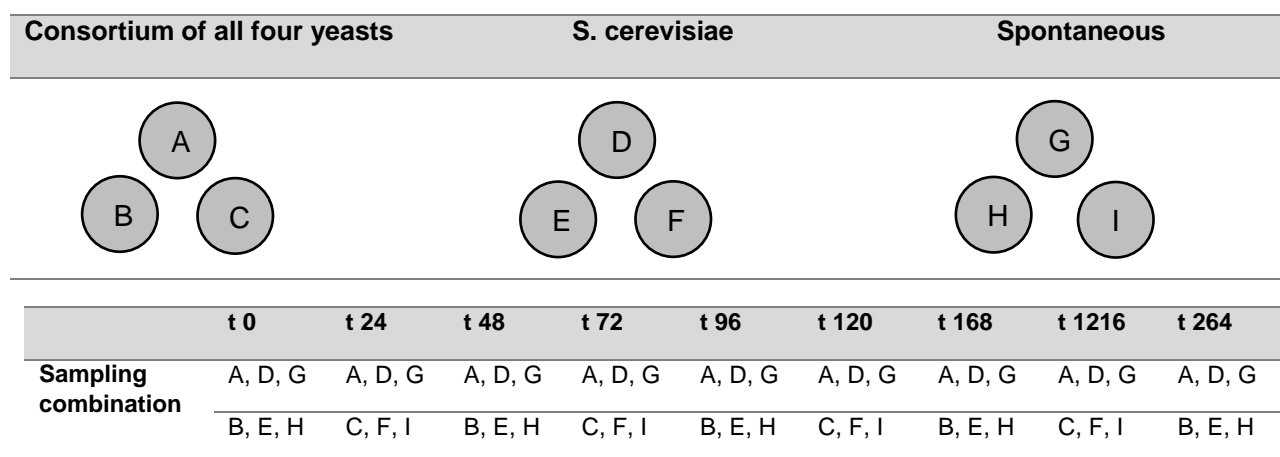


Figure 4.1 (above) Layout of tanks with inoculation strategy. (below) Sampling procedure used.

After the fermentations where complete, the wine from each individual tank, was racked into two 18 L canisters (Example – tank A was racked to two separate canisters marked A1 and A2) and the wine sulphured up with 50ppm SO₂ (thus 18 canisters in total). The wine then underwent three weeks cold stabilization at 0°C. After cold stabilization a sample was taken to determine to SO₂ content of the wine and SO₂ adjustments were made prior to bottling. The wine from each canister was filtered through a mat filter with diatomaceous earth. The wines were then left in a 15°C room for two months before using it for sensory as to ensure the bottle-shock has passed.

4.2.3. Monitoring population dynamics and fermentation

During the fermentation of the wines, cell viability was determined by plating out 0.1mL aliquots at every sampling point on Wallerstein Laboratory Nutrient (WLN) agar (BioLab, Merck, South Africa) containing biphenyl and chloramphenicol. A sample of the initial grape must (from the master tank) was plated using appropriate dilutions. From the plates, 21 colonies with different morphologies were picked and plated on WLN agar plates and it was left to incubate for five days at 30°C. After five days, a single colony from each plate was inoculated into a test tube containing 5 mL YPD broth. After 24 hours a 1 mL sample was taken and placed into 1,5 mL microcentrifuge tubes. The

microcentrifuge tubes were centrifuged for 10 minutes at 13000rpm after which the supernatant was discarded, and the pellets stored at -20°C until later use for PCR and ARISA.

4.2.4. DNA extractions

DNA extractions were performed on all the samples, taken throughout the fermentation cycle was stored at -20°C until needed. For extraction, the pellets were resuspended in 100µL 1x TE buffer (pH 7.6) and the sample was centrifuged for 3 min at 13000rpm after which the supernatant was discarded. Thereafter, 200 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 200µL glass beads and 80 µL 1x TE buffer (pH 7.6). In a fumehood, 240 µL PCI (phenol: chloroform: isoamyl alcohol; in the ratio of 25:24:1) was added to each sample. The mixture was vortexed for 4 min at medium speed after which it was centrifuged for 5 min at 10000 rpm. The top layer from each sample was aspirated into a microcentrifuge tube containing 100 µL chloroform. The samples were vortex for 4 min at medium speed where after it was centrifuged for 10 min at 5000rpm. The top layer from was aspirated into a new microcentrifuge tube containing 20µL NaOAc and 400µL 100% ethanol. The samples were left to incubate at room temperature for 20 min, where after they were centrifuged for 10 min at 10000rpm and the supernatant was discarded. after discarding the supernatant, 400µL 70% ethanol was added and centrifuged for 3 min at 8000rpm and the supernatant was discarded. The samples were dried in a Savant SpeedVac® DNA110 (Thermo Scientific). After the pellets dried, 35 µL 1x TE and 7 µL RNase was added to each sample and was left to incubate at room temperature for 8 h after which the samples were frozen at -20°C.

4.2.5. Species identification (PCR and ARISA)

To identify the species, present in the wine we sequenced the 5.8S-ITS rDNA region. For the 21 yeast isolations done from master wine tank WL agar plate, PCR was performed using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers described by Wilson *et al.* (2016). The final PCR product was 25µL (table 4.1). The mixture was subject to initial denaturation of 5 min at 94°C; thereafter, 40 cycles consisting of denaturation of 30 s at 94°C, annealing of 30 s at 51°C, extension of 45 s at 72°C and a final extension of 7 min at 72°C. For ARISA, the same method mentioned above was used, but PfaM ITS1 primer was used instead of ITS1. Sequence results were compared to the NCBI nucleotide database using BLAST algorithm and identification were confirmed when sequence coverage and maximum percentage of identification were higher the 98%, the norm is 98% (query cover > 98%, Max ID % > 98%).

Table 4.1 PCR master mixture composition

Reagent	[Stock]	[Required]	Volume / reaction
dNTP	2.5 mM	400 µM	3.2 µL
ITS1*	2.5 µM	0.25 µM	2 µL
ITS4	2.5 µM	0.25 µM	2 µL
Taq buffer	10x	1x	2 µL

MgCl	25mM	1mM	0.8 µL
xTaq	5u/µL	1u	0.2 µL
d.dH ₂ O			13.8 µL
DNA sample			1 µL
Total			24 µL

*Pfam ITS1 was used for ARISA of pellets from wine.

4.3. Results

4.3.1. Isolation and identification of yeasts

From the main tank containing the grape must, a sample was plated out on WL agar and 31 colonies was observed. Of the 31 colonies a total of 21 yeast colonies showed different colony morphologies and only these were isolated and streaked out on WL agar plates for identification. Of these 21 colonies seven different species were identified according to their ITS-5.8S sequence analysis (table 4.2). The colonies per species (after identification) were then counted on the plate containing the 31 colonies and yeast percentage dominance were calculated (Table 4.3) NS yeasts were dominant in the grape must.

Table 4.2 Yeast species and their accession number in GeneBank. Size in base pair (bp)

Species	5.8S-ITS region		
	Accession number	Base-pair	% Identity
<i>Hanseniaspora guilliermondii</i>	KY103524	818	99
<i>Hanseniaspora vineae</i>	KY103580	735	99
<i>Lachancea thermotolerans</i>	KY103994	673	99
<i>Starmerella bacillaris</i>	KY076623	459	100
<i>Hanseniaspora. uvarum</i>	KY103569	746	99
<i>Pichia kudriavzevii</i>	KY104575	532	99
<i>Zygoascus meyeriae</i>	KY106012	575	99

Of these seven different species, *L. thermotolerans* was the most dominant followed by *H. vineae* and *H. uvarum*. The remaining species was present at much lower concentrations. Table 4.3 shows the percentile presence of each of the seven yeasts in the must before fermentation.

Table 4.3 Percentage yeast distribution in Chenin blanc grape must according to the 31 colonies observed on WL agar plate.

Species	Number of colonies (from the 31 colonies)	Percentage abundance (%) = number of colonies / 31 * 100
<i>Hanseniaspora guilliermondii</i>	1	3.23
<i>Hanseniaspora vineae</i>	6	19.35
<i>Lachancea thermotolerans</i>	15	48.38
<i>Starmerella bacillaris</i>	2	6.45
<i>Hanseniaspora. uvarum</i>	5	16.13
<i>Pichia kudriavzevii</i>	1	3.23
<i>Zygoascus meyeriae</i>	1	3.23

4.3.2. Fermentation performance

4.3.2.1. Fermentation rate

Figure 4.2 show the averaged results for the three treatments with standard deviations. The barrels, inoculated with all four yeasts, started fermentation quicker (48 hours) than the other two treatments. This is to be expected as the initial yeast biomass was greater than that of the other two treatments. The treatment of *S. cerevisiae* lagged an extra two days (96 hours) before fermentation started, but at 144 hours the *S. cerevisiae* and consortium treatment fermented at the same rate until the end of fermentation. The onset of fermentation for the spontaneous fermentation was apparent from 120 hours onwards, but the fermentation lasted seven days longer than that of the inoculated treatments due to slower fermentation kinetics and a longer latency period. The spontaneous fermentation was also stopped at a higher balling than the other treatments as the fermentation started to produce H₂S on the noise which was indicative of a sluggish fermentation.

