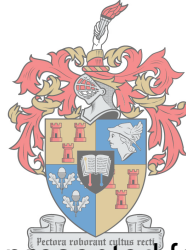


# **Effect of non-*Saccharomyces* yeasts and lactic acid bacteria interactions on wine flavour**

by

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## Declaration

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## Summary

Wine aroma and flavour are important indicators of quality and are primarily determined by the secondary metabolites of the grape, by the yeast that conducts the primary fermentation and also the lactic acid bacteria (LAB) that performs malolactic fermentation (MLF). This is a complex environment and each microorganism affects the other during the wine production process. Therefore, the overall aim of this study was to investigate the interactions between *Saccharomyces*, non-*Saccharomyces* yeasts and LAB, and the effect these interactions had on MLF and wine flavour.

Contour-clamped homogeneous electric field gel electrophoreses (CHEF) and matrix-assisted laser desorption ionization using time-of flight mass spectrometry (MALDI-TOF MS) were useful tools for identifying and typing of *Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Candida zemplinina* (synonym: *Starmerella bacillaris*) and *Torulaspora delbrueckii* strains. *Hanseniaspora uvarum* strains had  $\beta$ -glucosidase activity and *Metschnikowia pulcherrima* strains had  $\beta$ -glucosidase and protease activity. Only *Schizosaccharomyces pombe* and *C. zemplinina* strains showed mentionable malic acid degradation. *Candida stellata*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *Sc. pombe* strains were slow to medium fermenters, whereas *L. thermotolerans* and *T. delbrueckii* strains were found to be medium to strong fermenters, comparable to *S. cerevisiae*. The effect of non-*Saccharomyces* yeast species on MLF varied and inhibition was found to be strain dependent.

In a Shiraz winemaking trial where seven non-*Saccharomyces* strains were evaluated in combination with *S. cerevisiae* and three MLF strategies, the *C. zemplinina* and the one *L. thermotolerans* isolate slightly inhibited LAB growth in wines where yeast and LAB were inoculated simultaneously. However, the same effect was not observed during sequential inoculation of LAB. Mixed culture fermentations using non-*Saccharomyces* yeasts contained lower alcohol levels, and were more conducive to MLF than wines produced with *S. cerevisiae* only. Yeast treatment and MLF strategy resulted in wines with significantly different flavour and sensory profiles. Yeast selection and MLF strategy had a significant effect on berry aroma, but MLF strategy also had a significant effect on acid balance and astringency of wines.

In a follow up trial, *H. uvarum* was used in combination with two *S. cerevisiae* strains, two LAB (*Lactobacillus plantarum* and *Oenococcus oeni*) species and three MLF strategies. One of the *S. cerevisiae* strains had an inhibitory effect on LAB growth, while *H. uvarum* in combination with this *S. cerevisiae* strain had a stimulatory effect on MLF. Simultaneous MLF completed faster than sequential MLF and wines differed with regard to their chemical and sensory characteristics. Isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, 2-phenyl acetate, isobutanol, 3-methyl-1-pentanol, hexanoic acid and octanoic acid were important compounds in discriminating between the different wines. Yeast

treatment had a significant effect on fresh vegetative and spicy aroma, as well as body and astringency of the wines. The LAB strain and MLF strategy had a significant effect on berry, fruity, sweet associated and spicy aroma, as well as acidity and body of the wines.

Mid-infrared (MIR) spectroscopy was used to differentiate between wines produced with the selected *Saccharomyces* and non-*Saccharomyces* yeast combinations, LAB species and MLF strategies.

This study provides valuable information about the interactions between non-*Saccharomyces*, *Saccharomyces* yeast, LAB and MLF strategies, and how important pairing of strains are to ensure successful AF and MLF. Furthermore, the results also showed how these interactions can be applied to diversify wine flavour.

## Opsomming

Wynaroma en geur is belangrike aanwysers van kwaliteit en word hoofsaaklik bepaal deur die sekondêre metaboliete van die druif, deur die gis wat die alkoholiese gisting uitvoer en ook deur die melksuurbakterieë (MSB) wat appelmelksuurgisting (AMG) uitvoer. Die omgewing tydens wynproduksie is kompleks en elke mikroörganisme beïnvloed die ander. Die oorhoofse doel was om die interaksies tussen *Saccharomyces*, nie-*Saccharomyces* giste en MSB te ondersoek en om te bepaal watter effek hierdie interaksies op AMG en wynaroma het.

Kontoer toegeslane homogene elektriese veld gel elektroforese (KHEV) en matriks geassosieerde laser desorpsie ionisasie met tyd van vlug massa spektrometrie (MALDI-TVV MS) was nuttige tegnieke om *Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Candida zemplinina* (sinoniem: *Starmerella bacillaris*) en *Torulaspota delbrueckii* rasse te identifiseer en te karakteriseer. *Hanseniaspora uvarum* rasse het  $\beta$ -glukosidase aktiwiteit getoon en *Metschnikowia pulcherrima* rasse het  $\beta$ -glukosidase en protease aktiwiteit gehad. Slegs *Schizosaccharomyces pombe* en *C. zemplinina* rasse het noemenswaardige appelsuur afbraak getoon. *Candida stellata*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *Sc. pombe* rasse was stadig tot middelmatige fermenteerders, maar *L. thermotolerans* and *T. delbrueckii* rasse was middelmatige tot sterk fermenteerders en vergelykbaar met *S. cerevisiae*. Die effek wat nie-*Saccharomyces* gisspesies op die verloop van AMG gehad het, het gevarieer en inhibisie was ras afhanklik.

Vir die Shiraz wynmaak proef waar sewe nie-*Saccharomyces* rasse in kombinasie met 'n *S. cerevisiae* en drie AMG strategieë geëvalueer is, het die *C. zemplinina* en die een *L. thermotolerans* isolaat MSB groei effens geïnhibeer, toe die gis en MSB gelyktydig bygevoeg was. Dieselfde effek was nie by wyne wat opvolgende AMG ondergaan het, waargeneem nie. Gemengde fermentasies deur van nie-*Saccharomyces* giste gebruik te maak, het laer alkoholvlakke getoon en was meer bevorderlik vir AMG as wyne waar net *S. cerevisiae* gebruik is. Gisbehandeling en AMG strategie het wyne geproduseer wat betekenisvol verskil het in hul geur en sensoriese profiele. Gisseleksie en AMG strategie het 'n betekenisvolle effek op bessie aroma gehad, maar AMG strategie het ook 'n betekenisvolle effek op suurbalans en vrankheid van wyne gehad.

In 'n opvolgende proef, was *H. uvarum* gebruik in kombinasie met twee *S. cerevisiae* rasse, twee MSB spesies (*Lactobacillus plantarum* en *Oenococcus oeni*) en drie AMG strategieë. Een van die *S. cerevisiae* rasse het 'n inhiberende effek op MSB groei gehad, terwyl hierdie *S. cerevisiae* ras in kombinasie met *H. uvarum* 'n stimulerende effek op AMG getoon het. Appelmelksuurgisting was vinniger voltooi in wyne wat gelyktydige AMG ondergaan het as wyne wat opvolgende AMG ondergaan het en die wyne het ook verskil ten opsigte van chemiese en sensoriese eienskappe. Isoamielasetaat, etielheksanoaat, etieloktanoaat, etiel-3-

hidroksibutanoaat, etielfenielasetaat, 2-fenielasetaat, isobutanol, 3-metiel-1-pentanol, heksanoësuur en oktanoësuur was belangrike verbindings wat gebruik is om tussen die wyne te onderskei. Gisbehandeling het 'n betekenisvolle effek op vars vegetatiewe en spesery aromas gehad, sowel as mondgevoel en vrankheid van die wyne. Die MSB ras en AMG strategie het 'n betekenisvolle effek op bessie, vrugtig, soet geassosieerde en spesery aromas, sowel as suurbalans en mondgevoel van wyne gehad.

Mid-infrarooi spektroskopie was gebruik om tussen wyne wat met die geselekteerde *Saccharomyces* en nie-*Saccharomyces* giskombinasies, MSB spesie en AMG strategieë geproduseer is, te onderskei.

Hierdie studie verskaf waardevolle inligting oor die interaksies tussen nie-*Saccharomyces*, *Saccharomyces* giste, MSB en AMG strategieë, en hoe belangrik die regte kombinasies is vir suksesvolle alkoholiese gisting en AMG. Verder het resultate ook gewys hoe bogenoemde interaksies toegepas kan word om wyngneur te diversifiseer.

This dissertation is dedicated to my loving family and my late father Harry Andrew du Plessis.

## Biographical sketch

Heinrich du Plessis was born in Paarl, South Africa on 3 November 1975. He attended Paarlzicht Primary School and matriculated at Noorder Paarl Secondary School in 1993. He enrolled at Stellenbosch University in 1994 and obtained his BSc degree in 1997, majoring in Microbiology and Genetics. He completed his HonsBSc in Wine Biotechnology in 1998 and his MSc degree *cum laude* in 2002. He was appointed as a junior researcher at ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council) in 2000 and is currently working as a Microbiologist in the Post-Harvest and Agro-processing Technologies Division at ARC Infruitec-Nietvoorbij. His current research fields include the interactions between *Saccharomyces*, non-*Saccharomyces* yeasts and lactic acid bacteria in wine production, and the role of yeast and lactic acid bacteria in the production of volatile phenols associated with smoke taint.



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## Preface

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the South African Journal of Enology and Viticulture, except for Chapter 4, which is written according to the style of Fermentation, where it was published.

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# Chapter 1

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## **General introduction and project aims**



# 1. General introduction and project aims

## 1.1 Introduction

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About 300,000 people were employed both directly and indirectly in the South African wine industry in 2015, including farm labourers, those involved in packaging, retailing and wine tourism (Conningarth economists, 2015). The study also concluded that of the R36.1 billion gross domestic product (GDP) contributed by the wine industry to the regional economy, about R19.3 billion eventually would remain in the Western Cape to the benefit of its residents. Improving wine quality and reducing wine production costs will contribute to the long term sustainability of the South African wine industry.

Wine is the product of a complex biochemical process, which starts with the grapes, continues with the alcoholic and malolactic fermentations, maturation and bottling (Romano *et al.*, 2003). The compounds that define the appearance, aroma and taste properties of wines can be derived from three sources, *i.e.* grapes, microorganisms and wood, when used (Swiegers *et al.*, 2005). The aroma of wine is due to the volatile compounds that are detectable by the human nose and small differences in the concentration of these volatile aroma compounds can mean the difference between a world-class and an average wine. Wine aroma and flavour are important indicators of quality (Bartowsky *et al.*, 2002, 2015) and the yeast and bacteria involved and their interactions are important tools to modify wine flavour and improve quality (Swiegers *et al.*, 2005).

Winemaking involves two fermentation processes: alcoholic fermentation (AF), conducted by yeasts and malolactic fermentation (MLF), conducted by lactic acid bacteria (LAB), with considerable interactions occurring (Wibowo *et al.*, 1985, Lonvaud-Funel, 1995; Fleet, 2003). During AF, sugars are converted to ethanol and carbon dioxide, but also a range of sensorially important volatile compounds are produced. These volatile compounds, which include esters, higher alcohols, aldehydes, carbonyls, volatile fatty acids, sulphur compounds, monoterpenes and others are derived from components already present in the grapes, or are formed during fermentation or aging of the wines (Swiegers *et al.*, 2005; Concurso *et al.*, 2016).

The yeast associated in winemaking can be divided into two groups, *Saccharomyces* and non-*Saccharomyces* yeasts. *Saccharomyces cerevisiae*, also known as the 'wine yeast' is usually used to initiate the AF (Pretorius, 2000; Swiegers *et al.*, 2005). The ability of *S. cerevisiae* to rapidly complete the AF, while producing important volatile metabolites without producing off-flavour, has been well established. *S. cerevisiae* is tolerant to stresses associated with wine conditions, *e.g.* alcohol, presence of sulphur dioxide (SO<sub>2</sub>) and anaerobiosis (Pretorius, 2000; Fleet, 2008). The benefits of using commercial *S. cerevisiae* cultures are the production of uniform and predictable quality wines (Degré, 1993, Pretorius, 2000). However, lack of aromatic complexity, stylistic distinction and unique regional characteristics are

associated with using commercial *S. cerevisiae* cultures (Pretorius, 2000; Beltran *et al.*, 2002; Jolly *et al.*, 2014).

The non-*Saccharomyces* yeasts, also known as 'wild yeasts' are derived primarily from the grapes (vineyard), where they occur in higher numbers than the *S. cerevisiae* yeasts, and secondly from the cellar environment and equipment (Peynaud & Domercq, 1959; Martini *et al.*, 1996; Ribéreau-Gayon *et al.*, 2006; Alessandria *et al.*, 2015; Capozzi *et al.*, 2015). Non-*Saccharomyces* genera frequently found on grapes and in must, include *Hanseniaspora* (*Kloeckera*), *Metschnikowia* (*Candida*), *Pichia*, *Starmerella* (*Candida*), *Lachancea* (*Kluyveromyces*), *Torulaspota* (*Candida*), *Saccharomycodes*, *Dekkera* (*Brettanomyces*), *Zygosaccharomyces*, *Schizosaccharomyces*, *Rhodotorula* and *Cryptococcus* (Fleet *et al.*, 2002; Jolly *et al.*, 2003; Romano *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006; Jolly *et al.*, 2014; Alessandria *et al.*, 2015). Most non-*Saccharomyces* yeasts are slow fermenters, sensitive to SO<sub>2</sub> and alcohol, do not always finish alcoholic fermentation, and consequently have to be used in combination with *S. cerevisiae* (Fleet, 2008; Capozzi *et al.*, 2015).

Non-*Saccharomyces* yeasts can be beneficial or detrimental to wine production, depending on the species and strain present. Research over the last two decades has shown that non-*Saccharomyces* yeasts in combination with *S. cerevisiae* can be used to add flavour and improve wine quality (Comitini *et al.*, 2011; Jolly *et al.*, 2014; Benito *et al.*, 2015; Renault *et al.*, 2015). Non-*Saccharomyces* yeasts produce varying higher alcohol levels (n-propanol, isobutanol, isoamyl alcohol, active amyl alcohol) (Romano *et al.*, 1992; Lambrechts & Pretorius, 2000). 2-Phenylethanol has a floral aroma (Lambrechts & Pretorius, 2000) and higher levels have been reported in wines produced by *Candida zemplinina*, *Lachancea thermotolerans* and *Metschnikowia pulcherrima* (Clemente-Jimenez *et al.*, 2004; Andorra *et al.*, 2010; Whitener *et al.*, 2015). *M. pulcherrima* has also been reported to produce high concentrations of esters (Bisson & Kunkee, 1991; Rodríguez *et al.*, 2010), especially ethyl octanoate, which is associated with pear and pineapple aroma (Lambrechts & Pretorius, 2000; Clemente- Jimenez *et al.*, 2004). In mixed fermentations with *S. cerevisiae*, *Hanseniaspora uvarum* has been reported to produce increased concentrations of higher alcohols, acetate- and ethyl esters and medium-chain fatty acids (Andorra *et al.*, 2010). Wines produced with mixed cultures of *Torulaspota delbrueckii* and *S. cerevisiae* have enhanced complexity and fruity notes compared to wines produced with a *S. cerevisiae* pure culture (Renault *et al.*, 2015). Mixed fermentations of non-*Saccharomyces* yeasts in combination with *S. cerevisiae* can therefore be used as a tool to modulate flavour profiles and improve aromatic complexity (Liu *et al.*, 2016; Whitener *et al.*, 2016, 2017).

Malolactic fermentation is an enzymatic reaction performed by LAB, whereby malic acid is decarboxylated to lactic acid and CO<sub>2</sub> (Lonvaud-Funel, 1995). This process is often desired in the production of some red, white and sparkling wine styles (Wibowo *et al.*, 1985; Lerm *et al.*, 2010; Bartowsky *et al.*, 2015). Malolactic fermentation increases microbiological stability,

enhances aroma and flavour, and decreases the acidity of wine (Davis et al., 1985; Versari et al., 1999; Bartowsky et al., 2002; Sumbly et al., 2014). Lactic acid bacteria can affect wine aroma and flavour through the production or liberation of metabolites such as esters, higher alcohols, acids, carbonyl compounds, terpenes, nor-isoprenoids and phenolic compounds (Liu, 2002; Hernandez-Orte et al., 2009). The LAB species *Oenococcus oeni* is probably the best adapted to overcome the harsh wine conditions and therefore represents the majority of commercial MLF starter cultures. However, recently commercial *Lactobacillus plantarum* starter cultures have also become available (Du Toit et al., 2011; Bartowsky et al., 2015). *Lb. plantarum* has been shown to efficiently induce and complete MLF under high pH conditions. In addition, *Lb. plantarum* produces a broader range of extracellular enzymes, including glycosidases and esterases, than *O. oeni* (Guerzoni et al. 1995, Grimaldi et al., 2005, Mtshali et al., 2010), which could be applied to improve sensory properties of wine. Clear differences between the primary and secondary metabolites produced by *O. oeni* and *Lb. plantarum* have been reported by Lee et al. (2009). Besides the differences with regard to volatile aroma compounds, the two aforementioned species were also perceived to confer different sensory profiles to wine (Du Toit et al., 2011).

Malolactic fermentation usually follows alcoholic fermentation, but can be induced prior to alcoholic fermentation or simultaneously with alcoholic fermentation (Bartowsky et al., 2015). Simultaneous inoculation of LAB can result in wines having different flavour profiles than wines that underwent sequential MLF (Massera et al., 2009; Abrahamse, & Bartowsky 2012a, b). Yeast and LAB interactions may also differ between different timings of MLF inoculation and there is growing evidence that optimal yeast and LAB combinations may differ for simultaneous and sequential fermentations (Bartowsky et al., 2015).

The interaction between LAB and yeasts during AF and/or MLF will have a direct effect on LAB growth and malolactic activity (Lerm et al., 2010). Yeast can have an inhibiting, stimulating, or neutral (no) effect, depending on the yeast and LAB pairing (Alexandre et al., 2004). The antagonistic effect of *S. cerevisiae* against LAB is well known (Edwards & Beelman, 1987; Capucho & San Romao, 1994; Alexandre et al., 2004; Comitini et al., 2005; Nehme et al., 2010). Certain non-*Saccharomyces* yeasts can also have an antagonistic effect against LAB (Fornachon, 1968). Mendoza et al. (2010) found that *S. cerevisiae*, *M. pulcherrima*, *Candida stellata*, *Candida parapsilosis* and *P. fermentans* inhibited *O. oeni* growth, but varied with regard to the degree of inhibition. These authors also found that *H. uvarum* (*Kloeckera apiculata*) strains had no effect or stimulated the growth of *O. oeni*. *Cryptococcus* also had a stimulatory effect on *O. oeni* growth. Mendoza et al. (2011) investigated the interactions between *H. uvarum*, *S. cerevisiae* and *O. oeni* during mixed fermentations and found that the interactions between these yeasts did not affect the fermentation kinetics of *O. oeni*.

The use of state of the art analytical tools to ensure high quality standards and process control during wine production is crucial in a competitive wine market (Cusmano et al., 2010).

Analytical technologies combine several components, including physical, chemical, mathematical, statistical and other resources to provide a complete understanding of product properties (Aleixandre-Tudo *et al.*, 2018). The information obtained can be used for benchmarking, decision making, grading, process control, adulteration or geographical identification tasks, among others (Gishen *et al.*, 2005; Dambergs *et al.*, 2015). Infrared spectroscopy (IR) can be used to provide information of wine biochemical components, and is a non-destructive, fast and easy to perform analytical technique (Cozzolino *et al.*, 2006; Ricci *et al.*, 2013).

For wine producers to be successful in competitive global wine markets a better understanding of the biology of human perception, olfactory and flavour preferences, the relationship between composition and the sensorial quality of wine, and the production of wine to changing market specifications and sensory preferences is required (Swiegers *et al.*, 2005). The winemaker employs a variety of techniques and tools to produce wines with specific flavour profiles, which include the choice of microorganisms. The interactions between *S. cerevisiae*, non-*Saccharomyces* yeasts and the LAB, as well as their impact on AF, MLF, flavour and quality has also received limited attention. With the increasing number of non-*Saccharomyces* yeasts commercially available there is a need to better understand the interactions that occur between *S. cerevisiae*, non-*Saccharomyces* yeasts and LAB, and the effect these interactions have on MLF, wine flavour and quality. A better understanding of wine production components can be used to manipulate wine attributes such as aroma, flavour, body or mouthfeel, to produce a targeted wine style (Lesschaeve, 2007).

## 1.2 Aims and objectives of the study

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This study forms part of an extensive Winetech (Wine Industry Network for Expertise and Technology) strategy aimed at the production of quality South African wines and other grape-based products through the application of environmentally friendly practices and the best technologies. Part of the aforementioned Winetech strategy, is a long-term programme investigating yeast biodiversity and yeast development, which started in the mid-nineties. Some of long-term objectives of the programme as detailed by Pretorius *et al.* (1999) include the characterisation, evaluation, and utilisation of the natural yeast biodiversity occurring in the wine producing regions of the Western Cape.

Aligned to the aforementioned programme, the aim of this study was to investigate the interactions between *Saccharomyces*, non-*Saccharomyces* yeasts and LAB, and the effect these interactions had on MLF and wine flavour. This was done by the following objectives:

- (i) characterisation of 37 non-*Saccharomyces* yeast strains by means of CHEF karyotyping, MALDI-TOF bio-typing, enzyme activity, malic acid degradation, fermentation activity, and compatibility with MLF;
- (ii) evaluation of five non-*Saccharomyces* yeast species in combination with one *S. cerevisiae* and three MLF strategies for the production of Shiraz wines;
- (iii) investigation of the interactions between one non-*Saccharomyces* yeast, two *S. cerevisiae* strains and two LAB species (*Lb. plantarum* and *O. oeni*), and three MLF strategies during wine production; and
- (iv) exploration of mid-infrared (MIR) spectroscopy, in combination with pattern recognition methods, as a rapid and inexpensive tool to distinguish between wines produced with selected non-*Saccharomyces* and *S. cerevisiae* yeasts, LAB strains and MLF strategies.

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# Chapter 2

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## Literature review

**Characterisation of non-*Saccharomyces* yeast  
and the contribution of non-*Saccharomyces*  
yeast and lactic acid bacteria during wine  
production**



## 2. Literature review

### Characterisation non-*Saccharomyces* yeasts and contribution of non-*Saccharomyces* yeasts and lactic acid bacteria during wine production

#### 2.1 Introduction

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Winemaking or vinification, starts with the selection of grapes, continues with the processing and the fermentation and ends with bottling of the finished wine. Winemaking is a complex ecological niche where biochemical and microbiological interactions are important with regard to the quality of the final product (Du Toit & Pretorius, 2000). Wine composition is determined by a number of factors, including topography, soils, and viticultural and oenological practices (Lambrechts & Pretorius, 2000; Fleet, 2003). Wine quality is determined by the appearance, aroma, flavour and taste of the final product. Volatile compounds affect wine aroma, which is perceived by the sense of smell, while wine flavour refers to the combination of both aroma and taste (Francis & Newton, 2005). Although wine flavour is directly determined by grape variety, microorganisms can also affect wine flavour, thus wine quality (Bartowsky & Henschke 1995, Fleet, 2003; Lambrechts & Pretorius, 2000; Swiegers *et al.*, 2005, Bartowsky *et al.*, 2002, 2015).

The different microorganisms that play a role include fungi, yeasts, acetic acid bacteria and lactic acid bacteria (LAB) (Fleet, 2003). The yeasts associated with winemaking can be divided into *Saccharomyces* and non-*Saccharomyces* yeasts. Non-*Saccharomyces* yeasts refer to all yeast species, excluding *Saccharomyces* spp. that play a positive role in wine production (Jolly *et al.*, 2014). In this study, yeast species that are generally associated with spoilage were omitted from the non-*Saccharomyces* yeast group. During fermentation, there may be a succession of the various non-*Saccharomyces* yeasts, followed by *Saccharomyces cerevisiae*, which completes the fermentation. However, certain non-*Saccharomyces* yeasts can persist to the end of fermentation. During alcoholic fermentation, primarily sugars are fermented to ethanol, while the major flavour compounds such as esters, higher alcohols, aldehydes and fatty acids are also produced (Swiegers *et al.*, 2005; Du Toit *et al.*, 2011; Conurso *et al.*, 2016).

At the end of alcoholic fermentation the yeast numbers decrease and LAB numbers increase (Lonvaud-Funel, 1999; Ribéreau-Gayon *et al.*, 2006). Lactic acid bacteria are responsible for conducting malolactic fermentation (MLF), which is a secondary fermentation, that usually takes place during alcoholic fermentation or at the end of alcoholic fermentation and is carried out by one or more species (Ribéreau-Gayon *et al.*, 2006; Du Toit *et al.*, 2011). This fermentation involves the conversion of L-malic acid to L-lactic acid and CO<sub>2</sub> (Davis *et al.*, 1985; Ribéreau-Gayon *et al.*, 2006; Lerm *et al.*, 2010). Apart from an increase in pH, additional sugars

are fermented and aromatic compounds are produced which change the organoleptic profile of the wine (Bauer & Dicks, 2004).

Techniques for investigating non-*Saccharomyces* strain diversity and the role of non-*Saccharomyces* and LAB in wine production will be discussed in the following sections.

## 2.2 Classification

### 2.2.1 Yeast classification

Yeasts are unicellular ascomycetous or basidiomycetous fungi that have vegetative states and predominantly reproduce by budding or fission, and do not form their sexual states within or on a fruiting body (Barnett, 1992; Kurtzman & Fell, 1998; Kurtzman *et al.*, 2011a). Currently, there are about 149 yeast genera comprising more than 1500 species (Kurtzman *et al.*, 2011b), but only 40 of these are relevant to wine production (Jolly *et al.*, 2006; Ciani *et al.*, 2010). Yeasts previously had two classification names, *i.e.* the teleomorphic name referring to the sexual state producing ascospores (Kurtzman *et al.*, 2011a), and the anamorphic name referring to the asexual state that does not form ascospores. This type of classification was difficult because some yeasts do not sporulate or do not sporulate easily and the ability to form ascospores can be lost during long-term storage (Kurtzman *et al.*, 2011c). Some of the yeast species relevant to winemaking are listed in Table 2.1. Since the advent of molecular techniques it has become easier today to identify yeast and in general, the teleomorphic names are mostly used.

TABLE 2.1. Anamorphic, teleomorphic and synonyms of non-*Saccharomyces* yeast species relevant to wine production (Romano *et al.*, 2003; Jolly *et al.*, 2006, 2014; Vaudano *et al.*, 2014; Whitener *et al.*, 2015; Ciani *et al.*, 2016a, b; Jood *et al.*, 2017). The yeasts listed in this table are not comprehensive and only include ascomycetous yeasts.

Teleomorphic yeast	Anamorphic yeast	Synonyms <sup>1</sup>
<i>Citeromyces matritensis</i>	<i>Candida globosa</i>	
<i>Debaryomyces hansenii</i>	<i>Candida famata</i>	
<i>Debaryomyces vanriijiae</i>	NA <sup>3</sup>	<i>Schwanniomyces vanriijiae</i>
<i>Dekkera anomala</i>	<i>Brettanomyces anomalus</i>	
<i>Dekkera bruxellensis</i>	<i>Brettanomyces bruxellensis</i>	
<i>Hanseniaspora guilliermondii</i>	<i>Kloeckera apis</i>	
<i>Hanseniaspora occidentalis</i>	<i>Kloeckera javanica</i>	
<i>Hanseniaspora osmophila</i>	<i>Kloeckera corticis</i>	
<i>Hanseniaspora uvarum</i>	<i>Kloeckera apiculata</i>	
<i>Hanseniaspora vineae</i>	<i>Kloeckera africana</i>	
<i>Issatchenkia occidentalis</i>	<i>Candida sorbosa</i>	
<i>Issatchenkia orientalis</i>	<i>Candida krusei</i>	<i>Saccharomyces krusei</i>
<i>Issatchenkia terricola</i>	NA <sup>3</sup>	<i>Pichia terricola</i>
<i>Kazachstania aerobia</i>	NA <sup>3</sup>	
<i>Kazachstania exigua</i>	NA <sup>3</sup>	<i>Saccharomyces exiguus</i>
<i>Kazachstania gamospora</i>	NA <sup>3</sup>	

TABLE 2 (continued)

Teleomorphic yeast	Anamorphic yeast	Synonyms <sup>1</sup>
<i>Kazachstania hellenica</i>	NA <sup>3</sup>	
<i>Kazachstania servazii</i>	NA <sup>3</sup>	<i>Saccharomyces servazii</i>
<i>Kazachstania solicola</i>	NA <sup>3</sup>	
<i>Kazachstania unisporus</i>	NA <sup>3</sup>	<i>Saccharomyces unisporus</i>
<i>Lachancea fermentati</i>	NA <sup>3</sup>	<i>Zygosaccharomyces fermentati</i>
<i>Lachancea kluyveri</i>	NA <sup>3</sup>	<i>Saccharomyces kluyveri</i>
<i>Lachancea thermotolerans</i>	NA <sup>3</sup>	<i>Kluyveromyces thermotolerans</i> , <i>Candida dattlia</i>
NT <sup>2</sup>	<i>Kluyveromyces wickerhamii</i>	<i>Saccharomyces wickerhamii</i>
<i>Metschnikowia pulcherrima</i>	<i>Candida pulcherrima</i>	<i>Torulopsis pulcherrima</i>
<i>Meyerozyma guilliermondii</i>	<i>Candida guilliermondii</i>	<i>Pichia guilliermondii</i>
<i>Milleronzyma farinosa</i>	NA <sup>3</sup>	<i>Pichia farinosa</i>
<i>Pichia anomala</i>	<i>Candida pelliculosa</i>	<i>Hansenula anomala</i>
<i>Pichia fermentans</i>	<i>Candida lambica</i>	
<i>Pichia kluyveri</i>	NA <sup>3</sup>	<i>Hansenula kluyveri</i>
<i>Pichia kudriavzevii</i>	<i>Candida krusei</i>	<i>Candida solicola</i>
<i>Pichia membranifaciens</i>	<i>Candida valida</i>	
<i>Saccharomycodes ludwigii</i>	NA <sup>3</sup>	
<i>Schizosaccharomyces pombe</i>	NA <sup>3</sup>	<i>Schizosaccharomyces malidevorans</i>
<i>Starmerella bacillaris</i>	NA <sup>3</sup>	<i>Candida zemplanina</i> , <i>Saccharomyces bacillaris</i>
<i>Starmerella bombicola</i>	<i>Candida bombicola</i>	<i>Torulopsis bombicola</i>
<i>Tetrapisispora phaffii</i>	NA <sup>3</sup>	<i>Kluyveromyces phaffii</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>Hansenula anomala</i>
<i>Zygoascus hellenicus</i>	<i>Candida hellenica</i>	
<i>Zygosaccharomyces bailii</i>	NA <sup>3</sup>	<i>Saccharomyces bailii</i>
<i>Zygosaccharomyces bisporus</i>	NA <sup>3</sup>	<i>Zygosaccharomyces bisporus</i>
<i>Zygosaccharomyces kombuchaensis</i>	NA <sup>3</sup>	
<i>Zygosaccharomyces sapae</i>	NA <sup>3</sup>	
NT <sup>2</sup>	<i>Candida stellata</i>	<i>Torulopsis stellata</i>

<sup>1</sup>Names sometimes found in older literature. <sup>2</sup>No teleomorphic form. <sup>3</sup>No anamorphic form.

## 2.2.2 Lactic acid bacteria classification

Lactic acid bacteria play a role in many food fermentations and are closely associated with the human environment. Lactic acid bacteria are Gram-positive, catalase-negative, non-motile, non-spore forming rods, cocci or coccobacilli and produce mainly lactic acid from the fermentation of carbohydrates (Stiles & Holzapfel, 1997; Ribéreau-Gayon *et al.*, 2006; Holzapfel & Wood, 2012). They can be divided into three groups according to their metabolic activity, *i.e.* homofermentative, facultatively heterofermentative or obligately heterofermentative. Homofermentative LAB produce more than 85% lactic acid from glucose. Heterofermentative

LAB produce CO<sub>2</sub>, ethanol and acetic acid, in addition to lactic acid (Stiles & Holzapfel, 1997, Ribéreau-Gayon *et al.*, 2006; Holzapfel & Wood, 2012). LAB from the genera *Leuconostoc* and *Oenococcus* are obligately heterofermentative and those from the genus *Pediococcus* obligately homofermentative. The genus *Lactobacillus* contains both homo- and heterofermentative species.

The obligately homofermentative LAB ferment glucose to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway and do not ferment pentoses (Fig. 2.1a). Homofermentative LAB produce two molecules of lactic acid and two molecules of ATP from one molecule of glucose (hexose) via the EMP pathway (Fugelsang, 1997; Fugelsang & Edwards, 2006). Depending on the species, either the L- or D-lactic acid isomer is formed. *Oenococcus oeni* produces only D (-)-lactate, whereas *Pediococcus* spp. produce either D- or L- (+)-lactate, and *Lactobacillus* spp. produce both D- (-) and L- (+)-lactate (Fugelsang, 1997; Fugelsang & Edwards, 2006; Ribéreau-Gayon *et al.*, 2006).

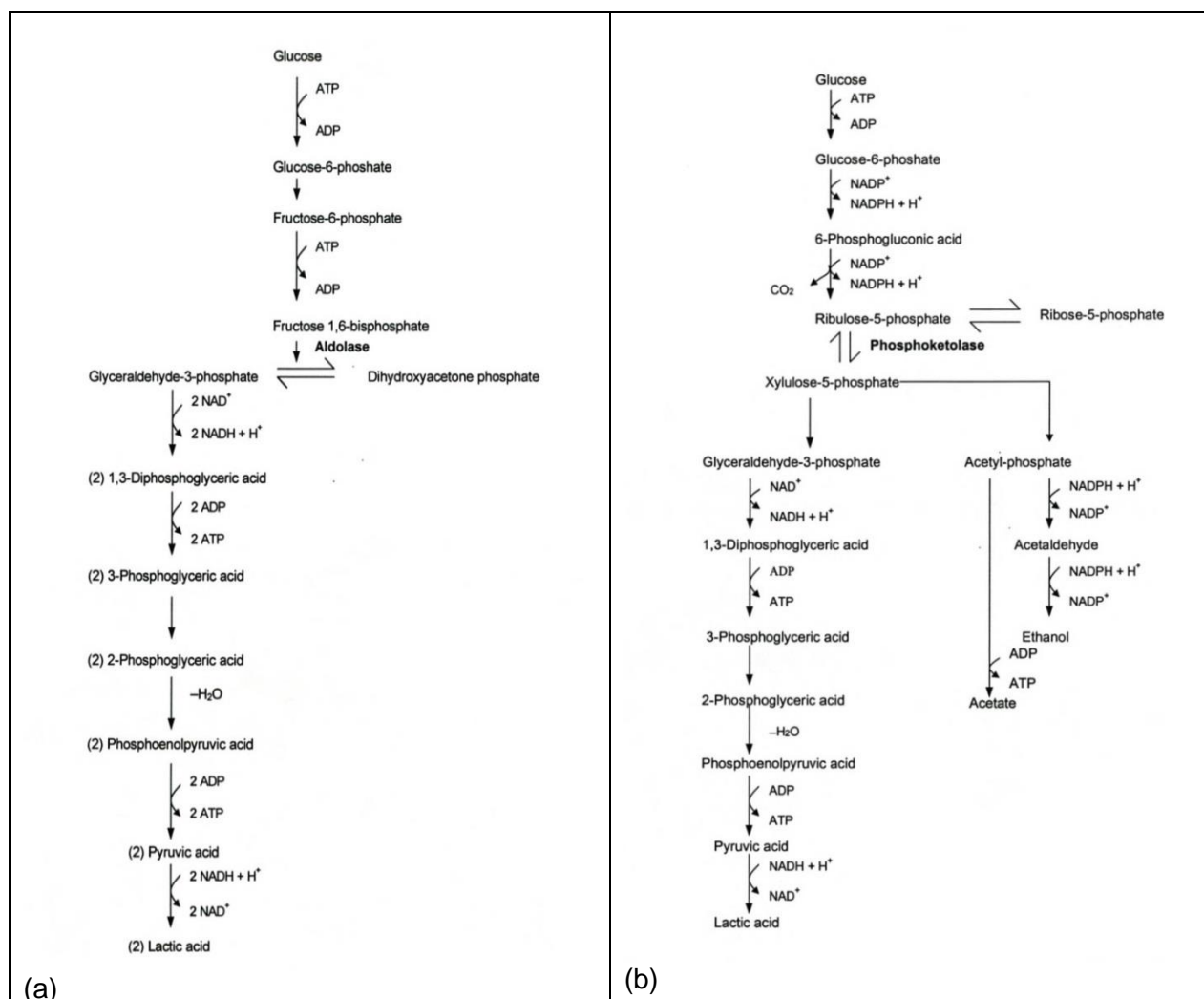


FIGURE 2.1 (a) Embden-Meyerhof-Parnas (EMP) pathway for the metabolism of glucose by obligately homofermentative LAB and (b) pentose phosphate (6-phosphogluconate) pathway for the metabolism of glucose by heterofermentative lactic acid bacteria.

In facultatively heterofermentative lactobacilli, glucose is metabolised to lactic acid, but pentoses are fermented into lactic acid and acetic acid via the pentose phosphate pathway (Fig. 2.1b). The obligately heterofermentative LAB lack the fructose diphosphate aldolase enzyme of the EMP pathway and ferment glucose to CO<sub>2</sub>, lactic acid, acetic acid and ethanol via the pentose phosphate pathway (Ribéreau-Gayon *et al.*, 2006). Similarly as facultatively heterofermentative LAB, pentoses are fermented into lactic acid and acetic acid. Some of the LAB associated with grapes, must and wine are listed in Table 2.2.

TABLE 2.2. The lactic acid bacteria species relevant to wine production (Dicks & Endo, 2009; Du Toit *et al.*, 2011).

<b>Genus</b>	<b>Species</b>	<b>Reference</b>
<i>Lactobacillus</i>	<i>Lb. brevis</i>	Vaughn (1955), Du Plessis and Van Zyl (1963), Ribéreau-Gayon <i>et al.</i> (2006)
	<i>Lb. bobalius</i>	Mañes-Lázaro <i>et al.</i> (2008a)
	<i>Lb. buchneri</i>	Vaughn (1955), Du Plessis and Van Zyl (1963)
	<i>Lb. casei</i>	Vaughn (1955), Carre (1982), Lonvaud-Funel <i>et al.</i> (1991), Izquierdo <i>et al.</i> (2009), Ruiz <i>et al.</i> (2010)
	<i>Lb. collinoides</i>	Carr & Davies (1972), Couto and Hogg (1994)
	<i>Lb. fermentum</i>	Vaughn (1955), O'Leary and Wilkinson (1988)
	<i>Lb. fructivorans</i>	Amerine and Kunkee (1968), Couto & Hogg (1994)
	<i>Lb. hilgardii</i>	Douglas and Cruess (1936), Vaughn (1955), Carre (1982), Couto and Hogg (1994), Ribéreau-Gayon <i>et al.</i> (2006), Izquierdo <i>et al.</i> (2009), Ruiz <i>et al.</i> (2010)
	<i>Lb. kunkeei</i>	Edwards <i>et al.</i> (1998), Bae <i>et al.</i> (2006)
	<i>Lb. lindneri</i>	Bae <i>et al.</i> (2006)
	<i>Lb. mali</i>	Carr & Davies (1970), Couto and Hogg (1994), Bae <i>et al.</i> (2006)
	<i>Lb. nagelii</i>	Edwards <i>et al.</i> (2000)
	<i>Lb. oeni</i>	Mañes-Lázaro <i>et al.</i> (2009)
	<i>Lb. paracasei</i>	Du Plessis <i>et al.</i> (2004)
	<i>Lb. paraplantarum</i>	Curk <i>et al.</i> (1996), Krieling (2003)
	<i>Lb. plantarum</i>	Carre (1982), Wibowo <i>et al.</i> (1985), Lonvaud-Funel <i>et al.</i> (1991), Johansson <i>et al.</i> (1995), Du Plessis <i>et al.</i> (2004), Beneduce <i>et al.</i> (2004), Bae <i>et al.</i> (2006), Ribéreau-Gayon <i>et al.</i> (2006), Izquierdo <i>et al.</i> (2009), Ruiz <i>et al.</i> (2010)
	<i>Lb. uvarum</i>	Mañes-Lázaro <i>et al.</i> (2008b)
<i>Lb. vini</i>	Rodas <i>et al.</i> (2006)	
<i>Leuconostoc</i>	<i>Lc. mesenteroides</i>	Garvie (1979, 1983), Lonvaud-Funel & Strasser De Saad (1982), Lonvaud-Funel <i>et al.</i> (1991), Ribéreau-Gayon <i>et al.</i> (2006), Izquierdo <i>et al.</i> (2009), Ruiz <i>et al.</i> (2010)
	<i>Lc. paramesenteroides</i>	Garvie (1983)
<i>Oenococcus</i>	<i>O. oeni</i> (previously <i>Lc. oenos</i> )	Garvie (1967), Lonvaud-Funel <i>et al.</i> (1991), Du Plessis <i>et al.</i> (2004), Ribéreau-Gayon <i>et al.</i> (2006), López <i>et al.</i> (2007), Ruiz <i>et al.</i> (2008, 2010)

TABLE 2.2 (continued)

<i>Pediococcus</i>	<i>Ped. acidilactici</i>	O'Leary and Wilkinson (1988)
	<i>Ped. damnosus</i>	Back (1978), Lonvaud-Funel <i>et al.</i> (1991), Dueñas <i>et al.</i> (1995), Beneduce <i>et al.</i> (2004), Ribéreau-Gayon <i>et al.</i> (2006)
	<i>Ped. inopinatus</i>	Back (1978), Edwards and Jensen (1992)
	<i>Ped. parvulus</i>	Edwards and Jensen (1992), Davis <i>et al.</i> (1986a, b), Rodas <i>et al.</i> (2003)
	<i>Ped. pentosaceus</i>	Lonvaud-Funel <i>et al.</i> (1991), Salado and Strasser De Saad (1995), Rodas <i>et al.</i> (2003), Ribéreau-Gayon <i>et al.</i> (2006)
<i>Weissella</i>	<i>Weissella paramesenteroides</i>	Dicks and Endo (2009)

## 2.3. Identification and characterisation

It is important to be able to distinguish between different yeast and LAB species and even different strains to follow their evolution during wine production. There are various techniques that can be used to characterise microorganisms and most of them are applicable to yeast and LAB. Characterisation techniques vary, but can broadly be divided into non-molecular (physiological and biochemical) and molecular (based on DNA composition) methods. Application of some non-molecular methods can be cumbersome, labour-intensive and cannot be used for inter- and intra-species differentiation. In general, molecular techniques have made the identification at genus, species and even strain level more accurate and reliable. Some of these characterisation techniques and their application to non-*Saccharomyces* yeasts will be briefly discussed.

### 2.3.1 Non-molecular characterisation techniques

Non-molecular techniques include morphology, physiology and biochemical assimilation of a broad range of substrates and the nature of these metabolic products.

#### 2.3.1.1 Morphological and physiological tests

Colony descriptions for yeast may comprise texture, colour, surface, elevation and margin (Kurtzman *et al.*, 2011a). Biochemical and physiological tests include fermentation of different carbohydrates, growth on specific carbon and nitrogen sources, as well as other tests that assess vitamin requirements, hydrolysis of arbutin, acid production from glucose, lipase activity and various others (Kurtzman *et al.*, 2011a). Physiological features include the ability to grow at different temperatures, pH values, salt concentrations and atmospheric conditions, and growth in the presence of different chemicals (e.g. antimicrobial agents). Examples of biochemical features are the presence and activity of different enzymes and the metabolism of different

compounds (Vandamme *et al.*, 1996). Positive or negative results can be visualised by inspecting plates or tubes for growth, formation of gas or the change in pH indicators depending on the test employed (Verweij *et al.*, 1999; Kurtzman *et al.*, 2011a). Commercial kits for biochemical and enzymatic profiling are available, but these kits are usually designed for clinical microbiology and their databases are often limited with regard to yeasts associated with wine. Nonetheless, these kits have been used with varying levels of success for wine yeasts. Biochemical profiling and enzyme activity is quite useful for characterisation of yeasts when used in combination with other identification and typing techniques (Fernandez *et al.*, 1999, 2000; Jolly *et al.*, 2003a; Ortiz *et al.*, 2013; Ženišová *et al.*, 2014; Englezos *et al.*, 2015; Belda *et al.*, 2016).

#### 2.3.1.2 Fatty acid analysis

Fatty acid analysis has been used for yeast and LAB characterisation and taxonomic purposes. Polar lipids and sphingolipids are present in a restricted number of taxa are examples of fatty acids (Jones & Krieg, 1984). Fatty acids have variability of chain length, double bond position and substituent groups (Suzuki *et al.*, 1993). However, standardisation of experimental conditions and techniques is necessary for obtaining reproducible results (Augustyn & Kock, 1989; Degré *et al.*, 1989). As a result, this method was replaced by other methods. This technique has been used to distinguish between wine yeast strains (Tredoux *et al.*, 1987; Augustyn, 1989; Augustyn & Kock, 1989).

#### 2.3.1.3 Fourier transform-infrared spectroscopy

Fourier-Transform infrared (FTIR) spectroscopy is a rapid and inexpensive method that can be used to identify microorganisms (Naumann *et al.*, 1991a, b). Absorption of infrared light by cellular compounds results in a fingerprint-like spectrum that can be identified by comparison to reference spectra. Due to the ease of use and rapidity (2 to 10 minutes), a large number of yeast samples can be processed on a day (Kümmerle *et al.*, 1998, Wenning *et al.*, 2006). A disadvantage is that sophisticated, very expensive equipment is necessary. Identification is limited only by the quality of the reference spectrum library, which can be improved steadily by adding further yeast isolates to the database. Wenning *et al.* (2002) used FTIR to differentiate among *Debaryomyces hansenii* and *S. cerevisiae* strains. Grangeteau *et al.* (2016) used FTIR to study inter- and intraspecific biodiversity of non-*Saccharomyces* yeasts.

FTIR spectroscopy has also been used to find differences between yeast strains, grape cultivars and also different wines (Cozzolino *et al.*, 2006a; Osborne, 2007). Combining of FTIR spectroscopy with mathematics and chemometrics makes it possible to investigate correlations between strains, as well as their environment (Osborne, 2007). Near infrared (NIR) and mid-infrared (MIR) spectroscopy provide information about the NIR (14,000 to 4000  $\text{cm}^{-1}$ ) and MIR

(4000 to 400  $\text{cm}^{-1}$ ) regions, respectively (Smith, 2011). MIR spectra contain information arising from fundamental molecular vibrational frequencies, while in the NIR region; information arises from overtones and combinations of such vibrations, making interpretation more difficult (Cozzolino *et al.*, 2012). MIR spectroscopy has been used to detect compositional differences between food samples on the basis of molecular vibrations of various chemical groups at specific wavelengths in the MIR region of the spectrum (Cozzolino *et al.*, 2012). NIR spectroscopy has been successfully applied in authentication studies of various food types including wine (Bevin *et al.* 2006; Subramanian & Rodriguez-Saona, 2009). Cozzolino *et al.* (2012) used NIR and MIR spectroscopy coupled with pattern recognition methods to classify grape juice samples of different varieties. Several authors also used FTIR to monitor or quantify phenolic compounds during winemaking (Cozzolino *et al.*, 2004, Fragoso *et al.*, 2011; Aleixandre-Tudo *et al.*, 2018). Culbert *et al.* (2015) used attenuated total reflection (ATR) and MIR spectroscopy to classify sparkling wines on production method and style. The use of NIR to monitor alcoholic fermentation in a diverse group of beverages such as table wine, fortified wine, champagne and beer has been reported by several authors (Roger *et al.*, 2002; Blanco *et al.*, 2004; Cozzolino *et al.*, 2004, 2006a, b). In Australia vis-NIR spectroscopy has also been used to predict wine quality (Damsberg *et al.*, 2001; Cozzolino *et al.*, 2006a). MIR spectroscopy has been used to explore the possibility of grading wine samples from the Qualified Denomination of Origin (QDO) "Rioja" (Lleti *et al.*, 2005).