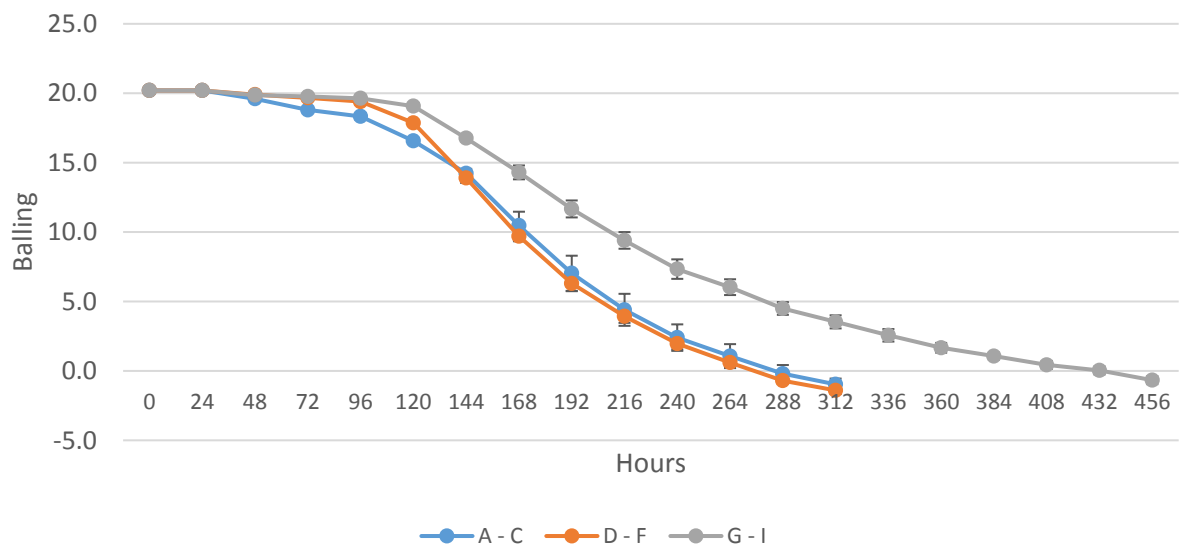


Figure 4.2 Average fermentation rates, according to balling measurements for each treatment with standard deviation from the three biological repeats.

4.3.2.2. Population monitor

Figures 4.3 and 4.4 show the percentage yeast dominance during fermentations. For full detailed values with standard deviations, please consult Table 1,2 and 3 in Appendix B. In all cases, the legend marked “rest” refers to the indigenous yeasts that were present but was little to count individually, and for this reason was pooled together. We combined the plate counts for B+C; E+F; G+I as they followed the same population dynamics. From Figure 4.3 we can see both the graphs for A and B+C follow the same trend with the same yeast species participating in the fermentation. The inoculated *T. delbrueckii* dominated fermentation until 168 hours when *S. cerevisiae* became the dominant specie until the end of fermentation. however, *T. delbrueckii* also persisted until the end. Both *S. cerevisiae* and *T. delbrueckii* followed the same trend in the fermentations A – C. *M.*

pulcherrima was detected in fermentations A, B and C albeit at low percentages and *H. uvarum*, an indigenous species in this must, was present until 96 hours. *P. kluyveri* followed similar trends in the fermentations, but was present for longer in the fermentation B+C. Figure 4.4 (a) and (b) show the abundance of yeast species for D and combined E and F respectively. *H. uvarum* and *S. bacillaris* dominated fermentation for the first 72 hours after which *S. cerevisiae* completed fermentation. Treatment C follows a similar trend to treatment B. During the early stages of fermentation (Fig 4.5 (a) and (b)), *S. bacillaris* and *H. uvarum* dominated fermentation for the first 120 hours after which *S. cerevisiae* and *S. bacillaris* remained the dominant species to end fermentation. The spontaneous fermentation also showed the most yeast species present at the end of fermentation with *S. cerevisiae*, *S. bacillaris* and *H. uvarum* at CFU.mL⁻¹ between 10⁵ and 10⁷.

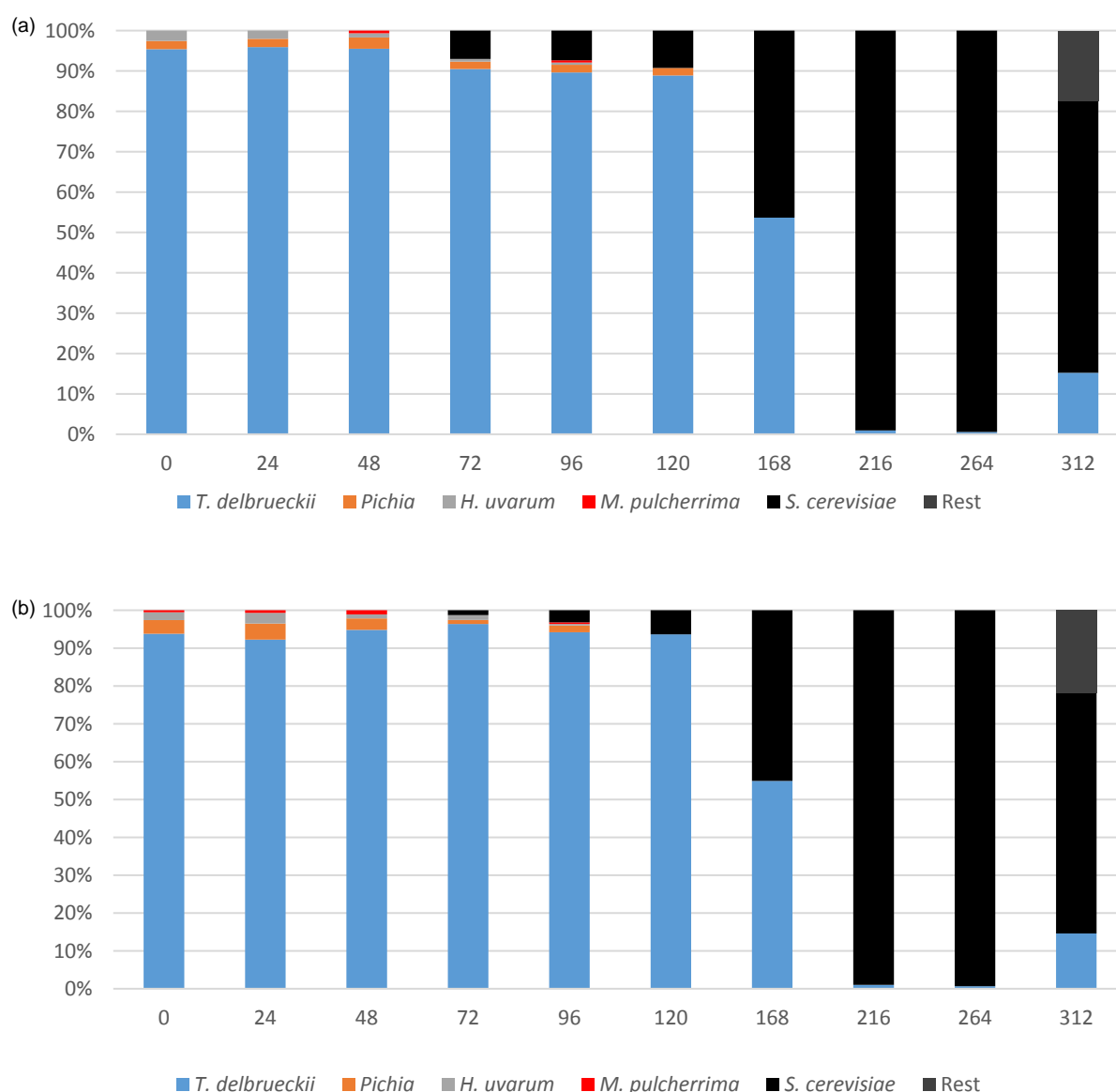


Figure 4.3 Percentage cell dominance during fermentation for (a) tank A – treatment 1 (b) combined percentage cell dominance for tank B (0, 48h, 96h, 168h and 216h) and C (24h, 72h, 120h and 192h).

Note: x-axis display hours and y-axis percentile yeast dominance.

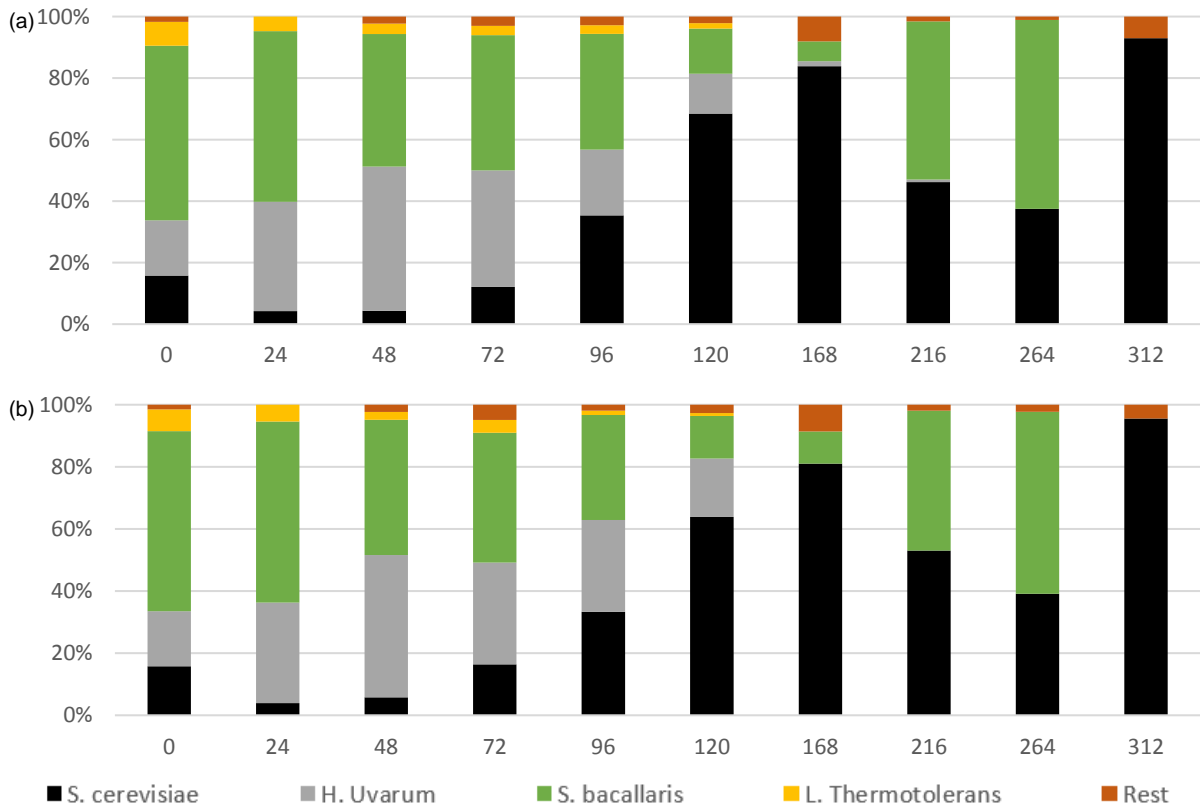


Figure 4.4 Percentage yeast dominance during fermentation for (a) tank D and (b) combined tank E (0, 48h, 96h, 168h and 216h) and F (24h, 72h, 120h and 192h) – treatment 2

Note: x-axis display hours and y-axis percentile yeast dominance.

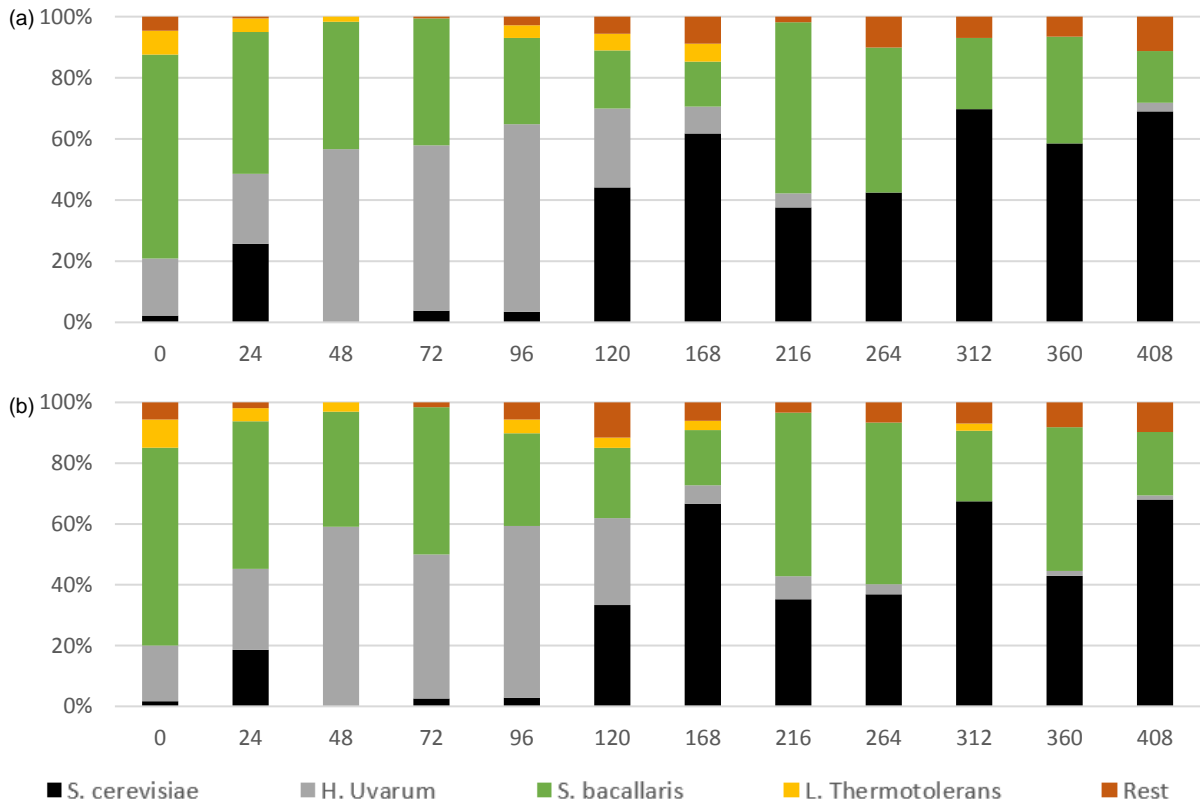


Figure 4.5 Percentage yeast dominance during fermentation for (a) tank G and (b) combined tank H (0, 48h, 96h, 168h and 216h) and I (24h, 72h, 120h and 192h) – treatment 3.

Note: x-axis display hours and y-axis percentile yeast dominance.

4.3.3. Influence of mixed starter culture and spontaneous fermentation on ester formation

Table 4.4 presents the GC-FID results for wines that were used for sensory evaluation. A1 and A2 show the results for wine from treatment 1 (consortium of all 4 yeasts) but was bottled from two different canisters. The same counts for the rest were treatment 1 was A – C, treatment 2 (*S. cerevisiae*) was D – F and treatment 3 (spontaneous) G – I. From table 4.6 we see the ethyl acetate production for the wines was more for treatment 3, second highest for treatment 2 and lowest for treatment 1. For both treatment 1 and 2 the amount of ethyl caprylate was similar, but was noticeably lower in treatment 3. The isoamyl acetate production for treatment 3 wine was noticeably lower with an average of 3.93 mg.L⁻¹ and for treatment 1 and 2, 6.87 mg.L⁻¹ and 5.86 mg.L⁻¹ respectively. Despite the lower production of some esters, the total amount of esters produced by treatment 3 are more than the other two fermentations and this is thus driven by the higher amount of ethyl acetate production.

4.3.4. Influence of mixed starter culture and spontaneous fermentation on higher alcohol formation

We see from Figure 4.6 (a) the amount of 3-ethoxy-1-propanol produced in treatment 3 was more than seven times lower than the *S. cerevisiae* fermentation and almost seven times less than in the consortium. The hexanol (Fig. 4.6 (b)) production for treatment 3 was more than 15 times greater than that produced by treatment 1 and 2.

We do see two bottles from treatment 2 also showing higher levels of hexanol, but the other four bottles showed the same as treatment 1. The propanol production (Fig. 4.6 (c)) in the spontaneous fermented wines were almost half of that from the consortium fermented wines.