### 2.3.2 Molecular characterisation techniques

A number of DNA-based techniques for identification and characterisation of yeast have been developed, e.g. pulsed-field gel electrophoresis (PFGE), polymerase chain reaction (PCR) based techniques e.g. random amplified polymorphic DNA-PCR (RAPD-PCR) and ribotyping. However, most of the molecular methods require expensive equipment and require special software for comparison and analysis of generated products.

#### 2.3.2.1 Pulsed-field gel electrophoresis

PFGE is a technique whereby the intact chromosomes are subjected to alternating electric fields in an agarose gel. As the direction of the electric field changes (pulsed), chromosomal DNA is separated according to size. Due to chromosomal length polymorphism each individual strain has a unique banding pattern. Genome digestion with low frequency restriction endonucleases can also be used to investigate strain diversity and were successfully applied to distinguish between clinical *Candida albicans* isolates (Shin *et al.*, 2004; Chen *et al.*, 2005). The resultant banding pattern can be visualised by staining with ethidium bromide and viewed under ultra-violet light (Carle & Olson, 1985; Van der Westhuizen & Pretorius, 1992). This technique is reliable and can be used for differentiation on species and strain level. This technique is



typically used for differentiation of *S. cerevisiae* and is more difficult for other yeast strains. Disadvantages of this technique are that it can be labour-intensive, time-consuming (up to five days to get results) and expensive. Sipiczki (2004), and Csoma and Sipiczki (2008) used electrophoretic karyotyping to differentiate among *Candida* species, but more specifically between *C. stellata* and *C. zemplinina*. *Hanseniaspora*, *Pichia* and *Lachancea* species and strains could also be differentiated by karyotyping (Cadez *et al.*, 2002; Naumova *et al.*, 2007; Naumov & Naumova, 2009). Mitrakul *et al.* (1999) were able to differentiate between *Brettanomyces/Dekkera* strains and van Breda *et al.* (2013) were able to distinguish among *Torulaspota delbrueckii* strains using this technique.

### 2.3.2.2 Ribotyping or PCR-RFLP of rDNA

Ribotyping involves the fingerprinting of genomic DNA restriction fragments that contain all or part of the genes coding for 16S and 23S rRNA of bacteria, and 5.8S, 18S and 26S rRNA of yeast (Fig. 2.2). These areas or regions include ribosomal genes, which are grouped in tandem to form transcription units. These transcription units are repeated between 100-200 times in the genome. Other regions that are included are the internal transcribed spacer (ITS) and external transcribed spacers (ETS), which are areas that are transcribed, but not processed. The transcription units are also separated by intergenic spacers (IGS). These ribosomal regions have become the tools for identifying phylogenetic relationships between all living organisms (Kurtzman *et al.*, 2011b). This technique was used to study wine yeasts isolated from vineyards (Constanti *et al.*, 1997) and for species and intra-species differentiation of non-*Saccharomyces* yeasts (Fernández *et al.*, 1999, 2000; Capece *et al.* 2003). *Hanseniaspora* species were identified using ITS PCR (Cadez *et al.*, 2002). Beltran *et al.* (2002) and Ocón *et al.* (2010a, b) used PCR RFLP to investigate the diversity of non-*Saccharomyces* yeasts found in wineries. This technique is rapid and reliable.



FIGURE 2.2. Schematic representation of the rRNA gene cluster of yeasts with the approximate binding sites of the ITS primers (Kráková *et al.*, 2012).

### 2.3.2.3 Random amplified polymorphic DNA (RAPD)-PCR

This PCR based technique uses arbitrary primer(s), with characteristically low hybridization temperature to amplify different sequences of DNA to give variety of different fragments. Quick fingerprinting profiles are obtained, which in turn, can be used for analysis of yeast genetic relatedness or relationships (Fernandez-Espinar *et al.*, 2006). This is a fast and a straight-

forward technique that requires low amounts of genetic material and no previous knowledge of DNA sequences is needed. Disadvantages of this technique are that software analysis is needed to assist with interpreting complicated fingerprints and low reproducibility can also be a problem. Strain diversity of *S. cerevisiae* isolates from the Chianti area was investigated using primer 1283 (Sebastiani *et al.*, 2004). This technique was used to differentiate among non-*Saccharomyces* yeast species and strains isolated from spontaneous fermentations (Di Maro *et al.*, 2007; Tofalo *et al.*, 2009, 2011, 2012). This technique was also used to investigate inter- and intraspecific relationships of *Hanseniaspora* species (Cadez *et al.*, 2002; De Benedictis *et al.*, 2011), *I. occidentalis* (Di Maro *et al.*, 2007), *I. terricola* (Di Maro *et al.*, 2007), and *T. delbrueckii* strains (Canonico *et al.*, 2015).

#### 2.3.2.4 Automated ribosomal intergenic spacer analysis

Automated ribosomal intergenic spacer analysis (ARISA), also described as fluorescence PCR and capillary electrophoresis (f-ITS PCR) (Brezna *et al.*, 2010), is a culture independent PCR method that relies on the heterogeneity of the rDNA spacer region (Fisher & Triplett, 1999; Brezna *et al.*, 2010; Ghosh *et al.*, 2015). This technique has been used for yeast identification and investigation of yeast diversity in grape must and wine (Brezna *et al.*, 2010; Zenisova *et al.*, 2014; Bagheri *et al.*, 2015; Ghosh *et al.*, 2015). This technique is sensitive, suitable for rapid analysis of a large number of samples and can be used to monitor yeast population dynamics during fermentation (Brezna *et al.*, 2010; Ghosh *et al.*, 2015, Bagheri *et al.*, 2017). Disadvantages of this technique are that microorganisms cannot be identified if the DNA fragments cannot be retrieved from capillary electrophoresis; the reliable taxonomic assignment of the peaks remains a challenge (Ghosh, 2015) and specialised software are needed.

#### 2.3.2.5 High-throughput sequencing

High-throughput sequencing is revolutionizing microbial ecology studies (Caporaso *et al.*, 2010). This technique allows for the precise analysis of RNA transcripts for gene expression, reliable and precise quantification of transcripts as a tool for identification, analysis of DNA regions interacting with regulatory proteins in functional regulation of gene expression (Ansorge, 2009). The next-generation sequencing technologies also offer novel and rapid ways for genome-wide characterisation and profiling of mRNAs, small RNAs, transcription factor regions, structure of chromatin and DNA methylation patterns. Microbiology and metagenomics DNA is directly extracted from the matrices and the rRNA-encoding genes amplified for taxonomical classification (Ansorge, 2009). Yeast diversity on grapevine leaves and grapes, as well as grape must and wine has been investigated using different sequencing platforms (Bokulich *et al.*, 2014; David *et al.*, 2014; Pinto *et al.*, 2014). This technique revealed greater diversity compared to other culture-independent studies.

Whole metagenome sequencing provides the opportunity to capture all genetic information available, which include the identities of the microbial populations and provides a better understanding about the microbial community structure and function (Gosh, 2015). Disadvantages of this technique are that it can be expensive and specialised equipment and software is needed.

#### 2.3.2.6 Matrix-assisted laser desorption/ionization mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid and inexpensive method for identifying microorganisms in clinical microbiology (Posteraro *et al.*, 2013). When using MALDI-TOF for bacterial identification, no strain pretreatment is required, but pretreatment is required for yeast identification. A formic acid extraction is recommended to penetrate the thick chitinous cell walls of the yeast. In order to optimize the workflow of microbial laboratories as well as the accuracy rate, various protocols have been tested to simplify the MALDI-TOF MS pretreatment for yeast identification (Gouriet *et al.*, 2016). Besides being rapid and reliable, MALDI-TOF MS is also considered to be cost effective. However, expensive instrumentation and software are needed to interpret results. Not all wine associated yeast species are included in the MALDI-TOF database, which could limit the identification of certain yeast species. This technique has a high resolution at genus and species level and can be used for qualitative DNA analysis. These include, single nucleotide polymorphism (SNP) analysis (Little *et al.*, 1997), microsatellite analysis (Braun *et al.*, 1997), DNA sequencing (Koster *et al.*, 1996; Kirpekar *et al.*, 1998), and quantitative analysis such as allele frequency determination and gene-expression analysis (Ross *et al.*, 2000; Buetow *et al.*, 2001; Ding & Cantor, 2003). This technique has been used to identify non-*Saccharomyces* yeasts from clinical studies (Marklein *et al.*, 2009) and in wine samples (Kántor & Kačániová, 2015) as well as to differentiate between commercial *S. cerevisiae* yeast strains (Moothoo-Padayachie *et al.*, 2013; Usbeck *et al.*, 2014).

#### 2.3.3 General remarks

In the past, phenotypical and morphological test methods were used to identify and characterise yeasts and LAB, but results were sometimes ambiguous. These techniques still provide valuable information, but should be used in conjunction with molecular methods. Molecular techniques might be faster and reliable for identifying and differentiating yeast and LAB, but the use of phenotypical characteristics is still crucial to supplement genotypical data. In general, the use of FTIR, ARISA, high-throughput sequencing and MALDI-TOF MS for identification and characterisation of wine microorganisms, especially non-*Saccharomyces* yeasts, needs further investigation. Most wine research has focused on identifying non-*Saccharomyces* to species level, but has not focused on strain diversity. In terms of managing and improving wine quality, it

is important to know what species are present in a wine and especially, if a spontaneous or a partially spontaneous fermentation is planned. Spontaneous alcoholic fermentation might produce more complex wine, but there is less control over the yeasts that dominate the fermentation and a greater risk of spoilage. Research has shown that there is great diversity among non-*Saccharomyces* species, and even among strains from the same species. With the potential contributions that non-*Saccharomyces* yeast strains can make, e.g. production of enzymes with commercial applications, ability to reduce ethanol, secretion of anti-microbial compounds, etc., it is important to be able to characterise these yeasts on strain level. With the advances in technology, research is focusing on understanding the whole genome of an organism, whether it is bacteria or yeasts. Current trends are investigating the metabolome, proteome and on transcriptome. However, with all technological advancements there are challenges as well, such as reproducibility and noise. “Omics” can generate so much data that noise overwhelms signal. More innovative and complex mathematical, informatics tools are required to analyse and assist with interpretation of data.

## 2.4 Ecology of yeast and bacteria

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The different yeast or bacterial species found in a habitat can either be autochthonous (those that are essential components of the community) or allochthonous (those that are transient or there by chance) (Lachance & Starmer, 1998). The component species within yeast communities are further defined by niches *i.e.* the physical, chemical and biotic attributes required by the yeast to survive and grow. Microorganisms found in many different habitats are considered generalists (broad niche), while those found in unique habitats are considered specialists (narrow niche) (Lachance & Starmer, 1998). Within the winemaking environment, the vineyard (grape surfaces and leaves) and cellar (equipment and grape must) can be considered specialised niches.

### 2.4.1. Evolution of non-*Saccharomyces* yeast during wine production

Initially, yeasts are found on unripe grapes at low numbers of ( $10^1$ - $10^3$  colony forming units/gram), but as the grapes ripen, the population increases to  $10^4$ - $10^6$  cfu/g (Fleet, 2003). During crushing, the non-*Saccharomyces* yeasts on the grapes, on cellar equipment and in the cellar environment are carried over to the must (Peynaud & Domercq, 1959; Bisson & Kunkee, 1991; Boulton *et al.*, 1996; Torok *et al.*, 1996; Constanti *et al.*, 1997; Mortimer & Polsinelli, 1999; Fleet, 2003; Alessandria *et al.*, 2015; Capozzi *et al.*, 2015). According to Jolly *et al.* (2014) the non-*Saccharomyces* yeasts found in grape must and during fermentation can be divided into three groups:

- (i) yeasts that are largely aerobic, for example, *Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Candida* spp., and *Cryptococcus albidus*;

(ii) apiculate yeasts with low fermentative activity, for example, *H. uvarum* (*K. apiculata*), *H. guilliermondii* (*K. apis*), *H. occidentalis* (*K. javanica*); and

(iii) yeasts with fermentative metabolism, for example, *Kluyveromyces marxianus* (*C. kefyr*), *T. delbrueckii* (*C. colliculosa*), *M. pulcherrima* (*C. pulcherrima*) and *Z. bailii*

During fermentation, especially spontaneous fermentations, which do not have the initial high-density of *S. cerevisiae*, there is a sequential succession of yeasts. Initially, species of *Hanseniaspora* (*Kloeckera*), *Rhodotorula*, *Pichia*, *Candida*, *Metschnikowia* and *Cryptococcus* are found at low levels in fresh must (Parish & Carroll, 1985; Bisson & Kunkee, 1991; Frezier & Dubourdieu, 1992; Granchi *et al.*, 1998; Fleet, 2003; Combina *et al.*, 2005). Frequently, *H. uvarum* is present at the highest numbers, followed by various *Candida* spp. This is usually more apparent in red must than white, possibly due to the higher pH of red must. However, there are exceptions and *Hanseniaspora* can be present at low levels or also be absent (Van Zyl & Du Plessis, 1961; Parish & Carroll, 1985; Jolly *et al.*, 2003a, 2006). Jolly *et al.* (2003a) reported that *H. uvarum*, *C. stellata*, *M. pulcherrima* and *T. delbrueckii* were predominant (>50%) before the start of fermentation in grape must samples of South Africa and ranged from  $2 \times 10^3$  to  $1 \times 10^6$  cells/mL. Di Maro *et al.* (2007) reported that *I. occidentalis*, *L. thermotolerans* (formerly *K. thermotolerans*), *Z. bailii* still occurred at the end of alcoholic fermentation. Despite the continued presence of certain non-*Saccharomyces* yeast, most disappear with the onset of fermentation (Fleet *et al.*, 1984; Henick-Kling *et al.*, 1998). This might be due to their slow growth and inhibition by the combined effects of low pH, sulphur dioxide (SO<sub>2</sub>), oxygen deficiency and high ethanol (Heard & Fleet, 1988; Combina *et al.*, 2005). Nutrient limitation and size or dominance of *S. cerevisiae* inoculum can also have a suppressive effect, sometimes separate from temperature or ethanol concentration (Granchi *et al.*, 1998). *T. delbrueckii* and *L. thermotolerans* are less tolerant to low oxygen levels and this, rather than ethanol toxicity, affects their growth and leads to their death during fermentation (Holm Hansen *et al.*, 2001; Lachance & Kurtzman, 2011).

#### 2.4.2 Evolution of lactic acid bacteria during wine production

In general, the LAB on the surface of grapes and vine leaves, occur at low numbers (<10<sup>4</sup> cfu/g), depending on the maturity and condition of the berries and vines (Wibowo *et al.*, 1985; Sponholz, 1993; Lonvaud-Funel, 1995, 1999). Grapes spoiled by acetic acid bacteria and fungi stimulate LAB growth (Fugelsang, 1997). There appears to be a link between grape cultivar and LAB species as shown by Bae *et al.* (2006). These authors investigated LAB associated with Australian wine grapes and found that *Lb. lindneri* was the main species isolated from Cabernet Sauvignon, Merlot and Shiraz, while *Lb. plantarum* and *Lb. mali* were also present on the Cabernet Sauvignon grapes. *Lactobacillus lindneri* and *Lb. kunkeei* were the main species present on Chardonnay, Semillon and Sauvignon blanc grapes. Winery equipment, such as

storage tanks, pumps, valves and transfer lines, wood barrels and bottling machines have also been implicated as sources of LAB (Webb & Ingraham 1960; Gini & Vaughn 1962; Wibowo *et al.*, 1985; Fugelsang, 1997; Ribéreau-Gayon *et al.*, 2006; Du Toit *et al.*, 2011).

Lactic acid bacteria are usually present at  $10^2$  to  $10^3$  cells/mL in grape must and may increase or decrease during fermentation (Fleet *et al.*, 1984; Ribéreau-Gayon *et al.*, 2006). In South African grape juice used for brandy base wines, LAB population were found to range between  $2 \times 10^2$  to  $8 \times 10^5$  cfu/mL (du Plessis *et al.*, 2002). During the first days of alcoholic fermentation, the LAB and yeasts multiply, but the yeasts are better adapted to grape must and rapidly dominate (Ribéreau-Gayon *et al.*, 2006). During this time, the LAB also multiplies, but their growth remains limited, reaching a maximum population of  $10^4$  to  $10^5$  cfu/mL. To a large extent LAB behaviour at this time depends on the pH of the must and the sulphur level (Ribéreau-Gayon *et al.*, 2006). After alcoholic fermentation, LAB can reach a level of  $10^7$  cfu/mL to conduct the MLF. LAB can still remain in the wine at  $10^{3-4}$  cfu/mL one to two months later. Malolactic fermentation only commences when the LAB population reaches  $10^6$  cfu/mL (Wibowo *et al.*, 1985; Lonvaud-Funel, 1999).

During fermentation, LAB populations evolve not only in numbers, but also in terms of species that may occur (Lonvaud-Funel *et al.*, 1991; Ribéreau-Gayon *et al.*, 2006). Grape juice contains diverse populations and the major species present at this stage include *Lb. plantarum*, *Lb. casei*, *Lb. hilgardii*, *Lb. brevis*, *Lc. mesenteroides*, *O. oeni*, *Ped. damnosus* and *Ped. pentosaceus* (Costello *et al.*, 1983; Lafon-Lafourcade *et al.*, 1983; Fleet *et al.*, 1984; Wibowo *et al.*, 1985; Fugelsang, 1997; Ribéreau-Gayon *et al.*, 2006; Du Toit *et al.*, 2011). All the LAB species are not always present, but the natural diversity changes during alcoholic fermentation. Usually, there is a successional growth of several LAB species during vinification (Wibowo *et al.*, 1985; Ribéreau-Gayon *et al.*, 2006). *Lactobacillus* species, *Pediococcus* and *Leuconostoc* progressively disappear during the alcoholic fermentation, or are present at a concentration that is too low to be detected. In most cases, *O. oeni* predominates at the end and after alcoholic fermentation and is responsible for conducting MLF (Wibowo *et al.*, 1985; Lonvaud-Funel 1999; Ribéreau-Gayon *et al.*, 2006; López *et al.*, 2007; Lerm *et al.*, 2010; Du Toit *et al.*, 2011). Several LAB species are able to perform MLF, but *O. oeni* is especially well adapted to tolerate the harsh conditions in wine, which include low nutrient content, low pH, high ethanol concentration and presence of  $\text{SO}_2$  (Versari *et al.*, 1999; Bartowsky *et al.*, 2002).

## 2.5 Malolactic fermentation

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The MLF reaction is called a secondary fermentation, but it is an enzyme-mediated decarboxylation of L-malic acid, a dicarboxylic acid, to L-lactic acid, a monocarboxylic acid, with the production of  $\text{CO}_2$  (Kunkee, 1967; Wibowo *et al.*, 1985; Lonvaud-Funel, 1995; Ribéreau-Gayon *et al.*, 2006; Lerm *et al.*, 2010). Lactic acid bacteria possess three possible enzymatic

pathways for the conversion of malic acid to lactic acid and CO<sub>2</sub> (Lerm *et al.*, 2010). Firstly, the direct conversion of L-malic acid to L-lactic acid via malate decarboxylase, also known as the malolactic enzyme (MLE), which requires NAD<sup>+</sup> and Mn<sup>2+</sup> as cofactors and produces no intermediates. Most wine LAB utilise this pathway to generate lactic acid (Lerm *et al.*, 2010). Secondly, L-malic acid is converted to pyruvic acid using the malic enzyme, which is subsequently reduced by L-lactate dehydrogenase to lactic acid. Thirdly, malate is reduced by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate and finally, reduction to lactic acid (Lonvaud-Funel, 1999). Unlike the formation of lactic acid from glucose, only the L-isomer is produced during MLF (Wibowo, 1985; Lonvaud-Funel, 1995, 1999).

The major physiological function of the malate fermentation pathway is to generate a proton motive force (PMF) as a means to acquire energy to drive essential cellular processes (Cox & Henick-Kling 1989, 1990; Henick-Kling, 1993; Fugelsang, 1997; Versari *et al.*, 1999; Lerm *et al.*, 2010).

### 2.5.1 Benefits of malolactic fermentation

Malolactic fermentation is necessary for most red wines and preferred for only some sparkling and white wines, such as Chardonnay (Bartowsky *et al.*, 2002, 2015). The main benefits of MLF are deacidification, improvement of microbiological stability and flavour modification. Deacidification of wine is beneficial and necessary in wines with high acid levels, but in countries and regions with high temperatures and low acid levels, reduction of the total acids could result in flat tasting wines and the growth of spoilage bacteria due to a higher pH (Davis *et al.*, 1985; Bauer & Dicks, 2004). Reduction in wine acidity by MLF may vary from 0.1 to 0.3% and the pH may increase by 0.1 to 0.3 of a unit (Davis *et al.*, 1985; Ribéreau-Gayon *et al.*, 2006). Reduction in acidity and increase in pH have a sensorial effect of softening the palate, improving smoothness and drinkability of the wines (Bartowsky *et al.*, 2002; Jackson, 2008).

Microbiological stability of the wines is obtained when all residual nutrients left after alcoholic fermentation are metabolised. Malic and citric acids are consumed and the more stable tartaric acid and lactic acid remains. In addition, the complex nutrient demands of LAB also reduce the concentrations of amino acids, other nitrogen compounds and vitamins (Davis *et al.*, 1985; Ribéreau-Gayon *et al.*, 2006; Jackson 2008).

Wine flavour can also be affected by the formation of organoleptically active compounds arising from LAB metabolism (Bartowsky *et al.*, 2002). The most frequently reported aroma modification associated with MLF consists of an increase of wine “buttery” character (Bartowsky & Henschke, 1995). Both, wine aroma and flavour can be affected by LAB via several mechanisms including (i) the removal of flavour compounds by metabolism and adsorption to the cell wall; (ii) the production of new volatiles from the metabolism of grape sugars, amino acids, organic acids and other nutrient compounds; and (iii) the metabolism or extracellular

modification of grape and yeast secondary metabolites, to either more or less flavoured metabolites (Bartowsky & Henschke, 1995). In support of these possible mechanisms, wine LAB have diverse genetic properties and possess a variety of enzymes that could potentially be involved in converting grape-derived (Hernandez-Orte *et al.*, 2009), yeast-derived (Ugliano & Moio, 2005) or wood-derived (de Revel *et al.*, 2005) precursor compounds into aroma compounds (Liu, 2002; Matthews *et al.*, 2004; Mtshali *et al.*, 2010). Many acids, alcohols, esters and carbonyl compounds have been associated with MLF and their production is greatly dependent on strain characteristics, cultivar selection and fermentation conditions (Bartowsky & Henschke, 1995; Ugliano & Moio, 2005; Lerm *et al.*, 2010).

Several attributes, such as an increased buttery and a reduced vegetative character and improved mouth feel and flavour persistence, are associated with MLF (Laurent *et al.*, 1994; Bartowsky & Henschke, 1995). Sauvageot and Vivier (1997) reported that MLF increased the hazelnut, fresh bread and dried fruit aromas of Chardonnay wines, whereas Pinot Noir wines partially lost their berry notes in favour of animal and vegetative aromas. Other attributes associated with MLF are caramel, fruity and sweaty flavour (Bartowsky & Henschke 1995). Increases in descriptors, such as buttery, were commonly observed for wines that completed MLF, while burnt sweet aroma, citrus, fruity, maple syrup and sweaty were less common. Attributes that were reported to decrease during MLF are banana, burnt sweet, buttery, citrus, fruity and floral (Bartowsky & Henschke, 1995). Increased or decreased fruity notes in red wines was shown to be dependent on the LAB strain used (Antalick *et al.*, 2012). Shiraz wines that underwent MLF contained higher concentrations of compounds associated with fruity descriptive attributes than wines that did not undergo MLF (Abrahamse & Bartowsky, 2012a). Cabernet Sauvignon wine where *Lb. plantarum* was used to induce MLF had prominent 'berry-fruity' sensory attributes (Bartowsky *et al.*, 2012). Costello *et al.* (2012) reported that MLF had a significant effect on dark fruit aroma, viscosity and astringency of Cabernet Sauvignon wine. Different LAB strains may increase or decrease the intensity of wine aroma/flavour attributes (Antalick *et al.*, 2012, 2013; Capello *et al.*, 2017).

### **2.5.2 Induction of malolactic fermentation**

Despite the considerable amount of published research, MLF still remains a difficult process to control and at times inducing MLF can be a problem (Krieger & Arnink, 2003). One possible explanation for this difficulty is that the wine may be lacking essential nutrient factors needed for LAB growth. Another argument is that inhibitory compounds are produced and accumulate during fermentation. Factors such as SO<sub>2</sub>, alcohol concentrations and pH are among the most significant parameters affecting LAB growth.



### 2.5.2.1 Spontaneous malolactic fermentation

Malolactic fermentation can occur spontaneously, induced by the wine LAB that are naturally present in the grape must or wine (Lafon-Lafourcade *et al.*, 1983). The advantage of spontaneous MLF is that the naturally occurring LAB should be better adapted to wine conditions. The potential risks associated with spontaneous MLF include the presence of unidentified/spoilage LAB that can produce undesirable or off-flavours, the production of biogenic amines (Davis *et al.*, 1985; Lonvaud-Funel, 1999), delays in the onset or completion of MLF (Nielsen *et al.*, 1996) and the development of bacteriophages (Bauer & Dicks, 2004); all of which contribute to a decrease in wine quality.

### 2.5.2.2 Use of starter cultures

The use of commercial starter cultures reduces the risk of potential spoilage LAB or bacteriophages, promotes the rapid start and completion of MLF and also results in a positive flavour contribution by the LAB (Krieger-Weber, 2009). Industrial MLF starter cultures of *O. oeni* have been available for some years (Davis *et al.*, 1985; Henick-Kling, 1993; Krieger-Weber, 2009; Lerm *et al.*, 2010; Bartowsky *et al.*, 2015). In review articles by Du Toit *et al.* (2011) and Bartowsky *et al.* (2015) the use of alternative LAB species are discussed. The use of a *Lactobacillus* strain ML-30 was successfully used in inoculation timing trials in Pinot Noir in the early 1960s (Bartowsky *et al.*, 2015), and a commercial *Lb. plantarum* strain (Viniflora plantarum, CHR Hansen) was promoted in the late 1980s (Henschke, 1989) for inoculation prior to alcoholic fermentation (Prahl, 1988). Suppliers of *Lb. plantarum* starters recommended pre-alcoholic inoculation of the starter cultures. However, there is no peer-reviewed literature comparing this inoculation regime with co- or sequential inoculation (Bartowsky *et al.*, 2015). A *Lb. plantarum*, V22, starter culture of was released to market around 2010 by Lallemand Inc. and was recommended for use in high pH red wines (Fumi *et al.*, 2010; Du Toit *et al.*, 2011). This *Lb. plantarum* strain was initially selected for its ability to reduce ochratoxin A and it efficiently conducted MLF in high pH wines (Fumi *et al.*, 2010). The *Lb. plantarum* strain V22 was included in a genetic screening of winemaking LAB, mostly *O. oeni* for relevant enzymatic activities and it was shown to possess more diverse enzymatic profiles related to aroma than *O. oeni* (Mtshali *et al.*, 2010). Therefore, *Lb. plantarum* may have a different impact than *O. oeni* on certain sensory properties of wine (Du Toit *et al.*, 2011). Another *Lb. plantarum* strain (NoVA) from Chr. Hansen has recently been released to the market (Saerens *et al.*, 2015).

Even with the use of commercial starter cultures, complete and successful MLF is not always guaranteed, especially under very difficult wine conditions (*i.e.* low pH, high ethanol) (Guerzoni *et al.*, 1995; Krieger & Arnink, 2003). Some of the factors affecting successful MLF include adhering to the instructions of the manufacturers. Winemakers should also check that

the commercial starter culture to be used can tolerate the physiochemical properties of the wine (e.g. the ability to tolerate high alcohol concentrations and sensitivity to pH) (Lerm *et al.*, 2010).

### 2.5.3 Timing of inoculation

A major consideration for optimising MLF has been to determine the optimal point for inoculation (Bartowsky *et al.*, 2015). Currently, there are four inoculation scenarios for MLF: (i) simultaneous inoculation for AF and MLF (co-inoculation), (ii) inoculation during AF, (iii) inoculation after the completion of AF (sequential inoculation) and (iv) inoculation prior to yeast (pre-AF) (Lerm *et al.*, 2010; Bartowsky *et al.*, 2015). The practice in industry has largely been to use sequential inoculations. However, the simultaneous inoculation strategy has received more attention recently, because of potential advantages this strategy has (Liu, 2002; Costello, 2006; Abrahamse & Bartowsky 2012a, b; Muñoz *et al.*, 2014; Guzzon *et al.*, 2015; Izquierdo Cañas *et al.*, 2015; Tristezza *et al.*, 2016; Versari *et al.*, 2016). Inoculation of certain *Lb. plantarum* strains in grape must prior to yeast inoculation has also received some attention (Bartowsky *et al.*, 2015), but it is not a common practice.

One of the main advantages of using simultaneous instead of sequential inoculation is the reduction in overall vinification time (Edwards & Beelman, 1989; Abrahamse & Bartowsky 2012a, b; Bartowsky *et al.*, 2015). This means that the wines can be stabilised sooner, which reduces wine production time. A shorter production time reduces the risk of spoilage and ensures that winery resources (e.g. tank space) are freed up, thereby minimising bottlenecks in processing (Bartowsky *et al.*, 2015). Other advantages of simultaneous inoculation are that there is no or very low levels of alcohol and the nutrient content of the juice or must should still be high, making it easier for the LAB to grow. The possible risks of simultaneous inoculation are the development of undesirable/antagonistic interactions between yeast and/or bacteria, stuck AF and the production of possible off-flavours (Henick-Kling & Park, 1994; Alexandre *et al.*, 2004). Muñoz *et al.* (2014) compared two commercial *S. cerevisiae* yeast strains in simultaneous and sequential inoculations with a commercial *O. oeni* culture and all sequential fermentations went to completion. Wine produced with simultaneous inoculation finished MLF in a much shorter time than those that underwent sequential inoculation. However, one of *S. cerevisiae* strains did not complete the alcoholic fermentation. This shows how important it is to choose the correct yeast and LAB combination for co-inoculated fermentations. However, this is not only applicable to simultaneous inoculations, but to sequential inoculations as well (Bartowsky *et al.*, 2015).

Several researchers have used simultaneous inoculation in the production of many red and some white wines and simultaneous inoculation strategies have been found to benefit production of Pinot Noir (Krieger, 2002; Christen & Mira de Orduña, 2010), Shiraz (Abrahamse & Bartowsky, 2012b), Cabernet Sauvignon (Guzzon *et al.*, 2013), Tannat (Muñoz *et al.*, 2014),

Merlot (Izquierdo Cañas *et al.*, 2012, Antalick *et al.*, 2013), Cabernet Franc (Izquierdo Cañas *et al.*, 2015), Tempranillo (Izquierdo Cañas *et al.*, 2012), Chardonnay and Riesling (Knoll *et al.*, 2011, 2012), Teroldego and Marzemino (Guzzon *et al.*, 2013), Malbec (Massera *et al.*, 2009), Amarone (Zapparoli *et al.*, 2009) and Nero di Troia (Garofalo *et al.*, 2015). Researchers have reported MLF completing in a shorter time period compared to that of sequential inoculation, no negative impact on fermentation success or kinetics and no difference in the final wine quality (Jussier *et al.*, 2006; Zapparoli *et al.*, 2009; Abrahamse & Bartowsky, 2012a, b; Lerm *et al.*, 2012; Guzzon *et al.*, 2015; Versari *et al.*, 2016). It has also been shown that wines that have undergone simultaneous AF/MLF tend to be less buttery, retain more fruitiness and are therefore more complex and better structured (Henick-Kling, 1993; Jussier *et al.*, 2006; Krieger, 2006; Versari *et al.*, 2016). Additionally to improving MLF efficiency, the wine sensory profile following simultaneous inoculation of LAB and wine yeast can differ from that of sequential inoculation (Bartowsky *et al.*, 2015). Mendoza *et al.* (2011) investigated the interaction between a strain of *H. uvarum* (*K. apiculata*), *S. cerevisiae* and *O. oeni*, as simultaneous and sequential inoculations, in Malbec wine. These authors reported that wines that were simultaneously fermented by yeasts and *O. oeni* scored the highest for phenolic aroma and consequently obtained the lowest score for most of the sensory descriptors. Whereas, wines that were sequentially inoculated with *O. oeni* had the highest acceptance, better fruity and floral aromas and highest score for equilibrium-harmony.

Muñoz *et al.* (2014) proposed that specific yeast and LAB interactions may differ between different timings of LAB inoculation. In addition, co- and sequential fermentations have the potential to affect wine style by modifying the profiles of wine volatiles and sensory properties. Yeast strain selection is an important consideration for successful wine production, but there is growing evidence that optimal combinations may indeed differ for co- and sequential fermentations (Bartowsky *et al.*, 2015). However, there are contradicting data about what MLF inoculation strategy to follow to improve wine flavour and quality.

## **2.6. Factors affecting lactic acid bacteria growth and malolactic fermentation**

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### **2.6.1. Physicochemical factors**

#### 2.6.1.1 pH

The pH of the must or wine impacts on the growth of LAB, with values above pH 3.5 favouring the growth of *Lactobacillus* and *Pediococcus* species, whereas *O. oeni* strains tend to dominate at lower pH values (Davis *et al.*, 1986b; Henick-Kling, 1993). *O. oeni* exhibits the greatest tolerance to pH, with strains being able to degrade L-malic acid at a pH below 3.0 (Davis *et al.*, 1988; Henick-Kling *et al.*, 1989; Ribéreau-Gayon *et al.*, 2006). Lactic acid bacteria can grow at a pH of 2.9-3.0, but their growth is slow (Ribéreau-Gayon *et al.*, 2006). The optimum pH for

growth and catabolism of glucose by *O. oeni* and *Lb. plantarum* are between 4.5 and 6.0 (Henick-Kling, 1986). In addition, *Lb. plantarum* has a preference for malate as an energy source at low pH, even in the presence of glucose (Guerzoni *et al.*, 1995). Vailiant *et al.* (1995) studied the effect of some physicochemical factors on malolactic activity and found that the *Lb. plantarum* strain was affected more by a low pH than *O. oeni*. *Lb. plantarum* and *O. oeni* strains were both able to grow at a pH 3.2 (G-Alegría *et al.*, 2004). Wines in the pH range of 3.5-4.0 generally show a more rapid onset and completion of MLF than wines in the pH range of 3.0-3.5 (Costello *et al.*, 1983; Wibowo *et al.*, 1985). The pH not only affects growth, but also the malolactic activity of the entire cell. The optimum malolactic activity of *O. oeni* strains is at a pH between 3.0 and 3.2 and around 60% of its maximum activity at pH 3.8 (Ribéreau-Gayon *et al.*, 2006). Gockowiak and Henschke (2003) showed that the interaction between pH, alcohol and cultivar (wine matrix) had notable effect on the rate of malic acid degradation, but the effect was also dependent on the LAB strain used. These authors showed that the wine matrix (grape cultivar or wine composition) had a greater impact than pH and alcohol on progression of MLF, followed by pH and alcohol, respectively. These authors also showed that the rate of malic acid degradation varied between LAB strains and that red wines had a higher rate of malic acid degradation than white wines.

#### 2.6.1.2 Sulphur dioxide

Sulphur dioxide is used in winemaking as an antioxidant and to control the growth of wild yeast and spoilage bacteria. In wine, SO<sub>2</sub> enters a pH-dependent equilibrium consisting of bound SO<sub>2</sub>, molecular SO<sub>2</sub>, bisulphite (HSO<sub>3</sub><sup>-</sup>) and sulphite ions (SO<sub>3</sub><sup>2-</sup>). Together, these different forms represent the total level of SO<sub>2</sub> (Wibowo *et al.*, 1985; Fugelsang & Edwards, 2006; Ribéreau-Gayon *et al.*, 2006; Du Toit *et al.*, 2011). Molecular SO<sub>2</sub> is most effective antiseptic form at lower pH values and the only form of SO<sub>2</sub> that can cross bacterial cell walls via diffusion (Carr *et al.*, 1976; Romano & Suzzi, 1993b; Ribéreau-Gayon *et al.*, 2006). Inside the cells, the molecular SO<sub>2</sub> is converted to bisulphite and may react with various cell components like proteins and affect the growth of LAB (Carreté *et al.*, 2002; Bauer & Dicks, 2004). Wells and Osborne (2011) investigated the impact of the production of SO<sub>2</sub> and SO<sub>2</sub>-binding compounds by wine yeast on MLF and significant differences between the yeast strains in the amount of SO<sub>2</sub>, acetaldehyde and pyruvic acid produced were found. These authors reported that high total SO<sub>2</sub> concentrations inhibited MLF and that the free SO<sub>2</sub> was insignificant. Bound SO<sub>2</sub> rather than free SO<sub>2</sub> was responsible for MLF inhibition and acetaldehyde-bound SO<sub>2</sub> was determined to be the likely source of inhibition (Wells & Osborne, 2011). In a follow-up study, it was found that acetaldehyde-bound and pyruvic acid-bound SO<sub>2</sub> had a bacteriostatic rather than bacteriocidal effect (Wells & Osborne, 2012). These authors also reported that *O. oeni* was the most sensitive of the LAB tested against pyruvic acid-bound SO<sub>2</sub>. Bacterial inhibition by bound SO<sub>2</sub> can be

attributed to degradation of the binding compound and the subsequent release of free SO<sub>2</sub> (Osborne *et al.*, 2000, 2006).

Total SO<sub>2</sub> concentrations of >100 mg/L, bound SO<sub>2</sub> of >50 mg/L or free SO<sub>2</sub> levels of 1 to 10 mg/L, is sufficient to inhibit the growth of LAB (Rankine & Bridson 1971; Somers & Wescombe 1982; Wibowo *et al.*; 1985; Lerm *et al.*, 2010). These values vary depending on the species (Fornachon, 1963), wine pH (Liu & Gallander, 1983). A combination of low pH (pH 3.2) and high SO<sub>2</sub> concentration (26 mg/L) had a strong inhibitory effect on *O. oeni* MLF starter cultures (Nielsen *et al.*, 1996).

#### 2.6.1.3 Temperature

Temperature affects the growth rate of all microorganisms, chemical and biochemical reactions (Ribéreau-Gayon *et al.*, 2006). The optimum growth temperature for *O. oeni* is reported as 27 to 30°C, but due to the presence of alcohol in wine, the optimum growth temperature in wine decreases to between 20 and 23°C (Britz & Tracey, 1990; Henick-Kling, 1993; Bauer & Dicks, 2004; Ribéreau-Gayon *et al.*, 2006). The optimal decarboxylation of malic acid occurs between 20 and 25°C (Ribéreau-Gayon *et al.*, 2006). G-Alegría *et al.* (2004) found that both *O. oeni* and *Lb. plantarum* are able to survive at 18°C, but temperatures below 18°C delay the onset of MLF and increase the duration of MLF, whereas temperatures below 16°C inhibit the growth of *O. oeni* as well as leading to a decrease in cellular activity (Henick-Kling, 1993; Ribéreau-Gayon *et al.*, 2006). Growth and MLF are strongly inhibited by a low temperature and only a few strains of *O. oeni* can conduct MLF below 15°C (Wibowo *et al.*, 1985). Ribéreau-Gayon *et al.* (1975) proposed that once MLF has started at 15 °C it may proceed at lower temperatures but at a slower rate. Guerzoni *et al.* (1995) studied the effects of pH, SO<sub>2</sub>, ethanol concentration and temperature on *Lb. plantarum* and *O. oeni* growth and malolactic activity. These authors found that an increase in temperature positively affected the lag phase of *O. oeni*, but not *Lb. plantarum*. Malolactic fermentation is rarely observed at temperatures below 10°C (Wibowo *et al.*, 1985).

#### 2.6.1.4 Ethanol

Lactic acid bacteria isolated from wine can be inhibited at alcohol levels around 8–10% v/v (Ribéreau-Gayon *et al.*, 2006). In their review, Wibowo *et al.* (1985) reported that the ability of LAB to survive and grow in wine decreases as the alcohol concentration increases above 10% v/v. Alcohol tolerance decreases with an increase in temperature and at low pH values (Lafon-Lafourcade *et al.*, 1983; Knoll *et al.*, 2011). Knoll *et al.* (2011) showed that duration of MLF for different ethanol and pH combinations varied, dependent on the *O. oeni* strain used. According Henick-Kling (1993) optimum LAB growth in the presence of 10% to 14% v/v occurred between 18 and 20°C, whereas optimum growth at 30°C is achieved at 0% to 4% v/v ethanol,

demonstrating the strong impact of higher temperatures on ethanol toxicity. *Lactobacillus* and *Pediococcus* species are known to be more ethanol tolerant than *O. oeni* (Wibowo *et al.*, 1985; Davis *et al.*, 1988). Guerzoni *et al.* (1995) found that *Lb. plantarum* was more resistant than *O. oeni* to the combined action of various stresses (pH, temperature, ethanol and malate concentrations) when ethanol was lower than 6% (v/v). These authors showed that *Lb. plantarum* is more competitive in the early stages of alcoholic fermentation, but ethanol concentrations above 6% (v/v) favour *O. oeni*. Berbegal *et al.* (2016) evaluated 62 *Lb. plantarum* for their abilities to grow and conduct MLF under different conditions and investigated the sugar, pH, and ethanol tolerance of these strains. These authors showed that growth was strain dependent, but none of the strains could grow at 10% or 12% v/v ethanol.

#### 2.6.1.5 Nutritional requirements

The nutritional status of wine is crucial for LAB growth and availability of some nutrients is therefore more important than others (Fugelsang & Edwards, 2006; Théodore *et al.*, 2005). Lactic acid bacteria have been described as 'fastidious' with regard to their nutritional requirements as a result of their limited biosynthetic capabilities (Fugelsang & Edwards, 2006; Théodore *et al.*, 2005; Terrade *et al.*, 2009). Amino acids, and often peptides, supply LAB with their assimilable nitrogen requirements (Fugelsang, 1997). Amino acid requirements vary with respect to the species and even the strain. These amino acids may be strictly indispensable or simply growth activators (Fugelsang, 1997). Terrade and Mira de Orduña (2009) investigated the essential nutrient requirements of wine-related strains from the genera *Oenococcus* and *Lactobacillus*. Essential nutrient requirements were found to be strain specific and 10 compounds were essential for all wine LAB tested: carbon and phosphate sources, manganese, amino acids (proline, arginine, valine, leucine and iso-leucine) and vitamins (nicotinic and pantothenic acids). These authors also showed that *O. oeni* strains revealed a greater number of auxotrophies and had a higher degree of nutritional similarity than *Lactobacillus* species. The two *Lactobacillus* spp. were auxotrophic for riboflavin, which was not needed by the *O. oeni* strains (Terrade & Mira de Orduña, 2009). These authors showed that the *O. oeni* strains were more sensitive to amino acid deficiency than the *Lactobacillus* strains, which were more reliant on vitamin supply.

#### 2.6.1.6 Phenolic compounds

Certain grape tannins can have an inhibitory effect on LAB and therefore on MLF. Some research has indicated that certain red cultivars, such as Merlot, can have great difficulty undergoing successful MLF (Du Toit *et al.*, 2011). Polyphenols tested alone or in a mixture had an inhibitory effect on LAB and gallic acid stimulated yeasts and LAB, while different phenolic acids (coumaric, protocatechic acid, etc.) and condensed anthocyanins inhibited these

microorganisms (Ribéreau-Gayon *et al.*, 2006). Vivas *et al.* (1997) reported that vanillic acid inhibited cell viability and that free anthocyanins stimulated LAB and MLF. Caffeic acid at a concentration of 50 and 100 mg/L had a positive effect on bacterial growth and degradation of malic acid (Du Toit *et al.*, 2011). On the other hand, ferulic acid can affect bacterial growth and malic acid degradation detrimentally, but is strain dependent. The inhibitory effect of *p*-coumaric acid was the greatest and increased with concentration. Campos *et al.* (2009a, b) investigated the effect of phenolic acids on glucose and organic acid metabolism of an *O. oeni* and *Lb. hilgardii* strain. These authors found that most of the phenolic acids tested, strongly inhibited the growth of *O. oeni*, but that the malolactic activity of this strain was not affected. However, *Lb. hilgardii* was less affected in its growth, but the capacity to degrade malic acid was clearly diminished.

Oligomer procyanidins, which are extracted from grape seeds, are powerful inhibitors and affected LAB viability in non-growing conditions, during LAB growth as well as malolactic activity (Vivas *et al.*, 2000). These authors also showed that pure ellagitannins were beneficial to the viability of *O. oeni*, while the total oak extract was also a powerful inhibitor. Curiel *et al.* (2010) reported that quercetin had a positive effect on the fermentation capacity of *Lb. plantarum*. Quercetin was not catabolised by *Lb. plantarum*, so the antioxidant properties of the flavonol were protected against degradation. Landete *et al.* (2007) showed that *Lb. plantarum* was able to grow in the presence of high concentrations of some wine phenolic compounds. Of the ten compounds analysed, only the hydroxycinnamic acids, gallic acid and methyl gallate were metabolised by the *Lb. plantarum* strains studied.

Hernández *et al.* (2006) reported that during MLF, the LAB hydrolysed the tartaric esters of caffeic acid (caftaric acid) and *p*-coumaric acid (coutaric acid), which coincided with the increase in the corresponding free acids. Hernández *et al.* (2007) examined the effect different *O. oeni* and *Lb. plantarum* starter cultures had on non-anthocyanin phenolic composition during red wine production. Differences in malolactic behaviours for *O. oeni* and *Lb. plantarum* were observed in relation to wine phenolic compositions. The hydroxycinnamic acids and their derivatives, the flavonols and their glycosides, the flavanol monomers and oligomers, and trans-resveratrol and its glucoside were the main compounds modified by the different LAB (Hernández *et al.*, 2007). These authors also reported that the natural occurring LAB population exerted a greater effect on some of these phenolic compounds in the wines than the inoculated starter cultures.

## 2.6.2 Biological factors

### 2.6.2.1 Interactions between yeasts

Winemakers conducting spontaneous fermentations (comprising mixed and sequential dominance of non-*Saccharomyces* and *Saccharomyces* yeasts), view indigenous yeasts as

integral to the authenticity of their wines imparting desired regional characteristics (Amerine *et al.*, 1972; Jolly *et al.*, 2014). The role that non-*Saccharomyces* yeasts play in winemaking has changed over the last few years and several studies have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeast combinations (Lema *et al.*, 1996; Ciani & Maccarelli, 1998; Heard, 1999; Jolly *et al.* 2003b, c; Ciani *et al.*, 2010; Ciani *et al.*, 2011; Comitini *et al.*, 2011). When yeasts develop together under fermentation conditions, they do not passively co-exist but interact and produce different fermentation compounds and/or at different levels, which can affect the chemical and aromatic composition of wines (Howell *et al.*, 2006; Anfang *et al.*, 2009). Several types of interactions may occur and include: commensalism, synergism and antagonism.

Several studies have shown that microbial interactions play an important role in the dominance of *S. cerevisiae* during mixed-culture alcoholic fermentations and consequently in death or inhibition of non-*Saccharomyces* yeasts (Nissen & Arneborg, 2003; Nissen *et al.*, 2003; Pérez-Navado *et al.*, 2006; Albergaria *et al.*, 2010; Renault *et al.*, 2013; Branco *et al.*, 2014; Taillandier *et al.*, 2014; Kemsawasd *et al.*, 2015a, b; Wang *et al.*, 2015). Non-*Saccharomyces* yeasts can survive and ferment sugars for much longer when in single-culture than in mixed culture fermentations with *S. cerevisiae* (Nissen & Arneborg, 2003; Pérez-Navado *et al.* 2006; Albergaria, 2007). Different death-inducing mechanisms exist, which include cell-to-cell contact (Nissen & Arneborg, 2003; Nissen *et al.*, 2003) and secretion of antimicrobial compounds (Pérez-Navado *et al.*, 2006; Albergaria *et al.*, 2010). The involvement of these mechanisms in antagonistic interactions by *S. cerevisiae* against non-*Saccharomyces* yeasts during wine fermentations has been confirmed by other researchers (Renault *et al.*, 2013; Branco *et al.*, 2014; Kemsawasd *et al.*, 2015a). *M. pulcherrima* can have an antagonistic effect on several yeasts including *S. cerevisiae* which leads to delays in fermentation (Panon, 1997; Nguyen & Panon, 1998). This phenomenon was due to a killer effect, although not the same as the classical *S. cerevisiae* killer phenomenon, and was linked to pulcherrimin pigment produced by *M. pulcherrima*. Contradicting reports on the interactions between *M. pulcherrima* and other yeasts may be due to strain related differences (Pallmann *et al.*, 2001).