4.3.5. Influence of mixed starter culture and spontaneous fermentation on volatile fatty acid formation

The consortium wine produced the least amount of acetic acid with the spontaneously fermented wine producing the highest amount, roughly three and a half times more. For butyric and decanoic acid production however, the spontaneously fermented wine produced less than the other fermentations, although not by much. The difference observed in the concentrations for valeric acid productions were little, but still showed; treatment 1 with levels between 0.30 – 0.35 mg.L⁻¹, treatment 2 producing concentrations between 0.35 – 0.40 mg.L⁻¹ and treatment 3 with the lowest levels, between 0.25 – 0.30 mg.L⁻¹. The production levels of the remaining volatile fatty acids were similar between fermentations, the spontaneously fermented wine produced the highest total amount of volatile fatty acids and the consortium wines the least, more than three times less.

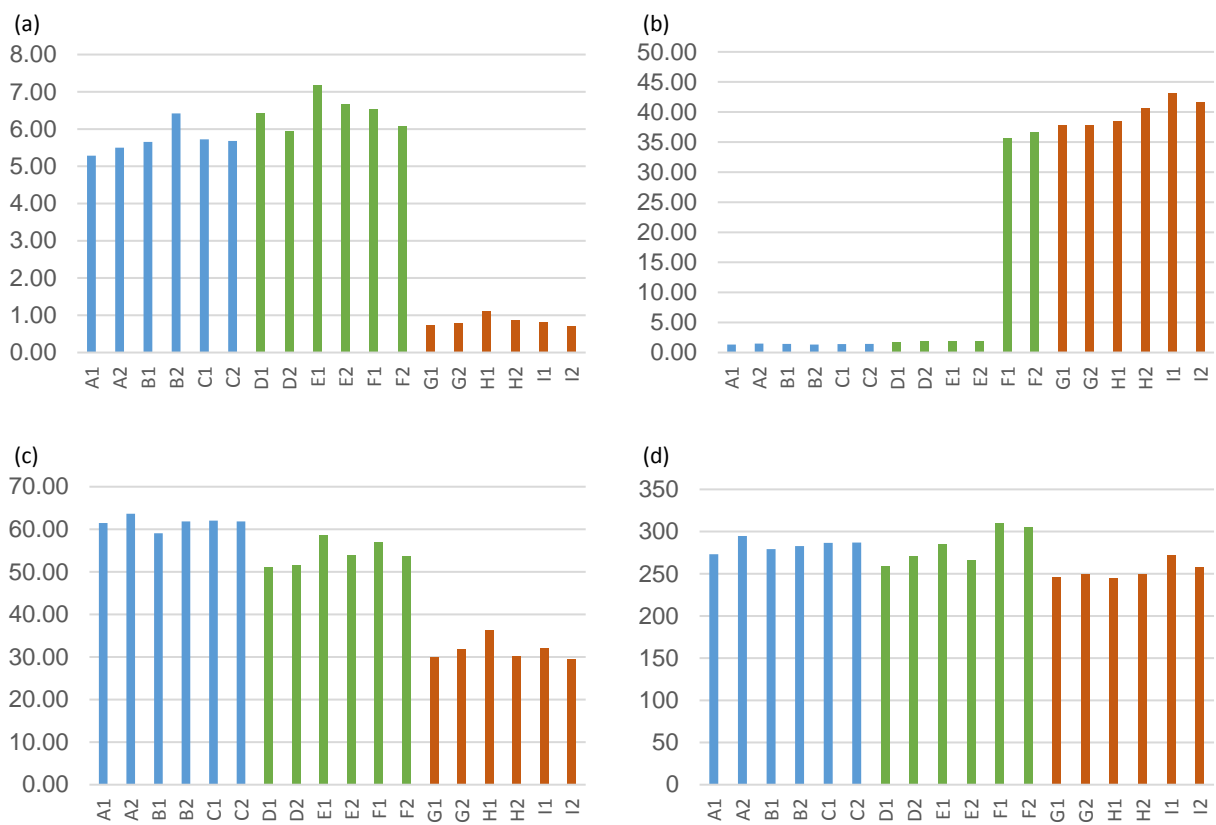


Figure 4.6 Show that GC-FID results for treatment 1 (blue), 2 (green) and 3 (orange). (a) Represents the levels of 3-ethoxy-1-propanol, (b) hexanol, (c) propanol and (d) the total amount of higher alcohols produced.

Note: All y-axis values in mg.L⁻¹

**Table 4.4 GC-FID results for wines used in sensory evaluation. The table only presents the data that fell within machine limit calibration. All values displayed in mg.L⁻¹
Note: SD are shown below each value**

	A1	A2	B1	B2	C1	C2	Avg.	D1	D2	E1	E2	F1	F2	Avg.	G1	G2
Higher alcohols																
2-Phenyl Ethanol	14.72 0.00	16.09 0.05	15.51 0.08	13.95 0.08	15.73 0.01	15.51 0.01	15.25 0.04	14.65 0.03	15.96 0.07	15.60 0.01	14.61 0.08	15.11 0.06	14.99 0.04	15.15 0.05	14.30 0.07	13.03 0.08
3-ethoxy-1-propanol	5.28 0.01	5.50 0.00	5.65 0.01	6.42 0.02	5.72 0.02	5.68 0.02	5.71 0.01	6.44 0.05	5.94 0.01	7.18 0.01	6.68 0.11	6.54 0.04	6.07 0.01	6.48 0.04	0.75 0.01	0.79 0.01
Hexanol	1.33 0.00	1.49 0.00	1.44 0.01	1.33 0.01	1.42 0.00	1.43 0.00	1.41 0.00	1.83 0.00	1.90 0.01	1.97 0.00	1.87 0.00	35.63 0.19	36.74 0.03	13.32 0.04	37.78 0.08	37.88 0.03
Butanol	1.47 0.00	1.53 0.00	1.44 0.00	1.53 0.00	1.52 0.01	1.50 0.01	1.50 0.00	1.63 0.01	1.61 0.00	1.80 0.05	1.67 0.02	1.75 0.00	1.69 0.01	1.69 0.01	1.38 0.01	1.41 0.00
Isoamyl alcohol	161.36 0.25	177.54 0.01	169.69 0.67	170.46 0.06	171.86 0.02	172.88 0.75	170.63 0.29	161.58 0.08	168.60 0.14	175.32 1.07	164.94 0.55	169.78 0.52	168.64 0.02	168.14 0.40	139.85 0.09	142.66 0.24
Isobutanol	27.34 0.05	28.81 0.01	26.21 0.08	26.98 0.05	28.03 0.26	27.95 0.20	27.55 0.11	21.33 0.00	24.45 0.26	24.03 0.31	22.30 0.08	24.08 0.05	23.17 0.05	23.23 0.13	21.28 0.06	21.87 0.11
Propanol	61.46 0.07	63.66 0.09	59.05 0.39	61.85 0.42	62.03 1.43	61.84 1.10	61.65 0.58	51.19 0.04	51.57 0.90	58.72 1.03	53.87 0.22	57.06 0.13	53.64 0.10	54.34 0.40	29.99 0.23	31.73 0.03
Total Higher Alcohols	272.97	294.62	278.99	282.53	286.32	286.79	283.70	258.64	270.05	284.62	265.94	309.95	304.93	282.36	245.32	249.37
Esters																
Ethyl Acetate	95.92 1.29	98.63 1.07	86.19 1.34	73.82 2.17	94.93 1.27	97.56 0.36	91.17 1.25	103.23 1.30	111.65 0.76	135.90 1.94	126.77 0.27	113.88 3.02	118.95 2.08	118.40 1.56	121.35 0.50	122.61 2.37
Ethyl Caprylate	0.82 0.00	0.84 0.00	0.91 0.01	0.67 0.00	0.77 0.00	0.64 0.00	0.78 0.00	0.81 0.00	0.71 0.00	0.92 0.00	0.94 0.00	0.48 0.00	0.49 0.00	0.72 0.00	0.46 0.00	0.44 0.00
Hexyl Acetate	0.58 0.00	0.62 0.00	0.60 0.00	0.59 0.00	0.59 0.00	0.60 0.00	0.60 0.00	0.61 0.00	0.60 0.00	0.69 0.00	0.66 0.00	0.59 0.00	0.63 0.00	0.63 0.00	0.52 0.00	0.51 0.00
Isoamyl Acetate	6.40 0.02	7.37 0.01	6.79 0.03	6.79 0.02	6.84 0.01	7.05 0.02	6.87 0.02	5.25 0.00	5.49 0.00	6.20 0.04	5.88 0.00	5.80 0.01	6.52 0.01	5.86 0.01	3.94 0.00	4.00 0.02
Total Esters	103.71	107.45	94.49	81.87	103.13	105.85	99.42	109.90	118.45	143.71	134.26	120.75	126.60	125.61	126.27	127.57

Volatile Fatty acids																
Acetic Acid	111.98 12.53	106.34 1.43	89.15 0.13	87.94 0.10	95.51 0.47	95.32 0.54	97.71 2.53	209.47 1.28	235.80 0.27	213.54 0.50	197.72 0.99	243.60 6.24	222.78 0.83	220.49 1.69	325.14 2.45	325.99 0.61
Butyric Acid	2.14 0.02	2.30 0.02	2.22 0.00	2.14 0.01	2.27 0.00	2.25 0.01	2.22 0.01	2.24 0.00	2.16 0.01	2.38 0.01	2.25 0.01	2.25 0.00	2.17 0.00	2.24 0.01	2.00 0.01	2.01 0.00
Decanoic Acid	2.70 0.99	2.18 0.04	1.94 0.00	1.90 0.02	2.07 0.01	2.17 0.01	2.16 0.18	2.17 0.00	1.19 0.01	2.35 0.01	2.21 0.00	2.48 0.68	2.16 0.01	2.09 0.12	1.82 0.01	1.69 0.00
Hexanoic Acid	4.31 0.03	4.97 0.02	4.96 0.02	4.18 0.03	4.75 0.01	4.78 0.01	4.66 0.02	4.50 0.01	4.07 0.02	4.72 0.00	4.48 0.02	4.33 0.02	4.49 0.01	4.43 0.01	3.96 0.02	3.87 0.02
Octanoic Acid	6.66 0.99	7.82 0.04	7.76 0.00	6.40 0.02	7.38 0.01	7.58 0.01	7.27 0.18	8.28 0.00	6.43 0.01	8.59 0.01	8.06 0.00	7.69 0.68	8.24 0.01	7.88 0.12	6.77 0.01	6.53 0.00
Total Volatile Fatty Acids	127.79	123.60	106.02	102.57	111.99	112.09	114.01	226.66	249.65	231.58	214.71	260.35	239.84	237.13	339.70	340.08

4.3.6. Wine sensory and chemical analysis

For the sensorial analysis, Napping, was used to group wines according to their similarities. The wines that were used for tasting was analysed by using Grapescan 2000 to make sure the duplicate bottles used, showed similar results based on parameters in Table 4, Appendix B.

A trained tasting panel of 15 people was used to do two rounds of tasting. Each round consisted of tasters grouping wine according to aroma and writing down aroma attributes with intensity, on a A2 size paper. The panel could use their own descriptors, but a page with descriptors was also provided which is standard from the sensory laboratory. They then grouped the wine together according to taste and wrote down a minimum of three descriptors with intensity for each (on the back of the same A2 size paper). Each wine was numbered, and the taster marked on the page with a “x” where they placed each wine. After the first round was complete, tasters took a break and repeated the process. After the tasting was done each page was measured for the x-axis and the y-axis. The bottom left corner of the page was used as the (0, 0) start point. After each point was measured for all the sheets (aroma and taste) the values were entered into an Excel spread sheet.

4.3.6.1. *Correspondence analysis – Sensory*

From the sensory data gathered no definitive conclusion could be made regarding aroma and taste attributes for each treatment. The correspondence analysis showed no clear groupings per treatment and attributes were scattered (Figure 1, Appendix B). With no clear results obtainable from the sensory data we rather looked at the chemical analysis from the GC-FID.

4.3.7. Comparing GC-FID results for real wine and synthetic wine

The GC-FID results from both synthetic wine and real wine was combined, using only the compounds found in both (Table 4.5). This resulted in less compounds to compare the two different environments, but was still useful. With almost all compounds in Table 4.3 no correlation was found between results obtained from the synthetic wine compared to real wine fermentations at the same temperature of 15°C. With the exception to butanol and propanol, the amount 2-phenylethanol, 3-etoxy-1-propanol, isoamyl alcohol and butanol was substantially lower in wine to the synthetic wine. Ethyl acetate production in the synthetic grape juice was more than two times, that of the amount in the wine. The ethyl caprylate was higher in the real wine, but the Isoamyl acetate levels were similar. The most profound difference between the two, were the acetic acid levels. For the real wine the acetic acid production was six times less than for the synthetic wine. For butyric, decanoic and octanoic acid, the real wine produced higher levels with the octanoic acid levels almost seven times higher in real wine.

Table 4.5 GC-FID results for the real wine (Wine) and the synthetic wine at 15°C (TPMS_15) with standard deviation (SD; n=2). All values displayed in mg.L⁻¹

	Wine	SD for Wine	TPMS_15	SD for TPMS_15
Higher alcohols				
2-Phenyl Ethanol	15.25	0.78	55.33	3.89
3-ethoxy-1-propanol	5.71	0.38	15.63	0.87
Butanol	1.50	0.04	0.55	0.03
Isoamyl alcohol	170.63	5.31	199.99	11.90
Isobutanol	27.55	0.91	64.30	5.74
Propanol	61.65	1.49	35.16	2.31
Esters				
Ethyl Acetate	91.17	9.58	233.95	20.40
Ethyl Caprylate	0.78	0.10	0.17	0.01
Isoamyl Acetate	6.87	0.32	8.10	0.60
Volatile Fatty acids				
Acetic Acid	97.71	9.56	596.63	51.47
Butyric Acid	2.22	0.07	1.26	0.07
Decanoic Acid	2.16	0.29	0.78	0.01
Octanoic Acid	7.27	0.59	1.11	0.02

4.4. Discussion

The natural yeasts isolated from the grape must sample showed natural species composition similar to those that have been reported before, with the presence of genera such as *Lachancea*, *Starmerella* and *Hanseniaspora* (Jolly *et al.*, 2014; Bagheri *et al.*, 2015). However, the number was low, and the inoculated yeasts dominated fermentation from the start with very little natural species being detected on plate counts. However, the yeasts were able to rapidly grow and ferment in the spontaneously fermented. The fermentation was dominated by *S. cerevisiae*, *H. uvarum* and *S. bacillaris*.

A faster fermentation rate was observed for inoculated wines when compared to spontaneous fermentation. The onset of fermentation for *S. cerevisiae* (1 g.hL⁻¹ inoculation, treatment B) was delayed, but once *S. cerevisiae* reached 1,00E+06 at 72 hours, fermentation proceeded faster, and fermentation rate proceeded faster than that of treatment A. We also observed a suppression by *S. cerevisiae* in treatment B, where *S. bacillaris*, *H. uvarum* and *L. thermotolerans* performed similar in treatment B compared to treatment C, but in treatment C we see a general higher concentration of other NS. The suppression of certain NS yeasts was also observed by Bagheri *et al.*, (2017). The

overall presence of *S. bacillaris* and *H. uvarum* throughout the spontaneous fermentations is similar to the findings of, Englezos *et al.*, (2015) and Wang *et al.* (2016).

This study proves how wine profiles are influenced due to the use of yeasts and the effect certain yeasts have on the formation of esters, higher alcohols and volatile fatty acids. We proved that in the presence of *L. thermotolerans* (native yeasts) in treatments B and C, higher levels of ethyl acetate were observed. The longer persistence of *L. thermotolerans* in treatment C, accompanied with slightly higher levels of ethyl acetate, proves the effect on ethyl acetate formation *L. thermotolerans* have on wines (Gobbi *et al.*, 2013). The overall concentration of esters in the spontaneously fermented wines were higher than that of the inoculated wines and this result is largely driven by the higher levels of ethyl acetate production in treatment C. Higher alcohol concentrations in treatment A was largely driven by the higher levels of propanol and isobutanol in the wine due to the presence of *T. delbrueckii*, *M. pulcherrima* and *S. cerevisiae*. Acetic acid formation had the biggest influence in the higher levels of volatile fatty acids in the wines. Treatment C had substantially higher amounts of volatile fatty acids compared to the other treatments. The higher levels of acetic acid produced in treatment C and B was due to the presence of *L. thermotolerans* (Gobbi *et al.*, 2013) (present longer in C than B) and the lower level of acetic acid in treatment was due to the presence of *T. delbrueckii*, which is considered a low acetic acid producer and was present throughout the fermentation of treatment A.

Comparing the results for the consortium fermentations in chapter 3 with the consortium fermentation (treatment A), it was clear that no real comparison could be drawn between the results obtained from real grape must compared to that obtained in the synthetic grape must. This could be due to the difference in media composition and due to the presence natural yeasts present in the real grape must compared to the sterile synthetic grape juice. The sensory data obtained through the rapid analysis method also showed no real definitive groupings of treatments. Although there were differences observed on aroma, though not well grouped within treatment, the taste resulted in most of the wines grouped together not showing much influence of the different treatments on the mouthfeel of the wine.

4.5. References

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4.6. Appendix B

Table 1 (A) Average population count per yeast in tank A, (B) Average population count of combined tank B and C.