Ethanol production by *S. cerevisiae* can affect non-*Saccharomyces* species development during fermentation (Fleet, 2003). Other compounds produced during fermentation that affect yeast-yeast interactions include medium-chain fatty acids, such as hexanoic, octanoic and decanoic acids, which can become inhibitory to *S. cerevisiae* above certain thresholds (Bisson, 1999). In wine fermentations, the consumption of amino acids and vitamins by non-*Saccharomyces* yeasts (either inoculated or occurring naturally) can impede the growth of *S. cerevisiae* strains (Fleet, 2003; Lleixà *et al.*, 2016). Taillandier *et al.* (2014) reported that *S. cerevisiae* was unable to develop in a medium containing 176 mg/L of initial assimilable nitrogen, due to nitrogen exhaustion by *T. delbrueckii* growth during the first 48 h, leading to a



sluggish fermentation. Bisson (1999) also reported that *K. apiculata* could deplete thiamine and other micronutrients of grape juices leading to deficient growth of *S. cerevisiae*.

The physiological and biochemical basis for the overall antagonistic interactions among wine yeasts are still unclear, but environmental factors, the production of bioactive yeast metabolites or yeast–yeast interaction could be involved (Ciani *et al.*, 2016a). In this context, the management of mixed fermentations, such as cell concentration, inoculation modalities (pure or mixed fermentation), and timing of sequential fermentations, require more knowledge on environmental factors and metabolic activities affecting these interactions. Some interactions that have been described in mixed fermentations of wines are shown in Table 2.3.

TABLE 2.3. Interactions between *Saccharomyces* and non-*Saccharomyces* yeasts in mixed culture wine fermentations (Adapted from Ciani *et al.*, 2010).

Species used	Compound or behaviour	Interactions	References
<i>S. cerevisiae</i> <i>H. uvarum</i>	Growth and viability	Persistence of non- <i>Saccharomyces</i>	Ciani <i>et al.</i> (2006); Mendoza <i>et al.</i> (2007)
<i>S. cerevisiae</i> <i>T. delbrueckii</i>	Cell-to-cell contact	Increase in death rate of non- <i>Saccharomyces</i>	Nissen and Arneborg (2003); Nissen <i>et al.</i> (2003)
<i>S. cerevisiae</i> <i>C. stellata</i>	Acetaldehyde, acetoin, glucose and fructose	Complementary consumption	Ciani and Ferraro (1998)
<i>S. cerevisiae</i> <i>H. uvarum</i> / <i>guillermondii</i>	Ethyl acetate Esters	Reduction Increase	Moreira <i>et al.</i> (2008)
<i>S. cerevisiae</i> <i>P. anomala</i>	Isoamyl acetate (EAHase)	Increase in production by <i>S. cerevisiae</i>	Kurita (2008)
<i>S. cerevisiae</i> <i>P. kluyveri</i>	3-Mercaptohexyl acetate	Increase in thiols	Anfang <i>et al.</i> (2009)
Mixed 'wild' yeasts	Volatile compounds	Increased and more complex aroma	Garde-Cerdán and Ancin-Azpilicueta (2006); Varela <i>et al.</i> (2009)

#### 2.6.2.2 Interactions between yeasts and lactic acid bacteria

The factor the winemaker has the most control over is the selection of the yeast and LAB culture for AF and MLF, respectively. The interaction between LAB and yeast during AF and/or MLF will have a direct effect on LAB growth and malolactic activity (Lerm *et al.*, 2010). Yeast can have an inhibiting, stimulating, or neutral effect, depending on the yeast and LAB pairing (Alexandre *et al.*, 2004). These authors proposed that the degree and complexity of interactions could be caused by three factors, *i.e.* the yeast/bacteria strain combination involved, the uptake and release of nutrients by yeast, and the ability of the yeast to produce toxic metabolites. Nehme *et al.* (2008) investigated the interactions between *S. cerevisiae* and *O. oeni* during the

winemaking process and found that inhibition between these microorganisms is largely dependent on the selected strains of yeast and LAB, and that inhibition correlated with a decrease in bacterial growth, rather than a decrease in malolactic activity. Most studies on yeast and LAB interactions have been done with *O. oeni* strains. However, Fumi *et al.* (2010) investigated the compatibility of a *Lb. plantarum* strain with various wine yeast strains as simultaneous or sequential inoculations. These authors found that yeast strains compatible with *O. oeni* starter cultures were also compatible with *Lb. plantarum*.

At the beginning of AF, *O. oeni* may be inhibited by *S. cerevisiae* due to the rapid uptake of certain grape metabolites from the must by the yeast. These compounds include sterols, amino acids and vitamins (Larsen *et al.*, 2003), which result in a nutrient diminished environment for the bacteria (Lerm *et al.*, 2010). The autolytic activity of yeast at the end of alcoholic fermentation modifies the concentration of amino acids, peptides and proteins, including mannoproteins and polysaccharides, in the wine (Alexandre *et al.*, 2001, 2004). The levels are dependent on the yeast strain (Escot *et al.*, 2001) and winemaking practices. Mannoproteins can stimulate LAB through two mechanisms: adsorption of medium chain fatty acids that detoxifies the medium or enzymatic hydrolysis by LAB can provide a source of nitrogen (Guilloux-Benatier & Chassagne, 2003; Alexandre *et al.*, 2004). Released vitamins, nucleotides and long chain fatty acids could also encourage the growth of LAB (Du Toit *et al.*, 2011).

Ethanol produced by yeast during alcoholic fermentation affects the growing ability rather than the malolactic activity of LAB (Capucho & San Romao, 1994). Sulphur dioxide is another factor that is regularly associated with inhibition by yeasts and the ability of *S. cerevisiae* to produce SO<sub>2</sub> is dependent upon various factors, including the strain involved and medium composition (Eschenbruch, 1974; Romano & Suzzi, 1993b).

Most strains produce <30 mg/L SO<sub>2</sub>, although some have been reported to produce >100 mg/L (Rankine & Pocock, 1969; Eschenbruch, 1974; Dott *et al.*, 1976; Suzzi *et al.*, 1985). Medium chain fatty acids, such as decanoic acid, can inhibit both yeast and LAB, and their formation has been suggested to cause antagonism between the yeast and LAB (Edwards & Beelman, 1987; Edwards *et al.*, 1990; Lonvaud-Funel *et al.*, 1988). In addition to limiting LAB growth, medium chain fatty acids can reduce the ability of LAB cells to degrade malic acid (Lafon-Lafourcade *et al.*, 1983; Capucho & San Romao, 1994). These effects are highly dependent upon the type and concentration of fatty acid present. Edwards and Beelman (1987) showed that addition of 5–10 mg/L decanoic acid to grape juice suppressed LAB growth and MLF, whereas 30 mg/L was lethal to the test LAB and completely inhibited MLF. The first three are the compounds most commonly studied with regard to LAB growth inhibition (Alexandre *et al.*, 2004). Several authors reported on proteinaceous or peptidic fractions of *S. cerevisiae* that inhibited LAB growth (Comitini *et al.*, 2005; Osborne & Edwards, 2007; Nehme *et al.*, 2010).

*Saccharomyces ludwigii*, *M. pulcherrima* and *Pichia* species were shown to have an antagonistic effect on the growth of *Lb. brevis*, *Lb. hilgardii* and *Lc. mesenteroides* strains (Fornachon, 1968). Mendoza *et al.* (2010) investigated the effect of different yeasts on *O. oeni* and *Lb. hilgardii* growth and found that *S. cerevisiae*, *M. pulcherrima*, *C. stellata*, *C. parapsilosis* and *P. fermentans* inhibited *O. oeni* growth. These authors showed that *Lb. hilgardii* was only inhibited by *S. cerevisiae* and *M. pulcherrima* yeasts and that *H. uvarum* (*K. apiculata*) and *Cryptococcus* stimulated the growth of *O. oeni*. Mendoza *et al.* (2011) investigated the interaction between a strain of *H. uvarum* (*K. apiculata*), *S. cerevisiae* and *O. oeni* in Malbec wine and reported that the interaction between these yeasts did not affect the fermentation kinetics of *O. oeni*.

Growth of certain LAB species has been shown to contribute to slow or stuck fermentations (Edwards *et al.* 1998, 1999a, b). Acetic acid and associated products of the LAB metabolism represent potent inhibitors to the fermentation of *Saccharomyces* yeasts, delaying the onset of fermentation with the potential of causing stuck fermentations (Edwards *et al.* 1999b). A *Lb. plantarum* strain was shown to produce a stable, but heat inactivated extracellular protein that was active against *Saccharomyces*, *Zygosaccharomyces* and *Schizosaccharomyces* species (Ribéreau-Gayon *et al.*, 2006). Lactic acid bacteria can also accelerate yeast death by producing glucosidases and proteases that are responsible for the hydrolysis of the yeast cell wall and lead to the lysis of the entire cell (Ribéreau-Gayon *et al.*, 2006). At the end of alcoholic fermentation, LAB therefore accelerate yeast autolysis. Lactic acid bacteria may also produce yeast inhibitors, because grape must fermented with certain LAB does not support yeast growth as well as unfermented (control) must (Ribéreau-Gayon *et al.*, 2006).

To add to the complexity of these interactions, some yeast strains can be both stimulatory and inhibitory, certain LAB strains are capable of inhibiting wine yeast and the composition of the must, as well as vinification practices affect these interactions (Lerm *et al.*, 2010).

#### 2.6.2.3 Interactions between lactic acid bacteria

There is a succession of LAB species and even strains during alcoholic fermentation, which can be explained by a difference in the sensitivity of the LAB to the conditions, but also interactions with yeast. Certain LAB can produce antimicrobial compounds (bacteriocins) to inhibit other bacteria (Ribéreau-Gayon *et al.*, 2006). Bacteriocins belong to a class of proteins whose bactericidal activity generally have a narrow range of action and sometimes even limited to the same species as the producing strain. Rammelsberg & Radler (1990) described bacteriocins from *Lb. brevis* (Brevicin) and *Lb. casei* (Caseicin), which inhibited important wine associated LAB. Brevicin, had broad spectrum activity and inhibited *Lb. brevis*, *O. oeni* and *Ped. damnosus* strains. Caseicin was only active against *Lb. casei* strains.

Navarro *et al.* (2000) screened 42 LAB strains (*Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Lactococcus*) isolated from Rioja red wines for antimicrobial activity. Nine of the 42 strains showed activity and they were all *Lb. plantarum*. These authors found that the *Lb. plantarum* strains produced antimicrobial peptides, which gave them evolutionary advantage over other strains that dominate in wine. Rojo-Bezares (2007) isolated a *Lb. plantarum* strain from grape must that showed antimicrobial activity against *Lactobacillus* and *Pediococcus* species, as well as *O. oeni*. Lonvaud-Funel & Joyeux (1993) identified small peptides from *Ped. pentosaceus* and *Lb. plantarum* that strongly inhibited the growth of *O. oeni*. These small peptides were thermostable and degraded by protease. However, the toxic effect was only temporary. The peptides did not kill *O. oeni*, but merely lowered the growth rate and final population. Another *Ped. pentosaceus* strain produced a bactericidal protein that was active against strains of *Lb. hilgardii*, *Ped. pentosaceus* and *O. oeni* (Ribéreau-Gayon *et al.*, 2006). This bacteriocin was stable in the acidic conditions and ethanol concentrations of wine.

In a study by Knoll (2007), 330 wine isolates were screened for inhibitory activity against wine related and non-wine related strains. Twenty seven strains belonging to the species, *Lb. plantarum*, *Lb. paracasei*, *Lb. hilgardii* and *O. oeni* were shown to have some inhibitory activity. These authors also reported on plantaricin producing *Lb. plantarum* strain that could inhibit the growth of *Enterococcus faecalis*.

## 2.7 Manipulation of wine aroma and flavour

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### 2.7.1 Compounds affected by yeast

Yeasts have a prominent role in determining the chemical composition of wine. Some compounds are directly derived from grapes, others are produced by yeast and some compounds are transformed/modified by yeast.

#### 2.7.1.1 Non-volatile acids

Tartaric acid and malic acid account for 90% of the titratable acidity (TA) of grape juice. Citric acid and lactic acid also contribute to the acidity of grape juice; succinic and keto acids are present only in trace amounts in grapes, but concentrations are higher in wines as a result of fermentation (Whiting, 1976; Fowles, 1992; Radler, 1993; Boulton *et al.*, 1996). These organic acids are important to wine pH, flavour, as well as the maintenance of colour and significantly affect the sensory properties and qualities of wines (Beelman & Gallander, 1979; Henick-Kling, 1993; Radler, 1993; Gao & Fleet, 1995). Low wine pH also restricts the growth of spoilage microorganisms, thus any changes to the concentration of the organic acids will affect wine quality (Wibowo *et al.*, 1985).

Tartaric acid is essentially stable, and little change in its concentration occurs during fermentation (Swiegers *et al.*, 2005). However, some *Candida*, *Cryptococcus* and *Rhodotorula*

strains have been reported to break down D (+)-tartrate and certain wine yeasts are also able to produce tartaric acid and concentrations that may vary from 100-520 mg/L (Whiting, 1976).

Various yeast strains have been investigated as alternative agents for malic acid degradation in wine. Malic acid is converted to ethanol by several yeasts; thus, the reduction in acidity is more acute than in MLF wines (Seo *et al.*, 2007). *Saccharomyces* species normally used for the wine fermentation do not degrade malic acid effectively during alcoholic fermentation and their low malic acid utilisation is well documented (Gao & Fleet, 1995; Volschenk *et al.*, 1997; Subden *et al.*, 1998; Volschenk *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006). Strains of *Sc. pombe* (*Sc. malidevorans*) and *Z. bailii* can degrade high concentrations of malic acid (Baranowski & Radler, 1984; Rodriguez & Thornton, 1989; Benito *et al.*, 2013, 2014). More recently, several *Sc. pombe* strains were screened for the ability to degrade malic acid, without producing unacceptable levels of acetic acid (Benito *et al.*, 2012, 2013, 2014, 2015a). These authors found a *Sc. pombe* strain that could complete the alcoholic fermentation and degrade malic acid (malaloalcoholic fermentation) without producing any off-flavours. Wines produced with the aforementioned *Sc. pombe* strain scored higher for aroma intensity and quality than the wines produced with *S. cerevisiae* (Benito *et al.*, 2014). Other non-*Saccharomyces* yeasts that can degrade malic acid are *I. orientalis* (Seo *et al.*, 2007) and *P. kudriavzevii* (Kim *et al.*, 2008; Seo *et al.*, 2007). Citric acid can be formed initially in fermentations by yeasts and later taken into the cell and catabolised (Whiting, 1976).

Succinic acid production is common amongst yeasts and is the main carboxylic acid produced during fermentation and can accumulate up to 2 g/L (Thoukis *et al.*, 1965, Radler, 1993; Coulter *et al.*, 2004). Its production is highly variable amongst strains of *S. cerevisiae* but *S. uvarum* or *S. bayanus* strains tend to produce higher concentrations (Heerde & Radler, 1978; Giudici *et al.*, 1995; Eglinton *et al.*, 2000; de Klerk, 2010). Some non-*Saccharomyces* yeasts can also produce succinic acid and production is correlated with high ethanol production and ethanol tolerance (Ciani & Maccarelli, 1998; Ferraro *et al.*, 2000). Succinic acid production can have a positive effect on the analytical profile of wines by contributing to the total acidity in wines with insufficient acidity, but it has a 'salt-bitter-acid' taste (Amerine *et al.*, 1972) and excessive levels will have a negative impact on wine quality (de Klerk, 2010).

#### 2.7.1.2 Volatile acids

Acetic acid is the most abundant volatile acid found in wine and accounts for 90% of the volatile acids (Fowles, 1992; Henschke & Jiranek, 1993; Radler, 1993). Other volatile acids such as propionic and hexanoic acids are products of yeast and bacteria metabolism (Swiegers *et al.*, 2005). Acetic acid is the main contributor to volatile acidity (VA) and above the sensory threshold of 0.7-1.1 g/L acetic acid/VA can impart a vinegar aroma (Lambrechts & Pretorius, 2000; Romano *et al.*, 2006); while at levels of between 0.2 to 0.7 g/L it can be considered as

acceptable (Corison *et al.*, 1979). Non-*Saccharomyces* yeasts produce variable levels of acetic acid, for example *M. pulcherrima* strains produce between 0.1 and 0.15 g/L, while *H. uvarum* (*K. apiculata*) produce between 1.0 and 2.5 g/L (Fleet & Heard, 1993; Renault *et al.*, 2009). Mixed culture fermentation of non-*Saccharomyces* and *S. cerevisiae* can result in lower levels of the acetic acid (Maturano *et al.*, 2012; Rantsiou *et al.*, 2012; Benito *et al.*, 2015a, b), but higher levels have also been reported (Rodríguez *et al.*, 2010; Romboli *et al.*, 2015). Whitener *et al.* (2016) reported high acetic acid levels in Sauvignon blanc wines produced with *C. zemplinina* in combination with *S. cerevisiae*. Combinations of *S. cerevisiae* with *L. thermotolerans* or *T. delbrueckii* and certain strains of *M. pulcherrima* have similarly showed a reduction in VA (Ciani & Maccarelli, 1998; Bely *et al.*, 2008; Comitini *et al.*, 2011; Sadoudi *et al.*, 2012; Benito *et al.*, 2015a, b). In Shiraz, combinations of *S. cerevisiae* and *T. delbrueckii*, *M. pulcherrima* and *P. kluyveri*, respectively, produced similar VA levels as *S. cerevisiae*, while *C. zemplinina*, *K. apiculata* and *L. thermotolerans* in combination with *S. cerevisiae* produced significantly higher VA levels (Whitener *et al.*, 2017).

#### 2.7.1.3 Alcohols

The presence of ethanol is essential to enhance the sensory attributes of other wine components. However, excessive ethanol levels can produce a perceived 'hotness' and mask the overall aroma and flavour of the wine (Guth & Sies, 2002). Significantly lower ethanol concentrations (0.2%  $v/v$  to 0.7%  $v/v$ ) have been reported in wines produced by mixed fermentation with non-*Saccharomyces* yeast and wines produced by *S. cerevisiae* monocultures, (Ferraro *et al.*, 2000; Soden *et al.*, 2000; Comitini *et al.*, 2011; Di Maio *et al.*, 2012; Sadoudi *et al.*, 2012; Benito *et al.*, 2013; Gobbi *et al.*, 2013). Erten and Campbell (2001) produced wines containing 3%  $v/v$  after fermentation of grape must by *Williopsis saturnus* and *Pichia subpelliculosa* under intensive aerobic conditions. These reduced alcohol wines were judged to be of an acceptable quality.

Glycerol, the next major yeast metabolite produced during wine fermentation after ethanol, is important in yeast metabolism for regulating redox potential in the cell (Scanes *et al.*, 1998; Prior *et al.*, 2000). Glycerol is a polyol with a colourless, odourless and highly viscous character (Swiegers *et al.*, 2005). It contributes to smoothness (mouthfeel), sweetness and complexity in wines (Ciani & Maccarelli, 1998), but the grape variety and wine style will determine the extent to which glycerol affects these properties. Glycerol is present in dry and semi-sweet wines in concentrations ranging from 5 to 14 g/L, and red wines typically have higher concentrations of glycerol than white wines (6.82 g/L versus 10.49 g/L; Nieuwoudt *et al.*, 2002), while botrytised wines frequently have concentrations up to 25 g/L (Rankine & Bridson, 1971, Ough *et al.*, 1972; Nieuwoudt *et al.*, 2002). *Candida stellata* is known as a high glycerol producer with concentrations reported in wine up to 14 g/L (Ciani & Picciotti, 1995; Ciani & Ferraro, 1998;

Ciani & Maccarelli, 1998). *Lachancea thermotolerans* and *C. zemplinina* have also been reported to produce high glycerol levels during wine fermentation (Ciani & Ferraro, 1998; Soden *et al.*, 2000; Comitini *et al.*, 2011; Zara *et al.*, 2014; Romboli *et al.*, 2015). Unfortunately, increased glycerol production is usually linked to increased acetic acid production (Prior *et al.*, 2000), which can be detrimental to wine quality. In contrast, *S. cerevisiae* has been reported to produce between 4 and 10.4 g/L of glycerol (Radler & Schütz, 1982; Ciani & Maccarelli, 1998; Prior *et al.*, 2000; de Klerk, 2010).

Higher alcohols (also known as fusel alcohols) are secondary yeast metabolites, and can have both positive and negative impacts on the aroma and flavour of wine. High concentrations of higher alcohols can result in a strong, pungent smell and taste, whereas optimal levels impart fruity characters and can add to wine complexity (Table 2.4) (Nykänen *et al.*, 1977; Romano & Suzzi, 1993a; Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005). The aliphatic alcohols include propanol, isoamyl alcohol, isobutanol and active amyl alcohol. The aromatic alcohols consist of 2-phenylethyl alcohol and tyrosol. It has been reported that concentrations below 300 mg/L add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg/L can have a detrimental effect (Rapp & Versini, 1995). Non-*Saccharomyces* yeasts produce different levels of higher alcohols (Romano *et al.*, 1992; Lambrechts & Pretorius, 2000) and often form lower levels of these alcohols than *S. cerevisiae*, but there is great strain variability (Romano *et al.*, 1992, 1993a; Zironi *et al.*, 1993). *L. thermotolerans* has been reported to produce high concentrations of lactic acid, glycerol and 2-phenylethanol during mixed fermentations (Kapsopoulou *et al.*, 2007; Comitini *et al.*, 2011; Gobbi *et al.*, 2013).

#### 2.7.1.4 Esters

Esters produced by the yeast during fermentation can have a significant effect on the fruity flavour in wine. Esters also have a positive effect on wine quality, especially in wine from varieties with neutral flavours (Lambrechts & Pretorius, 2000; Sumby *et al.*, 2010). Important esters are ethyl acetate (fruity, solvent-like aromas), isoamyl acetate (isopentyl acetate) (pear-drops aroma), isobutyl acetate (banana aroma), ethyl caproate (ethyl hexanoate) (apple aroma) and 2-phenylethyl acetate (honey, fruity, flowery rose aromas) (Lambrechts & Pretorius, 2000; Swiegers & Pretorius, 2005; Swiegers *et al.*, 2005). Flavour-producing yeasts such as *P. anomala* (*H. anomala*) and *H. uvarum* (*K. apiculata*) are known to be a high producer of esters (Bisson & Kunkee, 1991; Clemente-Jimenez *et al.*, 2004). *Metschnikowia pulcherrima* also produces high ester levels (Bisson & Kunkee, 1991; Rodríguez *et al.*, 2010; Sadoudi *et al.*, 2012), especially the pear-associated ester, ethyl octanoate (Lambrechts & Pretorius, 2000; Clemente-Jimenez *et al.*, 2004). *H. vineae* (formerly *H. osmophila*) and *H. guilliermondii* have been reported to produce increased amounts of 2-phenyl-ethyl acetate during fermentation (Rojas *et al.*, 2003; Viana *et al.*, 2009).

Wines produced with *H. guilliermondii* in combination with *S. cerevisiae* had higher concentrations of hexyl acetate, ethyl acetate and isoamyl acetate than wines produced with *S. cerevisiae* on its own (Moreira *et al.*, 2008). Mixed culture fermentations by *H. guilliermondii* and *P. anomala* in combination with *S. cerevisiae* showed increased acetate ester concentrations compared to fermentations with *S. cerevisiae* only, and had a significant affect on acetaldehyde, acetic acid, glycerol and total higher alcohol levels (Rojas *et al.*, 2003). Mixed culture fermentations of *L. thermotolerans* and *S. cerevisiae* contained higher levels of ethyl-2-methyl propanoate and 1-phenylethyl 2-methylpropanoate than *S. cerevisiae* on its own (Whitener *et al.*, 2017). These esters are characterised by sweet, floral and fruity aromas.

#### 2.7.1.4 Other volatile compounds

Sauvignon blanc wines produced with *C. zemplinina* and *S. cerevisiae* were characterized with a larger number of terpenes and sesquiterpenes than the *S. cerevisiae* only wines (Whitener *et al.*, 2016). Whitener *et al.* (2017) reported that all non-*Saccharomyces* fermentations displayed higher levels of geraniol, trans- $\beta$ -ocimene, linalool and  $\alpha$ -terpinene than *S. cerevisiae*. The aforementioned terpenes are associated with floral, sweet, rosy, fruity, sweet herbal, citrus and woody aroma. The aforementioned authors also reported that the wines produced with *L. thermotolerans* in combination with *S. cerevisiae* contained the highest linalool concentrations.

### 2.7.2 Compounds affected by lactic acid bacteria

#### 2.7.2.1 Non-volatile acids

Lactic acid bacteria can metabolise some organic acids, resulting in end-products that may enhance MLF flavour or lead to off-flavours (Du Toit *et al.*, 2011). Tartaric acid is the strongest and most important acid in wine and affects the acidity and pH of wine (Silva & Álvares-Ribeiro, 2002; Ribéreau-Gayon *et al.*, 2006). Some wine LAB are able to degrade tartaric acid, mostly *Lactobacillus* spp. (Wibowo *et al.*, 1985; Ribéreau-Gayon *et al.*, 2006). However, LAB that are able to degrade tartaric acid do not occur frequently (Krumperman & Vaughn, 1966; Radler, 1975; Sponholz, 1993). Wine spoilage by the degradation of tartaric acid lowers the fixed acidity and is accompanied by an increase in volatile acidity (Ribéreau-Gayon *et al.*, 2006). The degradation can be total or partial, depending on the species and the level of development, but it always lowers the wine quality (Ribéreau-Gayon *et al.*, 2006).

The removal of malic acid from the wine matrix ensures that the risk of spoilage post-bottling is minimised. The degradation of malic acid affects wine flavour. As the strong and sharp green taste of malic acid is replaced by the less aggressive and milder taste of lactic acid, an improved and softer mouthfeel can be expected (Lonvaud-Funel, 1999). However, MLF in wine with a high pH could lead to the survival and growth of spoilage microorganisms and could



also cause a loss of red colour intensity in red wines (Volschenk *et al.*, 2006). Lactic acid, a by-product of MLF, is a registered antibacterial agent and has the potential to inhibit growth of unwanted organisms (Swiegers *et al.*, 2005; Du Toit *et al.*, 2011).

Among the species found in wine, *Lb. plantarum*, *Lb. casei*, *O. oeni* and *Lc. mesenteroides* can utilise citric acid (Lonvaud-Funel 1999), but strains of the genus *Pediococcus* and of the species *Lb. hilgardii* and *Lb. brevis* cannot (Ribéreau-Gayon *et al.*, 2006). Citric acid metabolism is likely to occur after malic acid has been degraded (Nielsen & Richelieu, 1999). The metabolites produced during citrate metabolism are acetic acid, lactic acid, acetoin, 2,3-butanediol, diacetyl, as well as aspartic acid. Most of these products are of sensorial importance in wine (Du Toit *et al.*, 2011). Pyruvic acid can be metabolised to lactic acid, acetic acid and  $\alpha$ -acetolactic acid. The fate of pyruvate depends on conditions such as pH, aeration and carbohydrate availability (Starrenburg & Hugenholtz, 1991).  $\alpha$ -Acetolactic acid can be decarboxylated to acetoin, which accumulates in the medium or could be further reduced to 2,3-butanediol. Diacetyl is produced by a spontaneous decarboxylation of  $\alpha$ -acetolactic acid in the presence of oxygen and/or low pH (Ramos *et al.*, 1995).

The most important significance of citric acid metabolism is the production of diacetyl (Du Toit *et al.*, 2011). Diacetyl (2,3-butanedione) is a diketone that contributes buttery, nutty and butterscotch characters to the wine, as well as a yeasty character to sparkling wines, during MLF (Bartowsky & Henschke, 1995; Martineau *et al.*, 1995; Bartowsky & Henschke, 2004). At concentrations of 1 to 4 mg/L, diacetyl contributes positively to wine aroma, but higher concentrations (5 mg/L and or higher) give an undesirable butter-like flavour (Davis *et al.*, 1985; Bartowsky & Henschke, 1995, 2004; Swiegers *et al.*, 2005). Diacetyl can be further reduced to acetoin and 2,3-butanediol by yeast and LAB (Martineau & Henick-Kling, 1995; Nielsen & Richelieu, 1999; Bartowsky *et al.*, 2002; Costello, 2006). Acetoin and 2,3-butanediol can also contribute to the flavour profile, when present at levels higher than their respective sensory thresholds of ca. 150 and 600 mg/L (Bartowsky & Henschke, 2004).

#### 2.7.2.2 Volatile acids

Acetic acid production by heterofermentative LAB during MLF can occur via two potential mechanisms; (i) the conversion of hexoses to produce ethanol, CO<sub>2</sub>, acetic acid and D-lactic acid via the phosphoketolase pathway (Lonvaud-Funel, 1999; Swiegers *et al.*, 2005), or (ii) the formation during the first reaction of citric acid metabolism catalyzed by the citrate lyase enzyme (Bartowsky & Henschke, 2004). Usually, the acetic acid concentrations can increase with 0.1 to 0.2 g/L as a result of MLF (Bartowsky & Henschke, 1995). Recent studies have shown that co-inoculation of LAB and yeast do not lead to higher acetic acid concentrations (Zapparoli *et al.*, 2009; Izquierdo Cañas *et al.*, 2015; Guzzon *et al.*, 2015).

Wine consists of a mixture of straight chain fatty acids and branched chain fatty acids. The straight chain fatty acids are usually referred to as short chain (C2-C4), medium chain (C6-C10) or long chain (C12-C18) fatty acids (Ugliano & Henschke, 2008). As the chain length of fatty acids increase, the volatility decreases and the odour changes from sour to rancid and cheesy (Francis & Newton, 2005; Ugliano & Henschke, 2008). Fatty acids have low perception thresholds and therefore have the ability to add complexity when present in lower quantities or be detrimental to wine quality when present at higher concentrations, because they impart unpleasant, rancid, pungent, cheese, sweaty odours and fat-like aromas (Francis & Newton, 2005). Maicas *et al.* (1999) found that capric acid and caprylic acid levels were higher, but no significant increase in isovaleric, isobutyric and hexanoic acids in wines after MLF. Herjavec *et al.* (2001) reported an increase in the concentrations of octanoic, hexanoic and decanoic acids after completion of MLF. Pozo-Bayón *et al.* (2005) reported significant differences in the concentrations of octanoic and decanoic acids depending on the MLF culture that was used.

#### 2.7.2.3 Alcohols

Wine LAB are able to use glycerol as a carbon source to maintain viability when the fermentable sugars have been exhausted after alcoholic fermentation. This can have a negative impact on wine quality, depending on the pathway used for glycerol degradation (Du Toit *et al.*, 2011). Degradation of glycerol can lead to the formation of acrolein, which reacts with wine phenolics, particularly in red wines, to form a bitter tasting complex (Lonvaud-Funel, 1999; Swiegers *et al.*, 2005).

Jeromel *et al.* (2008) found that MLF had an insignificant effect on the higher alcohol concentration of wine, except for significant increases in isobutanol and 2-phenylethanol. Maicas *et al.* (1999) found that the production of isobutanol, 1-propanol, 1-butanol and isoamyl alcohol to be strain dependent. Herjavec *et al.* (2001) found no change in the levels of 1-propanol, isobutanol, isoamyl alcohol or 2-phenylethanol after MLF. Pozo-Bayón *et al.* (2005) reported increased levels of higher alcohols after MLF, but none of the increases were significant.

#### 2.7.2.4 Esters

The majority of wine esters are produced by yeast during alcoholic fermentation. However, esters can also be derived from the grape, the chemical esterification of alcohols and acids during wine ageing (Rapp & Mandery 1986; Etiévant, 1991; Younis & Stewart, 1998; Lambrechts & Pretorius, 2000). The two main groups of esters associated with wine fruitiness are acetate esters and ethyl esters of fatty acids. Increases in ethyl ester concentration (ethyl acetate, ethyl hexanoate, ethyl lactate, and ethyl octanoate) in wine following MLF, as well as decreases in some other esters have been reported (Zeeman *et al.* 1982; Laurent *et al.*, 1994;

de Revel *et al.*, 1999; Delaquis *et al.*, 2000; Gambaro *et al.*, 2001). Ethyl lactate, which has been described as giving a broader, fuller taste to wine, is one of the main esters produced during MLF (Bartowsky & Henschke, 1995). It may be formed at concentrations exceeding its flavour threshold of 60 to 110 mg/L. Sumbly *et al.* (2010) summarised the strain specific changes observed in ester concentrations during MLF, which included increases in ethyl-2-methylpropanoate (fruity, strawberry, lemon), ethyl 2-methylbutanoate (apple, berry, sweet, cider, anise), ethyl 3-methylbutanoate (sweet fruit, pineapple, lemon, anise, floral), ethyl 2-hydroxypropanoate (milk, soapy, buttery, fruity), ethyl 3-hydroxypropanoate (fruity, green, marshmallow), ethyl hexanoate (fruity, strawberry, green apple, anise), 3-methylbutyl acetate (banana, fruity), ethyl 2-phenylacetate (rose, floral), 2-phenylethyl acetate (flowery, rose) and hexyl acetate (green, herbaceous, fruit, grape).

Matthews *et al.* (2006) investigated 50 LAB isolates comprising of *Lactobacillus*, *Oenococcus* and *Pediococcus* spp. and all were found to hydrolyse esters. Genetic studies identified and characterised genes involved in the esterase activity of *O. oeni* (Sumbly *et al.*, 2009) and wine-associated *Lactobacillus* spp. (Mtshali *et al.*, 2010). These findings indicate that LAB possess the ability to synthesise and hydrolyse esters (Liu, 2002; Matthews *et al.*, 2004), which highlights the potential of LAB to contribute to wine aroma.

## 2.8 Conclusions

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It is clear that non-*Saccharomyces* yeasts are diverse and their characteristics vary among species and even strains. Non-*Saccharomyces* yeasts have different attributes than *Saccharomyces* yeast and can be used as a tool by the winemaker to manipulate flavour, to modify wine style and even improve wine quality. The benefits different non-*Saccharomyces* yeast species and strains have on wine production, as well as the effect winemaking practices have on these yeasts, still need further investigation. The non-*Saccharomyces* yeast metabolism and metabolic products have not received as much attention as those of *Saccharomyces* yeasts or LAB. Research on the use of non-*Saccharomyces* yeast to reduce ethanol and characterisation of volatile compounds of these yeasts need to be encouraged, so that their contribution to wine production can be managed better. The interactions between non-*Saccharomyces* and *Saccharomyces* also need further investigation, especially with regard to the impact on non-volatile and volatile compounds produced. The effect that non-*Saccharomyces* yeasts have on LAB and MLF also need to be elucidated. Very little research has focused on the interactions between non-*Saccharomyces* yeast and LAB. How they impact on each other's growth and also the metabolic compounds produced during these interactions. Understanding how the various yeast species and their metabolites interact with each other and with LAB will be invaluable for improving wine flavour and quality. Currently, there is no consensus with regard to which MLF inoculation strategy to use for optimal flavour production.

The use of different non-*Saccharomyces* in combination with *S. cerevisiae* yeast will produce wine with varying flavour profiles, but what impact different MLF inoculation strategies and LAB stains will have on wine flavour and quality is unclear. In the following sections, the interactions between *Saccharomyces*, non-*Saccharomyces*, LAB and MLF strategies will be investigated and how these interactions affect MLF and wine flavour.

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# Chapter 3

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## Research results I

**Characterisation of non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation**

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## CHAPTER 3

### Characterisation of non-*Saccharomyces* yeasts using different methodologies and evaluation of their compatibility with malolactic fermentation

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#### ABSTRACT

Although *Saccharomyces cerevisiae* is predominantly used for alcoholic fermentation, non-*Saccharomyces* yeast species are also important because they produce secondary metabolites that can contribute to the final flavour and taste of wines. In this study, 37 strains representing seven non-*Saccharomyces* species were characterised and evaluated for potential use in wine production, as well as their effects on malolactic fermentation (MLF). Contour-clamped homogeneous electric field gel electrophoreses (CHEF) and matrix-assisted laser desorption ionization using a time-of flight mass spectrometer (MALDI-TOF MS) were used to verify species identity and to determine intra-species variation. Extracellular enzyme production, malic acid degradation and the fermentation kinetics of the yeasts were also investigated. CHEF karyotyping and MALDI-TOF MS were useful for identifying and typing of *Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Candida zemplinina* (synonym: *Starmerella bacillaris*) and *Torulaspota delbrueckii* strains. Only *H. uvarum* and *Metschnikowia pulcherrima* strains were found to have  $\beta$ -glucosidase activity. In addition, *M. pulcherrima* strains also had protease activity. Most of the strains showed limited malic acid degradation and only *Schizosaccharomyces pombe* and the *C. zemplinina* strains showed mentionable degradation. In synthetic wine fermentations, *C. stellata*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *Sc. pombe* strains were shown to be slow to medium fermenters, whereas *L. thermotolerans* and *T. delbrueckii* strains were found to be medium to strong fermenters. The effect of the yeasts on MLF varied, but inhibition was strain dependent.



### 3.1 INTRODUCTION

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Yeasts play a key role in wine production. They are present on the grapes, winery equipment or added as starter cultures and are responsible for alcoholic fermentation, whereby the grape must is transformed into wine. These yeasts can be arbitrarily divided into two categories: *Saccharomyces* and non-*Saccharomyces* (wild yeasts). *Saccharomyces cerevisiae* may be present at very low numbers on the grape skins, but are normally found in greater numbers on the winery equipment (Fleet *et al.*, 2002; Ribéreau-Gayon *et al.*, 2006). Non-*Saccharomyces* yeast genera frequently found on grapes and in must, include *Hanseniaspora* (*Kloeckera*), *Candida*, *Metschnikowia*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula*, *Zygosaccharomyces*, *Cryptococcus* and the black pigmented yeast-like fungus, *Aureobasidium pullulans* (Fleet *et al.*, 2002; Jolly *et al.*, 2003a; Ribéreau-Gayon *et al.*, 2006; Romano *et al.*, 2006; Jolly *et al.*, 2014; Alessandria *et al.*, 2015; Capozzi *et al.*, 2015). In the initial phase of spontaneous fermentations, strains from the genera *Kloeckera* and *Candida* usually dominate (Ribéreau-Gayon *et al.*, 2006; Romano *et al.*, 2006). As the ethanol levels increase, the more ethanol tolerant, *Saccharomyces* yeast strains dominate.

Malolactic fermentation (MLF) is a secondary but important fermentation process conducted by lactic acid bacteria (LAB), usually *Oenococcus oeni* (Bauer & Dicks, 2004; Lerm *et al.*, 2010). Malolactic fermentation is not a true “fermentation”, but rather an enzymatic reaction whereby malic acid is decarboxylated to lactic acid and CO<sub>2</sub>. This process is often desired in the production of red wines, certain white and sparkling wine styles (Wibowo *et al.*, 1985; Bartowsky *et al.*, 2015), because it increases wine microbiological stability and enhances aroma and flavour (Davis *et al.*, 1985; Bartowsky *et al.*, 2002; Lerm *et al.*, 2010; Sumby *et al.*, 2014).

In the last decades, research has focused on the role that non-*Saccharomyces* yeasts play in wine production. The use of controlled mixed cultures of selected non-*Saccharomyces* and *Saccharomyces* strains can have advantages over fermentations inoculated with pure cultures of *S. cerevisiae*. These mixed fermentations lead to the production of wines with more desirable characteristics and starter cultures containing non-*Saccharomyces* yeasts, namely *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Pichia kluyveri* and *Metschnikowia pulcherrima*, are commercially available (Jolly *et al.*, 2014). Specific compounds produced by non-*Saccharomyces* yeasts that can affect wine aroma and flavour include: acetaldehyde, acetic acid, esters, glycerol, higher alcohols, terpenoids and other by-products (Romano *et al.*, 1997; 2003; Jolly *et al.*, 2006; Comitini *et al.*, 2011; Jolly *et al.*, 2014). Non-*Saccharomyces* yeasts also possess various degrees of  $\beta$ -glucosidase activity, which play a role in releasing volatile compounds from non-volatile precursors (Rosi *et al.*, 1994; Hernandez-Orte *et al.*, 2008). Extracellular proteolytic and pectinolytic enzymes of non-*Saccharomyces* yeasts might also be beneficial by improving wine processing through facilitation of juice extraction and clarification, wine filtration and colour extraction (van Rensburg & Pretorius, 2000; Strauss, 2003; Reid,

2012). Strains of *Candida stellata*, *C. zemplinina* (synonym: *Starmerella bacillaris*), *Hanseniaspora uvarum*, *M. pulcherrima* and *P. anomala* have been found to produce a variety of extracellular enzymes (Charoenchai *et al.*, 1997; Strauss, 2003; Mostert, 2013).

Considering the great diversity and potential applications of different non-*Saccharomyces* yeast strains within the same species, it is important to devise simple and reliable molecular typing techniques to discriminate at the subspecies level. Application of karyotyping electrophoresis techniques, such as contour-clamped homogeneous electric field (CHEF) gel electrophoresis, has been useful to differentiate non-*Saccharomyces* yeasts at species and strain level (Esteve-Zarzoso *et al.*, 2001; Sipiczki, 2004; Alcoba-Flórez *et al.*, 2007; Van Breda *et al.*, 2013). Its high discriminatory power and repeatability also justify why this technique is often considered favourably in comparison with other typing methods. Matrix-assisted laser desorption ionization using a time-of flight mass spectrometer (MALDI-TOF MS) is a 'soft' or non-destructive method that can be used for identification of yeasts and bacteria at the genus and species level (van Veen *et al.*, 2010). Studies using MALDI-TOF MS to identify yeasts have focused more on clinical *Candida* strains (Marklein *et al.*, 2009) than on wine associated yeasts (Moothoo-Padayachie *et al.*, 2013; Kántor & Kačániová, 2015).

The interactions between different non-*Saccharomyces* yeasts (naturally present and inoculated) and LAB, as well as their impact on MLF have received little attention. The resulting impact on wine aroma/flavour is also uncertain. With the increasing number of non-*Saccharomyces* yeasts commercially available, the need for a better understanding of the interactions between the wine yeast, *S. cerevisiae*, the non-*Saccharomyces* yeasts and LAB is critical. Therefore, the aims of this study were to characterise strains from seven non-*Saccharomyces* species by means of CHEF karyotyping, MALDI-TOF bio-typing, enzyme activity and malic acid degradation, to investigate their use in wine production and to evaluate their compatibility with MLF.

## 3.2 MATERIALS AND METHODS

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### 3.2.1 Characterisation

#### 3.2.1.1 Isolation and cultivation of microorganisms

The yeast strains used in this study are listed in Table 3.1 and included one *C. stellata*, seven *C. zemplinina* (synonym: *St. bacillaris*), 11 *H. uvarum* (anamorph: *Kloeckera apiculata*), two *L. thermotolerans* (previously *Kluyveromyces thermotolerans*), seven *M. pulcherrima* (anamorph: *Candida pulcherrima*), one *Schizosaccharomyces pombe*, eight *Torulaspora delbrueckii* (anamorph: *Candida colliculosa*) and six *S. cerevisiae* strains. Strain *L. thermotolerans*, Viniflora® Rhythm™ (Chr. Hansen, Denmark) and *T. delbrueckii* strains, Viniflora® Harmony™ (Chr. Hansen), (Level<sup>2</sup> TD™ (Lallemand Inc., France) and Zymaflore®

Alpha<sup>TD n. Sacc.</sup> (Laffort Oenologie, France), were isolated from commercial active dried yeast blends (Van Breda *et al.*, 2013 and this study) and included as reference strains. All the yeasts were stored under cryo-preservation at  $-80^{\circ}\text{C}$ . When required the yeasts were grown on yeast peptone dextrose agar (YPDA, Merck, South Africa) at  $28^{\circ}\text{C}$  for 48 hours or until sufficient growth was observed. Single colonies were then selected and transferred to 10 mL YPD broth and grown for 24 hours at  $28^{\circ}\text{C}$  before inoculation. *Oenococcus oeni* (Viniflora® oenos, Chr. Hansen) was used to induce MLF according to the supplier's instructions.

### 3.2.1.2 Electrophoretic karyotyping

Contour-clamped homogeneous electric field gel electrophoresis (CHEF) was used to investigate strain diversity of the non-*Saccharomyces* yeasts and the intact chromosomal DNA was prepared using the embedded agarose technique described by Hoff (2012). A CHEF DRIII electrophoretic apparatus (Bio-Rad Laboratories, Inc., Richmond, USA) and the method described by Hoff (2012) was used with the following changes to the running conditions: 34-hour programme, initial pulse was 30 s and final pulse was 215 s at an angle of 120 degrees at a constant 6 Volt; the 72-hour programme, initial and final pulse of 1800 s at an angle of 106 degrees at a constant 2.5 Volt. *Saccharomyces cerevisiae* reference strain CBS 432 was used as the standard reference strain for all CHEF gels and was loaded on the outer lanes of each gel. Agarose gels at a concentration of 1.2% and 0.8% were used to separate yeasts run on the 34 and 72 hour programmes, respectively.

Chromosomal banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% (v/v) ethidium bromide (Bio-Rad Laboratories, Inc.). Normalisation of gels and comparison of banding patterns were performed using FPQuest<sup>TM</sup> software (Bio-Rad Laboratories, Inc.) and the normalised electrophoretic patterns were grouped. Similarities (s) were obtained, using the Dice coefficient before cluster analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA).

### 3.2.1.3 MALDI-TOF bio-typing

Single colonies of each yeast strain were selected for identification and bio-typing by MALDI-TOF MS. One microliter of wine yeast protein extract was spotted onto a MTP 384 polished steel target plate as described by Moothoo-Padayachie *et al.* (2013) and Deak *et al.* (2015). Thereafter, the spotted target plate was inserted into a Bruker UltrafleXtreme MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) apparatus. Generation of yeast protein mass spectra using MALDI-TOF/TOF MS was conducted according to the standard National Agricultural Proteomics Research & Services Unit method (obtainable from the National Agricultural Proteomics Research & Services Unit (NAPRSU), University of the Western Cape, South Africa). Mass spectra for all strains were acquired in triplicate. The spectrum acquired for each

sample was compared to the Bruker reference database containing 4110 microorganisms (NAPRSU, May 2015).

#### 3.2.1.4 Enzyme screening and malic acid degradation

Polygalacturonase/pectinase activity was determined as described by McKay (1988),  $\beta$ -glucosidase activity through the screening method of Strauss *et al.* (2001) and acid protease activity was determined following the method of Charoenchai *et al.* (1997). The ability of yeasts to degrade malic acid was determined using a plate assay method described by Mocke (2005). The medium used for malic acid degradation was also modified slightly by excluding the agar and bromocresol green to determine malic acid degradation in a liquid medium. Aliquots of 10 mL medium were dispensed into 42 test tubes and autoclaved. Where after, single colonies of the yeast strains were inoculated into the test tubes containing the MLF broth and kept at an ambient temperature of 22°C for up to 40 days. Malic acid concentration was measured by enzymatic analysis (Arena 20XT enzyme robot, Institute for Wine Biotechnology, Stellenbosch University).

### 3.2.2 Evaluation of yeasts

#### 3.2.2.1 Fermentation trial

Laboratory-scale alcoholic fermentation trials were conducted in a chemically defined grape juice as described by Costello *et al.* (2003). Yeasts were grown in 10 mL YPD broth at 30°C prior to inoculation. Pure cultures of the different yeast strains were inoculated into sterilised 375 mL glass bottles containing 250 mL of filter-sterilised synthetic grape juice and fermented to dryness. Each yeast strain had three biological repeats. After the alcoholic fermentation (AF), the resultant synthetic wine of each yeast treatment was pooled, aseptically filtered (0.22  $\mu$ m) and used for the MLF trial. Fifty millilitres of the synthetic wine were aliquoted into sterilised 250 mL bottles before inoculating with LAB. Two treatments were applied, *i.e.* (1) addition of *O. oeni* only and (2) addition of nutrients as described by Costello *et al.* (2003) prior to addition of *O. oeni* (Viniflora® oenos). Alcoholic and malolactic fermentations were conducted at  $\pm$ 22°C.

#### 3.2.2.2 Chemical analyses

The Ripper method as described by Iland *et al.* (2000) was used to determine free and total SO<sub>2</sub>. Sugar concentration, pH, malic acid, total acidity (TA), alcohol and volatile acidity (VA) of synthetic wines were determined using an OenoFoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark).

TABLE 3.1. Yeasts used in this study.

Species name	Strain code	Strain, origin and source information	References <sup>1</sup>
<i>Saccharomyces cerevisiae</i>	S1	N 96, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012
	S2	VIN 13, commercial yeast from Anchor Wine Yeast, South Africa	Jolly <i>et al.</i> , 2003b, c; Hoff 2012; Van Breda <i>et al.</i> , 2013; Minnaar <i>et al.</i> , 2015
	S3	NT 112, commercial yeast from Anchor Wine Yeast, South Africa	Hoff 2012
	S4	NT 202, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012; Scholtz, 2013
	S5	VIN 7, commercial yeast from Anchor Wine Yeast, South Africa	Hoff, 2012
	S6	CBS 432, from Centraalbureau of Schimmelcultures (CBS), Netherlands	
<i>Candida stellata</i>	Cs	CBS 157 <sup>T</sup> , from CBS, Netherlands	Sipiczki, 2004; Csoma & Sipiczki, 2008
<i>Candida zemplinina</i> (synonym: <i>Starmerella bacillaris</i> )	C1	CBS 9494, type strain from CBS, Netherlands	Sipiczki, 2004; Csoma & Sipiczki, 2008, Magyar <i>et al.</i> , 2014
	C2	VEN 2097, from University of California, Davis	Bokulich <i>et al.</i> , 2012
	C3	770 <sup>2</sup> , from ARC Infruitec-Nietvoorbij, South Africa	Jolly <i>et al.</i> , 2003b <sup>2</sup>
	C4	788, from ARC Infruitec-Nietvoorbij, South Africa	This study
	C5	841, from ARC Infruitec-Nietvoorbij, South Africa	This study
	C6	971, from ARC Infruitec-Nietvoorbij, South Africa	This study
	C7	C2-19, from ARC Infruitec-Nietvoorbij, South Africa	This study
<i>Hanseniaspora uvarum</i> (anamorph: <i>Kloeckera apiculata</i> )	H1	752, from ARC Infruitec-Nietvoorbij, South Africa	Jolly <i>et al.</i> , 2003b
	H2	791, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H3	802, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H4	897, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H5	899, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H6	913, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H7	918, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H8	932, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H9	934, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H10	961, from ARC Infruitec-Nietvoorbij, South Africa	This study
	H11	980, from ARC Infruitec-Nietvoorbij, South Africa	This study

TABLE 3.1 (continued)

<i>Lachancea thermotolerans</i> (previously <i>Kluyveromyces thermotolerans</i> )	L1	Viniflora® Rhythm™, commercial yeast from Chr. Hansen, Denmark	This study
	L2	548, from ARC Infruitec-Nietvoorbij, South Africa	This study
<i>Metschnikowia pulcherrima</i> (anamorph: <i>Candida pulcherrima</i> )	M1	825, from ARC Infruitec-Nietvoorbij, South Africa	Jolly <i>et al.</i> , 2003b, c
	M2	C1/15, from ARC Infruitec-Nietvoorbij, South Africa	Jolly <i>et al.</i> , 2003c
	M3	780, from ARC Infruitec-Nietvoorbij, South Africa	This study
	M4	870, from ARC Infruitec-Nietvoorbij, South Africa	This study
	M5	950, from ARC Infruitec-Nietvoorbij, South Africa	This study
	M6	O2/16, from ARC Infruitec-Nietvoorbij, South Africa	This study
	M7	O2/17, from ARC Infruitec-Nietvoorbij, South Africa	This study
<i>Schizosaccharomyces pombe</i>	Sp	CBS 5557, CBS, Netherlands	This study
	T1	CBS 1146 <sup>1</sup> , CBS, Netherlands	Van Breda <i>et al.</i> , 2013
<i>Torulasporea delbrueckii</i> (anamorph: <i>Candida colliculosa</i> )	T2	CBS 4663, CBS, Netherlands	Van Breda <i>et al.</i> , 2013
	T3	Level <sup>2</sup> TD™, commercial strain from Lallemand Inc, France	This study
	T4	Zymaflore® Alpha <sup>TD n. Sacc.</sup> , commercial strain from Laffort, France	This study
	T5	Viniflora® Harmony™, commercial yeast from Chr. Hansen, Denmark	Van Breda <i>et al.</i> , 2013
	T6	M2/1, from ARC Infruitec-Nietvoorbij, South Africa	Jolly <i>et al.</i> , 2003b; Van Breda <i>et al.</i> , 2013
	T7	654, from ARC Infruitec-Nietvoorbij, South Africa	Van Breda <i>et al.</i> , 2013; Minnaar <i>et al.</i> , 2015
	T8	301, from ARC Infruitec-Nietvoorbij, South Africa	Van Breda <i>et al.</i> , 2013

<sup>1</sup>Publications where strains have been investigated.<sup>2</sup>Strain 770 was classified as *Candida stellata* in this paper.

### 3.3 RESULTS AND DISCUSSION

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The role of non-*Saccharomyces* yeasts in wine production is not as well researched as the role of *S. cerevisiae* (Jolly *et al.*, 2014). Although *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* are receiving much more attention due to the availability of commercial products, various other non-*Saccharomyces* yeast species have been investigated (Jolly *et al.*, 2003b; Comitini *et al.*, 2011; 2012; Jolly *et al.*, 2014; Padilla *et al.*, 2016). In this investigation, thirty-seven non-*Saccharomyces* strains representing seven different non-*Saccharomyces* species, *i.e.* *H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, *Sc. pombe*, *C. zemplinina*, *C. stellata* and *T. delbrueckii* were characterised by CHEF karyotyping, MALDI-TOF bio-typing, enzyme assays and malic acid degradation. The aforementioned non-*Saccharomyces* yeasts were compared to five commercial *S. cerevisiae* strains (N 96, NT 112, NT 202, VIN 7 and VIN 13) and their interactions with one *O. oeni* strain were investigated in synthetic grape juice. As the species level identities of the yeasts used in this study were already known, CHEF karyotyping and MALDI-TOF bio-typing were used to study strain diversity within the different species (Figs 3.1, 3.2 and 3.3).

#### 3.3.1 Electrophoretic karyotyping

Results of CHEF karyotyping of the 34 and 72 hour programmes are shown in Figs 3.1 and 3.2, respectively. The Dice coefficient was used to group the yeasts, based on the similarities of the electrophoretic banding patterns obtained. The 34 hr programme enabled the various yeasts to be separated to species and in some cases also to strain level (Fig. 3.1). The species could be separated into nine distinct clusters at a similarity (*s*) limit of 70%.

Cluster I was delineated at *s* = 75% and comprised two *H. uvarum* strains, H4 and H11, which were different from the other nine *H. uvarum* strains. Cluster II was delineated at *s* = 76% and included the remaining *H. uvarum* strains H1, H2, H3, H5, H6, H7, H8, H9 and H10. Within this cluster strains H1, H7, H9 and H10 had an almost identical karyotype and were delineated at *s* = 100%. Strains, H9 and H10 were isolated from grapes from the same location and may well be the same strain, but strains H1 and H7 were isolated from different areas within the Western Cape. This indicates that *H. uvarum* strains might not be as heterogeneous as *S. cerevisiae* strains. Cluster III comprised the two *L. thermotolerans* strains, L1 (Viniflora® Rhythm™) and L2, and delineated at *s* = 70%. There were clear differences between the karyotypes of these two strains. Seven *T. delbrueckii* strains, T2 (CBS 4663), T3 (Level 2<sup>Td</sup>), T4 (Zymaflore® Alpha <sup>TD n. Sacc.</sup>), T5 (Viniflora® Harmony™), T6, T7 and T8 formed cluster IV at *s* = 70%. *T. delbrueckii* type strain, T1 (CBS 1146), clustered alone in cluster V at *s* = 58%.

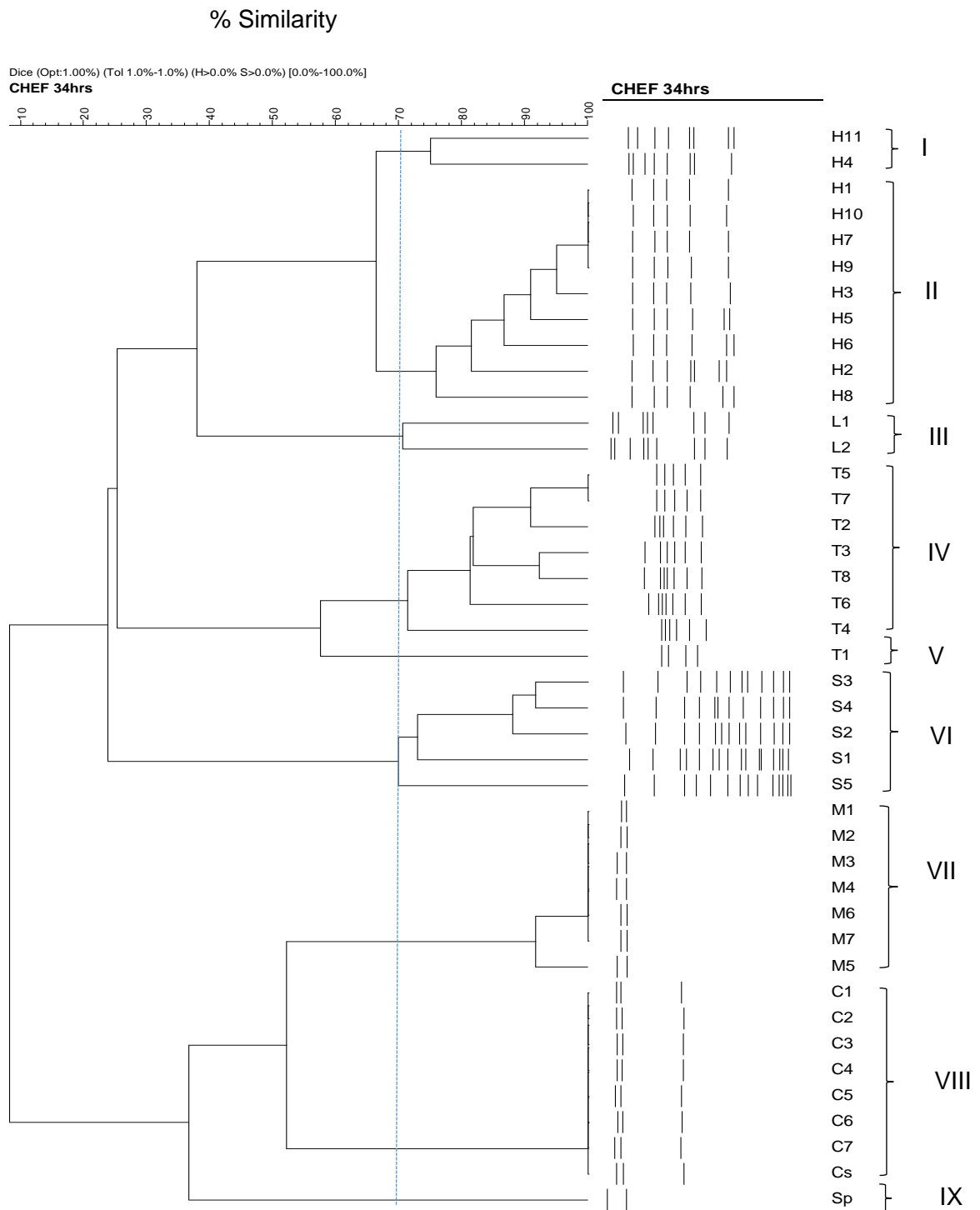


FIGURE 3.1. Dendrogram showing the clustering of yeast strains obtained by numerical analysis of CHEF karyotypes using a 34-hour programme. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cluster I and II: *Hanseniaspora uvarum* strains; III: *Lachancea thermotolerans* strains; IV and V: *Torulaspora delbrueckii* strains; VI: *Saccharomyces cerevisiae* strains; VII: *Metschnikowia pulcherrima* strains; VIII: *Candida zemplinina* (*Starmerella bacillaris*) and *Candida stellata* (*S. bombicola*) strains; and IX: *Schizosaccharomyces pombe*. Dashed blue line indicates a similarity limit of 70% that was used to define clusters.



Cluster VI comprised the five *S. cerevisiae* strains at  $s = 70\%$  and these strains showed a high level of heterogeneity. These results confirmed reports by Hoff (2012) and Moothoo-Padaychie *et al.* (2013) about the heterogeneity of *S. cerevisiae* wine yeast strains. The *M. pulcherrima* strains formed cluster VII at  $s = 92\%$ . All the strains had a similarity of 100%, except strain M5. The only difference for the *M. pulcherrima* karyotypes was the spacing between bands within the banding patterns. Cluster VIII was delineated at  $s = 100\%$ , comprised all the *C. zemplinina* strains, including the type strain (CBS 9494), and also contained the *C. stellata* type strain, Cs (CBS 157). These two species are closely related and were only reclassified as two different species when Sipiczki (2003, 2004) revealed the differences between these species. More recently, Duarte *et al.* (2012) recommended the reinstatement of *Starmerella bacillaris* comb. nov. with the name *C. zemplinina* as obligate synonym, which has not been widely accepted (Magyar *et al.*, 2014). As in the case of the *M. pulcherrima* cluster, the patterns of the *C. zemplinina* strains were very similar with small spacing differences. *Sc. pombe* grouped on its own to form cluster IX at  $s = 38\%$ , but showed some similarity with *M. pulcherrima* strains, which also had only two bands

The 34-hr CHEF programme was very useful for typing of the *S. cerevisiae* strains and strains within the *H. uvarum*, *L. thermotolerans* and *T. delbrueckii* clusters. However, it was not nearly as effective for typing *M. pulcherrima* and *C. zemplinina* strains. This confirms reports by van Breda (2012) about the usefulness of CHEF for typing of *T. delbrueckii* strains. However, the 34 hr programme could not be used to distinguish between *M. pulcherrima* and *C. zemplinina* at a strain level, therefore, an extended 72 hr CHEF programme was investigated.

The clustering analysis of the 72-hr programme is shown in Fig. 3.2. Nine clusters could be discerned at  $s = 70\%$ . Cluster I was delineated at  $s = 33\%$  and comprised of only the *M. pulcherrima* strain M5. The banding pattern of this strain was different to the other *M. pulcherrima* strains and this was also evident by the grouping of the strains using the 34 hr programme (Fig. 3.1, Cluster VII). Cluster II comprised of the one *C. stellata* type strain (Fig. 3.2). Cluster III contained three *C. zemplinina* strains, C3, C5 and C7 at  $s = 100\%$ . These *C. zemplinina* strains had identical karyotypes, indicating that these isolates are possibly the same strain. Strains C3 and C7 were isolated from grapes on the same farm and may well be the same strain. Despite being isolated from a different area, it is possible that strain C5 might be the same strain as C3 and C7. Cluster IV was delineated at  $s = 66\%$  and comprised only strain C1 (CBS 9494). Cluster V was delineated at  $s = 80\%$  and comprised of strains C4 and C6. Cluster VI was delineated at  $s = 40\%$  and comprised one strain C2. More differences were observed among the *C. zemplinina* strains with the 72-hr programme than with the 34-hr programme. The *M. pulcherrima* strains formed clusters VII (M3, M4, M6 and M7) and VIII (M1 and M2) at  $s = 100\%$ . Strains M4, M6 and M7 were isolated from the same location and could

possibly be the same strain. This would explain the similarity between these strains. However, strain M3 was isolated from a different area within the Western Cape (South Africa). As was observed with the 34-hr programme, the karyotypes of the different strains were very similar. This indicates that these strains had a few, but very long chromosomes. Cluster X contained the one *Sc. pombe* strain, which had a completely different banding pattern from the other species and this was also confirmed by a low similarity value.

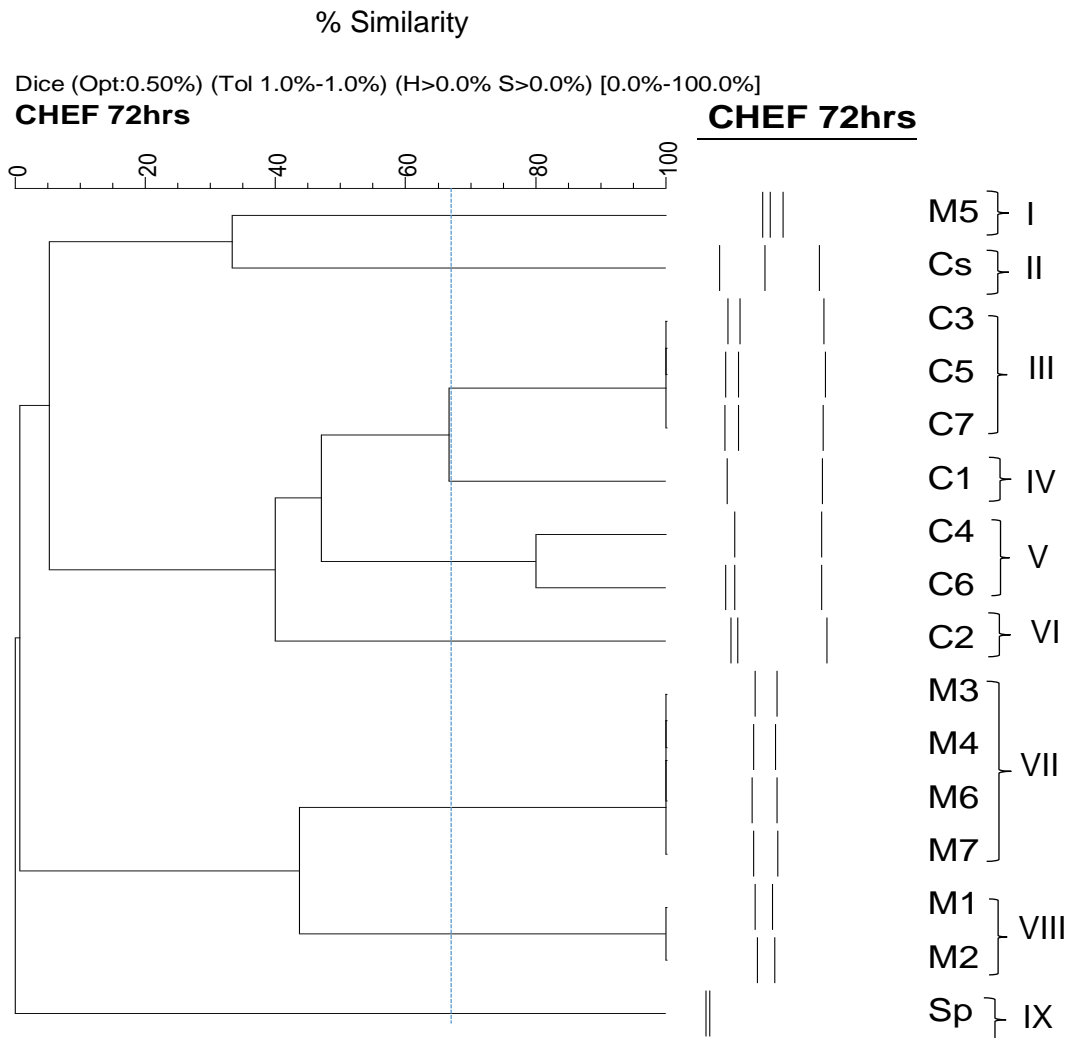


FIGURE 3.2. Dendrogram showing the clustering of yeast strains obtained by numerical analysis of CHEF karyotypes using the 72-hour programme. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Cluster I: *Metschnikowia pulcherrima*; II: *Candida stellata*; III, IV, V and VI: *C. zemplinina*; VII and VIII: *M. pulcherrima*; and IX: *Schizosaccharomyces pombe*.

More differences were observed between strains from *C. zemplinina* and *M. pulcherrima* clusters with the 72-hr programme than the 34-hr programme. *Candida zemplinina* strains showed a higher level of heterogeneity with the 72 hr programme than *M. pulcherrima* strains. This indicates that the CHEF programmes used in this study were not adequate for typing of

*M. pulcherrima* strains and more optimisation is required to obtain better separation of the chromosomes. Differences were observed between the karyotypes of *C. zemplinina* and *C. stellata* strains using the 72-hr programme, which is in agreement with findings of Sipiczki (2004) and Csoma & Sipiczki (2008) where electrophoretic karyotyping was performed over 99 and 96 hrs, respectively. Similar results were obtained in this study, but using a shorter running time (72 hrs). This study confirmed that CHEF is reliable technique for the identification of non-*Saccharomyces* yeast to species and strain level. However, more optimisation and refinement is required for typing of *M. pulcherrima* strains.

### 3.3.2 MALDI-TOF bio-typing

Results of MALDI-TOF MS analyses (Fig. 3.3) show that non-*Saccharomyces* and the *S. cerevisiae* yeasts formed distinct groups. The identity of *H. uvarum*, *M. pulcherrima*, *S. cerevisiae*, *Sc. pombe* and *T. delbrueckii* could all be verified to species level using the MALDI Biotyper database. As *L. thermotolerans*, *C. zemplinina* and *C. stellata* were not in the MALDI Biotyper database, it could not be used to identify these strains. However, the MALDI-TOF MS profiles could be used to differentiate between strains within a species. The six non-*Saccharomyces* species could be grouped into seven clusters following cluster analysis of the mass spectra obtained at a phylogenetic distance level of 0.3, indicated by the dotted line in Fig. 3.3. Cluster I and II comprised the *C. zemplinina* strains, with strain C2 positioning on its own. The strains in cluster I showed a high level of similarity and grouped closely together. The composition of the *C. zemplinina* groupings differed from the groupings obtained using the 72-hr CHEF programme. Cluster III consisted of the two *L. thermotolerans* strains, which clearly differed from each other. Cluster IV consisted of all the *M. pulcherrima* strains and also showed a high level of similarity and grouped closely together. Cluster V comprised of the *S. cerevisiae* strains and appear to be a heterogeneous cluster. The *T. delbrueckii* strains grouped together in cluster VI and three sub-groups can be differentiated within this cluster. These strains show a high degree of variation. Cluster VII comprised of the *H. uvarum* strains, which showed a high level of similarity, but four sub-groups could be differentiated. The *H. uvarum* strains H10 and H11 differed from the other strains and formed separate sub-groups. Strains H2, H6 and H9 also formed a separate sub-group. Strains H1, H3, H4, H5, H7 and H8 all grouped together and had a level of similarity. The sub-groups differed from the groupings obtained using CHEF karyotyping, indicating that isolates that were considered to be identical might be different strains.

MALDI-TOF MS results were easier and faster to obtain than the CHEF karyotyping results. In both cases, software was needed for normalisation and clustering analyses. Both CHEF and MALDI-TOF MS were useful for species identification and could clearly type strains from

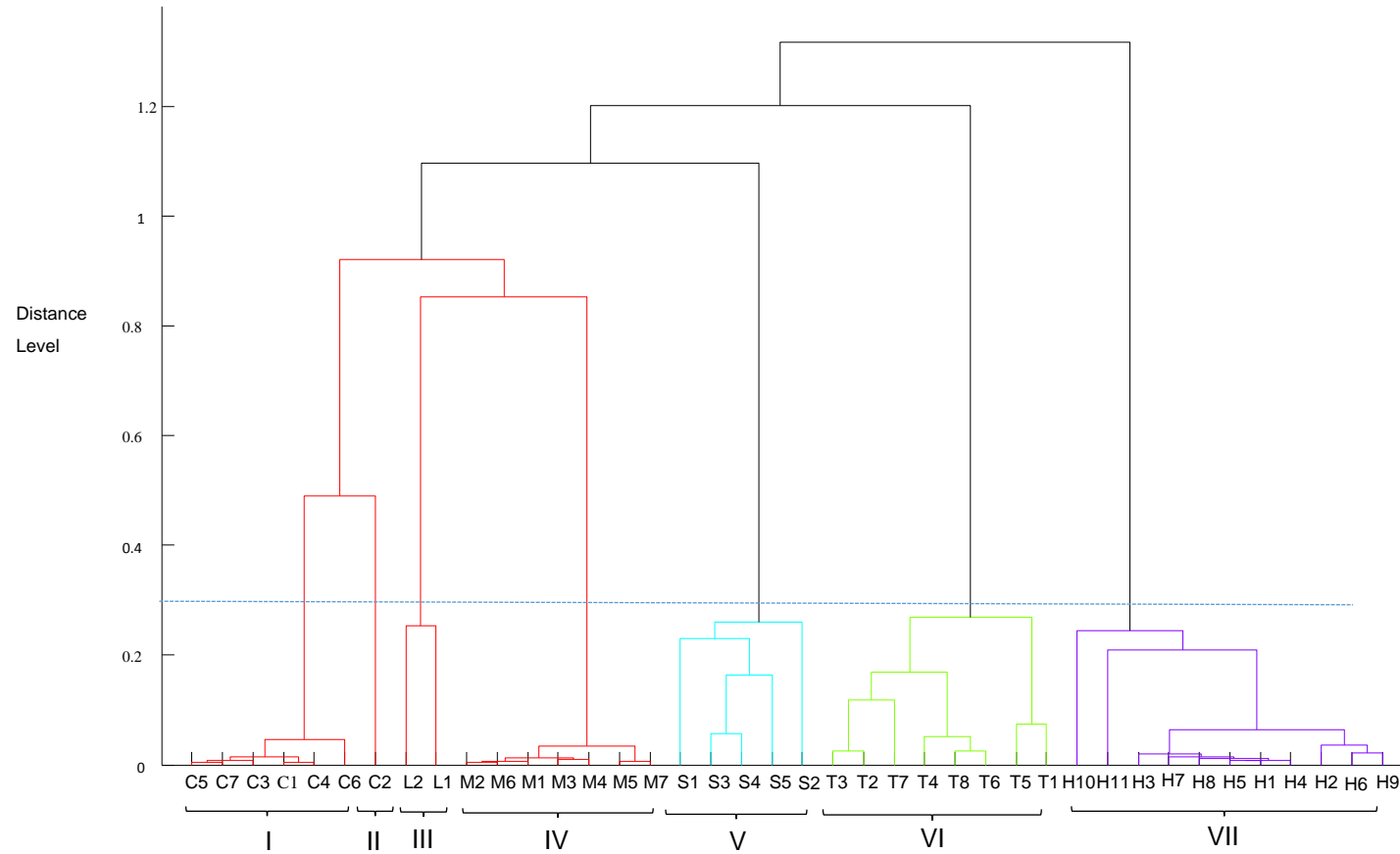


FIGURE 3.3. Dendrogram created from the mass spectral profiles of yeast strains using MALDI Biotyper software. Cluster I and II: *Candida zemplinina*; III: *Lachancea thermotolerans* strains; IV: *Metschnikowia pulcherrima*; V: *Saccharomyces cerevisiae* strains; VI: *Torulaspota delbrueckii* strains; and VII: *Hanseniaspora uvarum* strains.

*S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii* and *H. uvarum*, with MALDI-TOF MS profiles showing slightly more variation. Neither technique was effective for typing of *C. zemplinina* and *M. pulcherrima* strains, with MALDI-TOF MS revealing slightly more differences among the *M. pulcherrima* strains, and the 72-hr CHEF programme being more effective for typing of *C. zemplinina* strains. For typing of species with high genetic similarity, *i.e.* *M. pulcherrima* strains, alternative methods such as amplified fragment length polymorphism (Spadaro *et al.*, 2008) or tandem repeat-tRNA PCR (Barquet *et al.*, 2012) could be considered. This study showed that MALDI-TOF MS can be used for the identification and typing of non-*Saccharomyces* yeasts and confirms the findings of Kántor & Kačániová (2015) about the usefulness of MALDI-TOF MS to differentiate between wine yeast species. However, MALDI-TOF MS was not as effective for typing *C. zemplinina* and *M. pulcherrima* strains.

### 3.3.3 Enzyme production

The ability of the eight non-*Saccharomyces* yeast species to produce acid protease, polygalacturonase/pectinase and  $\beta$ -glucosidase enzymes and to degrade malic acid is shown in Table 3.2. The *S. cerevisiae* strains used in this study did not produce any extracellular enzymes. Charoenchai *et al.* (1997) reported some  $\beta$ -glucosidase activity in some *S. cerevisiae* strains, but Mostert (2013) found that the *S. cerevisiae* strain they tested did not have  $\beta$ -glucosidase or acid protease activity, but produced pectinase enzymes. The *C. stellata* strain was only positive for protease production and this is in agreement with the findings of Strauss (2003), who also showed that some *C. stellata* strains showed pectinolytic activity. Protease activity could be beneficial during fermentation by liberating assimilable nutrient sources, such as amino acids and peptides (Englezos *et al.*, 2015). All the *C. zemplinina* strains tested negative for all three enzyme activities. Di Maio *et al.* (2012) and Englezos *et al.* (2015) reported medium to low  $\beta$ -glucosidase activity for *C. zemplinina* strains. Englezos *et al.* (2015) reported protease activity in 48 of 63 *C. zemplinina* strains studied, but none of the strains had pectinase activity.

The *H. uvarum* strains tested positive for  $\beta$ -glucosidase and negative for the other two enzyme activities. This confirmed findings of Rodríguez *et al.*, 2004 and Hernández-Orte *et al.* (2008) that *H. uvarum* strains have  $\beta$ -glucosidase activity. Strauss (2003) and Mostert (2013) reported on *H. uvarum* strains that had protease and pectinase activity as well.

The two *L. thermotolerans* strains tested negative for all three enzyme activities. This is in contrast to Comitini *et al.* (2011) and Mostert (2013) who reported on two *L. thermotolerans* strains that showed  $\beta$ -glucosidase activity. As in the case with the other species, enzyme activity appears to be strain dependent. All the *M. pulcherrima* strains were positive for protease and  $\beta$ -glucosidase activity, which is in agreement with literature (Strauss, 2003; Mostert, 2013). The one *Sc. pombe* strain showed protease activity. Visintin *et al.* (2016) also reported on a

TABLE 3.2. Screening of *Saccharomyces* and non-*Saccharomyces* yeasts for production of extracellular enzymes and the ability to degrade malic acid.

Species name	Strain code	Enzyme activities			Malic acid degradation		
		Protease	Pectinase	$\beta$ -Glucosidase	Plate assay	Broth	% Utilised
<i>Saccharomyces cerevisiae</i>	S1	-	-	-	-	-	13
	S2	-	-	-	-	-	11
	S3	-	-	-	-	-	11
	S4	-	-	-	-	-	12
	S5	-	-	-	-	-	24
<i>Candida stellata</i>	Cs	+	-	-	+	-	9
<i>Candida zemplinina</i>	C1	-	-	-	+	+	54
	C2	-	-	-	+	+	34
	C3	-	-	-	+	+	37
	C4	-	-	-	+	+	33
	C5	-	-	-	+	+	34
	C6	-	-	-	+	+	51
	C7	-	-	-	+	+	47
<i>Hanseniaspora uvarum</i>	H1	-	-	+	+	+	10
	H2	-	-	+	+	+	30
	H3	-	-	+	+	+	9
	H4	-	-	+	+	+	11
	H5	-	-	+	+	+	12
	H6	-	-	+	+	+	14
	H7	-	-	+	+	-	8
	H8	-	-	+	+	-	7
	H9	-	-	+	+	-	9
	H10	-	-	+	+	-	10
	H11	-	-	+	+	-	7
<i>Lachancea thermotolerans</i>	L1	-	-	-	+	+	20
	L2	-	-	-	+	-	10

TABLE 3.2. (continued)

	M1	+	-	+	-	-	15
	M2	+	-	+	-	+	23
	M3	+	-	+	+	+	22
<i>Metschnikowia pulcherrima</i>	M4	+	-	+	-	+	24
	M5	+	-	+	-	+	28
	M6	+	-	+	-	+	26
	M7	+	-	+	-	+	20
<i>Schizosaccharomyces pombe</i>	Sp	+	-	-	+	+	78
<i>Torulasporea delbrueckii</i>	T1	-	-	-	-	-	14
	T2	-	-	-	-	-	11
	T3	-	-	-	-	+	19
	T4	-	-	-	-	+	31
	T5	-	-	-	-	+	18
	T6	-	-	-	-	-	8
	T7	-	-	-	-	+	18
	T8	-	-	-	-	-	11

*Sc. pombe* strain that had protease activity and a different *Sc. pombe* strain that produced pectinase. The results of this study confirmed the conclusion of Ganga & Martínez (2004) that enzyme production is not characteristic of a particular genus or species, but depends on the yeast strain analysed.

### 3.3.4 Malic acid degradation

The *S. cerevisiae* strains showed no malic acid degradation on the plate assay, but in the broth showed low activity, with S5 (VIN 7) utilising about 24% of the malic acid (Table 3.2). The low malic acid utilisation by *S. cerevisiae* is well documented (Gao and Fleet, 1995; Volschenk *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006). The ability of the non-*Saccharomyces* strains to degrade malic acid varied greatly and there were also clear differences between the results of the plate and broth assays. Results indicate that the plate assay for malic acid utilisation is not very reliable and gave a lot of negative results as well as false positives. The *C. stellata* strain produced a positive reaction for malic acid utilisation on the plate assay, but could only utilise 9% of the malic acid in the broth assay. All the *C. zemplinina* strains gave positive results for malic utilisation on the plate assay and in broth, with malic acid utilisation ranging from 33-54%.

All the *H. uvarum* strains also gave positive reactions for malic acid utilisation on the plate assay, but only strain H2 showed real malic acid utilisation (30%) in the broth. The other *H. uvarum* strains only utilised between 7 and 14% of the malic acid in broth. *T. delbrueckii* strains gave negative results for malic acid utilisation on the plate assay, but showed variable malic acid utilisation (11-31%) in the broth, with strain T4 (Zymaflore® Alpha <sup>TD n. Sacc.</sup>) showing the most activity (31%). Above results are in agreement with reports of low malic acid utilisation for *C. stellata*, *T. delbrueckii* and *H. uvarum* (Gao & Fleet, 1995; Saayman & Viljoen-Bloom, 2006). The *L. thermotolerans* strains were also able to degrade malic acid on the plate assay, but were not as efficient in the broth, with strain L1 (Vinflora® Rhythm™) managing to utilise 20% of the malic acid. Only strain M3 gave a positive reaction on the plate assay, but all the *M. pulcherrima* strains showed some malic acid utilisation (15-28%).

As expected, the *Sc. pombe* strain gave a positive reaction on the plate assay and utilized 78% of the malic acid in the broth. Strains of *Sc. pombe* can degrade high concentrations of L-malate, but only if glucose or another assimilable carbon source is present (Baranowski & Radler, 1984; Rodriguez & Thornton, 1989, Benito *et al.*, 2013, 2014).

### 3.3.5 Evaluation of yeasts

#### 3.3.5.1 Fermentation trial

The ability of the non-*Saccharomyces* yeast to ferment synthetic juice and progress of alcoholic fermentation are shown in Figs 3.4 to 3.8. The fermentations were monitored regularly for 40 days, but the final wine chemical analyses were carried out after 180 days, when the wines



produced with the slow-fermenting yeasts were found to be dry (glucose/fructose <4 g/L). *Candida zemplinina* strains showed variable fermentation abilities, with strains C1 (CBS 9494) and C2 (VEN 2097) standing out as the strongest fermenters, but still not comparable to the *S. cerevisiae* strains (Fig. 3.4). According to Csoma & Sipiczki (2008), *C. zemplinina* strains can be found throughout white and red wine fermentations and usually have sustained presence until the end of alcoholic fermentation. This study showed that some of the *C. zemplinina* strains have enough fermentation potential to be used in mixed culture fermentations.

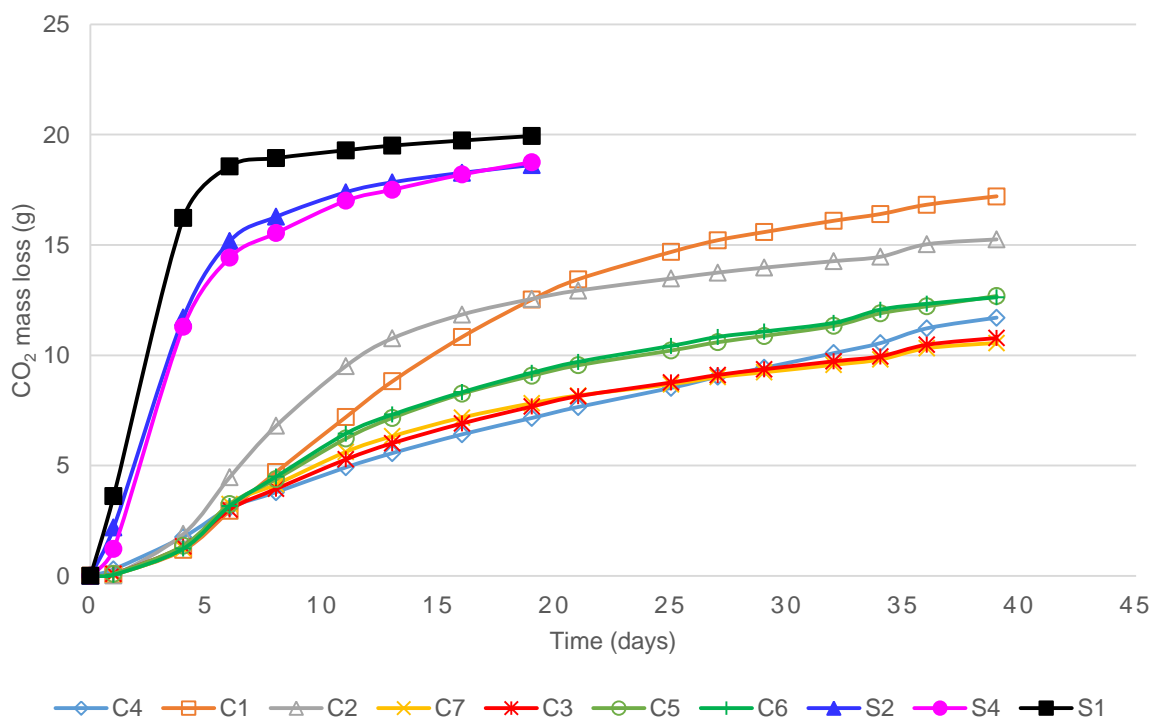


FIGURE 3.4. Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Candida zemplinina* strains in synthetic grape juice.

The *H. uvarum* strains were slow to moderate fermenters, with strain H11 being the strongest fermenter (Fig. 3.5). The low fermentation activity of *H. uvarum* is in agreement with Ciani & Maccarelli (1998). The *M. pulcherrima* strains were also slow fermenters and most were still fermenting after 40 days, the exception being strain M6 (Fig. 3.6). This concurs with reports from other studies (Jolly *et al.*, 2003c; Mostert & Divol, 2014). Strains H11 and M6 performed better than the other *H. uvarum* and *M. pulcherrima* strains, and there is a possibility that these fermentations may have been contaminated during sampling. No implantations were performed to verify that the inoculated yeast strains completed the alcoholic fermentations. The *T. delbrueckii* strains were strong fermenters and had comparable fermentation rates to the *S. cerevisiae* reference strains (Fig. 3.7). This concurs with reports of van Breda *et al.* (2013) and Renault *et al.* (2015). The two *L. thermotolerans* strains were also strong fermenters and comparable to the *S. cerevisiae* strains (Fig. 3.8).

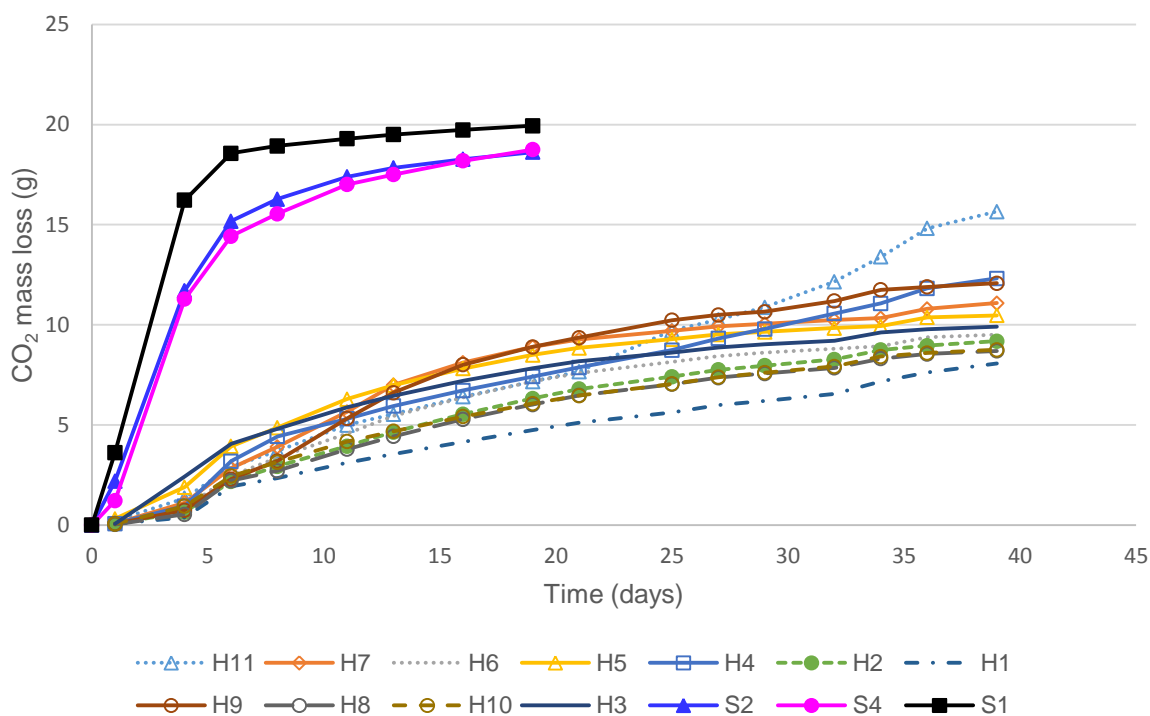


FIGURE 3.5. Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* strains in synthetic grape juice.

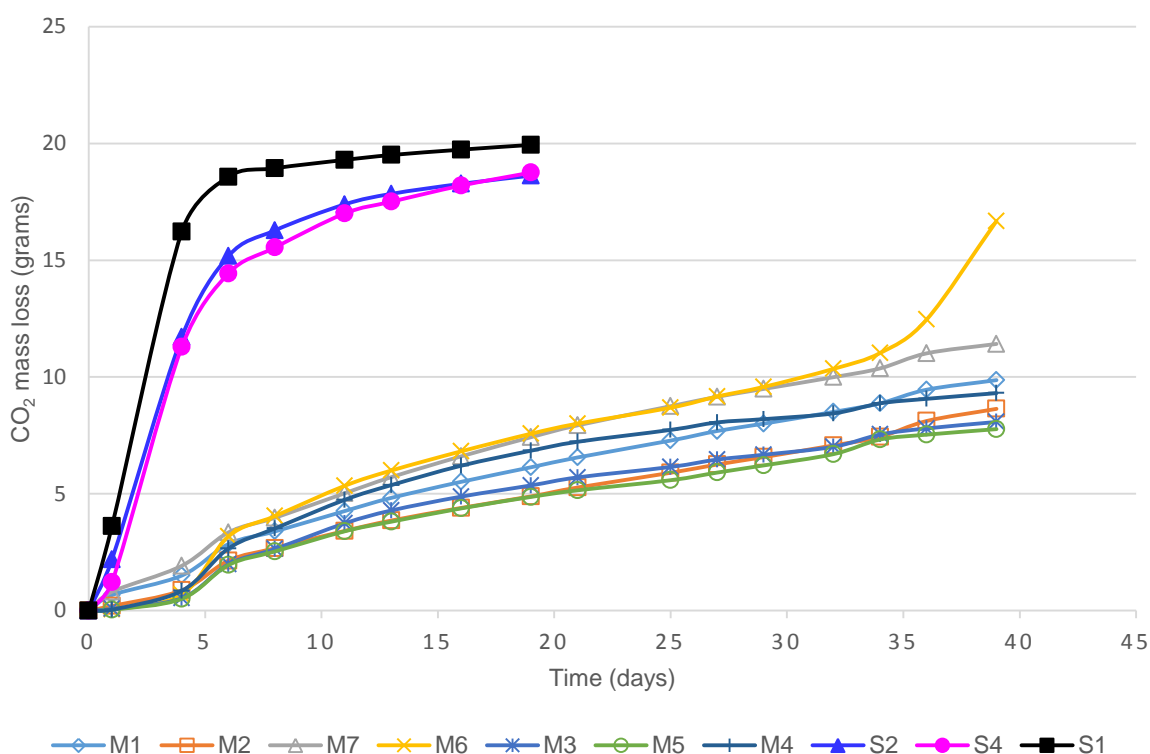


FIGURE 3.6. Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Metschnikowia pulcherrima* strains in synthetic grape juice.

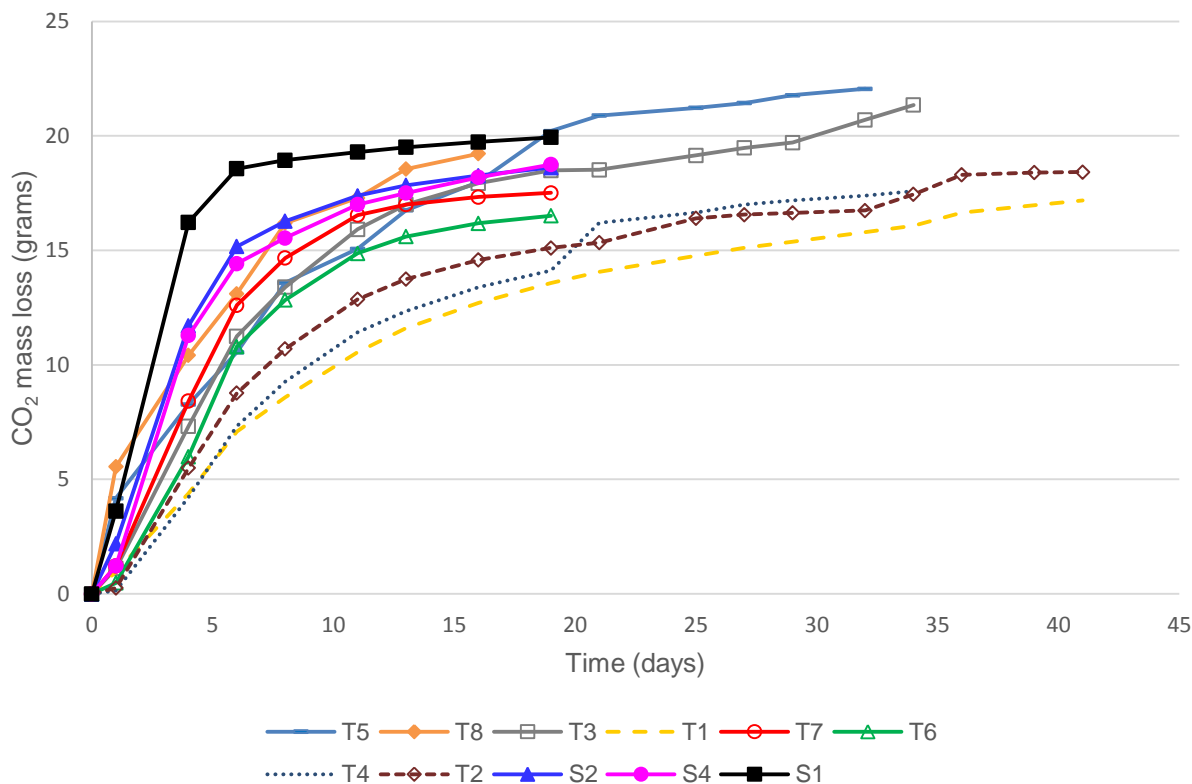


FIGURE 3.7. Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* strains in synthetic grape juice.