±SD displayed below each value, for the three biological repeats

A	<i>T. delbrueckii</i> SD	<i>P. kluyveri</i> SD	<i>H. uvarum</i> SD	<i>M. pulcherrima</i> SD	<i>S. cerevisiae</i> SD
0	9.30E+06 1.41E+00	2.00E+05 1.41E+00	2.50E+05 3.54E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00
24	1.42E+07 6.36E+00	3.00E+05 1.41E+00	3.00E+05 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00
48	2.24E+07 1.13E+01	6.50E+05 2.12E+00	2.50E+05 2.12E+00	1.50E+05 7.07E-01	0.00E+00 0.00E+00
72	2.00E+07 1.41E+01	4.00E+05 0.00E+00	1.50E+05 7.07E-01	0.00E+00 0.00E+00	1.55E+06 7.07E-01
96	2.38E+07 1.41E+01	5.00E+05 0.00E+00	1.50E+05 7.07E-01	1.50E+05 2.12E+00	1.95E+06 7.07E-01
120	2.40E+07 0.00E+00	5.00E+05 7.07E-01	0.00E+00 0.00E+00	0.00E+00 0.00E+00	2.50E+06 7.07E-01
168	2.20E+07 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.90E+07 4.24E+00
216	1.75E+06 2.12E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.83E+08 7.78E+00
264	1.00E+06 1.41E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.77E+08 0.00E+00
312	1.11E+06 7.07E-01	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	4.90E+06 2.12E+00
B	<i>T. delbrueckii</i> SD	<i>P. kluyveri</i> SD	<i>H. uvarum</i> SD	<i>M. pulcherrima</i> SD	<i>S. cerevisiae</i> SD
0	9.05E+06 7.78E+00	3.50E+05 7.07E-01	2.00E+05 0.00E+00	5.00E+04 7.07E-01	0.00E+00 0.00E+00
24	1.31E+07 1.70E+01	6.00E+05 0.00E+00	4.00E+05 1.41E+00	1.00E+05 0.00E+00	0.00E+00 0.00E+00
48	2.20E+07 2.83E+00	7.00E+05 2.83E+00	2.50E+05 7.07E-01	2.50E+05 7.07E-01	0.00E+00 0.00E+00
72	2.25E+07 2.12E+01	2.50E+05 7.07E-01	3.00E+05 2.83E+00	0.00E+00 0.00E+00	3.00E+05 7.07E-01
96	2.35E+07 1.91E+01	4.50E+05 7.07E-01	1.00E+05 0.00E+00	1.00E+05 1.41E+00	8.00E+05 4.24E+00
120	2.20E+07 1.41E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.50E+06 7.07E-01
168	1.95E+07 3.54E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.60E+07 1.41E+00
216	1.85E+06 7.07E-01	0.00E+00 0.00E+00	0.00E+00 0.00E+00	0.00E+00 0.00E+00	1.74E+08 3.54E+00
264	1.15E+06	0.00E+00	0.00E+00	0.00E+00	1.65E+08

	4.95E+00	0.00E+00	0.00E+00	0.00E+00	1.56E+01
312	1.11E+06	0.00E+00	0.00E+00	0.00E+00	4.82E+06
	1.73E+00	0.00E+00	0.00E+00	0.00E+00	9.57E-01

Table 2 (D) Average population count per yeast in tank D, (E) Average population count of combined tank E and F

±SD displayed below each value, for the three biological repeats

D	<i>S. cerevisiae</i>	<i>H. uvarum</i>	<i>S. bacillaris</i>	<i>L. thermotolerans</i>	Rest
	SD	SD	SD	SD	SD
0	6.00E+04	6.80E+04	2.16E+05	2.95E+04	6.50E+03
	1.70E+01	7.07E+00	1.20E+01	6.36E+00	4.27E+00
24	4.50E+04	3.75E+05	5.85E+05	5.00E+04	0.00E+00
	4.95E+00	7.07E-01	4.95E+00	1.41E+00	0.00E+00
48	9.50E+04	1.03E+06	9.45E+05	7.50E+04	5.00E+04
	7.07E-01	1.41E+00	4.95E+00	2.12E+00	3.32E+00
72	8.00E+05	2.50E+06	2.90E+06	2.00E+05	2.00E+05
	4.24E+00	4.24E+00	5.66E+00	1.41E+00	1.15E+00
96	3.15E+06	1.90E+06	3.35E+06	2.50E+05	2.52E+05
	7.07E-01	1.41E+00	2.12E+00	7.07E-01	9.57E-01
120	1.17E+07	2.20E+06	2.50E+06	3.00E+05	3.67E+05
	2.12E+00	1.41E+00	1.41E+00	2.83E+00	7.62E+00
168	2.60E+07	5.00E+05	2.00E+06	0.00E+00	2.50E+06
	2.83E+00	7.07E-01	0.00E+00	0.00E+00	1.29E+00
216	2.92E+07	5.00E+05	3.25E+07	0.00E+00	1.00E+06
	2.83E+00	7.07E-01	7.78E+00	0.00E+00	1.00E+00
264	1.74E+07	0.00E+00	2.85E+07	0.00E+00	5.00E+05
	2.83E+00	0.00E+00	7.07E-01	0.00E+00	5.00E-01
312	1.04E+07	0.00E+00	0.00E+00	0.00E+00	7.91E+05
	2.83E+00	0.00E+00	0.00E+00	0.00E+00	1.89E+00
E	<i>S. cerevisiae</i>	<i>H. uvarum</i>	<i>S. bacillaris</i>	<i>L. thermotolerans</i>	Rest
	SD	SD	SD	SD	SD
0	5.75E+04	6.45E+04	2.11E+05	2.55E+04	5.50E+03
	9.19E+00	2.12E+00	1.41E+00	6.36E+00	3.40E+00
24	4.00E+04	3.30E+05	5.95E+05	5.50E+04	0.00E+00
	1.41E+00	2.83E+00	1.34E+01	2.12E+00	0.00E+00
48	1.25E+05	9.95E+05	9.45E+05	5.50E+04	5.00E+04
	7.07E-01	2.12E+00	4.95E+00	7.07E-01	2.89E+00
72	1.00E+06	2.00E+06	2.55E+06	2.50E+05	3.00E+05
	9.90E+00	4.24E+00	4.95E+00	7.07E-01	1.73E+00
96	3.55E+06	3.15E+06	3.60E+06	1.50E+05	2.03E+05
	7.07E-01	2.12E+00	2.83E+00	7.07E-01	5.00E-01
120	1.07E+07	3.15E+06	2.30E+06	1.50E+05	4.53E+05
	1.41E+00	4.95E+00	5.66E+00	7.07E-01	1.89E+00
168	2.35E+07	0.00E+00	3.00E+06	0.00E+00	2.50E+06
	3.54E+00	0.00E+00	0.00E+00	0.00E+00	8.16E-01

216	2.77E+07	0.00E+00	2.35E+07	0.00E+00	1.00E+06
	9.19E+00	0.00E+00	3.54E+00	0.00E+00	5.77E-01
264	1.74E+07	0.00E+00	2.60E+07	0.00E+00	1.00E+06
	7.78E+00	0.00E+00	1.41E+00	0.00E+00	1.00E+00
312	1.02E+07	0.00E+00	0.00E+00	0.00E+00	4.74E+05
	4.43E+00	0.00E+00	0.00E+00	0.00E+00	1.16E+00

Table 3 (G) Average population count per yeast in tank G, (H) average population count per yeast in combined tank H and I.