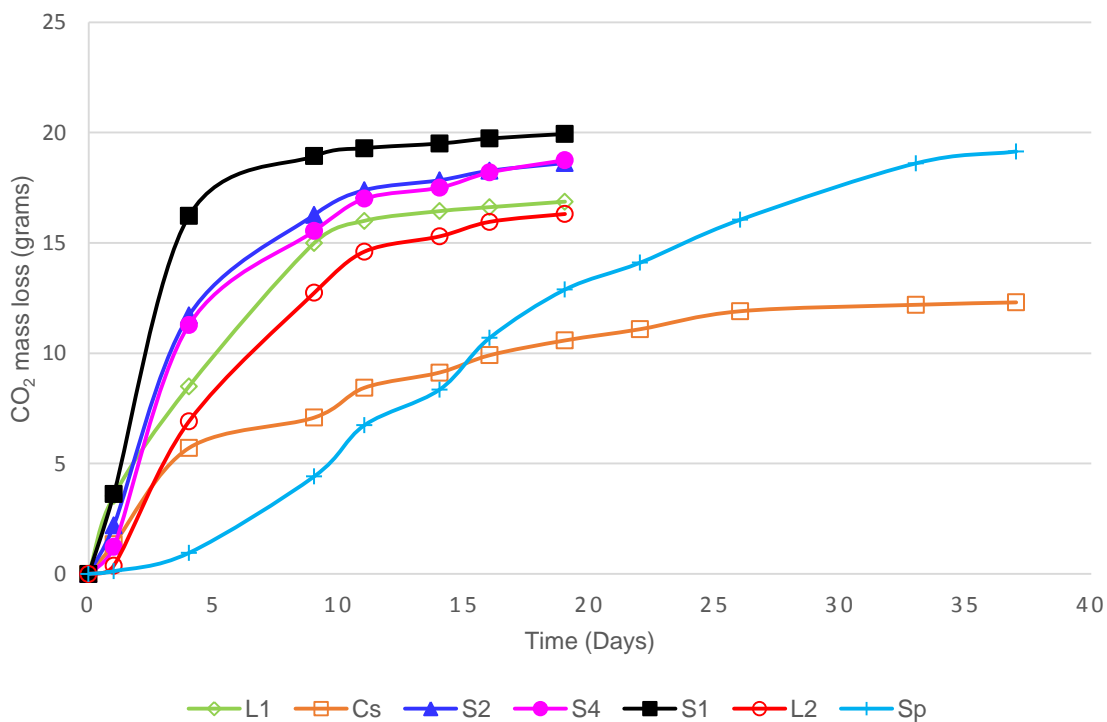


FIGURE 3.8. Fermentation kinetics of pure cultures of *Saccharomyces cerevisiae*, *Lachancea thermotolerans* and *Schizosaccharomyces pombe* strains in synthetic grape juice.

These results confirmed findings of Comitini *et al.* (2011) and Mostert & Divol (2014). The fact that both *T. delbrueckii* and *L. thermotolerans* are such strong fermenters is probably one of the reasons why strains from these species were selected for use as commercial starters in mixed culture fermentations with *S. cerevisiae* (Jolly *et al.*, 2014). The *Sc. pombe* strain was a moderate fermenter and fermentation activity may vary between strains (Benito *et al.*, 2012, 2013). The *C. stellata* strain was a slow fermenter.

#### 3.3.5.2 Chemical analyses

Results of chemical analyses of synthetic wines produced with the different yeast species are listed in Table 3.3. The fermentations conducted by the slow fermenting yeasts were considered to be dry (residual sugar < 4 g/L) after 180 days. A great degree of variation was observed among the ethanol, malic acid and volatile acidity (VA) levels for the different non-*Saccharomyces* yeast species and strains. The non-*Saccharomyces* yeast species and strains within a species also varied with regard to the amount of sugar utilised to produce 1% ( $v/v$ ) ethanol. *Candida zemplinina* strains produced low VA and were similar to the *S. cerevisiae* strains, although *C. zemplinina* strains can be low or high VA producers (Magyar & Toth, 2011; Magyar *et al.*, 2014; Englezos *et al.*, 2015). Synthetic wines produced with *H. uvarum* contained high VA levels, especially wines produced with strains H2, H3 and H10. In contrast, synthetic wines produced with strains H5, H6, H7, H8 and H9 had low VA levels, which indicate strain variation within this species. Wines produced by other non-*Saccharomyces* yeasts contained lower VA levels than *H. uvarum*, which is in agreement with findings by other researchers (Ciani & Picciotti, 1995; Rojas *et al.*, 2003). Wines produced with the *Sc. pombe* strain and *T. delbrueckii* strains contained the lowest VA levels. This is in agreement with Moreno *et al.* (1991) and Renault *et al.* (2009), who showed that pure cultures of *T. delbrueckii* produced lower VA levels than *S. cerevisiae*. Benito *et al.* (2012, 2013 and 2014) showed that *Sc. pombe* can be moderate to high VA producers, depending on the strain. Most of the *M. pulcherrima* strains produced low VA levels, except for strain M5 that produced slightly higher VA levels (0.52 g/L). *M. pulcherrima* is not normally associated with VA production, but with relatively high concentrations of esters (Bisson & Kunkee, 1991).

The malic acid levels were lower in all synthetic wines, indicating loss due to precipitation, but also some degradation (Table 3.3). In most cases, synthetic wines fermented with non-*Saccharomyces* yeasts had lower malic acid levels than synthetic wines fermented with *S. cerevisiae* strains. Wines fermented with *Sc. pombe* had a malic acid reduction of >77%, while the reduction by the other non-*Saccharomyces* yeast varied. These results are in agreement with those obtained for the malic acid utilisation in the malic acid broth.

TABLE 3.3. Chemical analyses and duration of alcoholic fermentation (AF) in synthetic wine produced with different yeast strains.

Species name	Strain code	Residual sugar (g/L)	Ethanol (% v/v)	Sugar consumed (g)/1% (v/v) alcohol produced	Total acidity (g/L)	pH	Malic acid (g/L)	Volatile acidity (g/L)	Duration of AF (days)
<i>Saccharomyces cerevisiae</i>	S1	1.90±0.25	9.93±0.03	17.94±0.08	3.54±0.02	3.48±0.01	2.35±0.04	0.40±0.01	20
	S2	1.76±0.19	9.88±0.14	18.05±0.25	3.43±0.03	3.56±0.03	2.56±0.22	0.34±0.04	19
	S3	1.56±0.13	10.02±0.25	17.81±0.30	3.52±0.04	3.53±0.02	2.29±0.12	0.29±0.05	20
	S4	1.66±0.55	10.07±0.19	17.70±0.28	3.54±0.04	3.54±0.04	2.36±0.07	0.30±0.10	20
	S5	1.27±0.13	9.37±0.23	19.09±0.49	3.37±0.03	3.59±0.03	1.96±0.22	0.57±0.08	22
<i>Candida stellata</i>	Cs	1.01±0.31	9.34±0.14	19.16±0.10	3.49±0.15	3.67±0.05	2.20±0.13	0.39±0.06	180
<i>Candida zemplinina</i>	C1	3.36±0.16	9.82±0.04	17.98±0.36	3.28±0.02	3.70±0.02	2.07±0.08	0.27±0.01	42
	C2	2.41±0.36	8.60±0.92	20.65±1.19	3.20±0.23	3.70±0.06	1.67±0.23	0.30±0.14	45
	C3	1.60±0.24	9.52±0.21	18.74±0.30	3.11±0.06	3.78±0.02	1.81±0.1	0.32±0.02	40
	C4	1.21±0.17	9.80±0.09	18.25±0.16	3.34±0.04	3.74±0.04	1.83±0.49	0.50±0.05	180
	C5	1.05±0.12	9.87±0.04	18.14±0.07	3.22±0.04	3.73±0.02	1.57±0.07	0.37±0.02	180
	C6	1.47±0.39	9.86±0.31	18.11±0.54	2.99±0.06	3.72±0.02	1.43±0.07	0.36±0.04	45
	C7	1.94±0.15	9.91±0.12	17.97±0.24	3.17±0.15	3.74±0.05	1.63±0.37	0.33±0.08	68
<i>Hanseniaspora uvarum</i>	H1	1.65±0.16	9.84±0.11	18.12±0.30	3.77±0.40	3.66±0.05	1.89±0.14	0.47±0.34	180
	H2	1.57±0.43	9.73±0.26	18.34±0.47	3.73±0.50	3.75±0.02	1.81±0.38	0.72±0.50	180
	H3	1.19±0.08	10.08±0.08	17.73±0.14	3.78±0.05	3.74±0.02	1.77±0.22	0.84±0.08	180
	H4	1.68±0.01	10.08±0.26	17.70±0.46	3.62±0.16	3.67±0.04	2.13±0.08	0.56±0.06	42
	H5	3.20±0.47	9.55±0.07	18.51±0.45	3.33±0.02	3.65±0.05	2.17±0.16	0.20±0.05	180
	H6	1.95±0.45	9.73±0.38	18.30±0.78	3.37±0.30	3.69±0.06	1.91±0.22	0.35±0.25	180
	H7	1.77±0.52	9.20±0.21	19.37±0.52	3.52±0.25	3.72±0.16	1.99±0.25	0.43±0.06	180
	H8	1.93±0.24	9.43±0.25	18.88±0.52	3.49±0.46	3.70±0.01	1.87±0.27	0.22±0.02	180
	H9	1.88±0.09	8.80±0.12	20.24±0.34	3.31±0.07	3.70±0.01	1.79±0.13	0.16±0.05	180
	H10	1.97±0.75	9.91±0.14	17.96±0.21	3.74±0.09	3.74±0.02	1.74±0.09	0.88±0.04	180
	H11	1.86±0.44	9.64±0.76	18.48±1.14	4.18±0.63	3.71±0.07	2.34±0.40	0.51±0.23	45

TABLE 3.3. (continued)

<i>Lachancea thermotolerans</i>	L1	1.12±0.10	9.31±0.02	19.22±0.05	2.84±0.02	3.59±0.01	1.81±0.22	0.18±0.04	22
	L2	2.29±1.20	10.35±0.12	17.17±0.32	3.10±0.08	3.51±0.02	2.12±0.18	0.10±0.07	25
<i>Metschnikowia pulcherrima</i>	M1	0.96±0.02	9.12±0.20	19.63±0.31	3.79±0.31	3.51±0.02	1.91±0.26	0.35±0.15	180
	M2	0.86±0.63	9.11±0.01	19.66±0.03	3.88±0.04	3.59±0.01	1.83±0.11	0.26±0.11	180
	M3	0.56±0.34	8.93±0.65	20.09±0.71	3.89±0.27	3.63±0.04	1.61±0.20	0.37±0.05	180
	M4	0	9.51±0.36	18.93±0.69	3.94±0.04	3.70±0.03	1.71±0.12	0.25±0.14	180
	M5	0	9.58±0.16	18.78±0.03	3.87±0.18	3.65±0.03	1.50±0.12	0.52±0.03	180
	M6	2.63±2.15	8.10±1.27	21.90±3.21	3.63±0.23	3.65±0.22	1.78±0.27	0.21±0.11	46
	M7	0.65±0.62	8.97±1.15	20.00±1.28	3.84±0.25	3.53±0.11	1.62±0.17	0.33±0.20	180
<i>Schizosaccharomyces pombe</i>	Sp	1.87±0.24	10.13±0.03	17.58±0.09	1.82±0.03	3.69±0.04	0.56±0.01	0.07±0.04	39
<i>Torulaspora delbrueckii</i>	T1	1.60±0.55	10.00±0.14	17.84±0.20	3.18±0.05	3.69±0.13	1.85±0.07	0.12±0.02	180
	T2	1.80±0.64	9.36±0.92	19.04±1.83	3.63±0.13	3.58±0.04	2.48±0.23	0.19±0.01	31
	T3	1.83±0.12	9.84±0.27	18.11±0.51	2.86±0.16	3.60±0.07	2.31±0.02	0.07±0.01	24
	T4	3.16±2.32	9.28±0.21	19.06±0.06	3.28±0.25	3.78±0.26	2.16±0.37	0.19±0.05	39
	T5	3.70±0.31	9.51±0.22	18.54±0.29	3.01±0.03	3.59±0.01	2.39±0.01	0.06±0.01	39
	T6	3.00±1.31	9.75±0.51	18.15±0.98	3.16±0.11	3.59±0.03	2.52±0.18	0.07±0.02	20
	T7	1.46±0.09	10.12±0.10	17.64±0.15	3.11±0.06	3.58±0.01	2.32±0.12	0.05±0.05	20
	T8	2.93±1.21	9.82±0.27	18.04±0.46	3.17±0.06	3.61±0.02	2.46±0.10	0.05±0.04	20

### 3.3.5.3 Malolactic fermentation

The effect of various yeast strains on *O. oeni* growth and its ability to complete MLF, with or without nutrient supplementation, prior to inoculation are presented in Table 3.4. There were clear differences between the MLF treatments that were applied. In most cases, MLF proceeded quickly and without delays. However, in some cases where delays occurred, nutrient supplementation improved the progress of MLF or completely eliminated the delays. None of the yeasts produced high levels of SO<sub>2</sub> that could inhibit LAB, but there were some variations between the species and among strains from the same species. Despite producing low levels of SO<sub>2</sub>, there were differences among the *S. cerevisiae* strains. Strains S1 and S5 had the least inhibitory effect on MLF, completed after 7 days (Table 3.4). Strain S3 had an inhibitory effect on MLF and this was evident in both treatments. In this case, inhibition could be due to SO<sub>2</sub>, but production of other inhibitory compounds is more likely. Yeasts can inhibit LAB and therefore MLF by depleting nutrients or by producing of toxic metabolites such as ethanol, SO<sub>2</sub>, medium chain fatty acids and proteins or peptides (Alexandre *et al.*, 2004, Comitini *et al.*, 2005; Nehme *et al.*, 2008). Strains S2 and S4 also had an inhibitory effect on MLF (treatment 1), but the inhibition could be overcome by nutrient supplementation (treatment 2). The antagonistic effect of some *S. cerevisiae* on MLF has been reported and yeast and LAB compatibility is an important factor to consider for successful MLF (Henick-Kling and Park 1994; Costello *et al.*, 2003).

The *C. stellata* strain (Cs) had an inhibitory effect on MLF (26 days) and resulted in MLF taking longer to complete (Table 3.4). However, delayed MLF could be partially alleviated by nutrient supplementation (treatment 2), but MLF still took 21 days. Inhibition by *C. stellata* could be partially due to nutrient depletion, but other inhibitory compounds are a more likely explanation. In general, the *C. zemplinina* strains did not have an inhibitory effect on MLF, except for strain C7, which took 20 days to complete MLF. The inhibitory effect of C7 was completely eliminated by nutrient supplementation.

*Hanseniaspora uvarum* strains H5 and H7 had a slight inhibitory effect on all MLF treatments. SO<sub>2</sub> levels were not excessively high in these wines, indicating that some other inhibitory compound/s was probably produced. Strains H3, H8 and H9 also had an inhibitory effect on MLF, but the inhibitory effect could be eliminated by nutrient supplementation. The *L. thermotolerans* and *M. pulcherrima* strains completed MLF quickly and were finished within 7 days. No variations with regard to MLF were observed for strains within these species. The *M. pulcherrima* strains had the highest total SO<sub>2</sub> levels of all the non-*Saccharomyces* yeast, but it did not affect the progression of MLF.

TABLE 3.4. Free and total SO<sub>2</sub> levels and duration of malolactic fermentation (MLF) in synthetic wines fermented with different yeasts.

Species name	Strain code	Free SO <sub>2</sub> (mg/L)	Total SO <sub>2</sub> (mg/L)	Duration of MLF (days)	
				Treatment 1 <sup>1</sup>	Treatment 2 <sup>2</sup>
<i>Saccharomyces cerevisiae</i>	S1	3	8	7	7
	S2	4	9	13	7
	S3	5	9	14	13
	S4	5	9	13	7
	S5	2	5	7	6
<i>Candida stellata</i>	Cs	2	9	26	21
<i>Candida zemplinina</i>	C1	2	4	7	7
	C2	2	5	7	7
	C3	2	5	7	7
	C4	2	6	7	7
	C5	2	5	7	7
	C6	2	5	7	7
	C7	2	6	20	7
<i>Hanseniaspora uvarum</i>	H1	2	10	7	7
	H2	1	8	7	7
	H3	2	8	10	7
	H4	2	9	7	7
	H5	2	8	13	13
	H6	1	8	7	7
	H7	2	6	14	14
	H8	2	6	13	7
	H9	1	8	13	7
	H10	2	9	7	7
	H11	2	8	7	7
<i>Lachancea thermotolerans</i>	L1	2	5	7	7
	L2	2	5	7	7
<i>Metschnikowia pulcherrima</i>	M1	3	9	7	7
	M2	3	10	7	7
	M3	3	9	7	7
	M4	2	9	7	7
	M5	2	10	7	7
	M6	3	10	7	7
	M7	3	10	7	7
<i>Torulaspora delbrueckii</i>	T1	2	10	7	7
	T2	2	8	14	14
	T3	2	4	13	7
	T4	2	5	7	7
	T5	2	5	7	7
	T6	3	5	7	7
	T7	3	5	7	10
	T8	2	5	7	7

<sup>1</sup>Treatment 1: Sequential inoculation with commercial *Oenococcus oeni* strain.<sup>2</sup>Treatment 2: Nutrient supplementation (Costello *et al.*, 2003) prior to sequential inoculation with *O. oeni* strain.



Duration of MLF varied between the yeasts used, but none of the yeasts completely inhibited MLF. In the cases of delayed MLF, it appears to be strain dependent. Results indicate that some of the yeast strains had a higher nutrient demand or uptake, or produced inhibitory compounds, which resulted in slower progression of MLF. SO<sub>2</sub> was ruled out as a reason for the delays, but other toxic metabolites were not investigated. The metabolites produced by these inhibitory strains need further investigation. The results obtained in synthetic wine should be confirmed in real grape juice and wine fermentations, because the interaction between the non-*Saccharomyces* yeast and LAB might be different in a real wine matrix.

### 3.4 CONCLUSIONS

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Both CHEF karyotyping and MALDI-TOF MS were effective techniques for identifying wine non-*Saccharomyces* yeast species and could also be used for typing of *C. zemplinina*, *H. uvarum*, *L. thermotolerans*, *T. delbrueckii* strains. Both techniques were unable to adequately type *M. pulcherrima* strains, but CHEF karyotyping showed more potential for typing of *M. pulcherrima* strains. Yeast enzyme activity appears to be strain dependent and most of the species investigated did not have extracellular  $\beta$ -glucosidase, pectinase and protease activity. In synthetic wine fermentations, *C. stellata*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *Sc. pombe* strains were shown to be slow to medium fermenters. The *L. thermotolerans* and *T. delbrueckii* strains were found to be medium to strong fermenters and comparable to *S. cerevisiae*. Further investigations are needed to evaluate the *L. thermotolerans* and *T. delbrueckii* strains in grape must as potential single inoculations or co-inoculations with *S. cerevisiae*, while the *H. uvarum* and *M. pulcherrima* strains need to be evaluated in co- or sequential inoculations with *S. cerevisiae*. The effect of non-*Saccharomyces* yeast species on MLF varied and inhibition was found to be strain dependent. All *M. pulcherrima* and *L. thermotolerans* strains used in this study were compatible with the *O. oeni* strain and conducive to MLF. In most cases, delays in MLF could be alleviated by nutrient supplementation. Many of the non-*Saccharomyces* yeast strains evaluated showed potential for use in wine production and warrant further investigation.

### 3.5 ACKNOWLEDGEMENTS

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# Chapter 4

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## Research results II

**Effect of *Saccharomyces*, non-*Saccharomyces* yeasts and malolactic fermentation strategies on fermentation kinetics and flavour of Shiraz wines**

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## CHAPTER 4

### Effect of *Saccharomyces*, non-*Saccharomyces* yeasts and malolactic fermentation strategies on fermentation kinetics and flavour of Shiraz wines

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**Abstract:** The use of non-*Saccharomyces* yeasts to improve complexity and diversify wine style is increasing, however, the interactions between non-*Saccharomyces* yeasts and lactic acid bacteria (LAB) have not received much attention. This study investigated the interactions of seven non-*Saccharomyces* yeast strains of the genera *Candida*, *Hanseniaspora*, *Lachancea*, *Metschnikowia* and *Torulaspora* in combination with *S. cerevisiae* and three malolactic fermentation (MLF) strategies in a Shiraz winemaking trial. Standard oenological parameters, volatile composition and sensory profiles of wines were investigated. Wines produced with non-*Saccharomyces* yeasts had lower alcohol and glycerol levels than wines produced with *S. cerevisiae* only. Malolactic fermentation also completed faster in these wines. Wines produced with non-*Saccharomyces* yeasts differed chemically and sensorially from wines produced with *S. cerevisiae* only. The *Candida zemplinina* and the one *L. thermotolerans* isolate slightly inhibited LAB growth in wines that underwent simultaneous MLF. Malolactic fermentation strategy had a bigger impact on sensory profiles than yeast treatment. Both yeast selection and MLF strategy had a significant effect on berry aroma, but MLF strategy also had a significant effect on acid balance and astringency of wines. Winemakers should apply the optimal yeast combination and MLF strategy to ensure fast completion of MLF and improve wine complexity.

**Keywords:** yeast selection; lactic acid bacteria; inoculation; volatile compounds; chemical profile; sensory evaluation; aroma

#### 4.1 Introduction

Shiraz, also known as Syrah (*Vitis vinifera* L.) is a red cultivar used internationally to produce dark-coloured and full-bodied wines that are suitable for ageing. Shiraz is cultivated in all wine producing regions of the world, including Australia, South Africa and South American countries [1]. Shiraz is renowned for 'spicy', 'dark fruit'- and 'berry'-like flavors and different wine styles can be produced, depending on the region of origin, viticultural and winemaking practices [2]. Wine flavor contributes to the final quality of wine and is the product of the combined effects of several volatile compounds, such as alcohols, aldehydes, esters, acids, monoterpenes and other minor components already present in the grapes, or that are formed during fermentation or maturation [1].

Wine production includes two important fermentation processes, *i.e.* alcoholic fermentation conducted by yeast, and malolactic fermentation (MLF) conducted by lactic acid bacteria (LAB) [3,4]. The

yeasts drive alcoholic fermentation by converting grape sugar to alcohol, carbon dioxide and volatile compounds that affect the aroma and taste of wine [3,5]. At the onset of alcoholic fermentation, a large number of non-*Saccharomyces* species may be present, but the final stage is dominated by alcohol-tolerant *Saccharomyces cerevisiae* strains [3,5-7].

Recent studies have shown that non-*Saccharomyces* yeasts have different oenological properties to those of *S. cerevisiae*, and can be used to modulate and improve the aroma and complexity of wines [8-11]. Most non-*Saccharomyces* yeasts are poor fermenters and therefore are used in combination with *S. cerevisiae* in sequential inoculations, to complete the fermentation [9].

In studies carried out with Shiraz, using *Candida zemplinina*, *Kazachstania aerobia*, *K. gamospora*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Torulaspora delbrueckii* and *Zygosaccharomyces kombuchaensis*, the wines produced with these non-*Saccharomyces* yeasts had distinct volatile chemical profiles that were different to the *S. cerevisiae* reference [10,11]. These non-*Saccharomyces* wines had lower concentrations of esters, alcohols and terpenes than the *S. cerevisiae* wines. In a study carried out in Sauvignon blanc, using some of the aforementioned non-*Saccharomyces* yeasts, the wines also showed distinct chemical and sensory profiles [12]. Sauvignon blanc wines produced with *S. cerevisiae* had guava, grapefruit, banana, and pineapple aromas, while *C. zemplinina* wines were driven by fermented apple, dried peach/apricot, and stewed fruit aromas, as well as a sour flavor.

Non-*Saccharomyces* yeast can also be used to reduce ethanol content and a reduction from 0.64% v/v at pilot scale in grape juice to 1.60% v/v in laboratory scale trials using synthetic grape juice was reported [13]. Sequential fermentation trials using *L. thermotolerans* (formerly *Kluyveromyces thermotolerans*) under industry conditions with a two day delay of the second inoculum (*S. cerevisiae*), resulted in an ethanol reduction of 0.7% v/v [8]. A sequential inoculation of *M. pulcherrima* AWRI 1149 followed by a *S. cerevisiae* wine strain lowered ethanol concentration to 0.9 and 1.6% v/v for Chardonnay and Shiraz wines, respectively [14].

Malolactic fermentation is an enzymatic decarboxylation of L-malic acid to L-lactic acid and CO<sub>2</sub>, and is required for the production of some red wines, full-bodied white and sparkling wines [4,15]. Malolactic fermentation increases microbiological stability and can affect wine flavor through the modification of compounds such as diacetyl, esters, higher alcohols and volatile acids by LAB [16-19]. *Oenococcus oeni* is the preferred LAB species for MLF due to its resistance to harsh conditions found in wine [17-19]. Various MLF strategies have been investigated with simultaneous (at the start of alcoholic fermentation) and sequential inoculation (after alcoholic fermentation) receiving the most attention [15]. Selecting compatible yeast and LAB strains are essential for successful alcoholic and malolactic fermentation, as certain yeast strains have been shown to have a negative effect on LAB growth and MLF [20,21]. However, some LAB strains can also cause slow or stuck fermentations [22]. Yeast and LAB interactions differ for the various MLF inoculation strategies, so the optimal yeast/LAB combinations may not be the same for simultaneous and sequential MLF [15,23]. Wine sensory profiles following simultaneous inoculation of LAB, can differ from those of sequential MLF inoculation [24,25]. The interactions between *S. cerevisiae*, non-*Saccharomyces* yeasts and LAB are not as well researched as the interactions between *S. cerevisiae* and LAB.

There is still a lack in understanding of how specific non-*Saccharomyces* yeasts alter the sensory properties of wine, as well as the interactions of these non-*Saccharomyces* with *S. cerevisiae* yeasts in wines from various grape cultivars [11]. Little is known about the interactions of *Saccharomyces*, non-*Saccharomyces* yeasts and lactic acid bacteria, and how their interactions affect wine aroma and flavor. In a previous study [26], 37 non-*Saccharomyces* yeast strains were evaluated for use in wine production. The current study narrowed the non-*Saccharomyces* yeasts down to seven strains from five species, i.e. *C. zemplinina*, *Hanseniaspora uvarum*, *M. pulcherrima*, *L. thermotolerans* and *T. delbrueckii*. These non-*Saccharomyces* strains were used in combination with *S. cerevisiae* and three MLF strategies in a small-scale Shiraz wine production trial. The aims were to investigate the interactions between *Saccharomyces*, non-*Saccharomyces* yeast and *Oenococcus oeni*, as well as the resulting effect of these interactions on duration of MLF and Shiraz wine flavor.



## 4.2 Materials and Methods

### 4.2.1 Cultivation and enumeration of microorganisms

The yeasts and LAB used in this study are listed in Table 4.1. The two commercial non-*Saccharomyces* yeast strains, *i.e.* *T. delbrueckii* (Level<sup>2</sup> TD<sup>TM</sup>, Lallemand Inc.) and *L. thermotolerans* (Viniflora<sup>®</sup> Rhythm<sup>TM</sup>, Chr Hansen) were isolated from active dried yeast blends [26] and used as wet cultures. All non-*Saccharomyces* yeasts were stored under cryo-preservation at -80°C. The non-*Saccharomyces* yeasts were propagated in a four step protocol: (i) on yeast peptone dextrose agar (YPDA, Merck, South Africa) at 28°C for 48 hours or until sufficient growth was observed, (ii) then single colonies inoculated into 10 mL YPD broth and grown for 24 hours at 28°C, (iii) transfer to 100 mL YPD broth and incubated for 24 hours at 28°C, and (iv) final transfer to containers holding 3-4 L YPD broth and incubated at 28°C for 24 hrs. The containers were shaken during propagation to ensure aerobic conditions. Non-*Saccharomyces* yeasts were inoculated into the Shiraz grape juice at a concentration of  $\sim 1 \times 10^6$  cells/mL. *S. cerevisiae* was used as an active dried yeast culture and rehydrated according to the supplier's recommendations and inoculated at 0.3 g/L. A commercial *O. oeni* culture was used to induce MLF (Table 4.1). This MLF culture was used at the dosage prescribed by the supplier for the simultaneous MLF treatment, but a higher dosage (15 mg/L) was used to induce sequential MLF due to higher alcohol concentrations of the wines.

**Table 4.1.** Yeasts and lactic acid bacterium used in this study.

Reference code	Species name	Source
Sc	<i>Saccharomyces cerevisiae</i>	VIN 13, commercial yeast strain from Anchor Wine Yeast, South Africa
C7	<i>Candida zemplinina</i> (synonym: <i>Starmerella bacillaris</i> )	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
H4	<i>Hanseniaspora uvarum</i>	Yeast isolate Y0858 from ARC Infruitec-Nietvoorbij culture collection, South Africa
L1	<i>Lachancea thermotolerans</i>	Viniflora <sup>®</sup> Rhythm <sup>TM</sup> , commercial yeast strain from Chr. Hansen A/S, Denmark
L2	<i>Lachancea thermotolerans</i>	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
M2	<i>Metschnikowia pulcherrima</i>	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
T3	<i>Torulaspora delbrueckii</i>	Level <sup>2</sup> TD <sup>TM</sup> , commercial yeast strain from Lallemand Inc
T6	<i>Torulaspora delbrueckii</i>	Yeast isolate from ARC Infruitec-Nietvoorbij culture collection
<i>O. oeni</i>	<i>Oenococcus oeni</i>	Commercial malolactic bacteria culture Viniflora <sup>®</sup> oenos from Chr. Hansen A/S

Yeast counts of Shiraz juice and wines were obtained by plating on Wallerstein Laboratory (WL) Nutrient medium (Biolab, Merck, South Africa) and bacterial counts by plating out on De Man Rogosa, Sharpe (MRS) agar (Biolab, Merck) supplemented with 25% (v/v) grape juice and 100 mg/L cycloheximide (Sigma-Aldrich, Germany). Yeast growth media were incubated aerobically and the LAB growth media were incubated under facultative anaerobic conditions at 28°C for 2-7 days, after which the colonies were counted. The naturally occurring non-*Saccharomyces* yeast populations were determined by counting the non-*Saccharomyces* yeast colonies present in the reference treatment, which only received a *S. cerevisiae* inoculum. The naturally occurring *Saccharomyces* yeast populations were determined by counting the *Saccharomyces* yeast colonies in the treatments that did not receive any *S. cerevisiae* inoculum. The development of the naturally occurring LAB during fermentation was monitored by sampling the wines

that did not undergo MLF and the sequential MLF treatments until day 19, when the commercial *O. oeni* starter culture was added to the sequential MLF wines.

#### 4.2.2 Wine production

Shiraz grapes, obtained from the Nietvoorbij research farm (Stellenbosch, South Africa), were crushed, the juice separated from skins and the volume measured. The skins were weighed and each 70 L fermentation container received the same volume and ratio of juice and skins. The method of grape must preparation ensured a homogenous matrix so that treatments could be compared. Fermentations were carried out at *ca.* 24°C using a standardized winemaking protocol as described by Minnaar *et al* [27]. Eight yeast strains in combination with three MLF strategies, *i.e.* (1) yeast treatment without MLF, (2) yeast treatment and LAB inoculated simultaneously (sim MLF) and (3) yeast treatment with sequential MLF (seq MLF), were investigated (Table 4.2). In total 72 wines were produced, which included 24 different treatments and each treatment had three replicates. *S. cerevisiae* (Sc) on its own served as the reference treatment. The non-*Saccharomyces* yeasts and the *S. cerevisiae* only treatment were inoculated on day 0. In the sequential yeast fermentations, the *S. cerevisiae* yeast was only inoculated 24 hrs after the non-*Saccharomyces* monocultures (day 1). For the wines that underwent the simultaneous MLF treatment, *O. oeni* was also added on day 1, but an hour after the addition of *S. cerevisiae*. For the sequential MLF treatments, *O. oeni* was added to the wines after alcoholic fermentation was completed. All treatments were racked, fined, cold stabilized and bottled as described by Minnaar *et al.* [27]. All wines were stored at 15°C until needed.

#### 4.2.3 Juice and wine analyses

The following were measured on the grape must: sugar in °Brix (Refractometer), free and total SO<sub>2</sub> (Ripper method), pH and titratable acidity analyses as described in the South African Wine Laboratories Association Manual (SALWA) [28]. Standard chemical parameters (glucose and fructose concentrations, pH, malic and lactic acid, total acidity (TA), alcohol, volatile acidity (VA) and glycerol) were determined for the bottled wine using a WineScan™ FT120 instrument (FOSS Analytical A/S, Denmark) at the Institute for Wine Biotechnology (Stellenbosch University, South Africa). Data were predicted from infrared spectra using in-house calibration models as described by Louw *et al.* [29]. The concentrations of major volatile compounds in bottled wines were determined by the Chemical Analytical Laboratory (Institute for Wine Biotechnology and Department of Viticulture and Oenology, Stellenbosch University), using a gas chromatograph coupled to a flame ionization detector (GC-FID) as described by Louw *et al.* [29].

#### 4.2.4 Sensory evaluation

A panel consisting of 15 experienced wine judges (3 women and 11 men, aged 22 to 50 years) evaluated the wines after 24 months of bottle ageing. The panelists were commercial winemakers or staff of ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council). Panel members were experienced in wine evaluation (from 2 to 20 years of experience) and did not receive collective training. Wines were evaluated during three sessions (24 wines per session) over three consecutive days in a temperature-controlled room at ±20°C. During each session, panel members had to take a compulsory break after tasting 12 wines. Each replicate was evaluated on a separate day. The descriptors were chosen from a predefined lexicon and the wines were subjected to classical profiling [30]. The panel members were asked to evaluate the wines orthonasally and to score the intensity of each descriptor individually on a 100 mm unstructured line scale. The descriptors were berry, fruity, fresh vegetative, cooked vegetative, floral, spicy, acid balance, body, astringency, bitterness and overall quality. Each panelist had a separate tasting booth and *ca.* 30 mL of the wine was presented, in a randomized order, in a standard international wine tasting glass, labeled with a three digit code. Research Randomizer (Version 4.0, <http://randomizer.org>) was used to generate the three digit code and to randomize the order in which the wines were presented to each panelist.

#### 4.2.5 Data and statistical analysis

The chemical and sensory data were tested for normality using the method of [31] and then subjected to analysis of variance (ANOVA) using the general linear means procedure of SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Student's *t* least significant difference (LSD) values were calculated at the 5% probability level ( $p = 0.05$ ) to facilitate comparison between treatment means [32]. Additionally, the sensory data were subjected to mixed model ANOVA using Statistica 13.0 (Quest software Inc., Aliso Viejo, California). Means within data sets that differed at the 5% probability level were considered significantly different. Principal component analysis (PCA) was performed, using XLSTAT software (Version 18.07.39157, Addinsoft, New York, USA), to examine the correlation between wine samples and the chemical compounds determined with GC-FID.

### 4.3 Results and Discussion

#### 4.3.1 Fermentation kinetics and progress of MLF

##### 4.3.1.1 Yeast growth in wines without MLF

Counts of the naturally occurring *Saccharomyces* and non-*Saccharomyces* yeast populations in the Shiraz juice were *ca.*  $2.1 \times 10^4$  and  $1.9 \times 10^5$  colony forming units (CFU)/mL, respectively (Figure 4.1). Monitoring the naturally occurring non-*Saccharomyces* yeasts population in the *S. cerevisiae* reference fermentation showed an increase over the first 24 hours, before decreasing to  $\sim 3.8 \times 10^5$  CFU/mL after 48 hours. This is a normal occurrence for natural non-*Saccharomyces* populations during fermentation [33]. The non-*Saccharomyces* yeast count decreased during the remainder of alcoholic fermentation and at the end of fermentation (18 days) the count was lower than  $1 \times 10^4$  CFU/mL. After 48 hours, the expected dominance by the inoculated *S. cerevisiae* was observed in all wines (Figure 4.1).

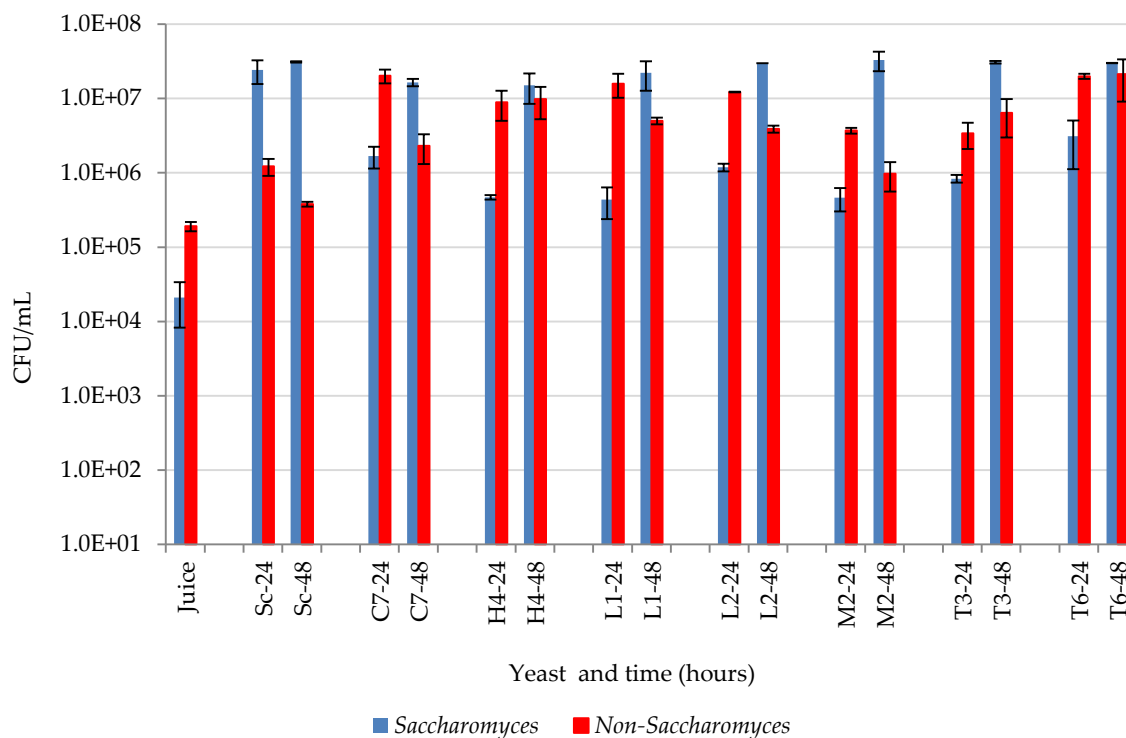
In the non-*Saccharomyces* inoculated wines, these yeasts were present at higher levels ( $10^6$ - $10^7$  CFU/mL) during the first two days of alcoholic fermentation than the naturally occurring non-*Saccharomyces* population in the *S. cerevisiae* reference wine. For the first 24 hours, the inoculated non-*Saccharomyces* yeasts were also present at higher levels than the naturally occurring *Saccharomyces* yeasts. It is expected that these yeasts could have made a notable contribution to the flavor profiles of the various wines [34].

##### 4.3.1.2 LAB growth

The naturally occurring LAB populations in the grape must were initially present at moderate numbers ( $6 \times 10^3$  CFU/mL) (Figure 4.2). Thereafter, the population size was either maintained at  $10^2$ - $10^4$  CFU/mL or decreased during fermentation, before increasing at the end of alcoholic fermentation. The decrease of LAB numbers during alcoholic fermentation, with the subsequent increase after fermentation [3,35], as well as the occurrence at low to moderate numbers and increasing during alcoholic fermentation [36,37], are both typical winemaking scenarios. Factors such as pH,  $\text{SO}_2$  concentration, ethanol levels, temperature, yeast strain, *etc.* are important and can affect the growth of LAB during wine production [3,4,36].

Individually, the numbers of naturally occurring LAB varied notably in wines, fermented with the selected non-*Saccharomyces* yeast combinations, which underwent sequential MLF (Figure 4.2a). The variation in LAB numbers can be ascribed to the effect of the different yeasts that conducted the primary fermentation and support the findings of Muñoz *et al.* [22]. Based on the LAB counts from day 1 to 12, it was observed that yeast strains *S. cerevisiae* (Sc), *T. delbrueckii* T3 and T6, *M. pulcherrima* M2 and *L. thermotolerans* L1 had a larger inhibitory effect on the levels of the naturally occurring LAB (decreased from  $6 \times 10^3$  to  $9 \times 10^1$  CFU/mL) than *C. zemplinina* C7, *H. woarum* H4 and *L. thermotolerans* L2 (decreased from  $6 \times 10^3$  to  $2.7 \times 10^2$  CFU/mL) (Figure 4.2a). However, as previously mentioned, all the LAB populations started to recover at the end of alcoholic fermentation (days 18-19). Inoculation with the commercial *O. oeni* strain on day 19 resulted in the dramatic and expected increase of LAB from  $\sim 1 \times 10^3$

to  $\geq 1 \times 10^6$  CFU/mL. During the subsequent sequential MLF, wines produced with *S. cerevisiae*, *M. pulcherrima* and *T. delbrueckii* T3 had the lowest *O. oeni* counts, indicating that these yeast strains had a negative effect on the viability of *O. oeni*, which also explains why MLF took longer to complete. Wines produced with *C. zemplinina* also had low *O. oeni* counts ( $7.9 \times 10^5$  CFU/mL on day 27), but this did not result in delays in MLF. In wines produced with *H. uvarum*, *O. oeni* counts remained high ( $>1.2 \times 10^6$  CFU/mL) throughout MLF, which explains why this treatment completed MLF the fastest (38 days) (Table 4.2).



**Figure 4.1.** *Saccharomyces* and non-*Saccharomyces* yeast counts in colony forming units/millilitres (CFU/mL) of Shiraz juice, wines inoculated with a commercial *Saccharomyces cerevisiae* (Sc) strain on its own and wines with *S. cerevisiae* in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6 were evaluated. The yeast counts were performed after 24 and 48 hours of the alcoholic fermentation. Values are averages of three replicates and the error bars indicate the standard deviation.

The naturally occurring LAB numbers (Figure 4.2a) in the simultaneous MLF treatments were notably lower than the inoculated *O. oeni* numbers (Figure 4.2b). This indicates that the inoculated *O. oeni* was probably responsible for completion of MLF. Non-*Saccharomyces* treatments, C7+Sc sim MLF and L2+Sc sim MLF had a negative (inhibitory) effect on *O. oeni*, resulting in lower counts for these wines (Figure 4.2b). Simultaneous MLF also took longer to complete than in wines produced with the other yeast strains (Table 4.2). The inhibitory effect of C7 was already noted in Chapter 3 (Du Plessis *et al.* [26]) and inhibition could be alleviated by nutrient supplementation. Therefore, it can be concluded that inhibition of *O. oeni* growth by C7 was due to competition for essential nutrients. These wines did not contain notably higher alcohol concentrations (Table 4.2) or SO<sub>2</sub> levels (supplementary Table S4.1) than the other yeast treatments that could also lead to inhibition.

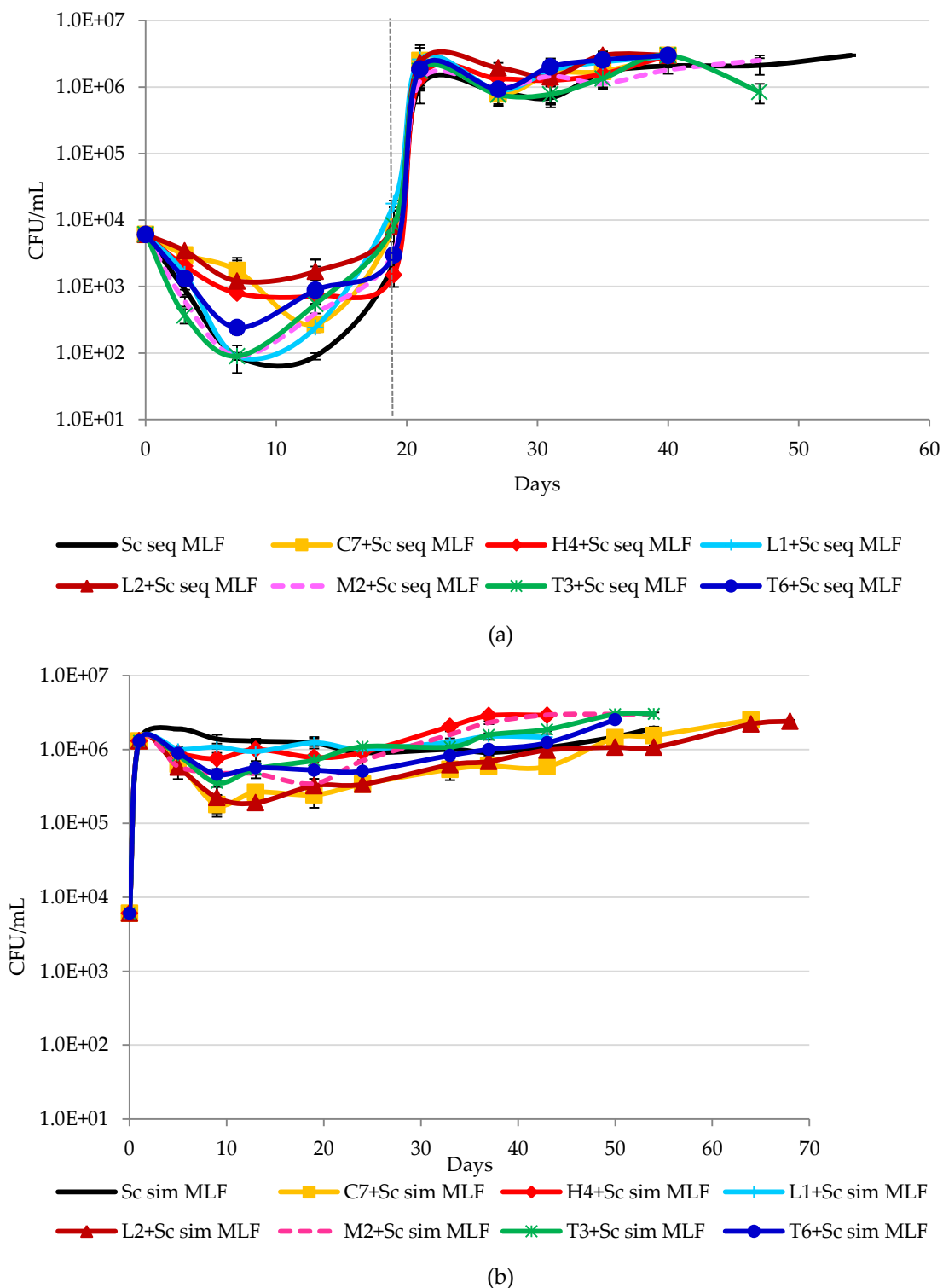


Figure 4.2. Cell counts (colony forming units per millilitres) of the naturally occurring lactic acid bacteria and inoculated *Oenococcus oeni* in Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, as well as three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). (a) Wines that underwent sequential malolactic fermentation (seq MLF) and the dashed vertical line at day 19 indicates when the commercial *O. oeni* was inoculated. (b) Wines where the commercial *O. oeni* was inoculated after 24 hrs (day 1) to induce MLF as a simultaneous inoculation (sim MLF). Values are averages of three replicates and the error bars indicate the standard deviation.

The inhibitory effect of *L. thermotolerans* strain L2 was not noted when it was evaluated in synthetic wine in Chapter 3 [26], but delays in MLF were observed for Chardonnay wines that underwent simultaneous MLF when the same *S. cerevisiae*/LAB combination was used (unpublished data). In the current study, the inhibition by L2 might be due to the combination of L2 with this specific *S. cerevisiae* strain, which resulted in the production of toxic metabolites or depletion of essential nutrients necessary for LAB growth. However, without further investigation it is difficult to draw a conclusion.

#### 4.3.1.3 Progression of MLF

In most cases, wines produced with non-*Saccharomyces* yeasts completed MLF in a shorter period than wines produced with *S. cerevisiae* only. Duration of MLF varied amongst the wines produced with the different non-*Saccharomyces* yeast strains, with sequential MLF taking less time to complete than simultaneous MLF (Table 4.2). However, the success of sequential MLF is mainly due to the higher *O. oeni* dosage applied, which resulted in higher concentrations of viable cells. Due to circumstances outside the control of the researcher, the Shiraz grapes were harvested at a different ripeness level than initially planned, resulting in higher sugar concentration (26.9°B) and wines with high alcohol levels (>15% v/v) (Table 4.2). As the supplier does not recommend the use of Viniflora® oenos in high alcohol wines, a higher dosage was used for the sequential MLF treatments to ensure the successful completion of MLF and to compensate for cell death due to alcohol toxicity.

The H4+Sc combination was most compatible with inoculated *O. oeni* and progress of simultaneous and sequential MLF. Results clearly show that there were differences between the non-*Saccharomyces* strains with regard to their effect on LAB growth and progress of MLF. The use of a different *S. cerevisiae* or LAB strain might have generated different results. These findings agree with reports of Bartowsky *et al.* [15] and Muñoz *et al.* [22] that optimal yeast LAB combinations may indeed differ for simultaneous and sequential MLF.

### 4.3.2 Standard oenological parameters

#### 4.3.2.1 Wines without MLF

The alcoholic fermentation was completed after 18 days and all treatments fermented to dryness (residual sugar <4 g/L) (Table 4.2). In most cases, wines produced with non-*Saccharomyces* yeast had lower alcohol levels (15.49 to 15.94% v/v) than wines produced with *S. cerevisiae* only (~16% v/v), except L2+Sc wines (16.04% v/v). A similar trend was observed by various authors [38,39]. Wines produced with *C. zemplinina* in combination with *S. cerevisiae* (C7+Sc) contained the lowest alcohol levels (15.49% v/v). In most of the wines produced with non-*Saccharomyces* yeasts, glycerol levels were significantly lower than wines produced with *S. cerevisiae* only. Mendoza and Farías [40] reported similar results, but Comitini *et al.* [38] and Benito *et al.* [39] reported the contrary. The differences in reports might be due to the fact that different yeast strains and different grape varieties were used.

Acetic acid is the main contributor to volatile acidity (VA) and above the sensory threshold of 0.7-1.1 g/L can impart a vinegar aroma [41]. Although the wines produced with non-*Saccharomyces* yeasts had significantly higher VA levels than wines produced with *S. cerevisiae* only, the levels were well below the sensory threshold and legal limit of 1.2 g/L [42]. This is similar to the findings of Mendoza *et al.* [43]. *T. delbrueckii* has been described as producing low to high VA levels [44,45]. In this study, *T. delbrueckii* wines contained higher VA levels than *S. cerevisiae* only wines (0.25 vs 0.4 g/L). The *H. uvarum* strain used in this study produced relatively low VA levels, confirming reports about the high strain variability of this species, and that some strains are comparable to *S. cerevisiae* with regard to levels of VA produced [46,47].

Malic acid levels varied significantly among the different yeast treatments and wines produced with *L. thermotolerans* L2 in combination with *S. cerevisiae* (L2+Sc) had the highest concentration (1.68 g/L),

**Table 4.2.** Standard chemical parameters and duration of malolactic fermentation (MLF) of Shiraz juice<sup>1</sup> and wines produced with *Saccharomyces* and non-*Saccharomyces* yeasts in combination with MLF strategies (none, simultaneous and sequential). Values are averages of three replicates.

Treatment <sup>2</sup>	Residual sugar (g/L)	pH	Volatile acidity (g/L)	Total acidity (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Alcohol (% v/v)	Glycerol (g/L)	Duration of MLF (days)
Sc	2.23±0.13ef <sup>3</sup>	3.66±0.01jkl	0.25±0.01k	6.19±0.04a	1.26±0.06c	<0.20i	15.99±0.03abcd	11.43±0.05fgh	No MLF
Sc+sim MLF	2.16±0.16ef	3.74±0.04defg	0.39±0.02gh	5.89±0.12cde	<0.20f	1.01±0.04b	16.09±0.12a	11.84±0.09ab	54
Sc+seq MLF	2.18±0.27ef	3.76±0.01cdef	0.39±0.02gh	5.52±0.03ij	<0.20f	0.86±0.02d	16.01±0.07abc	11.75±0.09bc	53
C7+Sc	2.23±0.13ef	3.59±0.01m	0.33±0.01i	6.21±0.03a	1.21±0.05c	<0.20i	15.49±0.01k	11.08±0.13k	No MLF
C7+Sc+sim MLF	2.20±0.25ef	3.67±0.0ijkl	0.40±0.01fg	6.03±0.13bc	<0.20f	0.95±0.04c	15.93±0.04bcdef	11.85±0.14ab	63
C7+Sc+seq MLF	2.32±0.22bcdef	3.70±0.01ghij	0.47±0.02c	5.65±0.04ghi	<0.20f	0.77±0.02g	15.54±0.03k	11.24±0.04ij	40
H4+Sc	2.77±0.16a	3.76±0.04cdef	0.37±0.01h	5.69±0.03gh	0.77±0.09e	<0.20i	15.94±0.03bcde	11.26±0.04ij	No MLF
H4+Sc+sim MLF	2.42±0.19bcde	3.73±0.04efgh	0.42±0.02ef	5.76±0.05efg	<0.20f	1.06±0.06a	15.82±0.09fghij	11.76±0.16bc	48
H4+Sc+seq MLF	2.78±0.18a	3.85±0.01b	0.52±0.01b	5.19±0.04m	<0.20f	0.81±0.02efg	15.96±0.04bcd	11.59±0.05de	38
L1+Sc	2.32±0.26bcdef	3.72±0.01efghi	0.30±0.02j	5.88±0.01def	1.12±0.02d	<0.20i	15.77±0.02ij	11.33±0.05hi	No MLF
L1+Sc+sim MLF	2.60±0.09ab	3.76±0.25def	0.43±0.01ef	5.58±0.06hij	<0.20f	1.00±0.02b	15.93±0.08bcdef	11.61±0.08cde	48
L1+Sc+seq MLF	2.22±0.27ef	3.80±0.01bcd	0.44±0.01de	5.35±0.02kl	<0.20f	0.86±0.02d	15.80±0.04hij	11.71±0.03bcd	48
L2+Sc	2.55±0.13abcd	3.83±0.0b	0.42±0.01ef	5.48±0.05jk	1.68±0.01a	<0.20i	16.04±0.04ab	11.06±0.05k	No MLF
L2+Sc+sim MLF	2.18±0.30ef	3.62±0.13lm	0.39±0.05gh	6.04±0.03b	<0.20f	0.82±0.07def	15.89±0.02defgh	11.62±0.26cde	68
L2+Sc+seq MLF	2.59±0.18abc	3.92±0.02a	0.56±0.01a	4.95±0.03n	<0.20f	0.70±0.04h	16.08±0.02a	11.37±0.06ghi	40
M2+Sc	2.20±0.28ef	3.67±0.02hijk	0.31±0.10ij	6.01±0.02bcd	1.21±0.03c	<0.20i	15.81±0.13ghij	11.32±0.03hi	No MLF
M2+Sc+sim MLF	2.08±0.22f	3.74±0.04defg	0.43±0.02de	5.76±0.12efg	<0.20f	0.96±0.012bc	15.81±0.09ghij	11.71±0.02bcd	52
M2+Sc+seq MLF	2.28±0.19cdef	3.77±0.02cde	0.48±0.01c	5.44±0.03jk	<0.20f	0.78±0.03fg	15.92±0.02cdefg	11.57±0.04def	48
T3+Sc	2.45±0.20bcde	3.63±0.01klm	0.31±0.01ij	6.08±0.07ab	1.25±0.08c	<0.20i	15.80±0.15hij	10.98±0.11k	No MLF
T3+Sc+sim MLF	2.26±0.11def	3.74±0.02defg	0.41±0.01fg	5.76±0.10efg	<0.20f	0.99±0.04bc	15.91±0.01cdefgh	11.98±0.07a	51
T3+Sc+seq MLF	2.29±0.21bcdef	3.73±0.01efg	0.46±0.01cd	5.48±0.03jk	<0.20f	0.85±0.04de	15.88±0.02defghi	11.25±0.78ij	48
T6+Sc	2.45±0.06bcde	3.72±0.02fghi	0.40±0.01fg	5.74±0.05fg	1.59±0.04b	<0.20i	15.84±0.14efghij	11.14±0.04jk	No MLF
T6+Sc+sim MLF	2.22±0.10ef	3.67±0.01ijkl	0.41±0.02fg	6.00±0.06bcd	<0.20f	0.95±0.04c	15.75±0.09j	11.75±0.09bc	53
T6+Sc+seq MLF	2.58±0.16abcd	3.82±0.01bc	0.54±0.01a	5.27±0.02lm	<0.20f	0.65±0.02h	15.94±0.02bcde	11.49±0.11efg	40

<sup>1</sup>Juice analysis: Balling = 26.9°B, pH = 3.41, total acidity = 6.5 g/L, malic acid = 1.80 g/L, free SO<sub>2</sub> = 16 mg/L and total SO<sub>2</sub> = 29 mg/L.

<sup>2</sup>*Saccharomyces cerevisiae* (Sc), *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, simultaneous (sim) MLF and sequential (seq) MLF induced with *Oenococcus oeni*.

<sup>3</sup>Values in the same column followed by the same letters did not differ significantly ( $p \leq 0.5$ ).

while wines produced with *H. uvarum* in combination with *S. cerevisiae* (H4+Sc) contained the lowest concentration (0.77 g/L) (Table 4.2). Significantly lower malic acid concentrations for the H4+Sc and L1+Sc treatments indicate possible malic acid degradation by these strains. The low lactic acid concentrations (0.2 g/L) and naturally occurring LAB levels ( $\sim 2 \times 10^3$  and  $2 \times 10^4$  CFU/mL, respectively) at the end of alcoholic fermentation, excludes the occurrence of spontaneous MLF in these wines. In Chapter 3 (Du Plessis *et al.* [26]), it was shown that strains H4 and L1 had limited malic acid degradation ability in MLF broth and synthetic media, but the ability of these strains to degrade malic acid was not tested in grape juice or must.

#### 4.3.2.2 Wines that underwent MLF

Wines that underwent MLF had significantly higher VA values (0.39 to 0.56 g/L) than the wines that did not undergo MLF (Table 4.2). Acetic acid, together with carbon dioxide, ethanol and lactic acid are produced by heterofermentative bacteria such as *O. oeni* during MLF [3], which impact on VA levels. In general, the sequential MLF wines contained higher VA levels than wines that underwent simultaneous MLF. This is similar to results reported by other researchers [48,49].

For most treatments, wines that did not undergo MLF had lower alcohol levels than wines that underwent MLF. Similar results have been reported by Benito *et al.* [48] and Izquierdo-Cañas *et al.* [50]. The *S. cerevisiae* simultaneous MLF treatment had the highest alcohol level (16.09% v/v), but no clear trend with regard to alcohol levels was observed in wines produced with non-*Saccharomyces* yeasts that underwent simultaneous or sequential MLF. However, there appeared to be an increase or decrease in the alcohol levels in wines that underwent MLF that was dependent on the yeast strain used. These results contradict those of Izquierdo-Cañas *et al.* [51], who found that sequential MLF wines had lower alcohol levels than simultaneous MLF wines.

Glycerol levels were significantly higher in wines that underwent MLF than in wines that did not and this is in agreement with the findings of Tristezza *et al.* [49] and Benito *et al.* [48]. In most cases, glycerol levels were also higher in wines that underwent simultaneous MLF than in wines that underwent sequential MLF, with the highest being 11.98 g/L for T3+Sc. These results confirm the findings of Mendoza and Farías [40] and Mendoza *et al.* [43], but contradict those of Tristezza *et al.* [49].

#### 4.3.3 Flavor compounds

ANOVA of volatile compounds showed that there was a significant interaction for all volatile compounds between wines produced with the three MLF strategies (none, simultaneous and sequential MLF) and eight yeast combinations (Table 4.3 and supplementary Table S4.2). This resulted in all 24 treatments delivering wines with significantly different volatile chemical profiles. These variations will have an impact on the perceived flavor profiles of the wines. The aforementioned results are in agreement with the findings of Whitener *et al.* [10-12], who reported that wines produced with different non-*Saccharomyces* and *Saccharomyces* yeast combinations had distinctive flavor profiles. However, unlike this investigation, those studies did not address yeast-LAB interactions.

To determine the potential contribution of the various volatile compounds to wine flavor, the odor activity values (OAVs) were determined. The OAV values were calculated by dividing the mean concentration of a compound by its odor threshold value (OTH, Table 4.4) as described by Guth [52]. Volatile compounds with OAV > 1 could potentially make an active contribution to wine aroma [52]. However, compounds with high OAVs do not always have an effect on wine aroma and the OAV is only an indication of the potential aroma contribution of individual compounds [53]. In a similar manner, the contribution by volatile compounds that are present at sub-threshold concentrations (*i.e.* OAVs < 1) should also not be excluded, as these aroma-active compounds can have additive, interactive effects, masking or suppressing effects [54].



**Table 4.3.** Concentrations of volatile compounds (mg/L) and their calculated odor activity values (OAV) of bottled Shiraz wines produced with different yeast<sup>1</sup> strains in combination with three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Values are averages of three replicates.

Treatment <sup>1</sup>	Ethyl acetate	OAV	Ethyl butanoate	OAV	Isoamyl acetate	OAV	Ethyl lactate	OAV	Ethyl-3-hydroxy butanoate	OAV	Diethyl succinate	OAV	Ethyl hexanoate	OAV	Ethyl octanoate	OAV
Sc	40.20p <sup>2</sup>	3.3	0.49kl	1.2	1.33ijk	8.3	1.59l	0.1	1.68efg	1.7	2.103f	1.8	0.77ij	9.6	0.33f	0.6
Sc+sim MLF	52.98m	4.4	0.49kl	1.2	1.05n	6.6	9.22b	0.7	1.68ef	1.7	2.466c	2.1	0.76j	9.4	0.25i	0.4
Sc+seq MLF	55.77l	4.6	0.48l	1.2	1.48def	9.3	6.48g	0.5	1.60j	1.6	2.076f	1.7	0.77ij	9.6	0.33f	0.6
C7+Sc	58.55jk	4.9	0.53efgh	1.3	1.06n	6.7	1.62l	0.1	1.76bc	1.8	1.941h	1.6	0.80fg	10.0	0.32f	0.6
C7+Sc+sim MLF	62.08ghi	5.2	0.53efgh	1.3	1.07n	6.7	8.81c	0.6	1.75cd	1.8	2.581b	2.2	0.80fg	10.0	0.28gh	0.5
C7+Sc+seq MLF	76.02b	6.3	0.55ab	1.4	1.20lm	7.5	6.77ef	0.5	1.79ab	1.8	2.002g	1.7	0.83cd	10.4	0.37e	0.6
H4+Sc	65.72f	5.5	0.52efgh	1.3	1.47defg	9.2	2.08k	0.1	1.67fgh	1.7	1.544m	1.3	0.81ef	10.1	0.37e	0.6
H4+Sc+sim MLF	64.15fg	5.3	0.50jk	1.3	1.23klm	7.7	7.54d	0.5	1.70e	1.7	2.486c	2.1	0.78hi	9.8	0.27h	0.5
H4+Sc+seq MLF	73.35c	6.1	0.54bcd	1.4	1.60abc	10.0	4.801j	0.3	1.64hi	1.6	1.586m	1.3	0.84c	10.5	0.41c	0.7
L1+Sc	45.83o	3.8	0.53cdef	1.3	1.44efgh	9.0	1.53l	0.1	1.59j	1.6	1.828k	1.5	0.80efg	10.0	0.36e	0.6
L1+Sc+sim MLF	63.54fgh	5.3	0.52ghi	1.3	1.39fghi	8.7	7.46d	0.5	1.67fgh	1.7	2.459c	2.0	0.79gh	9.9	0.29gh	0.5
L1+Sc+seq MLF	60.39ij	5.0	0.53cde	1.3	1.53cde	9.5	5.63hi	0.4	1.64i	1.6	1.854jk	1.5	0.81efg	10.1	0.38de	0.7
L2+Sc	69.35e	5.8	0.55ab	1.4	1.64ab	10.2	2.01k	0.1	1.74cd	1.7	1.710l	1.4	0.88a	11.0	0.43b	0.7
L2+Sc+sim MLF	72.04cd	6.0	0.52fghi	1.3	0.95o	5.9	9.64a	0.7	1.80a	1.8	2.595b	2.2	0.81ef	10.1	0.31f	0.5
L2+Sc+seq MLF	81.31a	6.8	0.56a	1.4	1.67a	10.5	5.03j	0.4	1.74cd	1.7	1.714l	1.4	0.88a	11.1	0.46a	0.8
M2+Sc	48.61n	4.1	0.54bc	1.4	1.52cde	9.5	1.39l	0.1	1.65ghi	1.7	1.870ijk	1.6	0.83c	10.4	0.40cd	0.7
M2+Sc+sim MLF	57.62kl	4.8	0.51ij	1.3	1.21lm	7.6	6.69fg	0.5	1.69ef	1.7	2.283e	1.9	0.77ij	9.6	0.27gh	0.5
M2+Sc+seq MLF	63.71fgh	5.3	0.53cdefg	1.3	1.44defgh	9.0	5.69hi	0.4	1.67fgh	1.7	1.852jk	1.5	0.82de	10.2	0.39cd	0.7
T3+Sc	51.47m	4.3	0.54bcd	1.3	1.32ijk	8.3	1.50l	0.1	1.73d	1.7	1.885ij	1.6	0.83cd	10.3	0.37e	0.6
T3+Sc+sim MLF	60.75ij	5.1	0.53cdefg	1.3	1.36hij	8.5	6.99e	0.5	1.66fghi	1.7	2.353d	2.0	0.79gh	9.9	0.29g	0.5
T3+Sc+seq MLF	63.41fgh	5.3	0.54bcd	1.4	1.29jkl	8.0	5.88h	0.4	1.73d	1.7	1.912hi	1.6	0.84c	10.5	0.40cd	0.7
T6+Sc	70.27de	5.9	0.55ab	1.4	1.38ghij	8.6	2.10k	0.1	1.67fgh	1.7	1.581m	1.3	0.86b	10.7	0.40cd	0.7
T6+Sc+sim MLF	61.55hi	5.1	0.52hi	1.3	1.18m	7.4	8.65c	0.6	1.75cd	1.8	2.717a	2.3	0.80fgh	10.0	0.28gh	0.5
T6+Sc+seq MLF	83.26a	6.9	0.56a	1.4	1.55bcd	9.7	5.59i	0.4	1.68efg	1.7	1.595m	1.3	0.88a	11.0	0.46a	0.8

Table 4.3 (Continued).

Treatment	Ethyl decanoate	OAV	Ethyl phenyl acetate	OAV	2-Phenyl ethyl acetate	OAV	Methanol	OAV	Propanol	OAV	Butanol	OAV	Isobutanol	OAV	Pentanol	OAV
Sc	0.097ij	0.2	0.61c	8.4	1.18jk	0.7	156.39de	0.3	47.65fg	0.2	3.34a	0.02	42.39hi	1.1	0.710j	0.01
Sc+sim MLF	0.123bc	0.2	0.64b	8.7	1.15k	0.6	184.84abc	0.4	53.65bc	0.2	2.62e	0.02	47.77cd	1.2	0.734b	0.01
Sc+seq MLF	0.096j	0.2	0.67a	9.2	1.18jk	0.7	146.68gh	0.3	43.27ij	0.1	3.15b	0.02	38.50k	1.0	0.706kl	0.01
C7+Sc	0.098hij	0.2	0.44n	6.1	1.31efg	0.7	178.68c	0.4	42.25j	0.1	2.07m	0.01	52.69b	1.3	0.723gh	0.01
C7+Sc+sim MLF	0.132a	0.3	0.49kl	6.8	1.27hi	0.7	184.83abc	0.4	51.70cd	0.2	2.50fg	0.02	51.95b	1.3	0.737ab	0.01
C7+Sc+seq MLF	0.125abc	0.2	0.48klm	6.6	1.38d	0.8	177.87c	0.4	42.76ij	0.1	2.15l	0.01	54.62a	1.4	0.733cd	0.01
H4+Sc	0.101ghij	0.2	0.51ij	7.0	1.27hi	0.7	179.10c	0.4	49.41ef	0.2	2.36i	0.02	38.02k	1.0	0.719h	0.01
H4+Sc+sim MLF	0.121bcd	0.2	0.59d	8.0	1.19j	0.7	182.98bc	0.4	43.93hij	0.1	2.35ij	0.02	49.38c	1.2	0.733cd	0.01
H4+Sc+seq MLF	0.111ef	0.2	0.55fg	7.6	1.37d	0.8	161.97d	0.3	44.95hi	0.1	2.26k	0.02	35.61l	0.9	0.723gh	0.01
L1+Sc	0.102ghij	0.2	0.50kl	6.8	1.28ghi	0.7	152.48efg	0.3	54.69ab	0.2	2.98c	0.02	34.72l	0.9	0.715i	0.01
L1+Sc+sim MLF	0.096j	0.2	0.57de	7.8	1.28fghi	0.7	190.70a	0.4	49.37ef	0.2	2.24k	0.01	52.49b	1.3	0.727ef	0.01
L1+Sc+seq MLF	0.105fghi	0.2	0.56ef	7.7	1.29fgh	0.7	156.00def	0.3	56.13a	0.2	3.03c	0.02	35.65l	0.9	0.721h	0.01
L2+Sc	0.113def	0.2	0.57de	7.8	1.47b	0.8	183.45abc	0.4	42.93ij	0.1	2.51fg	0.02	44.82efg	1.1	0.726efg	0.01
L2+Sc+sim MLF	0.117cde	0.2	0.48lm	6.6	1.29fgh	0.7	190.62a	0.4	50.31de	0.2	2.70d	0.02	56.45a	1.4	0.739a	0.01
L2+Sc+seq MLF	0.122bcd	0.2	0.67a	9.2	1.80a	1.0	186.66ab	0.4	43.49ij	0.1	2.51fg	0.02	45.19ef	1.1	0.732cd	0.01
M2+Sc	0.107fg	0.2	0.50jk	6.8	1.34de	0.7	143.03h	0.3	38.41k	0.1	2.52fg	0.02	39.81jk	1.0	0.704kl	0.01
M2+Sc+sim MLF	0.126ab	0.3	0.57ef	7.8	1.21j	0.7	183.91abc	0.4	43.72ij	0.1	2.40hi	0.02	49.32c	1.2	0.720h	0.01
M2+Sc+seq MLF	0.105fghi	0.2	0.54gh	7.4	1.32ef	0.7	148.78fgh	0.3	39.55k	0.1	2.56ef	0.02	40.80ij	1.0	0.706kl	0.01
T3+Sc	0.106fgh	0.2	0.47m	6.5	1.35e	0.7	149.18efgh	0.3	35.70l	0.1	2.22k	0.01	42.94gh	1.1	0.703l	0.01
T3+Sc+sim MLF	0.100ghij	0.2	0.53gh	7.3	1.25i	0.7	178.55c	0.4	49.52def	0.2	2.46gh	0.02	46.60de	1.2	0.729de	0.01
T3+Sc+seq MLF	0.103ghij	0.2	0.54gh	7.4	1.38d	0.8	150.39efgh	0.3	35.75l	0.1	2.28jk	0.02	43.44fgh	1.1	0.707jk	0.01
T6+Sc	0.107fg	0.2	0.47m	6.4	1.43c	0.8	179.67bc	0.4	46.10gh	0.2	2.35ij	0.02	38.90jk	1.0	0.721h	0.01
T6+Sc+sim MLF	0.126abc	0.3	0.53hi	7.2	1.28fghi	0.7	179.36bc	0.4	54.32ab	0.2	2.38hi	0.02	52.08b	1.3	0.740a	0.01
T6+Sc+seq MLF	0.120bcd	0.2	0.51ij	7.1	1.47b	0.8	181.11bc	0.4	46.09gh	0.2	2.40hi	0.02	39.14jk	1.0	0.725fg	0.01

Table 4.3 (Continued).

Treatment	Isoamyl alcohol	OAV	3-Ethoxy-1-propanol	OAV	3-Methyl-1-pentanol	OAV	Hexanol	OAV	2-Phenyl ethanol	OAV	Acetoin	OAV	Acetic acid	OAV
Sc	338.70ef	5.6	2.43jk	24.3	0.65d	0.6	37.73kl	4.7	79.45bc	5.7	5.26kl	0.04	180.02p	0.9
Sc+sim MLF	370.68c	6.2	2.71h	27.1	0.69a	0.7	45.05bc	5.6	75.78e	5.4	13.95de	0.09	269.45l	1.3
Sc+seq MLF	335.27f	5.6	2.41k	24.1	0.651d	0.7	36.92klm	4.6	77.00cde	5.5	12.59efg	0.08	278.61kl	1.4
C7+Sc	302.06h	5.0	3.86b	38.6	0.59i	0.6	39.42hi	4.9	57.37j	4.1	4.59l	0.03	272.79l	1.4
C7+Sc+sim MLF	381.58b	6.4	2.51ij	25.1	0.67b	0.7	44.20bcde	5.5	80.14b	5.7	13.57efg	0.09	298.04ij	1.5
C7+Sc+seq MLF	319.82g	5.3	4.06a	40.6	0.60h	0.6	42.16g	5.3	59.73ij	4.3	13.64def	0.09	385.22c	1.9
H4+Sc	228.40m	3.8	3.23f	32.3	0.56k	0.6	40.21h	5.0	39.14l	2.8	5.44kl	0.04	306.70ghi	1.5
H4+Sc+sim MLF	366.74cd	6.1	2.44jk	24.4	0.67b	0.7	45.58ab	5.7	76.33de	5.5	8.65ij	0.06	316.47gh	1.6
H4+Sc+seq MLF	228.74m	3.8	3.38e	33.8	0.56jk	0.6	43.17efg	5.4	39.77l	2.8	15.90b	0.11	384.48c	1.9
L1+Sc	285.62j	4.8	2.56i	25.6	0.62e	0.6	37.94jk	4.7	63.25gh	4.5	5.45kl	0.04	201.73o	1.0
L1+Sc+sim MLF	366.73cd	6.1	2.79h	27.9	0.62e	0.6	44.76bcd	5.6	74.72e	5.3	15.54bc	0.10	318.76fg	1.6
L1+Sc+seq MLF	290.64ij	4.8	2.57i	25.7	0.62e	0.6	38.13ijk	4.8	64.89g	4.6	12.25gh	0.08	313.81gh	1.6
L2+Sc	260.06k	4.3	3.34e	33.4	0.57j	0.6	43.55defg	5.4	44.24k	3.2	6.02k	0.04	350.01d	1.8
L2+Sc+sim MLF	400.33a	6.7	2.57i	25.7	0.65d	0.7	45.52ab	5.7	87.48a	6.2	11.78h	0.08	305.54hi	1.5
L2+Sc+seq MLF	259.57k	4.3	3.53d	35.3	0.57j	0.6	44.27bcde	5.5	43.81k	3.1	12.53fgh	0.08	468.89a	2.3
M2+Sc	290.00ij	4.8	3.56d	35.6	0.61ef	0.6	36.44lm	4.6	62.50gh	4.5	4.19l	0.03	219.49n	1.1
M2+Sc+sim MLF	346.22e	5.8	3.58cd	35.8	0.65d	0.7	43.28efg	5.4	69.30f	4.9	9.79i	0.07	290.89jk	1.5
M2+Sc+seq MLF	290.23ij	4.8	3.61cd	36.1	0.61fg	0.6	36.24m	4.5	61.54hi	4.4	7.94j	0.05	334.87e	1.7
T3+Sc	294.95hi	4.9	3.54d	35.4	0.60gh	0.6	38.02ijk	4.8	57.63j	4.1	4.28l	0.03	242.29m	1.2
T3+Sc+sim MLF	358.92d	6.0	2.56i	25.6	0.66c	0.7	42.66fg	5.3	78.99bcd	5.6	12.16h	0.08	298.90ij	1.5
T3+Sc+seq MLF	297.42hi	5.0	3.67c	36.7	0.61fg	0.6	39.18hij	4.9	57.92j	4.1	8.40ij	0.06	338.07de	1.7
T6+Sc	235.74m	3.9	3.23f	32.3	0.56jk	0.6	43.08efg	5.4	37.94l	2.7	6.49k	0.04	331.14ef	1.7
T6+Sc+sim MLF	401.63a	6.7	2.91g	29.1	0.70a	0.7	46.61a	5.8	86.07a	6.1	14.30cd	0.10	294.87ij	1.5
T6+Sc+seq MLF	237.91l	4.0	3.36e	33.6	0.56jk	0.6	43.86cdef	5.5	38.32l	2.7	17.89a	0.12	432.88b	2.2

Table 4.3 (Continued).

Treatment	Propionic acid	OAV	Butyric acid	OAV	Isobutyric acid	OAV	Valeric acid	OAV	Isovaleric acid	OAV	Hexanoic acid	OAV	Octanoic acid	OAV	Decanoic acid	OAV
Sc	3.89bcde	0.2	1.13ghi	0.5	1.524g	0.05	0.417ab	1.4	9.07i	6.0	0.64b	0.2	1.57j	0.2	1.09cd	0.2
Sc+sim MLF	3.92bcd	0.2	1.10ij	0.5	1.759e	0.06	0.416b	1.4	18.67g	12.4	0.52j	0.2	1.29m	0.1	1.04fgh	0.2
Sc+seq MLF	3.91bcd	0.2	1.09j	0.5	1.443h	0.05	0.423a	1.4	18.80g	12.5	0.64ab	0.2	1.57j	0.2	1.06efg	0.2
C7+Sc	3.52hi	0.2	1.21bc	0.6	1.930c	0.06	0.39kl	1.3	9.24i	6.2	0.55hi	0.2	1.64i	0.2	1.08d	0.2
C7+Sc+sim MLF	4.05ab	0.2	1.21c	0.6	2.168a	0.07	0.398ghij	1.3	22.58b	15.1	0.55i	0.2	1.43kl	0.1	1.06ef	0.2
C7+Sc+seq MLF	3.49ij	0.2	1.25ab	0.6	1.972c	0.07	0.395ijkl	1.3	22.82b	15.2	0.55i	0.2	1.77efg	0.2	1.09cd	0.2
H4+Sc	4.18a	0.2	1.15fgh	0.5	1.283k	0.04	0.404efg	1.3	8.78i	5.9	0.57fgh	0.2	1.67hi	0.2	1.06efg	0.2
H4+Sc+sim MLF	3.66efghi	0.2	1.12hij	0.5	1.818d	0.06	0.405ef	1.4	18.94g	12.6	0.58efg	0.2	1.37l	0.1	1.03h	0.2
H4+Sc+seq MLF	3.76defgh	0.2	1.15fgh	0.5	1.197l	0.04	0.406def	1.4	20.91ef	13.9	0.59de	0.2	1.86bcd	0.2	1.08d	0.2
L1+Sc	3.54ghi	0.2	1.17def	0.5	1.333ijk	0.04	0.406def	1.4	10.19h	6.8	0.66ab	0.2	1.73gh	0.2	1.08de	0.2
L1+Sc+sim MLF	4.09ab	0.2	1.16fg	0.5	2.039b	0.07	0.412bcd	1.4	20.31f	13.5	0.59de	0.2	1.45k	0.1	1.06ef	0.2
L1+Sc+seq MLF	3.77cdefg	0.2	1.20cde	0.5	1.282k	0.04	0.416b	1.4	24.19a	16.1	0.65ab	0.2	1.78efg	0.2	1.08d	0.2
L2+Sc	3.63fghi	0.2	1.25a	0.6	1.525g	0.05	0.410cde	1.4	9.07i	6.0	0.61c	0.2	1.92b	0.2	1.10bc	0.2
L2+Sc+sim MLF	4.07ab	0.2	1.26a	0.6	1.940c	0.06	0.405ef	1.3	22.59b	15.1	0.52j	0.2	1.44k	0.1	1.10bcd	0.2
L2+Sc+seq MLF	4.08ab	0.2	1.26a	0.6	1.453h	0.05	0.415bc	1.4	21.43de	14.3	0.58def	0.2	2.00a	0.2	1.09cd	0.2
M2+Sc	2.95l	0.1	1.15fgh	0.5	1.378i	0.05	0.394jkl	1.3	9.09i	6.1	0.66a	0.2	1.74g	0.2	1.10bcd	0.2
M2+Sc+sim MLF	3.55ghi	0.2	1.09j	0.5	1.676f	0.06	0.398ghij	1.3	20.78ef	13.9	0.56ghi	0.2	1.39kl	0.1	1.03h	0.2
M2+Sc+seq MLF	3.24jk	0.2	1.15g	0.5	1.302k	0.04	0.396hijk	1.3	22.33bcd	14.9	0.62c	0.2	1.80def	0.2	1.08d	0.2
T3+Sc	3.47ij	0.2	1.21cd	0.5	1.482gh	0.05	0.390l	1.3	9.21i	6.1	0.60cd	0.2	1.75fg	0.2	1.09cd	0.2
T3+Sc+sim MLF	3.85cdef	0.2	1.17ef	0.5	1.734e	0.06	0.407def	1.4	21.34e	14.2	0.59de	0.2	1.44k	0.1	1.04gh	0.2
T3+Sc+seq MLF	3.03kl	0.2	1.21c	0.5	1.440h	0.05	0.397hij	1.3	22.81b	15.2	0.58def	0.2	1.88bc	0.2	1.11ab	0.2
T6+Sc	4.01abc	0.2	1.17def	0.5	1.366ij	0.05	0.396ijkl	1.3	9.26i	6.2	0.56fghi	0.2	1.84cde	0.2	1.08cd	0.2
T6+Sc+sim MLF	3.43ij	0.2	1.14fgh	0.5	2.058b	0.07	0.401fghi	1.3	22.40bc	14.9	0.56fghi	0.2	1.45k	0.1	1.04h	0.2
T6+Sc+seq MLF	3.95abcd	0.2	1.20cde	0.5	1.321jk	0.04	0.403fgh	1.3	21.65cde	14.4	0.57fgh	0.2	2.04a	0.2	1.13a	0.2

<sup>1</sup>*Saccharomyces cerevisiae* (Sc), *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, simultaneous (sim) MLF and sequential (seq) MLF treatments induced with *Oenococcus oeni*.

<sup>2</sup>Values in the same column followed by the same letter did not differ significantly ( $p \leq 0.05$ ).

**Table 4.4.** Odor threshold (OTH) values (mg/L) and descriptions of aroma and flavor compounds found in various wine styles. Superscript values denote the appropriate reference.

Compounds	OTH Values (mg/L)	Aroma/Flavor Descriptors
<b>Esters</b>		
Ethyl acetate	12 [55]	Fruit, nail polish [41,56]
Ethyl butanoate (butyrate)	0.4 [57]	Strawberry [57], apple [56], fruity [21]
Isoamyl acetate	0.16 [57]	Banana, pear [16,41]
Ethyl lactate	14 [58]	Butter, cream, fruit [56]
Ethyl-3-hydroxy butanoate	1 [55]	Fruity, grape [55], strawberry [59]
Diethyl succinate	1.2 [57]	Fruity, melon [57], berry [56]
Ethyl hexanoate (ethyl caproate)	0.08 [57]	Apple [56], fruity, anise [53], strawberry [58]
Ethyl octanoate (ethyl caprylate)	0.58 [57]	Fruit [56], pear, pineapple [41]
Ethyl decanoate (ethyl caprate)	0.5 [57]	Floral [41,56], grape, soap [16,56]
Ethyl phenylacetate	0.073 [60]	Honey-like [60]
2-Phenylethyl acetate	0.25 [52]	Flowery, fruity, rose [16,41]
<b>Alcohols</b>		
Methanol	500 [57]	Alcohol [57]
N-Propanol	306 [57]	Alcohol, ripe fruit [57], pungent, harsh [16,56]
N-Butanol	150 [57]	Fusel, spirituous [16,56]
Isobutanol	40 [52]	Wine, solvent, fusel [16]
Pentanol	64 [61]	Fusel, alcoholic, fermented, pungent, bready, yeasty [11]
Isoamyl alcohol	60 [57]	Herbaceous [59], whiskey, malt, burnt [56]
3-Ethoxy-1-propanol	0.1 [55]	Fruity [57]
3-Methyl-1-pentanol	1 [55]	Green, pungent, solvent [55]
Hexanol	8 [52]	Herbaceous [55], grass [16,53], resin [53]
2-Phenylethanol	14 [62]	Floral, rose [16,41], honey, spice, lilac [56]
<b>Ketones</b>		
Acetoin	150 [57]	Buttery, cream [57]
<b>Acids</b>		
Acetic acid	200 [52]	Vinegar [41,62]
Propionic acid	0.42 [41]	Pungent, rancid [41,56], sweat [56]
N-Butyric acid	2.2 [55]	Cheese [53], pungent [41]
Isobutyric acid	30 [55]	Rancid, butter, cheese [56], pungent [41]
N-Valeric acid	3 [63]	unpleasant [41]
Isovaleric acid	1.5 [55]	Cheese [41,52], rancid, sweaty [41]
Hexanoic acid	3 [55]	Sweat [41,56], sour, vinegar, cheese, rancid, fatty, pungent [41]
Octanoic acid	10 [55]	Sweat, cheese [56], oily, fatty, rancid, soapy, sweet, faint fruity, butter [41]
Decanoic acid	6 [57]	Rancid, fat [41,56], unpleasant, citrus, phenolic [41]

Sixteen of the 31 quantified volatile compounds had OAVs > 1 (Table 4.3). They were ethyl acetate, ethyl hexanoate, ethyl butanoate, ethyl-3-hydroxybutanoate, isoamyl acetate, 2-phenylethyl acetate, ethyl phenylacetate, diethyl succinate, 2-phenylethanol, isoamyl alcohol, 3-ethoxy-1-propanol, hexanol, isobutanol, acetic acid, isovaleric acid and valeric acid.

Wines produced with *S. cerevisiae* only that did not undergo MLF contained higher diethyl succinate (fruity, melon, berry aroma) and 2-phenylethanol (floral, rose, honey, spice, lilac aroma) concentrations than wines produced with non-*Saccharomyces* yeasts that did not undergo MLF (Table 4.3). Whitener *et al.* [10] reported similar results.

The concentrations of MLF marker compounds such as diethyl succinate, ethyl lactate and ethyl acetate were higher in wines that underwent MLF, which is in agreement with literature [23,25]. In most cases, ethyl acetate concentrations were lower in wines that underwent simultaneous MLF than wines that underwent sequential MLF. This finding is in agreement with those of Abrahamse and Bartowsky [25] and Izquierdo-Cañas *et al.* [51], but contrary to findings of Antalick *et al.* [23]. Ethyl lactate and diethyl succinate concentrations were higher in wines that underwent simultaneous MLF than in wines that underwent sequential MLF. Izquierdo-Cañas *et al.* [51] reported similar results. The other ethyl and acetate esters are

known as odorant esters because of their impact on wine aroma, despite being present at low concentrations (g/L) [23]. The concentrations of these esters varied and some (ethyl-3-hydroxybutanoate, ethyl decanoate and ethyl phenylacetate) were higher in wines that underwent simultaneous MLF, while others (ethyl butanoate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and 2-phenylethyl acetate) were higher in wines that underwent sequential MLF.

Diacetyl is one of the most important compounds associated with MLF and contributes to buttery, nutty and butterscotch characters in wine [3,4,16]. However, diacetyl is chemically unstable and can be reduced to acetoin, which in turn can be reduced to 2,3-butanediol. Reduction of diacetyl to acetoin and 2,3-butanediol is advantageous because these products are less toxic to yeasts. Acetoin does not contribute to wine flavor due to its high aroma threshold of 150 mg/L [4]. In this study, only the concentration of acetoin was analyzed and, as expected, was significantly higher in wines that underwent MLF (Table 4.3).

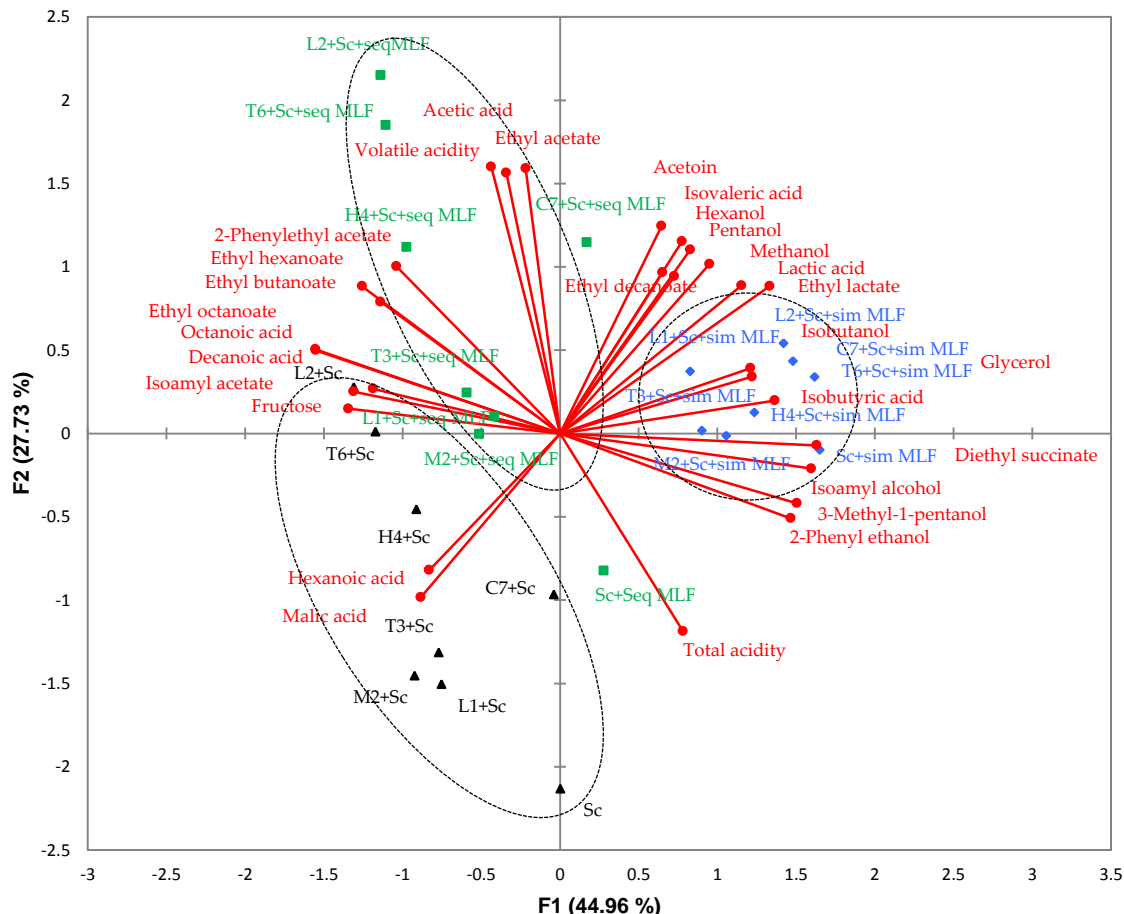
#### 4.3.4 Multivariate data analysis of wines

To investigate the correlation between the chemical composition of the Shiraz wines and the various yeast combinations and MLF strategies, a PCA was performed, using the data of the 31 volatile compounds (GC-FID analysis) and nine standard chemical parameters (glucose, fructose, pH, volatile acidity, total acidity, malic acid, lactic acid, ethanol and glycerol). The first two principal components explained 58.37% of the variance in the data set (Figure 4.3). Subsequently, 11 variables (pH, glucose, ethanol, propanol, butanol, 3-ethoxy-1-propanol, ethyl-3-hydroxybutanoate, ethyl phenylacetate, propionic acid, butyric acid and valeric acid) that did not contribute to the separation on PC1 and PC2 were removed. The PCA biplot of the 29 variables explained 72.69% (PC1 = 44.96% and PC2 = 27.73%) of the variance in the data set. Three groups were observed, *i.e.* the wines that underwent simultaneous MLF (top right quadrant of PC1), the wines that underwent sequential MLF (Top left quadrant of PC1) and those that did not undergo MLF (bottom left quadrant PC2). Not all of the wines from the aforementioned MLF strategies clustered with the other wines from the same group and a few outliers were observed. There was also some overlapping between wines that did not undergo MLF and wines that underwent sequential MLF. The clustering of the wines indicates that MLF strategy had a bigger effect on chemical composition of the wines than yeast treatment, but that yeast treatment also played a role with regard to the clustering. The effects of the yeast combinations can be seen in the variations within the three clusters. The association of the wines within the clusters indicates that there are similarities, but also some differences among the wines. Results also show that the chemical profiles of wines that underwent sequential MLF and wines that did not undergo MLF were similar and were notably different from wines that underwent simultaneous MLF. The association of *S. cerevisiae* only wines that did not undergo MLF and *S. cerevisiae* only wines that underwent sequential MLF is a good example of the aforementioned observation.

Based on the contribution and the squared cosines of the variables, the most important compounds for differentiating between wines produced with the selected yeast combinations and three MLF strategies were volatile acidity, acetic acid, ethyl acetate, isoamyl alcohol, 3-methyl-1-pentanol, ethyl octanoate, diethyl succinate, 2-phenyl ethanol and octanoic acid.

Most of the wines that did not undergo MLF were positively correlated with malic acid, hexanoic acid and total acidity. These wines were also negatively correlated with most of the other compounds. Wines that did not undergo MLF had higher total acidity than wines that underwent MLF and consequently, lower pH levels as shown in Table 4.2. The wines produced with *L. thermotolerans* L2 (ARC culture collection isolate) differed the most from the *S. cerevisiae* reference wines with regard to chemical composition. This is in agreement with the finding of Whitener *et al.* [10], who reported that Shiraz wines produced with *L. thermotolerans* in combination with *S. cerevisiae* were significantly different from wines produced with *S. cerevisiae* only. However, the finding of the aforementioned authors was for different *S. cerevisiae* and *L. thermotolerans* strains than reported in this study. Wines produced with the two *L. thermotolerans* strains (L1 and L2) were not closely associated, indicating that wines produced with L1 (commercial strain) is significantly different from wines produced with L2 (ARC culture collection isolate) with regard to chemical composition (Figure 4.3 and Table 4.3). Wines produced with L1+Sc contain significantly higher levels of diethyl succinate, isoamyl alcohol, 2-phenylethanol, propanol,

butanol, 3-methyl-1-pentanol and hexanoic acid than L2+Sc wines, while wines produced with L2+Sc contained significantly higher levels of most of the other volatile compounds. A similar trend with regard to differences in chemical composition was observed for wines produced with *T. delbrueckii* T3 (commercial strain) and T6 (ARC culture collection isolate) (Figure 4.3 and Table 4.3).



**Figure 4.3.** Principal component analysis (PCA) bi-plot derived from volatile compounds and standard chemical parameters of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, as well as three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Circles are for illustrative purpose only.

Most of the wines that underwent sequential MLF were positively correlated with volatile acidity, ethyl acetate, acetic acid, 2-phenylethyl acetate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, octanoic acid, isoamyl acetate, decanoic acid and fructose (Figure 4.3). Clear variation was observed with regard to the clustering of wines produced with the different yeast combinations that underwent sequential MLF, indicating that their chemical compositions differed from each other and the other MLF treatments. Similar to what was observed for the wines that did not undergo MLF, wines produced with M2+Sc and L1+Sc that underwent sequential MLF were similar.

Wines that underwent simultaneous MLF were closely associated and positively correlated with ethyl decanoate, hexanol, acetoin, methanol, pentanol, isovaleric acid, lactic acid, ethyl lactate,

isobutanol, isobutyric acid, glycerol, diethyl succinate, isoamyl alcohol, 3-methyl-1-pentanol and 2-phenyl ethanol. Despite the close association of wines that underwent simultaneous MLF, differences were observed for wines produced with the selected yeast combinations. Wines produced with M2+Sc and L1+Sc that underwent simultaneous MLF did not cluster together as observed for the no MLF and sequential MLF strategies. For wines that underwent simultaneous MLF, M2+Sc and T3+Sc wines clustered together.