±SD displayed below each value, for the three biological repeats

G	<i>S. cerevisiae</i>	<i>H. uvarum</i>	<i>S. bacillaris</i>	<i>L. thermotolerans</i>	Rest
	SD	SD	SD	SD	SD
0	6.50E+03	5.50E+04	1.97E+05	2.30E+04	1.35E+04
	4.95E+00	2.12E+01	1.91E+01	2.83E+00	7.89E+00
24	2.30E+05	2.05E+05	4.15E+05	4.00E+04	5.00E+03
	5.66E+00	3.54E+00	2.12E+00	0.00E+00	5.00E-01
48	0.00E+00	1.70E+06	1.25E+06	5.00E+04	0.00E+00
	0.00E+00	2.83E+00	3.54E+00	7.07E-01	0.00E+00
72	3.50E+05	4.95E+06	3.80E+06	0.00E+00	5.25E+04
	5.00E+04	7.07E-01	7.07E-01	0.00E+00	0.00E+00
96	2.50E+05	4.45E+06	2.05E+06	3.00E+05	2.04E+05
	7.07E-01	7.78E+00	7.07E-01	1.41E+00	9.57E-01
120	3.25E+06	1.90E+06	1.40E+06	4.00E+05	4.12E+05
	6.36E+00	5.66E+00	2.83E+00	0.00E+00	4.35E+00
168	1.05E+07	1.50E+06	2.50E+06	1.00E+06	1.50E+06
	7.07E-01	7.07E-01	2.12E+00	1.41E+00	5.77E-01
216	2.05E+07	2.50E+06	3.05E+07	0.00E+00	1.00E+06
	1.41E+00	7.07E-01	7.07E-01	0.00E+00	1.15E+00
264	1.48E+07	0.00E+00	1.65E+07	0.00E+00	3.51E+06
	3.54E+00	0.00E+00	2.12E+00	0.00E+00	2.71E+00
312	1.50E+07	0.00E+00	5.00E+06	0.00E+00	1.50E+06
	2.83E+00	0.00E+00	1.41E+00	0.00E+00	1.29E+00
360	1.34E+07	0.00E+00	8.00E+06	0.00E+00	1.50E+06
	1.41E+00	0.00E+00	2.83E+00	0.00E+00	9.57E-01
408	1.23E+07	5.00E+05	3.00E+06	0.00E+00	2.00E+06
	2.12E+00	7.07E-01	0.00E+00	0.00E+00	2.00E+00
Combined H and I	<i>S. cerevisiae</i>	<i>H. uvarum</i>	<i>S. bacillaris</i>	<i>L. thermotolerans</i>	Rest
	SD	SD	SD	SD	SD
0	5.00E+03	5.35E+04	1.90E+05	2.70E+04	1.65E+04
	1.41E+00	1.06E+01	1.41E+01	2.83E+00	9.74E+00
24	1.95E+05	2.80E+05	5.10E+05	4.50E+04	2.00E+04
	4.95E+00	2.83E+00	9.90E+00	7.07E-01	1.15E+00
48	0.00E+00	1.95E+06	1.25E+06	1.00E+05	0.00E+00
	0.00E+00	7.07E-01	2.12E+00	0.00E+00	0.00E+00
72	2.50E+05	4.55E+06	4.65E+06	0.00E+00	2.00E+05
	7.07E-01	3.54E+00	2.12E+00	0.00E+00	8.16E-01
96	2.50E+05	5.00E+06	2.70E+06	4.00E+05	8.00E+05
	2.12E+00	8.49E+00	2.83E+00	1.41E+00	1.41E+00
120	2.45E+06	2.10E+06	1.70E+06	2.50E+05	1.15E+06
	7.07E-01	0.00E+00	2.83E+00	7.07E-01	3.20E+00
168	1.10E+07	1.00E+06	3.00E+06	5.00E+05	2.50E+06
	1.41E+00	0.00E+00	1.41E+00	7.07E-01	5.00E-01

216	2.10E+07	4.50E+06	3.20E+07	0.00E+00	2.00E+06
	3.54E+00	7.07E-01	1.41E+00	0.00E+00	1.41E+00
264	1.67E+07	1.50E+06	2.40E+07	0.00E+00	5.00E+06
	4.95E+00	7.07E-01	1.41E+00	0.00E+00	1.29E+00
312	1.45E+07	0.00E+00	5.00E+06	5.00E+05	3.00E+06
	3.54E+00	0.00E+00	1.41E+00	7.07E-01	5.77E-01
360	1.32E+07	5.00E+05	1.45E+07	0.00E+00	3.50E+06
	3.54E+00	7.07E-01	7.07E-01	0.00E+00	9.57E-01
408	1.22E+07	2.50E+05	3.75E+06	0.00E+00	2.25E+06
	2.99E+00	5.00E-01	1.89E+00	0.00E+00	1.36E+00

Table 4 FOSS (grape scan) analysis on wine that was used for sensory

Wine	pH	Total Acid	Ethanol	Volatile Acid	Glucose	Fructose	Malic Acid	Glycerol
A1	3.45	5.2	11.73	0.22	-0.08	2.03	2.36	6.33
A2	3.44	5.22	11.8	0.23	0.28	2.07	2.36	6.37
B1	3.45	5.17	11.62	0.21	-0.22	4.4	2.43	6.35
B2	3.44	5.19	11.53	0.23	0.07	6.03	2.47	6.35
C1	3.45	5.21	11.76	0.22	0.15	2.77	2.36	6.44
C2	3.44	5.2	11.76	0.21	0.25	2.95	2.36	6.45
D1	3.44	5.2	11.74	0.31	-0.35	3.11	2.63	6.24
D2	3.46	5.2	12.11	0.33	0.11	3.02	2.41	6.61
E1	3.45	5.28	11.96	0.31	0.12	2.13	2.59	6.31
E2	3.46	5.26	11.95	0.32	0.07	2.12	2.6	6.3
F1	3.47	5.24	11.89	0.33	-0.18	2.27	2.59	6.21
F2	3.47	5.23	11.92	0.33	0.09	2.29	2.61	6.11
G1	3.45	5.22	11.3	0.38	0.03	6.69	2.48	5.99
G2	3.46	5.28	11.64	0.37	0.01	5.33	2.49	6.07
H1	3.43	5.31	11.5	0.38	0.37	7.2	2.44	6.3
H2	3.44	5.29	11.65	0.39	0.19	6.91	2.48	6.44
I1	3.45	5.28	11.63	0.39	0.18	7.42	2.54	6.21
I2	3.46	5.3	11.6	0.38	0.02	6.85	2.55	6.12

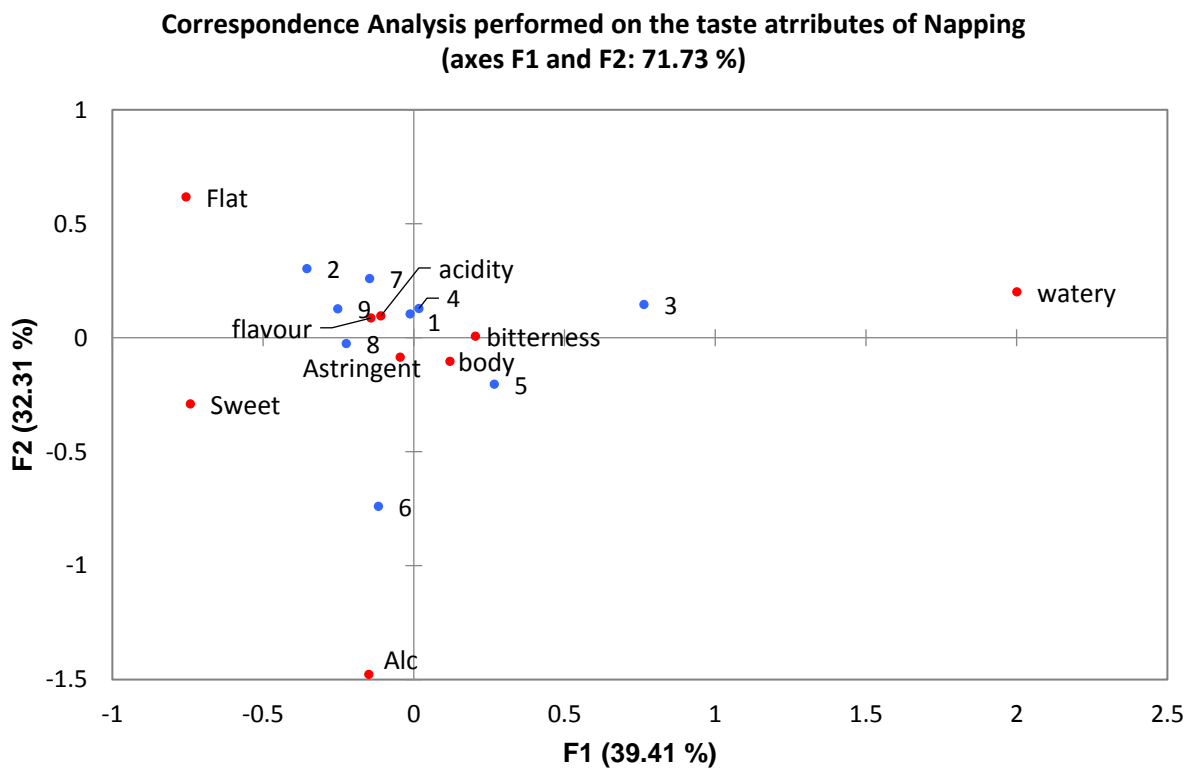
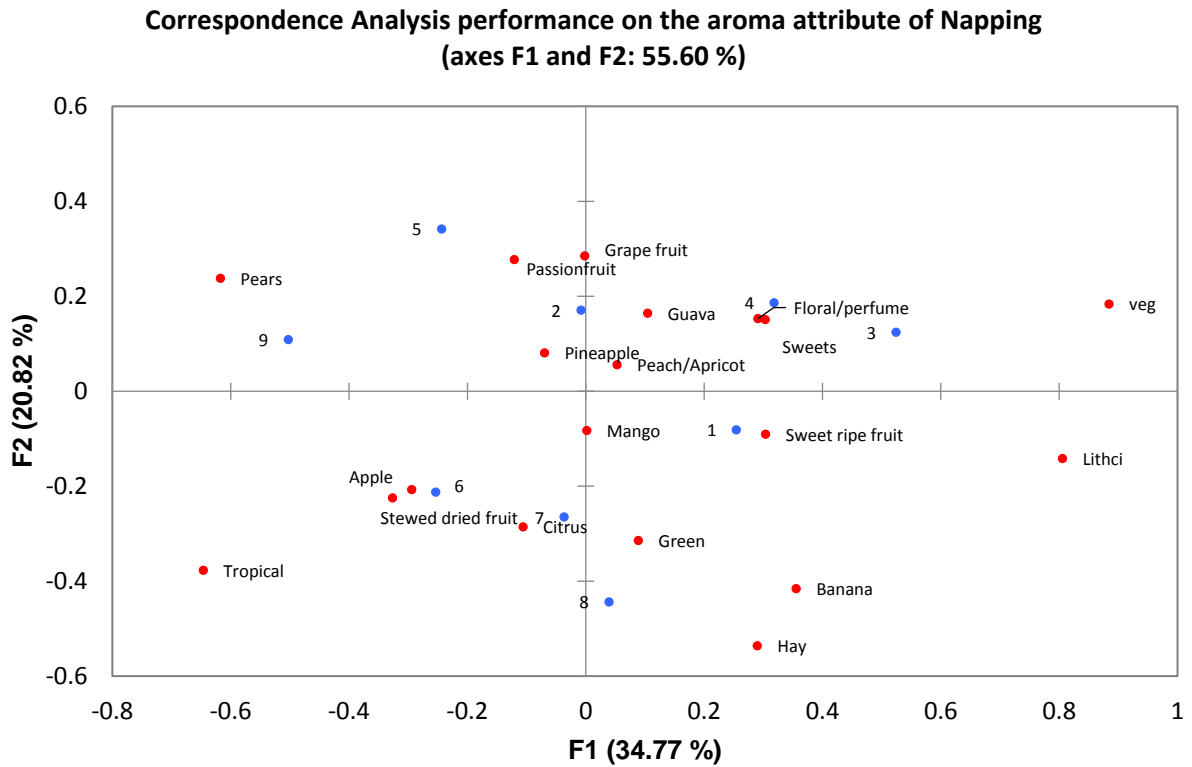