Results also showed that the variation in chemical composition of wines produced by strains from the same non-*Saccharomyces* species can be as significant as the variation between different non-*Saccharomyces* species, or as significant as the differences between non-*Saccharomyces* and *Saccharomyces* yeasts.

#### 4.3.5 Sensory evaluation

The sensory data show that the different yeast combinations had a significant effect on berry aroma ( $p = 0.0036$ ), while MLF strategy (none, simultaneous and sequential MLF) had a significant effect on berry aroma ( $p = 0.0053$ ), acid balance ( $p = 0.0447$ ) and astringency ( $p = 0.0271$ ) (Table 4.5). At the 90% confidence level ( $p \leq 0.1$ ) yeast treatment had a significant effect on fresh vegetative aroma and MLF strategy had a significant effect on fruity aroma. Overall, there was no significant interaction effect between yeast treatment and MLF strategy (Table 4.5), but for certain wines significant differences were observed (Table S4.3). Only the treatment effects for berry, acid balance and astringency are discussed, but the additional sensory data for all descriptors and treatment interactions are listed in the supplementary information (Table S4.3). Although the interactive effect of yeast treatment and MLF strategy was not significant, the effects of all the treatment combinations on the aforementioned descriptors are shown for illustrative purposes (Figures 4.4-4.6).

**Table 4.5.** Probability ( $p$ ) values<sup>1</sup> of Shiraz wines produced with the different yeast treatments and malolactic fermentation (MLF) strategies.

Descriptor	Treatments		
	Yeast	MLF strategy	Yeast $\times$ MLF strategy
Berry	0.0036	0.0053	0.1643
Fruity	0.1696	0.0857	0.4701
Fresh vegetative	0.0989	0.8366	0.9774
Cooked vegetative	0.6539	0.1068	0.9403
Spicy	0.1848	0.5088	0.2219
Floral	0.3241	0.6223	0.8284
Acid balance	0.2679	0.0447	0.5892
Body	0.4319	0.2718	0.1424
Astringency	0.1749	0.0271	0.2493
Bitterness	0.1547	0.3787	0.6995
Overall quality	0.2355	0.8938	0.2737

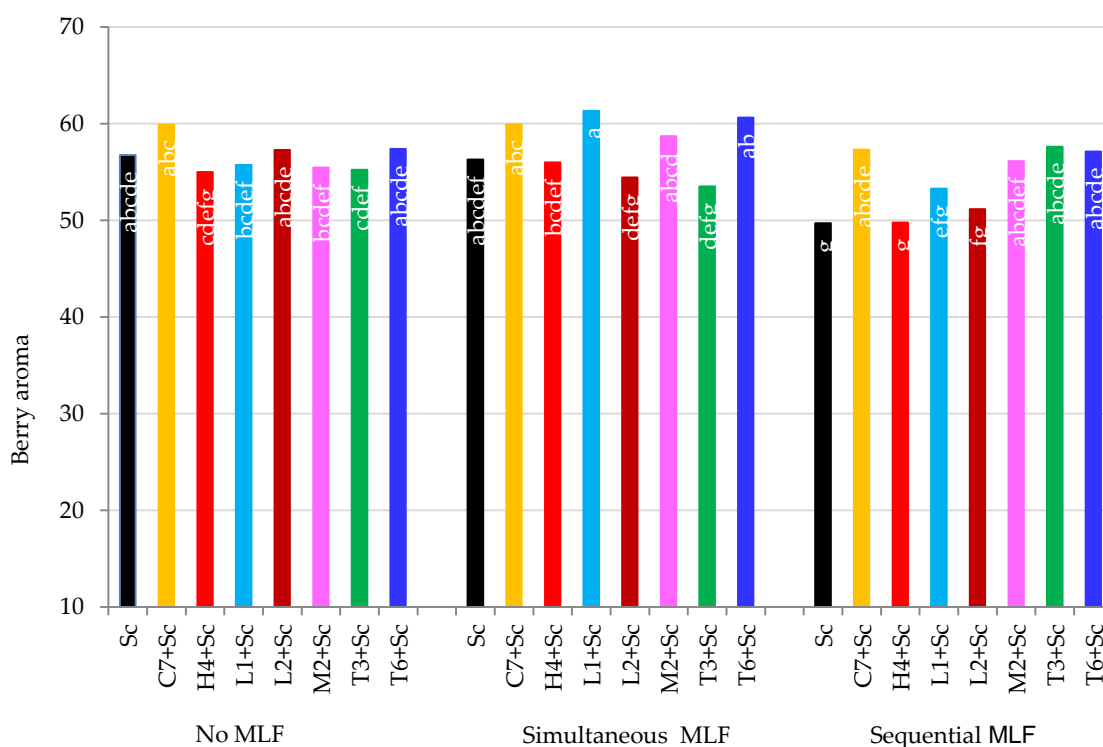
<sup>1</sup>Differences between treatments are significant if  $p \leq 0.05$ .



### 3.5.1. Berry aroma

Wines that underwent simultaneous MLF scored slightly higher for “berry” aroma than wines that did not undergo MLF, but both treatments scored significantly higher than wines that underwent sequential MLF (Figure 4.4 and Table S4.3). Of all the treatments, wines produced with L1+Sc that underwent simultaneous MLF scored the highest for berry aroma, and Sc and H4+Sc wines that underwent sequential MLF scored the lowest. The *S. cerevisiae* reference wines that underwent MLF scored less for berry aroma than the *S. cerevisiae* wines that did not undergo MLF. A similar trend was observed for wines produced with L2+Sc. Berry aroma increased in wines produced with M2+Sc that underwent MLF.

Even though wines that underwent sequential MLF contained higher concentrations of most of the various esters than wines that underwent simultaneous MLF and wines that did not undergo MLF (Table 4.3), it did not contribute to more perceivable berry aroma in those wines (Figure 4.4). Other compounds such as volatile acids possibly masked the contribution of the esters. Wines that underwent simultaneous MLF contained higher levels of diethyl succinate (fruity, melon, berry aroma), ethyl-3-hydroxybutanoate (fruity, grape, strawberry aroma) and ethyl decanoate (floral, grape, soap aroma) than wines that underwent sequential MLF. These compounds might have contributed to the perceived berry and fruity aroma of the wines. It is also possible that the perceived berry aroma could be due to enhancement of the aforementioned compounds by other volatile compounds, such as higher alcohols, or the synergistic interactions with other compounds. Another possibility is that compounds not quantified in this study might be responsible for perceived berry aroma.

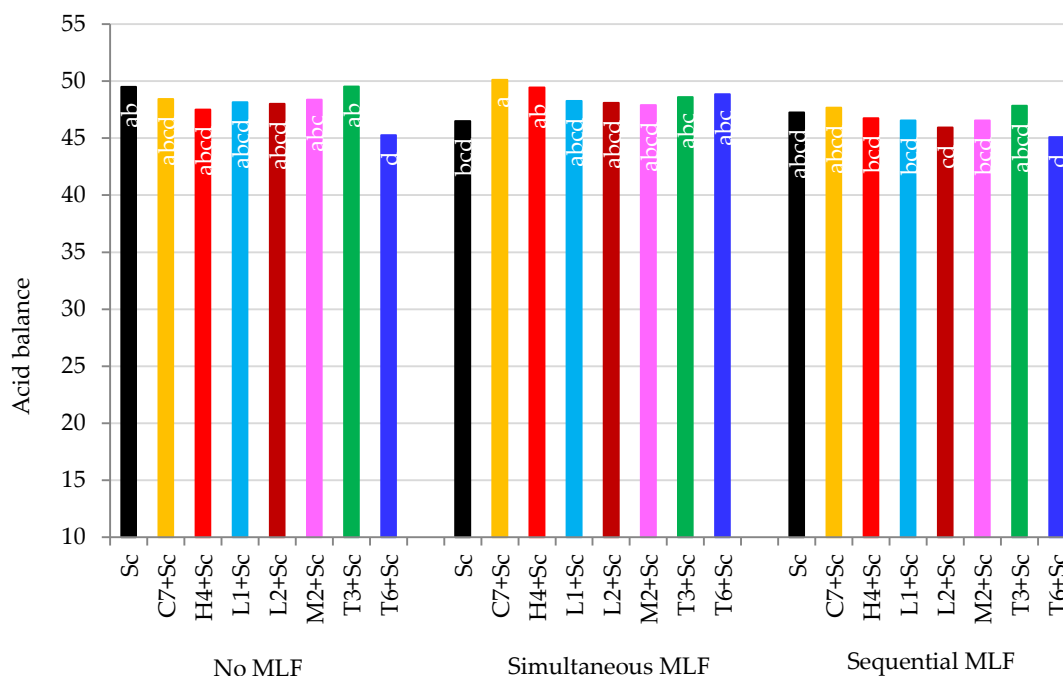


**Figure 4.4.** Percentage (%) berry aroma in Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, and three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Mean values followed by the same letter did not differ significantly ( $p \leq 0.5$ ).

Results show that wines produced with certain non-*Saccharomyces* yeast strains in combination with simultaneous MLF had more berry aroma than wines that did not undergo MLF, while wines produced with other non-*Saccharomyces* yeast strains had more berry aroma when MLF was induced as a sequential inoculation. This indicates that the effect of MLF strategy on berry aroma is strain dependent and that yeast and LAB strain combination needs further investigation.

#### 4.3.5.2 Acid balance

In general, wines that underwent sequential MLF were less balanced and scored lower for acid balance than wines that underwent simultaneous MLF and wines that did not undergo MLF (Figure 4.5). The lack of acidity was confirmed by the total acidity data, which showed that wines that underwent sequential MLF had significantly lower TA levels than wines that did not undergo MLF and wines that underwent simultaneous MLF (Table 4.2). However, the sequential MLF wines were perceived to be less balanced and did not have a clear negative effect on the perceived quality of these wines because the wines scored similar or better for overall quality than wines that did not undergo MLF (supplementary Table S4.3).

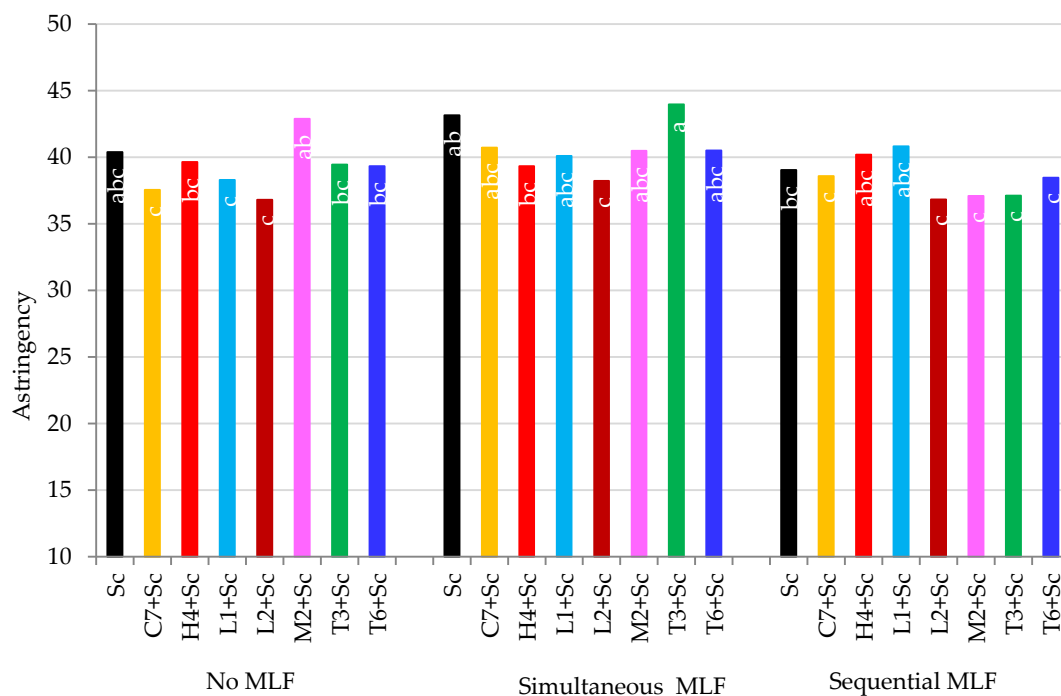


**Figure 4.5.** Acid balance (%) of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulaspora delbrueckii* strains T3 and T6, and three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Mean values of the various treatments followed by the same letter did not differ significantly ( $p \leq 0.05$ ).

#### 4.3.5.3 Astringency

Wines that underwent simultaneous MLF were perceived to be more astringent than wines that did not undergo MLF and significantly more astringent than wines undergoing sequential MLF (Figure 4.6). None of the treatments produced wines that were considered unacceptable with regard to astringency. Wines that underwent sequential MLF were the least astringent, which could be beneficial to winemakers who want to get their wines on the market quickly. If a wine is too astringent, it could have a negative effect on the overall quality of wine, which was not the case for wines that underwent simultaneous MLF (supplementary Table S4.3). Wines that underwent simultaneous MLF scored highest for overall quality for most of the yeast combinations, even though it was not significant (Table S4.3). Simultaneous MLF

might be beneficial for wines that are made to be aged for a long period, because astringency decreases over time and may contribute to the ageing potential of such wines.



**Figure 4.6.** Percentage (%) astringency of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc) on its own or in combination with *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2 and *Torulasporea delbrueckii* strains T3 and T6, as well as three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Mean values followed by the same letter did not differ significantly ( $p \leq 0.05$ ).

#### 4.3.6 Overall effects

The selected non-*Saccharomyces* yeasts were present at high levels and long enough to contribute to wine flavor and this is supported by chemical and sensory results. The non-*Saccharomyces* isolates in combination with *S. cerevisiae* and the three MLF strategies produced wines without any off-flavors. The aforementioned wines were different to wines produced with the *S. cerevisiae* reference and also the two commercial non-*Saccharomyces* yeast strains (L1 and T3). The non-*Saccharomyces* yeast isolates showed potential for producing wines with different styles and flavor profiles, but need further evaluation in different grape cultivars/varieties and in combination with different *S. cerevisiae* yeast strains.

The yeast treatment and the stage of MLF induction had a significant effect on the standard chemical parameters and volatile composition of the wines. However, the variation in wine composition did not always translate to perceivable sensory differences and neither did the contributions of volatile compounds with OAV's above 1.

#### 4.4 Conclusions

This is the first report on the use of the non-*Saccharomyces* yeast strains *C. zemplinina* C7, *H. uvarum* H4 and *L. thermotolerans* L2 in the production of Shiraz wines. Strains *C. zemplinina* C7 and *L. thermotolerans* L2 had a negative effect on LAB growth and the progress of MLF when LAB were used in a simultaneous inoculation, but the same effect was not observed for sequential MLF. Results indicated that non-*Saccharomyces* yeast strains had a beneficial effect on the progress of MLF. Therefore, if MLF is required, it is important to choose *Saccharomyces* and non-*Saccharomyces* strains that are compatible and promote MLF. On the contrary, spontaneous and inoculated MLF can be delayed if yeast strains or combinations are used that have a negative effect on LAB growth. Non-*Saccharomyces* yeasts can also be

used to reduce alcohol levels. Wines that did not undergo MLF were significantly different to wines that underwent MLF in terms of chemical and sensory properties. Time of MLF induction had a significant effect on the chemical and sensory properties of the wines and had a greater effect on the sensory properties than the yeast treatment alone. However, significant variation in wine composition did not always translate to perceivable sensory differences. Wine flavor profiles can be changed by using different non-*Saccharomyces* yeast strains and MLF strategies. Differences between strains from the same non-*Saccharomyces* species can be as significant as the variation between different non-*Saccharomyces* species, or as significant as the differences between non-*Saccharomyces* and *Saccharomyces* yeasts. Induction of simultaneous or sequential MLF can also result in significant changes to wine flavor profiles. In general, wines that underwent simultaneous MLF scored higher for certain sensory descriptors than wines that underwent sequential MLF, but some yeast combinations yielded better wines with sequential MLF. The optimal MLF strategy for each yeast strain or yeast combination to improve wine flavor and quality appears to be strain dependent. The interactions between *Saccharomyces*, non-*Saccharomyces* and LAB are complex and the resulting changes to wine composition need further investigation.

**Supplementary Materials:** The following are available online at [www.mdpi.com/link](http://www.mdpi.com/link), Table S4.1: Glucose, fructose and sulphur dioxide (SO<sub>2</sub>) concentrations of Shiraz wines produced with different yeast strains in combination with three malolactic fermentation (MLF) strategies (none, simultaneous or sequential). Values are averages of triplicate fermentations, Table S4.2: Probability (p) values of volatile compounds present in Shiraz wines produced with different yeasts in combination with three malolactic fermentation (MLF) strategies (none, simultaneous or sequential) and the interaction between yeast and MLF strategy, Table S4.3: Sensory data of Shiraz wines produced with different yeasts in combinations with three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Values are averages of triplicate fermentations.

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**Table S4.1.** Glucose, fructose and sulphur dioxide (SO<sub>2</sub>) concentrations of Shiraz wines produced with different yeast strains in combination with three malolactic fermentation (MLF) strategies (none, simultaneous or sequential). Values are averages of three replicates.

Treatment <sup>1</sup>	Glucose (g/L)	Fructose (g/L)	Free SO <sub>2</sub> (mg/L)	Total SO <sub>2</sub> (mg/L)
Sc	1.18±0.11def <sup>2</sup>	1.05±0.03cdefgh	16	29
Sc+sim MLF	1.20±0.18def	0.95±0.07hijk	19	35
Sc+seq MLF	1.18±0.23def	1.00±0.06fghij	16	28
C7+Sc	1.34±0.14bcdef	0.89±0.10klm	17	33
C7+Sc+sim MLF	1.30±0.21bcdef	0.89±0.04jklm	16	29
C7+Sc+seq MLF	1.33±0.02bcdef	0.99±0.07ghijk	16	30
H4+Sc	1.62±0.10a	1.16±0.07ab	18	33
H4+Sc+sim MLF	1.41±0.10abcde	1.02±0.08efghi	17	31
H4+Sc+seq MLF	1.55±0.16ab	1.20±0.02a	18	32
L1+Sc	1.16±0.27ef	1.16±0.02ab	18	30
L1+Sc+sim MLF	1.54±0.10ab	1.06±0.03bcdefgh	16	29
L1+Sc+seq MLF	1.08±0.16f	1.13±0.11abcd	17	31
L2+Sc	1.43±0.12abcd	1.12±0.04bcde	18	32
L2+Sc+sim MLF	1.38±0.24abcde	0.79±0.10m	16	30
L2+Sc+seq MLF	1.45±0.20abcd	1.14±0.03abc	18	32
M2+Sc	1.18±0.24def	1.03±0.04defghi	18	31
M2+Sc+sim MLF	1.14±0.15ef	0.94±0.07ijkl	16	30
M2+Sc+seq MLF	1.23±0.08cdef	1.04±0.11cdefghi	17	31
T3+Sc	1.40±0.18abcde	1.05±0.04cdefgh	16	30
T3+Sc+sim MLF	1.29±0.13bcdef	0.98±0.06hijk	17	31
T3+Sc+seq MLF	1.19±0.22def	1.10±0.57bcdef	16	27
T6+Sc	1.35±0.14abcdef	1.10±0.09bcdefg	17	34
T6+Sc+sim MLF	1.38±0.07abcde	0.84±0.06lm	16	31
T6+Sc+seq MLF	1.48±0.10abc	1.10±0.07bcdefg	16	32

<sup>1</sup>*Saccharomyces cerevisiae* (Sc), *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2, *Torulaspora delbrueckii* strains T3 and T6 and simultaneous (sim) MLF and sequential (seq) MLF induced with a commercial *Oenococcus oeni* culture.

<sup>2</sup>Values in the same column followed by the same letter did not differ significantly ( $p \leq 0.05$ ).



**Table S4.2.** Probability (p) values<sup>1</sup> of volatile compounds of Shiraz wines produced with different yeast strains in combination with three malolactic fermentation (MLF) strategies (none, simultaneous or sequential) and the interaction between yeast and MLF strategy.

Compounds	Treatment		
	Yeast	MLF strategy	Yeast × MLF strategy
Diethyl succinate	<0.0001	<0.0001	<0.0001
Ethyl acetate	<0.0001	<0.0001	<0.0001
Ethyl butanoate	<0.0001	<0.0001	0.0002
Ethyl decanoate	<0.0001	<0.0001	<0.0001
Ethyl hexanoate	<0.0001	<0.0001	<0.0001
Ethyl-3-hydroxybutanoate	<0.0001	<0.0001	<0.0001
Ethyl lactate	<0.0001	<0.0001	<0.0001
Ethyl octanoate	<0.0001	<0.0001	<0.0001
Ethyl phenylacetate	<0.0001	<0.0001	<0.0001
Isoamyl acetate	<0.0001	<0.0001	<0.0001
2-Phenylethyl acetate	<0.0001	<0.0001	<0.0001
Butanol	<0.0001	<0.0001	<0.0001
3-Ethoxy-1-propanol	<0.0001	<0.0001	<0.0001
Hexanol	<0.0001	<0.0001	<0.0001
Methanol	<0.0001	<0.0001	<0.0001
3-Methyl-1-pentanol	<0.0001	<0.0001	<0.0001
Isoamyl alcohol	<0.0001	<0.0001	<0.0001
Isobutanol	<0.0001	<0.0001	<0.0001
Pentanol	<0.0001	<0.0001	<0.0001
2-Phenylethanol	<0.0001	<0.0001	<0.0001
Propanol	<0.0001	<0.0001	<0.0001
Acetoin	<0.0001	<0.0001	<0.0001
Acetic acid	<0.0001	<0.0001	<0.0001
Butyric acid	<0.0001	<0.0001	0.0573
Decanoic acid	<0.0001	<0.0001	<0.0001
Hexanoic acid	<0.0001	<0.0001	<0.0001
Isobutyric acid	<0.0001	<0.0001	<0.0001
Isovaleric acid	<0.0001	<0.0001	<0.0001
Octanoic acid	<0.0001	<0.0001	<0.0001
Propionic acid	<0.0001	0.0002	<0.0001
Valeric acid	<0.0001	<0.0001	0.0049

<sup>1</sup>Values are significant if  $p \leq 0.05$ .

**Table S4.3.** Sensory data of Shiraz wines produced with different yeast strains in combinations with three malolactic fermentation (MLF) strategies (none, simultaneous and sequential). Values are averages of three replicates.

Treatment <sup>1</sup>	Berry	Fruity	Fresh Vegetative	Cooked vegetative	Spicy	Floral	Acidity	Body	Astringency	Bitterness	Overall quality
Sc	56.78abcde <sup>2</sup>	36.91de	33.00abc	16.09b	32.12abcde	14.96ab	49.51ab	58.40abcd	40.40abc	15.13bc	55.59abcd
Sc+sim MLF	56.26abcdef	36.60e	32.32abc	15.99b	34.68abcd	15.72ab	46.50bcd	59.74ab	43.16ab	15.99bc	57.61abc
Sc+seq MLF	49.71g	37.18cde	31.96abc	19.22ab	34.62abcd	14.33ab	47.27abcd	54.91cde	39.04bc	19.88ab	52.74d
C7+Sc	59.91abc	39.91abcde	32.93abc	16.60ab	32.69abcde	15.13ab	48.44abc	55.57bcde	37.54c	15.91bc	57.71abc
C7+Sc+sim MLF	59.96abc	40.91abcde	32.13abc	18.82ab	35.24ab	16.94a	50.13a	60.40a	40.73abc	18.72abc	59.45a
C7+Sc+seq MLF	57.11abcde	38.06cde	28.85abc	17.92ab	36.36ab	16.02ab	47.67abcd	58.13abcde	38.58c	15.75bc	57.62abc
H4+Sc	55.00cdefg	39.47abcde	30.44abc	18.13ab	28.63cde	14.69ab	47.51abcd	58.56abc	39.64bc	16.59bc	57.05abcd
H4+Sc+sim MLF	56.00bcdef	38.41bcde	27.78c	18.49ab	31.27bcde	15.62ab	49.46ab	59.32ab	39.33bc	17.89bc	57.14abcd
H4+Sc+seq MLF	49.77g	37.92cde	31.89abc	21.78ab	33.89abcde	16.31ab	46.76bcd	59.01ab	40.21abc	16.73bc	54.19bcd
L1+Sc	55.72bcdef	39.26abcde	34.39abc	17.13ab	34.25abcd	15.74ab	48.16abcd	54.43de	38.29c	16.67bc	54.23bcd
L1+Sc+sim MLF	61.33a	40.14abcde	35.28ab	16.36ab	38.06a	13.53ab	48.28abcd	57.98abcde	40.09abc	18.93abc	56.50abcd
L1+Sc+seq MLF	53.29efg	36.06e	31.46abc	18.51ab	32.22abcde	15.18ab	46.57bcd	56.42abcde	40.82abc	17.53bc	53.80cd
L2+Sc	57.29abcde	44.36a	29.58abc	21.04ab	33.94abcde	16.78ab	48.02abcd	58.55abcd	36.81c	16.96bc	57.94abc
L2+Sc+sim MLF	54.51defg	36.74e	32.14abc	17.41ab	31.64bcde	15.79ab	48.10abcd	57.31abcde	38.22c	14.51c	55.86abcd
L2+Sc+seq MLF	51.22fg	38.87abcde	29.17abc	23.10a	27.83e	12.54b	45.93cd	55.69bcde	36.83c	17.36bc	56.26abcd
M2+Sc	55.47bcdef	40.56abcde	32.07abc	17.42ab	32.08abcde	13.76ab	48.37abc	59.37ab	42.89ab	18.49abc	55.67abcd
M2+Sc+sim MLF	58.71abcd	42.67abc	32.29abc	17.79ab	34.02abcde	16.93a	47.90abcd	56.41abcde	40.49abc	19.81ab	57.45abcd
M2+Sc+seq MLF	56.12abcdef	37.34cde	34.02abc	23.08a	33.34abcde	13.53ab	46.56bcd	57.84abcde	37.09c	18.07bc	58.53ab
T3+Sc	55.24cdef	40.83abcde	35.70a	16.44ab	33.11abcde	13.13ab	49.54ab	57.37abcde	39.46bc	18.07bc	57.58abc
T3+Sc+sim MLF	53.64defg	36.59e	34.92abc	17.50ab	28.45cde	13.03ab	48.60abc	59.35ab	43.98a	23.55a	54.24bcd
T3+Sc+seq MLF	57.63abcde	38.73abcde	33.30abc	20.43ab	30.63bcde	14.13ab	47.83abcd	59.07abcd	37.13c	18.90abc	<b>59.85a</b>
T6+Sc	57.40abcde	43.93ab	27.83bc	17.60ab	34.62abcd	17.42a	45.27d	54.04e	39.33bc	16.07bc	56.19abcd
T6+Sc+sim MLF	60.64ab	42.36abcd	28.96abc	16.69ab	34.76abc	16.98a	48.87abc	59.38ab	40.51abc	16.82bc	56.77abcd
T6+Sc+seq MLF	57.13abcde	38.24cde	27.87bc	15.96b	28.33de	16.24ab	45.10d	57.84abcde	38.47c	15.00bc	58.49ab

<sup>1</sup>*Saccharomyces cerevisiae* (Sc), *Candida zemplinina* C7, *Hanseniaspora uvarum* H4, *Lachancea thermotolerans* strains L1 and L2, *Metschnikowia pulcherrima* M2, *Torulaspora delbrueckii* strains T3 and T6, simultaneous (sim) MLF and sequential (seq) MLF induced with a commercial *Oenococcus oeni* culture.

<sup>2</sup>Values in the same column followed by the same letter did not differ significantly ( $p \leq 0.05$ ).

# Chapter 5

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## Research results III

**Modulation of wine flavour using *Hanseniaspora uvarum* in combination with two *Saccharomyces cerevisiae* strains and three malolactic fermentation strategies**

## Chapter 5

### Modulation of wine flavour using *Hanseniaspora uvarum* in combination with two *Saccharomyces cerevisiae* strains and three malolactic fermentation strategies

#### ABSTRACT

The effects of *Hanseniaspora uvarum* (*Kloeckera apiculata*) on *Saccharomyces* yeast, lactic acid bacteria (LAB) growth and wine flavour have not been extensively studied, despite *H. uvarum* being the predominant non-*Saccharomyces* yeast species found on grapes and in juice. Therefore, the interaction between *H. uvarum*, two commercial *Saccharomyces cerevisiae* yeast strains, two LAB species (*Lactobacillus plantarum* and *Oenococcus oeni*) in combination with three malolactic strategies were investigated in small-scale Shiraz wine production trials. The evolution of the different yeasts and LAB were monitored, the levels of the standard wine chemical parameters and the volatile flavour compounds were measured, and the wines were also subjected to sensory evaluation. One of the *S. cerevisiae* strains had an inhibitory effect on LAB growth and progression of MLF, while wines produced with *H. uvarum* had a stimulatory effect on LAB growth. Wines produced with the simultaneous MLF inoculation strategy of *H. uvarum* in combination with *S. cerevisiae* completed MLF in a shorter period than wines produced with *S. cerevisiae* only. The wines produced with the aforementioned yeast, LAB combinations and MLF strategies were significantly different with regard to their flavour and sensory profiles. Isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, 2-phenyl acetate, isobutanol, 3-methyl-1-pentanol, hexanoic acid and octanoic acid were important compounds in discriminating between the different wines. Yeast treatment had a significant effect on fresh vegetative and spicy aroma, as well as body and astringency of the wines. The LAB strain and MLF strategy had a significant effect on berry, fruity, sweet associated and spicy aroma, as well as acidity and body of the wines. *H. uvarum* in combination with a MLF compatible *S. cerevisiae* yeast and can be used to reduce the duration of MLF and enhance wine flavour and complexity. Different LAB strains and MLF strategies can also be used to reduce duration of MLF and to diversify flavour profile of wines.

#### 5.1 INTRODUCTION

The contribution of yeasts to wine composition and quality is well-known (Fleet, 2003; Swiegers *et al.*, 2005; Jolly *et al.*, 2014). The *Saccharomyces* yeasts drive alcoholic fermentation by

converting the grape sugar to alcohol, carbon dioxide and other compounds affecting the wine aroma and taste (Fleet, 2003; Ribéreau-Gayon *et al.*, 2006). The other group of yeasts important to winemaking are the non-*Saccharomyces* yeasts, also known as “wild yeast”, which have different oenological characteristics to *S. cerevisiae* that can be used to improve wine quality in terms of enhanced wine aroma and complexity (Ciani *et al.*, 2010; Gobbi *et al.*, 2013; Jolly *et al.*, 2014). Non-*Saccharomyces* yeast species, *Hanseniaspora uvarum* (*Kloeckera apiculata*), frequently found on grapes and in grape must are known to dominate the initial phases of spontaneous fermentations (Jolly *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006; Romano *et al.*, 2006; Capozzi *et al.*, 2015). Some *H. uvarum* strains can produce high levels of acetic acid and ethyl acetate, but there is high variability among strains (Romano *et al.*, 2003; De Benedictis *et al.*, 2011; Tristezza *et al.*, 2016b). It has also been reported that *H. uvarum* can produce high levels of desirable compounds such as esters, higher alcohols and carbonyl compounds (Moreira *et al.*, 2008; Jolly *et al.*, 2014, Tristezza *et al.*, 2016b). Mendoza *et al.* (2011) and Tristezza *et al.* (2016b) both showed that mixed culture fermentations of *H. uvarum* and *S. cerevisiae* can be used to enhance wine aroma and quality.

Another process that plays an important role with regard to wine flavour and quality is the malolactic fermentation (MLF), which decreases acidity by converting L-malic acid to L-lactic acid and CO<sub>2</sub>. Malolactic fermentation can affect wine flavour through aroma impact compounds such as diacetyl, esters, higher alcohols and volatile acids (Davis *et al.*, 1985; Bartowsky *et al.*, 2002; Lerm *et al.*, 2010). While *Oenococcus oeni* has been the LAB of choice as a MLF starter, recently *Lactobacillus plantarum* starters have become available and produce a broader range of extracellular enzymes, including glycosidases and esterases, than *O. oeni*, which is beneficial to flavour development (Guerzoni *et al.*, 1995; Grimaldi *et al.*, 2005; Mtshali *et al.*, 2010). Different MLF inoculation strategies, *i.e.* simultaneous inoculation (at the start of alcoholic fermentation) and sequential inoculation (after alcoholic fermentation) have been shown to affect the flavour profiles of wines (Massera *et al.*, 2009; Mendoza *et al.*, 2011; Abrahamse & Bartowsky, 2012a, b; Tristezza *et al.*, 2016a; Versari *et al.*, 2016).

A better understanding of how wine production methodology can be manipulated to change wine attributes such as aroma, flavour, body or mouthfeel, is important for the production of a targeted wine style (Lesschaeve, 2007). In Chapter 4 (Du Plessis *et al.*, 2017b), five different non-*Saccharomyces* yeast species were evaluated in wine production using different MLF strategies. Results showed that MLF strategy had a greater impact on the chemical and sensory profiles of the wines than yeast combination used. Therefore, we wanted to investigate whether the use of a different *S. cerevisiae* strain would have the same outcomes. The impact of different LAB species on the chemical and sensory profiles was another research question that needed to be answered. The *H. uvarum* strain was shown to be compatible with MLF, had potential to enhance wine flavour and is the non-*Saccharomyces* species most frequently found on grapes and in must. Therefore, the aims of the current study were to investigate the

interactions between *H. uvarum*, two commercial *S. cerevisiae* strains and two LAB species (*Lb. plantarum* and *O. oeni*) and three MLF strategies, and to determine how these interactions affect fermentation kinetics and Shiraz wine flavour.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Cultivation and enumeration of microorganisms

The selected yeast and LAB strains used in this study are listed in Table 5.1. Similar culturing conditions and procedures were followed as described in Chapter 4 (Du Plessis *et al.*, 2017b). *H. uvarum* were inoculated into Shiraz grape juice at concentration of  $\sim 1 \times 10^6$  cells/mL. Commercial *S. cerevisiae* strains and LAB cultures (*O. oeni* and *Lb. plantarum*) were inoculated according to the manufacturer's recommendations.

TABLE 5.1. Microorganisms used in Shiraz wine production trials.

Reference code	Species name	Source
Sc1	<i>Saccharomyces cerevisiae</i>	VIN 13, commercial strain, Anchor Wine Yeast, South Africa
Sc2	<i>Saccharomyces cerevisiae</i>	NT 202, commercial strain, Anchor Wine Yeast
Hu	<i>Hanseniaspora uvarum</i>	Y0858, natural isolate, ARC Infruitec-Nietvoorbij culture collection
LAB1	<i>Oenococcus oeni</i>	Viniflora <sup>®</sup> oenos, commercial malolactic fermentation starter, Chr. Hansen A/S, Denmark
LAB2	<i>Lactobacillus plantarum</i>	Enoferm V22, commercial malolactic fermentation starter, Lallemand Inc., France

Total yeast counts for the Shiraz juice and wine were obtained by plating out on WL medium (Biolab, Merck, South Africa). Non-*Saccharomyces* yeast counts were obtained by plating out on Lysine medium (Biolab, Merck, South Africa). Bacterial counts were obtained by plating out on MRS agar (Biolab, Merck) supplemented with 25% (v/v) grape juice and 100 mg/L Natamycin (Danisco A/S, Denmark). Growth media were incubated at 28°C for 2-7 days, after which the colonies were counted. The natural occurring non-*Saccharomyces* yeast populations were determined by counting the non-*Saccharomyces* yeasts present in the reference treatments, which only received a *S. cerevisiae* inoculum. The naturally occurring *Saccharomyces* yeast populations were determined by counting the *Saccharomyces* yeasts in the treatments that did not receive any *S. cerevisiae* inoculum, *i.e.* *H. uvarum* treatments. However, this only applied to the counts for days 0 and 1. The development of the naturally occurring LAB during fermentation was monitored by sampling the treatments that were not inoculated for MLF and the sequential MLF treatments until day 5, when the commercial LAB cultures were added to the sequential MLF wines.

## 5.2.2 Wine production

The two commercial *S. cerevisiae* strains, Sc1 and Sc2, were used on their own or in combination with *H. uvarum* (Hu), resulting in four yeast combinations. These four yeast combinations were further evaluated in combination with two LAB species, LAB1 and LAB2, and three MLF strategies (none, simultaneous and sequential MLF), which resulted in 15 treatment combinations (Table 5.2). All treatments had three replicates. The MLF strategies were: (1) the yeast strains (*S. cerevisiae* only or in combination with *H. uvarum*) without MLF (no MLF), (2) yeast strains in combination with LAB1 or LAB2 as a simultaneous inoculation (simultaneous MLF) and (3) yeast strains in combination with LAB1 or LAB2 as a sequential inoculation (sequential MLF). The treatments with *S. cerevisiae* strains (Sc1 and Sc2) on their own, served as the reference treatments.

Shiraz grapes were obtained from the Nietvoorbij research farm (Stellenbosch, South Africa) and the same standardised small-scale (20 L) winemaking procedure was followed as described in Chapter 4 (Du Plessis *et al.*, 2017b). The *S. cerevisiae* strains Sc1 and Sc2 were inoculated on day 0 in the reference treatments. *H. uvarum* was inoculated on day 0 and Sc1 and Sc2 were inoculated after 24 hours (day 1) for the mixed yeast fermentation. The LAB in the simultaneous MLF samples were added 25 hours after the initial yeast inoculation on day 0. Fermentations were carried out at *ca.* 24°C and after completion of the alcoholic fermentation, the sequential MLF treatments were inoculated with LAB1 or LAB2. All treatments were racked, fined, cold stabilized and bottled as described by Minnaar *et al.* (2015). After bottling, all wines were stored at 15°C until needed.

## 5.2.3 Yeast isolation, identification and typification

Yeasts were isolated from juice and wine samples to verify successful implantation. Colonies were selected based on colour and morphological differences. Subsequently, yeast DNA was extracted using the method described by Lööke *et al.* (2011) and identification to species level were carried out by PCR amplification of the 5.8S-internal transcribed spacer (ITS) ribosomal region, followed by enzyme restriction with *CfoI*, as described by Esteve-Zarzoso *et al.* (1999). The identity of the implanted *H. uvarum* strain was verified with random amplified polymorphic DNA (RAPD) PCR, using primer 1283 as described by Pfliegler *et al.* (2014). This technique was chosen above the two typing techniques evaluated in Chapter 3, as it was better suited for rapid profiling of *Hanseniaspora* strains (Cadez *et al.*, 2002; De Benedictis *et al.*, 2011). Amplification products (ITS-RFLP and RAPD) were separated on 2% agarose gels and banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% ( $\frac{1}{100}$ ) ethidium bromide (Bio-Rad Laboratories, Inc., USA).

#### 5.2.4 Juice and wine analyses

The following parameters of the grape must were measured, *i.e.* sugar (Balling), free and total SO<sub>2</sub> (Ripper method), pH and titratable acidity (Mettler titrator) analyses as described in the South African Wine Laboratories Association manual (SALWA) (Anonymous, 2003). The progression of MLF was monitored with an OenoFoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) until the malic acid levels were below 0.2 g/L, the point where MLF was considered to be complete. Standard chemical parameters (glucose and fructose, pH, malic and lactic acid, total acidity (TA), alcohol, volatile acidity (VA) and glycerol) were determined on the bottled wines using a WineScan™ FT120 instrument (FOSS Analytical A/S) at the Institute for Wine Biotechnology (Stellenbosch University, South Africa) as described by Louw *et al.* (2009). The concentrations of major volatile compounds in wines were determined by the Chemical Analytical Laboratory (Institute for Wine Biotechnology and Department of Viticulture and Oenology, Stellenbosch University), using a gas chromatograph coupled to a flame ionization detector (GC-FID) as described by Louw *et al.* (2009).

#### 5.2.5 Sensory evaluation

A panel consisting of 22 experienced wine judges (13 men and 9 women, aged 22 to 50 years) evaluated the wines four months after bottling. The panellists were commercial winemakers or staff of ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council). Panel members did not receive collective training. Wines were evaluated during three sessions (15 wines per session) over two days in a temperature-controlled room at ±20°C. Panel members had to take a compulsory break between session 1 and 2. The descriptors were chosen from a predefined lexicon and the wines were subjected to classical profiling as described in Chapter 4 (Du Plessis *et al.*, 2017b). The panellists were asked to evaluate the aroma and taste of the wines and to score the intensity of each descriptor individually on a 100 mm unstructured line scale. The descriptors were berry, fruity, fresh vegetative, cooked vegetative, floral, sweet associated, spicy, acid balance, body (mouthfeel), astringency and bitterness. Each judge had a separate tasting booth and *ca.* 30 mL of the wine samples were presented in a randomised order in a standard wine glass, labelled with a three digit code. Research Randomizer (Version 4.0, <http://randomizer.org>) was used to generate the three digit code and to randomise the order in which the wines were presented to each panellist.

#### 5.2.6 Statistical analysis

Shiraz chemical and sensory data were tested for normality by the Shapiro-Wilk test then subjected to mixed model analysis of variance (ANOVA) using the general linear means procedure of SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Student's *t*-least significant difference (LSD) values were calculated at the 5% probability level ( $p = 0.05$ ) to



facilitate comparison between treatment means. Principal Component Analysis (PCA) was performed using XLSTAT software (Version 18.07.39157, Addinsoft, New York, USA) to evaluate relationships between sensory attributes and chemical compounds of the wines.

## 5.3 RESULTS AND DISCUSSION

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Two commercial *S. cerevisiae* strains, Sc1 and Sc2, were used on their own or in combination with *H. uvarum* (Hu) in small-scale Shiraz wine production trials. The different yeast combinations were subsequently used in combination with LAB1 (*O. oeni*) or LAB2 (*Lb. plantarum*) as simultaneous or sequential MLF inoculations. Resultant wines differed with regard to cell counts, duration of MLF, chemical composition and also the sensory profiles.

### 5.3.1 Fermentation kinetics

#### 5.3.1.1 Yeast growth

The naturally occurring *Saccharomyces* and non-*Saccharomyces* yeast populations in the Shiraz juice were ca.  $4.2 \times 10^5$  and  $4.1 \times 10^5$  colony forming units/mL (CFU/mL), respectively (Fig. 5.1). The naturally occurring non-*Saccharomyces* yeast populations decreased dramatically on day 1 in treatments inoculated with the commercial *S. cerevisiae* yeasts, before increasing again on day 2. The naturally occurring non-*Saccharomyces* yeast populations varied between  $1 \times 10^4$  to  $1 \times 10^5$  CFU/mL during alcoholic fermentation. The *S. cerevisiae* Sc1 had a negative effect on the growth of naturally occurring non-*Saccharomyces* yeasts and cell numbers were lower after five days for this treatment, than for wines fermented with Sc2. Alcoholic fermentation was completed within five days in all treatments.

Initial yeast counts of the wines inoculated with *H. uvarum* were just below  $1 \times 10^6$  CFU/mL, but increased to levels  $>10$  million CFU/mL after 24 hours. However, this trend changed after inoculation of commercial *S. cerevisiae* yeasts (day 1, Fig. 5.1), which resulted in the decrease of *H. uvarum* numbers. The same trend was observed with regard to the inhibitory activity of Sc1 on the natural non-*Saccharomyces* yeasts. At the end of alcoholic fermentation, inoculated and natural occurring non-*Saccharomyces* yeast populations were at a similar level.

The naturally occurring *Saccharomyces* yeast populations were present at moderately high numbers, which increased after 24 hours, but were clearly dominated by the inoculated *H. uvarum* populations. However, both aforementioned populations were dominated by the inoculated *S. cerevisiae* yeasts, following their addition after 24 hrs. These results indicate that the inoculated *S. cerevisiae* strains were responsible for completing the alcoholic fermentations. However, the inoculated *H. uvarum* populations were present at high levels ( $10^7$  to  $10^8$  CFU/mL) and long enough to potentially make a contribution to wine flavour. Similar trend to results observed in Chapter 4 (Du Plessis *et al.*, 2017b).

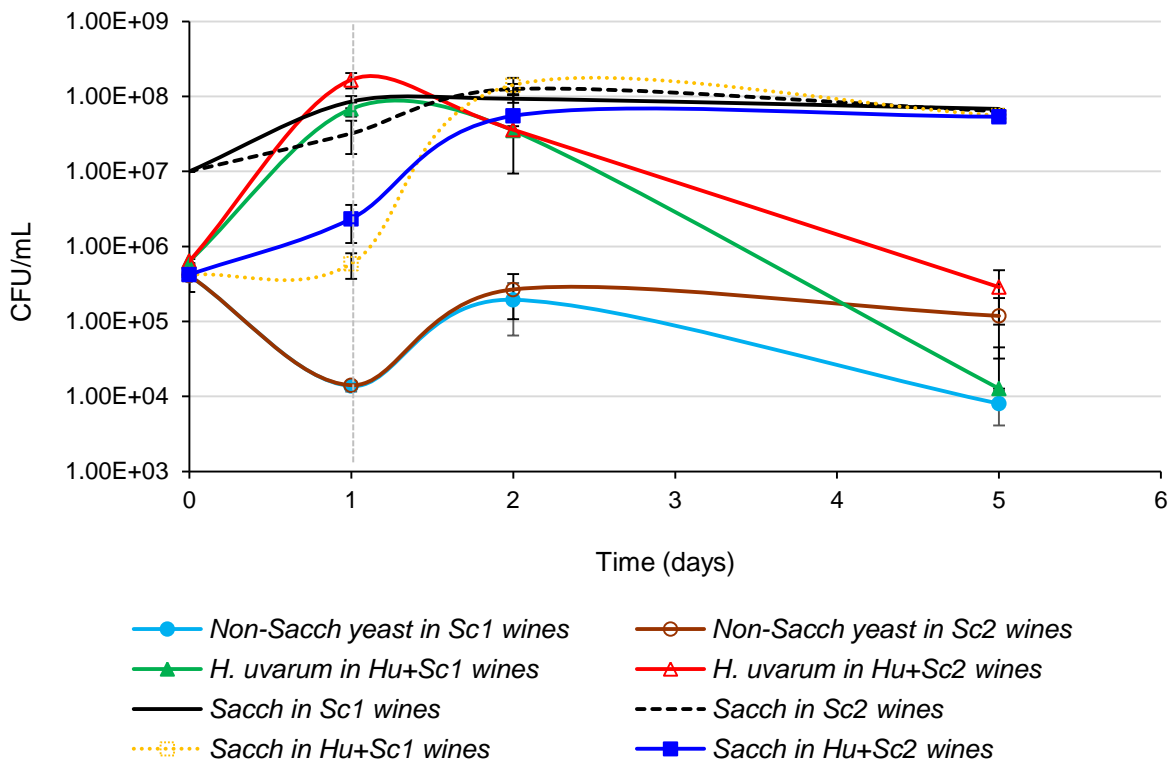


FIGURE 5.1. Cell counts (colony forming units/millilitres) of naturally occurring and inoculated *Saccharomyces cerevisiae* (Sacch), naturally occurring non-*Saccharomyces* (Non-Sacch) and inoculated *Hanseniaspora uvarum* (*H. uvarum*) yeasts during alcoholic fermentation. The dashed vertical line at day 1 indicates when commercial *S. cerevisiae* yeasts were added. Abbreviations: Sc1 = commercial *S. cerevisiae* strain 1, Sc2 = commercial *S. cerevisiae* strain 2, Hu = inoculated *H. uvarum* yeasts. Values are averages of three replicates and error bars indicate standard deviation.

#### 5.3.1.1.1 Verification of yeast implantations

A selection of yeast colonies from day 2 was identified by amplification of the ITS-5.8S region in combination with restriction analysis. The profiles obtained were compared to restriction profiles obtained by Esteve-Zarzoso *et al.* (1999). The dominant non-*Saccharomyces* yeast isolates from the Hu+Sc1 and Hu+Sc2 wines were identified as *H. uvarum*. These isolates were subsequently amplified using RAPD PCR and were compared to the reference *H. uvarum* strain (Table 5.1). All wine isolates had similar banding patterns as the *H. uvarum* reference (Fig. 5.2), indicating 100% successful implantation. The banding patterns of *H. uvarum* juice isolates (naturally occurring strains) differed from the *H. uvarum* reference, but were not detected in any of the implanted wines during the first two days of alcoholic fermentation.