Figure 1. Correspondence analysis performed on (a) aroma and (b) taste from the sensory panel, rapid analysis method.

Numbers: 1 – 3 (1 = tank A; 2 = tank B; 3 = tank C); 4 – 6 (4 = tank D; 5 = tank E; 6 = tank F); 7 – 8 (7 = tank G; 8 = tank H; 9 = tank I)

Chapter 5

General discussion and conclusion

Chapter 5 - General discussion and conclusion

5.1. Concluding remarks and future prospects

Technological advances are being made in more rapid methods of identifying the natural microbiota present in grape must. With this technology winemakers will soon be able to determine the yeasts and other microbial species present in the grape must. The question however will remain how to interpret such a data set: Given a specific microbiota, which species are likely to dominate? Will desirable yeasts be dominant, and lead to a wine of desirable quality when using spontaneous fermentation? Or would inoculation be the safer option, and if yes, which non-*Saccharomyces* species would have the most desirable impact. By evaluating how various yeasts interact with each other, the aim of this project was to provide a baseline data set to evaluate whether ecosystem dynamics in a simplified consortium are relatively predictable. The data will contribute to a better understanding regarding how the addition of one or more NS yeasts, to fermenting must, could aid the natural microbiota and ferment harmoniously. This knowledge would ultimately allow for winemakers to perform spontaneous fermentations without the need to use a *S. cerevisiae* starter culture. There is however little research focussing on looking at yeast interactions of more than two species. In order to have this knowledge available when the technology becomes available, we need to start looking into multispecies interaction (more than two yeasts) within a wine media environment.

NS yeasts have become a commonly used tool in the wine industry to enhance wine flavour and aroma. These yeasts are selected to achieve a specific desirable outcome, but anecdotal evidence suggests that the impact of these yeasts are not always predictable. This may in particular be due to interactions between various yeasts present in fermentation, influencing the metabolic production of one another. There is however little information available regarding how these interactions within a multispecies environment might affect the final wine quality. This project is but the start to better understand these interactions. By using various commercial NS yeast products in combination with each other we evaluated how four yeasts interacted within a wine like medium. Starting with mono-culture fermentations of the three commercially available NS yeasts and *S. cerevisiae* then looking at one-on-one, three and finally the consortium of all four yeasts, we hoped to start better understanding these interactions.

From the results in Chapter 3 it was clear that regarding the population dynamics for *T. delbrueckii* and *P. kluyveri* the performance and trends in mono-culture, co-culture, three combinations and the consortium remained similar irrespective of the addition of other yeasts. The fermentations dynamics of both *M. pulcherrima* and *S. cerevisiae* was somewhat unpredictable, and these yeasts showed to impact each other in unexpected ways both individually and in combination with the other yeasts. Temperature also played an important role on the performance of the yeasts with some yeast performing better at 15°C compared to 25°C. Temperature thus also had a major impact on the

production of chemical compounds by yeasts but more work will have to be done to understand how population dynamics affected by temperature will affect certain compound production levels.

Comparing fermentation results from both synthetic and real grape juice we saw no real correspondence between the consortium fermentation regarding population dynamics. This could be due to the presence of indigenous yeasts or many other parameters found in real grape must, that we cannot mimic in synthetic grape juice (Viana *et al.*, 2014). This was a problem regarding the results drawn from the findings as there was no way of comparing the two systems with each other. In the simplified synthetic media, the outcomes were somewhat predictable, but this was not the case for the grape must fermentations as the presence of indigenous microbiota had an influence on the population dynamics.

Overall the results obtained from the synthetic media fermentations was what was expected. Yeasts performed as was indicated by suppliers and some yeasts showed interesting interactions which will be of use for future research. Regarding the industrial scale fermentations, it was clear that the results obtained from synthetic grape juice fermentations was not the same and a different approach would have to be taken in the future in order to be able to compare the two systems with each other.

Future prospects for synthetic grape must trails will be to use more yeasts and also included other commercially available NS yeasts such as Viniflora® Concerto (Chr. Hansen Holding A/S, Denmark). Another short coming regarding comparing synthetic grape must fermentations with real grape must fermentations must be looked at, creating a synthetic grape must more similar to real grape must similar to the work of Viana *et al.*, (2014). Continuing this work and comparing the work to synthetic grape must, grape juice used for fermentation should be sterilized by means of thermovinification as to minimize/remove the amount of natural yeast present in the must which could compete in fermentation and skew the data. The effect of nitrogen sources on the performance of these NS yeasts have been studied (Koker, 2015; Rollero *et al.*, 2015; Rollero *et al.*, 2016). For future work, the results from such research should be incorporated into understanding the interactions and nutrient effects on yeast performance. All-in-all doing all lab fermentations (small scale) in thermovinified grape must would be best, as to get a more comparable data set to bigger/ large scale fermentations. For future sensory work on determining the aroma and taste attributes for wines made by these various fermentation combinations, descriptive analysis should be used together with a trained panel of tasters to get a more accurate description for the wines thus ensuring more concluding results.

The work conducted here will help us to start better understanding how yeasts interact within a multispecies medium and how interactions between these four yeasts influence their performance throughout fermentation. This work will also shed light on the possibilities of using more than two yeasts in laboratory environments to mimic how yeasts will interact within a spontaneously fermenting system. This is very important to start looking at such fermentations as the outcomes of mono-culture or co-culture fermentations are not always the same when placed into a multispecies

system and we need to start better understanding what interactions are at play in such cases. Although winemakers may not use three or even four yeasts to ferment wine, as it might be too costly, in laboratory environment this work is needed to start understanding the complex multispecies environment winemakers experience in real wine fermentations.

This work will open new possibilities for winemakers to create wines with new knowledge as to help improve wine quality and aroma. It will also allow winemakers to cut down on costs with regards to the amount of yeast products needed to inoculate wine, by allowing them to use the natural yeasts present in the grape must, to their full advantage whilst still ensuring fermentations will complete. This work is but the start of understanding these complex interactions between inoculated NS and the natural yeasts present in grape must.

5.2. References

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