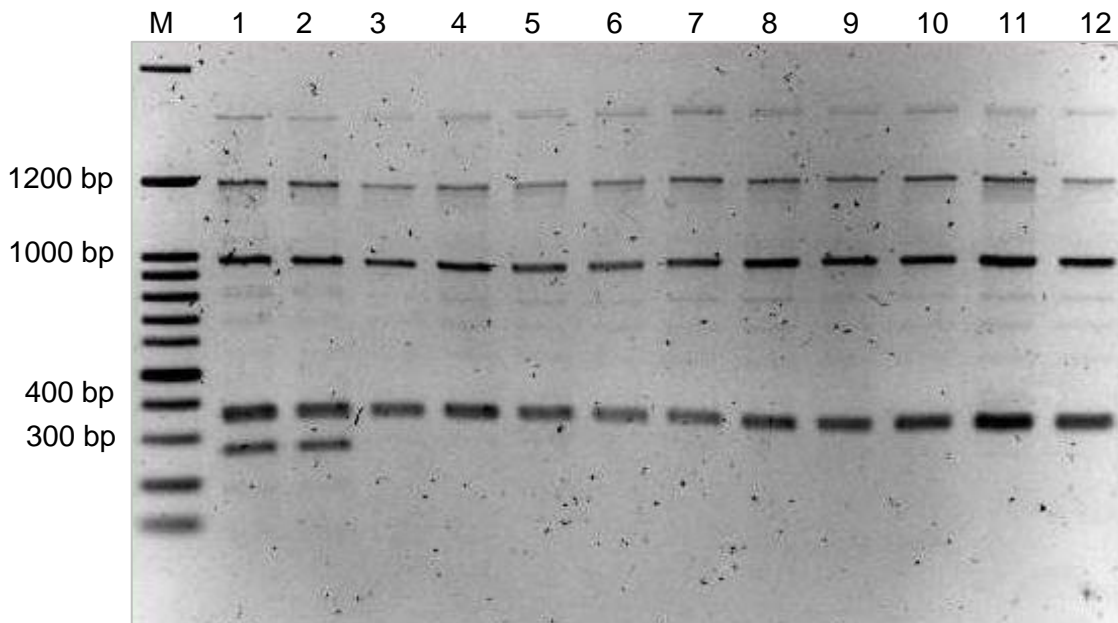


FIGURE 5.2. Random amplified polymorphic DNA products of Shiraz wines produced with *Hanseniaspora uvarum* in combination with *Saccharomyces cerevisiae* Sc1 and Sc2. M: 100 bp DNA ladder, lane1: *H. uvarum* strain isolated from juice, lane 2: *H. uvarum* strain isolated from juice, lane 3: *H. uvarum* reference used for implantations, lane 4 to 12: dominant non-*Saccharomyces* yeast isolated from wines inoculated with *H. uvarum* and *S. cerevisiae*.

### 5.3.1.2 Development of LAB and MLF progression

The growth and development of the naturally occurring and inoculated LAB are shown in Figure 5.3. The naturally occurring LAB were present at  $\sim 3.5 \times 10^4$  CFU/mL) in the grape must and decreased during AF in most of the treatments, with the increase in numbers at the end of alcoholic fermentation (day 5) (Fig. 5.3a). This is also the typical winemaking scenario (Ribéreau-Gayon *et al.*, 2006; Costantini *et al.*, 2009). Individually, the numbers of naturally occurring LAB varied notably in wines, fermented with the selected yeast combinations. Based on the LAB counts from day 2 to 5, Sc1 had a larger inhibitory effect on LAB growth (decreased from  $3.5 \times 10^4$  to  $8.8 \times 10^2$  CFU/mL) than Sc2 or *H. uvarum* in combination with Sc1 or Sc (decreased from  $3.5 \times 10^4$  to  $1.8 \times 10^3$  CFU/mL). This is in agreement with previous reports from Du Plessis *et al.* (2017b).

The alcoholic fermentation was completed after six days and the commercial LAB were inoculated on day 7 to induce sequential MLF in the selected treatments. The addition of commercial LAB resulted in a dramatic and expected increase of LAB numbers from  $\sim 1 \times 10^3 - 10^4$  to  $>7 \times 10^5$  CFU/mL (Fig. 5.3a). No notable delays in MLF was observed in sequentially inoculated wines, despite inoculated LAB2 and LAB1 counts decreasing from  $6.8$  to  $1.9 \times 10^5$  CFU/mL and  $5 \times 10^6$  to  $4.5 \times 10^5$  CFU/mL, respectively (Table 5.2). Wines produced with Hu+Sc1+LAB1 and Hu+Sc2+LAB2 completed MLF in the shortest time (18 days), while wines produced with Sc1+LAB1 and Sc1+LAB2 took the longest to complete MLF (34 days). The

delay in MLF of the Sc1+LAB2 wines can be correlated to lower LAB numbers ( $<1 \times 10^6$  CFU/mL), but the trend was not observed for Sc1+LAB1 wines, which contained high LAB numbers ( $>1 \times 10^6$  CFU/mL) throughout MLF (Fig. 5.3a).

Two distinct trends were observed with regard to the development of inoculated LAB in wines that underwent simultaneous MLF (Fig. 5.3b). Lactic acid bacteria numbers were high ( $>1 \times 10^6$  CFU/mL) in wines inoculated with LAB1 and completed MLF quickly (10 days) for all yeast combinations. On the other hand, LAB numbers in wines inoculated with LAB2 were initially above  $1 \times 10^6$  CFU/mL, but decreased below  $1 \times 10^6$  CFU/mL for all yeast combinations, which resulted in delays in MLF. For wines that underwent simultaneous MLF with LAB2, MLF completed in the shortest time in the Hu+Sc2 combination (15 days), while wines produced with Sc1+LAB2 took the longest (34 days, Table 5.2). Overall, simultaneous MLF completed in a shorter time than sequential MLF and LAB1 performed slightly better than LAB2. In general, *O. oeni* is known to be better suited to harsh conditions found in wine than *Lb. plantarum* and this explains why LAB1 performed better than LAB2. This trend of simultaneously inoculated wines completing MLF in less time than wines that were sequentially inoculated is in agreement with literature (Abrahamse & Bartowsky, 2012a, b; Izquierdo-Cañas *et al.*, 2015).

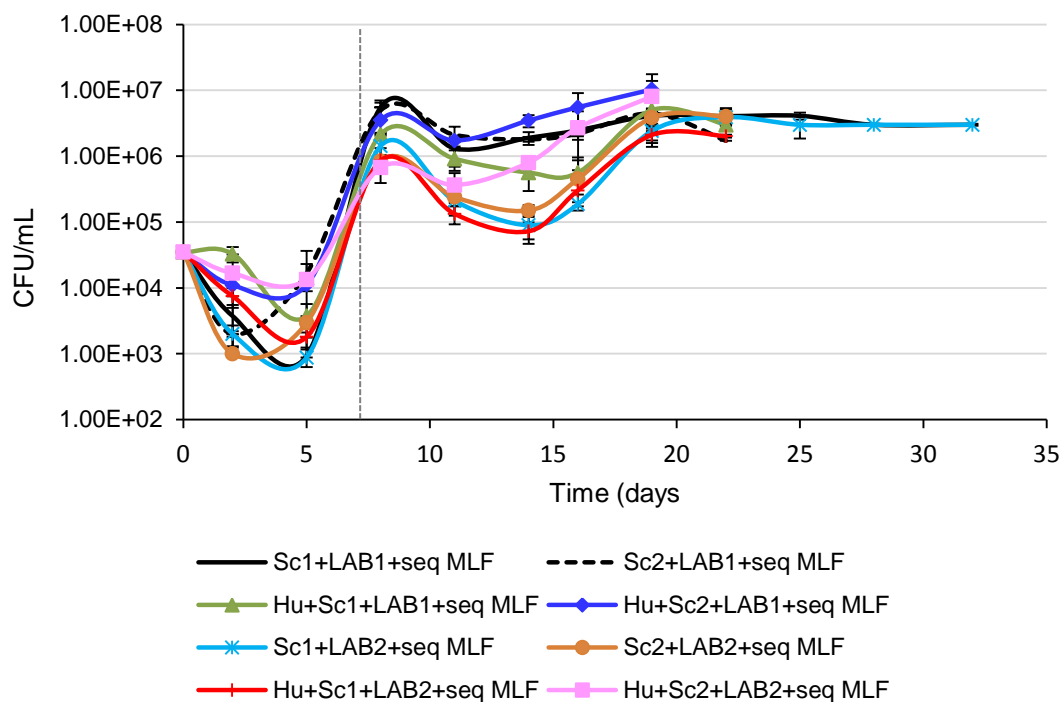
### 5.3.2 Standard oenological parameters

#### 5.3.2.1 Wines without MLF

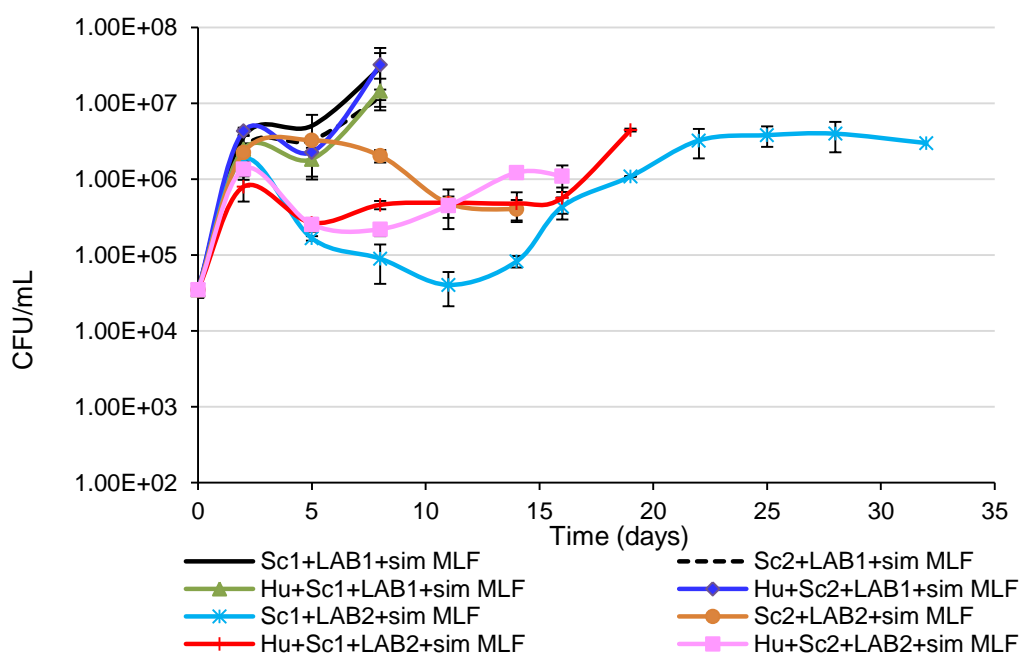
All wines fermented to dryness and contained residual sugar levels of less than 4 g/L (Table 5.2). Alcohol concentrations in wines produced with *H. uvarum* in combination with Sc1 (13.52%  $v/v$ ) and Sc2 (13.60%  $v/v$ ) were lower than wines produced with Sc1 and Sc2 (13.65%  $v/v$ ) on their own. This trend is in agreement with findings of Mendoza *et al.* (2007, 2011). Wines produced with only *S. cerevisiae* yeasts contained significantly higher glycerol concentrations than wines produced with the *H. uvarum* and *S. cerevisiae* combinations. Mendoza *et al.* (2011) reported similar findings, but Liu *et al.* (2016) reported the contrary, which indicate that this is not a species trait, but strain dependent.

None of the treatments produced above threshold concentrations of VA ( $>0.7$  g/L). However, VA concentrations in wines produced with *H. uvarum* in combination with Sc1 (0.29 g/L) and Sc2 (0.43 g/L) were slightly higher than wines produced with Sc1 (0.24 g/L) and Sc2 (0.35 g/L) on their own. This is in agreement with the findings of Mendoza *et al.* (2011) and also confirmed reports that some *H. uvarum* (*K. apiculata*) strains can produce lower VA levels comparable to that of *S. cerevisiae* (Romano *et al.*, 1992; Ciani *et al.*, 2006; Tristezza *et al.*, 2016b; Whitener *et al.*, 2017). Malic acid concentrations in wines produced with *H. uvarum* in combination with Sc1 (1.82 g/L) and Sc2 (1.69 g/L) were significantly lower than wines produced with Sc1 (2.81 g/L) and Sc2 (2.11 g/L) on their own. The ability of this *H. uvarum*

strain to degrade malic acid has been reported by in Chapters 3 and 4 (Du Plessis *et al.* 2017a, b).



(a)



(b)

FIGURE 5.3. Cell counts (colony forming units per millilitres, CFU/mL) of the naturally occurring and inoculated lactic acid bacteria (LAB) in Shiraz juice and wine produced with *Saccharomyces cerevisiae* (Sc1 or Sc2) on its own or in combination with *Hanseniaspora uvarum*, two LAB species (LAB1 or LAB2) and two malolactic fermentation (MLF) strategies. (a) Wines that underwent sequential (seq) MLF. The dashed vertical line at day 7 indicates when the commercial LAB was inoculated. (b) Wines that underwent simultaneous (sim) MLF. Values are averages of three replicates and error bars indicate standard deviation.

TABLE 5.2. Oenological parameters and duration of malolactic fermentation (MLF) of Shiraz juice<sup>1</sup> and wines produced with *Saccharomyces cerevisiae* (Sc1 or Sc2) only or in combination with *Hanseniaspora uvarum* (Hu), two lactic bacteria strains and three MLF strategies (none, simultaneous and sequential). Values are averages of triplicate fermentations.

Treatment	Residual sugar (g/L)	pH	Volatile acidity (g/L)	Total acidity (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Alcohol (% v/v)	Glycerol (g/L)	MLF duration (days)
Sc1	1.70 <sup>cdefg</sup> ±0.22	3.61 <sup>g</sup> ±0.00	0.24 <sup>k</sup> ±0.01	6.16 <sup>a</sup> ±0.07	2.81 <sup>a</sup> ±0.08	<0.2 <sup>j</sup> ±0.00	13.65 <sup>bcd</sup> ±0.02	10.62 <sup>fg</sup> ±0.03	No MLF
Sc1+LAB1 sim MLF <sup>2</sup>	1.60 <sup>fg</sup> ±0.22	3.75 <sup>cd</sup> ±0.01	0.38 <sup>hi</sup> ±0.01	5.36 <sup>defg</sup> ±0.03	<0.20 <sup>d</sup> ±0.00	1.33 <sup>fg</sup> ±0.01	13.80 <sup>abcde</sup> ±0.05	11.00 <sup>bcd</sup> ±0.06	10
Sc1+LAB1 seq MLF	1.69 <sup>defg</sup> ±0.16	3.80 <sup>ab</sup> ±0.02	0.45 <sup>def</sup> ±0.01	5.39 <sup>cde</sup> ±0.04	<0.20 <sup>d</sup> ±0.00	1.35 <sup>ef</sup> ±0.04	13.99 <sup>a</sup> ±0.09	11.13 <sup>b</sup> ±0.01	34
Sc1+LAB2 sim MLF	1.63 <sup>efg</sup> ±0.11	3.81 <sup>a</sup> ±0.01	0.46 <sup>de</sup> ±0.01	5.43 <sup>cd</sup> ±0.03	<0.20 <sup>d</sup> ±0.00	1.39 <sup>de</sup> ±0.03	13.92 <sup>ab</sup> ±0.02	11.13 <sup>b</sup> ±0.02	34
Sc1+LAB2 seq MLF	1.47 <sup>g</sup> ±0.18	3.77 <sup>bc</sup> ±0.01	0.43 <sup>efg</sup> ±0.01	5.50 <sup>c</sup> ±0.05	<0.20 <sup>d</sup> ±0.00	1.40 <sup>de</sup> ±0.02	13.87 <sup>abc</sup> ±0.11	11.09 <sup>bc</sup> ±0.09	34
Hu+Sc1	1.81 <sup>abc</sup> ±0.07	3.60 <sup>g</sup> ±0.00	0.29 <sup>j</sup> ±0.01	6.17 <sup>a</sup> ±0.02	1.82 <sup>c</sup> ±0.11	0.24 <sup>ij</sup> ±0.03	13.52 <sup>efg</sup> ±0.04	10.27 <sup>h</sup> ±0.06	No MLF
Hu+Sc1+LAB1 sim MLF	1.97 <sup>abc</sup> ±0.27	3.70 <sup>f</sup> ±0.01	0.41 <sup>gh</sup> ±0.01	5.35 <sup>defg</sup> ±0.03	<0.20 <sup>d</sup> ±0.00	1.50 <sup>a</sup> ±0.02	13.61 <sup>cdefg</sup> ±0.06	10.75 <sup>efg</sup> ±0.08	10
Hu+Sc1+LAB1 seq MLF	1.87 <sup>abcde</sup> ±0.19	3.75 <sup>cd</sup> ±0.02	0.44 <sup>ef</sup> ±0.02	5.27 <sup>fg</sup> ±0.02	<0.20 <sup>d</sup> ±0.00	1.40 <sup>cde</sup> ±0.04	13.66 <sup>bcd</sup> ±0.08	10.77 <sup>defg</sup> ±0.04	22
Hu+Sc1+LAB2 sim MLF	1.93 <sup>abcd</sup> ±0.14	3.73 <sup>de</sup> ±0.02	0.41 <sup>gh</sup> ±0.01	5.29 <sup>efg</sup> ±0.02	<0.20 <sup>d</sup> ±0.00	1.42 <sup>bcd</sup> ±0.03	13.61 <sup>cdefg</sup> ±0.01	10.82 <sup>defg</sup> ±0.02	19
Hu+ Sc1+LAB2 seq MLF	1.74 <sup>bcd</sup> ±0.32	3.76 <sup>cd</sup> ±0.03	0.43 <sup>fg</sup> ±0.02	5.25 <sup>g</sup> ±0.05	<0.20 <sup>d</sup> ±0.00	1.46 <sup>abc</sup> ±0.02	13.66 <sup>bcd</sup> ±0.10	10.86 <sup>cdef</sup> ±0.04	22
Sc2	1.78 <sup>abc</sup> ±0.23	3.58 <sup>g</sup> ±0.03	0.35 <sup>i</sup> ±0.01	6.04 <sup>b</sup> ±0.21	2.11 <sup>b</sup> ±0.43	<0.2 <sup>j</sup> ±0.00	13.65 <sup>bcd</sup> ±0.64	11.11 <sup>b</sup> ±0.46	No MLF
Sc2+LAB1 sim MLF	2.03 <sup>a</sup> ±0.20	3.68 <sup>f</sup> ±0.02	0.51 <sup>b</sup> ±0.03	5.36 <sup>defg</sup> ±0.04	<0.20 <sup>d</sup> ±0.00	1.24 <sup>h</sup> ±0.01	13.87 <sup>abc</sup> ±0.08	11.70 <sup>a</sup> ±0.09	10
Sc2+LAB1 seq MLF	1.98 <sup>ab</sup> ±0.10	3.74 <sup>cde</sup> ±0.01	0.51 <sup>b</sup> ±0.02	5.26 <sup>fg</sup> ±0.04	<0.20 <sup>d</sup> ±0.00	1.22 <sup>h</sup> ±0.08	13.99 <sup>a</sup> ±0.11	11.74 <sup>a</sup> ±0.05	22
Sc2+LAB2 sim MLF	1.99 <sup>ab</sup> ±0.24	3.68 <sup>f</sup> ±0.02	0.48 <sup>cd</sup> ±0.02	5.35 <sup>defg</sup> ±0.02	<0.20 <sup>d</sup> ±0.00	1.19 <sup>h</sup> ±0.02	13.95 <sup>a</sup> ±0.12	11.60 <sup>a</sup> ±0.17	20
Sc2+LAB2 seq MLF	1.95 <sup>abcd</sup> ±0.08	3.68 <sup>f</sup> ±0.02	0.50 <sup>bc</sup> ±0.03	5.34 <sup>defg</sup> ±0.02	<0.20 <sup>d</sup> ±0.00	1.2 <sup>h</sup> ±0.00	13.82 <sup>abcd</sup> ±0.23	11.73 <sup>a</sup> ±0.12	22
Hu+Sc2 no MLF	1.91 <sup>abcd</sup> ±0.25	3.58 <sup>g</sup> ±0.01	0.43 <sup>efg</sup> ±0.01	6.08 <sup>ab</sup> ±0.05	1.69 <sup>c</sup> ±0.00	0.26 <sup>i</sup> ±0.02	13.60 <sup>cdefg</sup> ±0.06	10.60 <sup>g</sup> ±0.05	No MLF
Hu+Sc2 +LAB1 sim MLF	1.94 <sup>abcd</sup> ±0.09	3.71 <sup>ef</sup> ±0.04	0.57 <sup>a</sup> ±0.03	5.37 <sup>def</sup> ±0.12	<0.20 <sup>d</sup> ±0.00	1.49 <sup>a</sup> ±0.07	13.35 <sup>g</sup> ±0.11	11.16 <sup>b</sup> ±0.07	10
Hu+Sc2 +LAB1 seq MLF	1.88 <sup>abcde</sup> ±0.07	3.71 <sup>ef</sup> ±0.03	0.58 <sup>a</sup> ±0.01	5.26 <sup>fg</sup> ±0.06	<0.20 <sup>d</sup> ±0.00	1.37 <sup>def</sup> ±0.04	13.47 <sup>fg</sup> ±0.05	10.97 <sup>bcd</sup> ±0.12	18
Hu+Sc2 +LAB2 sim MLF	1.82 <sup>abc</sup> ±0.16	3.76 <sup>ad</sup> ±0.03	0.57 <sup>a</sup> ±0.01	5.26 <sup>fg</sup> ±0.06	<0.20 <sup>d</sup> ±0.00	1.47 <sup>ab</sup> ±0.06	13.55 <sup>defg</sup> ±0.12	11.19 <sup>b</sup> ±0.02	15
Hu+Sc2 +LAB2 seq MLF	1.93 <sup>abcd</sup> ±0.20	3.71 <sup>ef</sup> ±0.02	0.53 <sup>b</sup> ±0.05	5.27 <sup>fg</sup> ±0.09	<0.20 <sup>d</sup> ±0.00	1.30 <sup>g</sup> ±0.03	13.56 <sup>defg</sup> ±0.04	10.99 <sup>bcd</sup> ±0.33	18

<sup>1</sup>Juice analysis: Balling = 23.0°B, pH = 3.57, total acidity = 7.43 g/L, malic acid = 3.1 g/L, free SO<sub>2</sub> = 4 mg/L and total SO<sub>2</sub> = 16 mg/L.

<sup>2</sup>LAB1: *Oenococcus oeni*, LAB2: *Lactobacillus plantarum*, simultaneous (sim) and sequential (seq) MLF.

### 5.3.2.2 Wines that underwent MLF

In most cases, wines that underwent MLF contained higher alcohol levels than wines that did not undergo MLF (Table 5.2). Ethanol is one of the byproducts of sugar metabolism by heterofermentative LAB, which explains why the wines that underwent MLF contained higher alcohol levels. These findings are contrary to other reports (Mendoza *et al.*, 2011; Abrahamse & Bartowsky, 2012b; Tristezza *et al.*, 2016b), but in agreement with results of Chapter 4 (Du Plessis *et al.*, 2017b). In general, the alcohol levels were lower for wines that underwent simultaneous MLF than for wines that underwent sequential MLF, which is in agreement with the findings of Mendoza *et al.* (2011), but contrary to the findings of Izquierdo-Cañas *et al.* (2015) and Tristezza *et al.* (2016b). Wines that underwent MLF had significantly higher glycerol levels than wines that did not undergo MLF. There was some variation with regard to the glycerol levels of wines that underwent simultaneous and sequential MLF. In most cases, wines that underwent simultaneous MLF contained slightly lower glycerol levels than those that underwent sequential MLF.

Overall, wines that underwent MLF contained significantly higher VA values (0.38 to 0.58 g/L) than wines that did not undergo MLF (0.24 to 0.43 g/L). Similar results have been reported by Mendoza *et al.* (2011) and Izquierdo-Cañas *et al.* (2016). Most of the wines that underwent simultaneous MLF had slightly lower VA levels than wines that underwent sequential MLF, which is similar to results reported by Tristezza *et al.* (2016a).

The conversion of malic acid to lactic acid resulted in a significant decrease in the total acidity levels of the wines that underwent MLF, with the expected increase in the pH of those wines. In most cases, wines that underwent simultaneous MLF had slightly higher total acidity levels than wines that underwent sequential MLF, which is similar to the findings of Mendoza *et al.* (2011).

### 5.3.3 Volatile compounds analysis

The ANOVA of the volatile compounds of wines produced with the selected yeasts, LAB strains and MLF strategies showed a significant interaction ( $p \leq 0.05$ ) and significant differences were observed for most compounds, except ethyl acetate (Table 5.3). Overall, yeast treatment had a significant effect on all volatile compounds. A similar observation was made for LAB strain and MLF strategy with the exception for the compound, ethyl butanoate.

TABLE 5.3. Probability (p) values obtained through the analysis of variance to show the effect that yeast strain, lactic acid bacteria (LAB) strain and malolactic fermentation (MLF) strategy (none, simultaneous and sequential) interactions, and yeast, LAB strain and MLF strategy interactions have on the volatile compounds of Shiraz wines.

Compound	Treatments		
	Yeast	LAB strain × MLF strategy	Yeast × LAB strain × MLF strategy
Ethyl acetate	0.0001	0.0068	0.1774
Ethyl lactate	0.0001	0.0001	0.0001
Ethyl butanoate	0.0001	0.6630	0.0003
Ethyl hexanoate	0.0001	0.0002	0.0001
Ethyl decanoate	0.0001	0.0001	0.0001
Ethyl octanoate	0.0001	0.0001	0.0001
Ethyl-3-hydroxybutanoate	0.0001	0.0001	0.0001
Isoamyl acetate	0.0001	0.0033	0.0001
Ethyl phenylacetate	0.0001	0.0001	0.0001
2-Phenylethyl acetate	0.0001	0.0001	0.0001
Diethyl succinate	0.0001	0.0001	0.0001
Acetoin	0.0001	0.0001	0.0001
Methanol	0.0001	0.0016	0.0001
Propanol	0.0001	0.0304	0.0001
Isobutanol	0.0001	0.0001	0.0001
Butanol	0.0001	0.1037	0.0001
Isoamyl alcohol	0.0001	0.0005	0.0001
Pentanol	0.0001	0.0013	0.0008
3-Methyl-1-pentanol	0.0001	0.0001	0.0001
Hexanol	0.0001	0.0001	0.0001
3-Ethoxy-1-propanol	0.0001	0.0001	0.0001
2-Phenyl ethanol	0.0001	0.0001	0.0001
Acetic acid	0.0001	0.0001	0.0001
Propionic acid	0.0001	0.0315	0.0001
Isobutyric acid	0.0001	0.0036	0.0001
Butyric acid	0.0001	0.0018	0.0001
Isovaleric acid	0.0001	0.0001	0.0001
Valeric acid	0.0001	0.0001	0.0001
Hexanoic acid	0.0001	0.0068	0.0001
Octanoic acid	0.0001	0.0001	0.0001
Decanoic acid	0.0001	0.0001	0.0001

<sup>1</sup>Probability (p) values ≤ 0.05 indicate significant differences between treatments.



TABLE 5.4. Concentration of volatile compounds (mg/L) of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc1 or Sc2) only, or in combination with *Hanseniaspora uvarum* (Hu), two lactic acid bacteria strains and three MLF strategies (none, simultaneous and sequential). Values are averages of three replicates.

Treatment <sup>1</sup>	Ethyl acetate	Ethyl butanoate	Isoamyl acetate	Ethyl hexanoate	Ethyl lactate	Ethyl octanoate	Ethyl-3-hydroxybutanoate	Ethyl decanoate	Diethyl succinate	2-Phenylethyl acetate
Sc1	41.760h <sup>2</sup>	0.455cd	1.449de	0.705bcd	12.085g	0.193de	1.464d	0.103a	3.263ab	1.129d
Sc1+LAB1 sim MLF	42.533gh	0.462bc	1.357ef	0.693def	63.545a	0.193de	1.508c	0.091f	3.233b	1.126de
Sc1+LAB1 seq MLF	47.859ef	0.447d	1.385ef	0.703cde	44.279f	0.212b	1.537ab	0.092ef	3.136c	1.176bc
Sc1+LAB2 sim MLF	47.249ef	0.483a	1.310f	0.721a	52.410cde	0.232a	1.533ab	0.099b	1.983f	1.214a
Sc1+LAB2 seq MLF	45.200fgh	0.459c	1.304f	0.703cde	49.183ef	0.209bc	1.530b	0.092def	3.334a	1.166c
Hu+Sc1	106.914a	0.467bc	1.703bc	0.690efg	15.704g	0.202bcd	ND	0.000g	2.274e	1.101def
Hu+Sc1+LAB1 sim MLF	107.237a	0.456cd	1.559cd	0.685fg	63.930a	0.209bc	ND	0.091f	2.350de	1.094ef
Hu+Sc1+LAB1 seq MLF	107.291a	0.458c	1.651bc	0.687fg	51.515de	0.208bc	ND	0.092ef	2.333de	1.100def
Hu+Sc1+LAB2 sim MLF	110.782a	0.464bc	1.658bc	0.686fg	55.265bcd	0.210bc	ND	0.093cdef	2.354de	1.095def
Hu+ Sc1+LAB2 seq MLF	107.148a	0.457cd	1.632bc	0.679g	46.051f	0.195cde	ND	0.094cde	2.329de	1.080f
Sc2	51.861d	0.430e	1.162ef	0.678ef	13.288h	0.171de	ND	0.0930c	2.328b	0.996fg
Sc2+LAB1 sim MLF	54.557d	0.453abc	1.129f	0.674fg	62.928b	0.169e	ND	0.092def	2.225cd	1.023efg
Sc2+LAB1 seq MLF	57.336d	0.441bcde	1.161f	0.678ef	50.370e	0.171de	ND	0.099b	2.312bc	1.033ef
Sc2+LAB2 sim MLF	54.102d	0.433de	1.108f	0.682ef	50.647e	0.182cd	ND	0.099b	2.493a	1.046de
Sc2+LAB2 seq MLF	57.009d	0.454ab	1.249e	0.689de	56.413d	0.187c	ND	0.101a	2.295bc	1.078b
Hu+Sc2	161.514a	0.440bcde	1.525bcd	0.660h	18.752g	0.168e	ND	ND	1.786f	0.980g
Hu+Sc2 +LAB1 sim MLF	165.411a	0.437cde	1.442d	0.662h	75.069a	0.166e	ND	ND	2.174d	1.054de
Hu+Sc2 +LAB1 seq MLF	166.347a	0.440bcde	1.448d	0.662h	59.345bcd	0.166e	ND	ND	2.016e	1.025ef
Hu+Sc2 +LAB2 sim MLF	167.476a	0.449abcd	1.450d	0.664hg	61.666bc	0.168e	ND	ND	1.839f	1.037def
Hu+Sc2 +LAB2 seq MLF	149.563b	0.452abc	1.528bcd	0.675fg	58.718bcd	0.186c	ND	ND	1.976e	1.055de

TABLE 5.4 (continued)

Treatment <sup>1</sup>	Methanol	Propanol	Isobutanol	Butanol	Isoamyl alcohol	3-Methyl-1-pentanol	Hexanol	3-Ethoxy-1-propanol	2-Phenylethanol
Sc1	93.995f	77.239b	49.449ef	1.960e	371.184ab	0.643a	2.541cd	4.305b	67.771def
Sc1+LAB1 sim MLF	105.161cde	75.252bc	48.662f	2.095b	357.399cde	0.643a	2.564bc	4.088bc	65.785fg
Sc1+LAB1 seq MLF	113.645ab	93.203a	52.197def	2.240a	377.309a	0.645a	2.567bc	4.951a	64.326gh
Sc1+LAB2 sim MLF	114.436ab	89.872a	53.955de	2.025cd	361.698bc	0.630b	2.633b	4.837a	62.443h
Sc1+LAB2 seq MLF	106.543cde	71.250c	56.593d	1.975de	378.915a	0.636ab	2.561bc	3.978c	68.164cdef
Hu+Sc1	103.116de	60.374de	68.742c	1.589ghi	340.810f	0.571de	2.543cd	3.250ef	66.185efg
Hu+Sc1+LAB1 sim MLF	101.715de	55.918ef	68.722c	1.648fg	362.400bc	0.571de	2.594bc	3.195ef	71.808b
Hu+Sc1+LAB1 seq MLF	100.545ef	54.082f	74.217b	1.564i	350.354def	0.570e	2.551bc	3.103fg	68.504cde
Hu+Sc1+LAB2 sim MLF	115.309a	61.823d	79.730a	1.687f	373.679a	0.573de	2.724a	3.576d	70.464bc
Hu+ Sc1+LAB2 seq MLF	108.099bcd	58.666def	69.902bc	1.691f	346.846f	0.570e	2.591bc	3.355de	69.893bcd
Sc2	130.243a	64.957b	47.083c	1.909a	226.117f	0.554def	2.619fg	3.003e	39.164f
Sc2+LAB1 sim MLF	124.205ab	75.640a	43.194d	1.816ab	230.999ef	0.554ef	2.769bcde	3.281d	38.941f
Sc2+LAB1 seq MLF	126.748ab	73.401a	43.968cd	1.867a	241.438ef	0.559cde	2.798bcd	3.347cd	39.935f
Sc2+LAB2 sim MLF	119.149bc	76.772a	42.029d	1.761b	240.076ef	0.571a	2.771bcde	3.491bc	45.512e
Sc2+LAB2 seq MLF	123.203ab	71.535a	45.205cd	1.850ab	244.585e	0.568ab	2.844b	3.400cd	41.520f
Hu+Sc2	110.077c	55.068cd	72.758b	1.478f	282.479d	0.538g	2.693def	3.460bcd	47.826e
Hu+Sc2 +LAB1 sim MLF	112.284c	54.496cde	71.894b	1.509ef	291.544cd	0.553ef	3.150a	3.542ab	54.237d
Hu+Sc2 +LAB1 seq MLF	117.014bc	56.486cd	74.759b	1.568cdef	297.784bcd	0.558cde	2.835bc	3.458bcd	58.493bc
Hu+Sc2 +LAB2 sim MLF	118.232bc	58.332c	73.753b	1.617cd	292.252cd	0.552f	3.022a	3.837a	55.797cd
Hu+Sc2 +LAB2 seq MLF	126.059ab	58.425c	74.977b	1.520def	348.898a	0.563bc	2.827bc	3.505bc	73.174a

TABLE 5.4 (continued)

Treatment <sup>1</sup>	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Hexanoic acid	Octanoic acid	Decanoic acid
Sc1	116.268h	2.594e	2.630de	1.009g	1.990f	0.516de	0.532e	1.241f	1.001d
Sc1+LAB1 sim MLF	257.076f	2.653de	2.707cd	1.008g	2.052f	0.523cd	0.510f	1.241f	1.008d
Sc1+LAB1 seq MLF	309.832ab	2.718cde	2.773c	1.057ef	7.711a	0.535b	0.514f	1.336bcd	1.046bc
Sc1+LAB2 sim MLF	307.411abc	2.932ab	3.173b	1.056ef	3.256e	7.577a	0.509f	1.419a	1.063ab
Sc1+LAB2 seq MLF	294.512bcd	2.652de	3.315a	1.042fg	7.226a	0.526bc	0.508f	1.319bcde	1.049bc
Hu+Sc1	139.936g	2.807bcd	2.524efg	1.093cd	2.073f	0.473hi	0.641a	1.295cdef	1.000d
Hu+Sc1+LAB1 sim MLF	254.802f	3.002a	2.417g	1.046ef	1.942f	0.472hi	0.621bc	1.323bcd	1.003d
Hu+Sc1+LAB1 seq MLF	291.950bcde	2.857abc	2.557ef	1.075def	5.494bc	0.471i	0.629ab	1.325bcd	1.036c
Hu+Sc1+LAB2 sim MLF	273.531ef	2.303f	2.712cd	1.097cd	4.018d	0.481gh	0.623bc	1.308bcde	1.047bc
Hu+ Sc1+LAB2 seq MLF	284.994de	2.901ab	2.530efg	1.079de	5.631b	0.479fgi	0.633ab	1.258ef	1.042c
Sc2	239.01f	3.494bc	2.191e	0.987g	1.914hi	0.481ef	0.505gh	1.096g	0.972g
Sc2+LAB1 sim MLF	399.50bcd	4.386a	2.147e	0.979g	1.902i	0.497ef	0.498h	1.113fg	0.970g
Sc2+LAB1 seq MLF	381.73d	4.188a	2.252e	0.995fg	4.073c	0.497ef	0.504gh	1.117fg	1.032c
Sc2+LAB2 sim MLF	387.86cd	3.724b	2.216e	1.033ef	4.065c	0.495ef	0.508gh	1.167de	1.059b
Sc2+LAB2 seq MLF	401.21bcd	3.527bc	2.311de	1.045de	6.003a	0.508e	0.518g	1.184cd	1.069b
Hu+Sc2	293.97e	2.891ef	2.600bc	0.973g	2.012ghi	0.851d	0.604bc	1.138efg	0.991ef
Hu+Sc2 +LAB1 sim MLF	450.26a	2.6093f	2.688bc	1.044de	2.037ghi	0.481ef	0.564f	1.111g	0.976fg
Hu+Sc2 +LAB1 seq MLF	454.02a	2.911ef	2.517cd	0.981g	2.020fgh	2.488b	0.581def	1.156def	0.986fg
Hu+Sc2 +LAB2 sim MLF	405.29bcd	2.943ef	2.560c	0.952g	2.134fg	2.147c	0.590cde	1.114fg	1.021cd
Hu+Sc2 +LAB2 seq MLF	419.14b	3.005de	2.483cd	0.994fg	2.218f	3.403a	0.609abc	1.216c	1.006de

<sup>1</sup>LAB1: *Oenococcus oeni*, LAB2: *Lactobacillus plantarum*, simultaneous (sim) and sequential (seq) MLF.<sup>2</sup>Values in a column followed by the same letter did not differ significantly ( $p \leq 0.05$ ).

### 5.3.4 Multivariate data analysis of wines

Principal component analysis (PCA), an unsupervised pattern recognition method, was used to investigate the correlation between yeast combinations, LAB strain, MLF strategies and volatile composition of Shiraz wines (Fig. 5.4). The biplot explain 65% of the variance in the data of the two principal components (PC1 = 38.78% and PC2 = 26.27%). Four distinct clusters (indicated by different colours) can be observed, *i.e.* Hu+Sc1 with or without MLF (top right quadrant), Hu+Sc2 with or without MLF (top left quadrant), Sc2 with or without MLF (bottom left quadrant) and Sc1 with or without MLF (bottom right quadrant). These results clearly show that the yeast combinations used had a significant effect on chemical composition of the wines (Fig. 5.4 and Table 5.3). The distribution of the data points within the aforementioned four clusters shows that there was some within-group variation, but there was no consistent pattern. These results indicate that yeast combination had the biggest impact on chemical composition, but LAB strain and MLF strategy also had played a role.

Based on contribution and squared cosines of the variables, the main compounds responsible for differentiating between wines produced with the selected yeast combinations, LAB strain and MLF strategies were, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, 2-phenyl acetate, isobutanol, 3-methyl-1-pentanol, hexanoic acid and octanoic acid (Fig. 5.4).

Wines produced with Sc1 with or without MLF show a positive correlation with 2-phenylethyl acetate, 3-methyl-pentanol, ethyl hexanoate, decanoic acid, ethyl-3-hydroxybutanoate, 3-ethoxy-1-propanol, isovaleric acid, diethyl succinate, ethyl decanoate, butanol and propanol. The aforementioned wines were negatively correlated with ethyl acetate.

The Sc2 wines with and without MLF show a positive correlation with methanol, propionic acid, pentanol and ethyl phenylacetate, and a negative correlation with isoamyl acetate, 2-phenyl ethanol, isoamyl alcohol, octanoic acid, isobutyric acid and ethyl butanoate.

Wines produced with Hu+Sc1 with or without MLF show a positive correlation with isoamyl acetate, 2-phenyl ethanol, isoamyl alcohol and butyric acid, and a negative correlation with propionic acid, methanol and acetic acid. Octanoic acid, ethyl butanoate, isobutyric acid, valeric acid and ethyl octanoate show a positive correlation with wines produced Sc1 only and wines produced with *H. uvarum* in combination with Sc1.

Wines produced with Hu+Sc2 with or without MLF show a positive correlation with ethyl acetate and a negatively correlated with ethyl decanoate, butanol, propanol, diethyl succinate, isovaleric acid, decanoic acid, ethyl-3-hydroxybutanoate. Isobutanol and hexanoic acid show a positive correlation with wines produced with *H. uvarum* in combination with Sc1 and Sc2. This indicates that these compounds are linked to the growth and metabolism of the *H. uvarum* strain.

Aforementioned results show that yeast combination has a significant impact on volatile composition of the wines and should produce wines that differ with regard to flavour profiles.

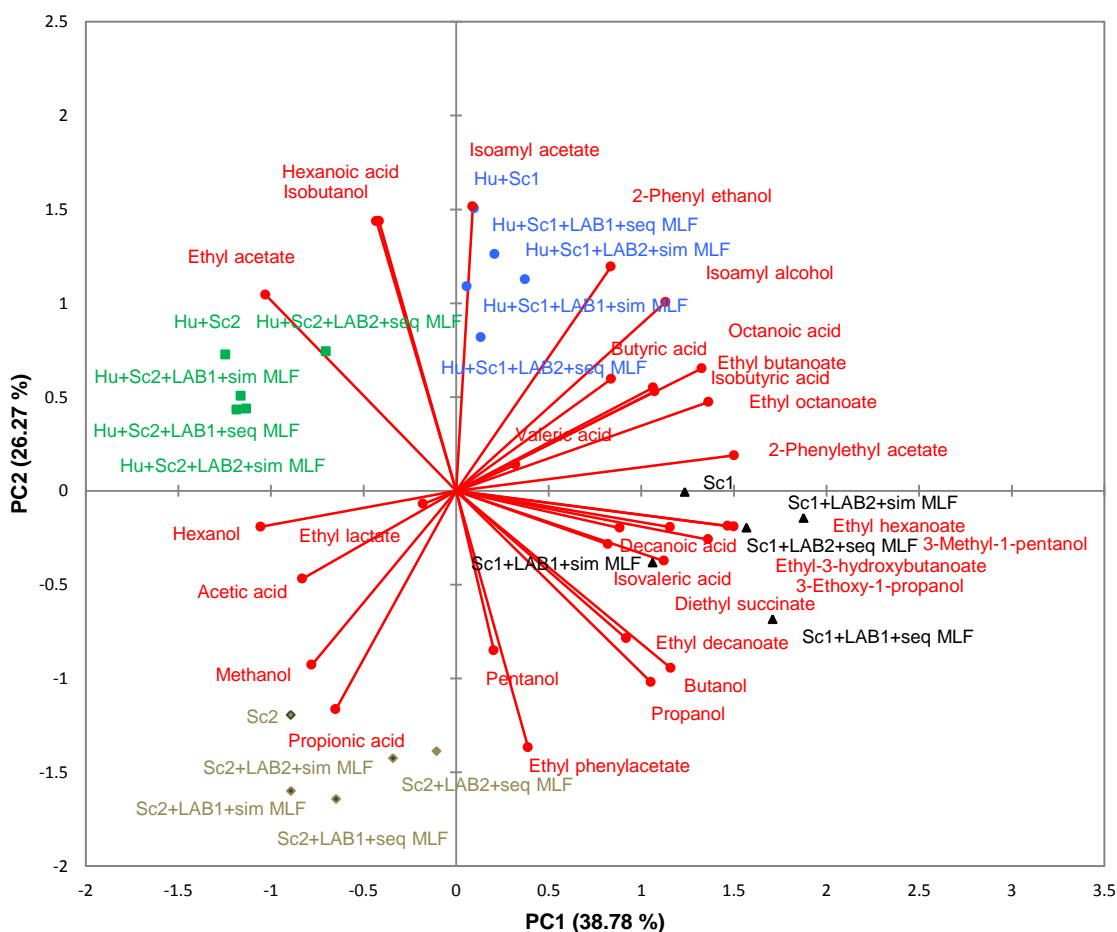


FIGURE 5.4. Principal component biplot of volatile compounds of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc1 and Sc2) in combination with *Hanseniaspora uvarum*, two lactic bacteria strains (LAB1 and LAB2) and three malolactic fermentation (MLF) strategies. Abbreviations: LAB1 = *Oenococcus oeni*, LAB2 = *Lactobacillus plantarum*, sim = simultaneous MLF and seq = sequential MLF.

### 5.3.5 Sensory evaluation

ANOVA of the sensory data showed that there was significant interaction ( $p \leq 0.05$ ) for fresh vegetative, cooked vegetative, spicy and floral aroma among the Shiraz wines produced with the selected yeast combinations, LAB strains and even MLF strategies (indicated in bold, Table 5.7). Wines produced with the selected yeast combinations varied significantly with regard to fresh vegetative and spicy aroma, as well as body and astringency of the wines. Wines produced with the selected LAB strains and MLF strategies were significantly different with regard to berry, fruity, sweet associated and spicy aroma, as well as acid balance and body. These results show how much yeast selection, LAB combination and MLF strategy can impact the volatile composition and sensory profiles of wines.

TABLE 5.7. Probability (p) values obtained through the analysis of variance to show the effect that yeast strain, lactic acid bacteria (LAB) strain and malolactic fermentation (MLF) strategy (none, simultaneous and sequential) interactions, and yeast strain, LAB strain and MLF strategy interactions have on the sensory descriptors of Shiraz wines.

Descriptor	Treatment		
	Yeast	LAB strain x MLF strategy	Yeast x LAB strain x MLF strategy
Berry	0.3042	<b>0.0004</b>	0.8400
Fruity	0.7647	<b>0.0191</b>	0.9095
Sweet associated	0.4417	<b>0.0023</b>	0.5761
Fresh vegetative	<b>0.0001</b>	0.1245	<b>0.0418</b>
Cooked vegetative	0.5094	0.2079	<b>0.0420</b>
Spicy	<b>0.0165</b>	<b>0.0009</b>	<b>0.0548</b>
Floral	0.0602	0.5104	<b>0.0159</b>
Acid balance	0.0905	<b>0.0001</b>	0.3488
Body	<b>0.0001</b>	<b>0.0020</b>	0.1454
Astringency	<b>0.0010</b>	0.0876	0.1182
Bitterness	0.7069	0.2683	0.0800

<sup>1</sup>Probability (p) values  $\leq 0.05$  indicate significant differences between treatments.

#### 5.3.5.1 Fresh vegetative aroma

For wines that did not undergo MLF, Hu had a clear impact on vegetative aroma (Fig. 5.4 and Table 5.8). Whereas for wines that underwent MLF, the *S. cerevisiae* strain had the largest impact on vegetative aroma. Wines that underwent sequential MLF scored higher for fresh vegetative aroma than wines that underwent simultaneous MLF and wines that did not undergo MLF. Of all the different treatments, Hu+Sc1+LAB2 seq MLF wines scored the highest (35.27) for fresh vegetative aroma (Table 5.8). The Hu+Sc1 combination consistently produced wines with high fresh vegetative aroma scores and this was observed for wine without and with MLF. The opposite trend was observed for wines produced with Sc2. These results indicate that this Hu+Sc1 combination can be used to enhance the fresh vegetative character in wines where this attribute is lacking or to produce a wine style with a predominant fresh vegetative flavour profile. On the other hand, if a wine with low fresh vegetative character is preferred, the use of yeast strain, Sc2 is recommended.

Differences in fresh vegetative aroma scores were observed for wines produced with the two LAB strains, and were affected by yeast combination and also whether simultaneous or sequential MLF occurred. In most cases, wines inoculated with LAB1 scored higher for vegetative aroma than wines inoculated with LAB2.

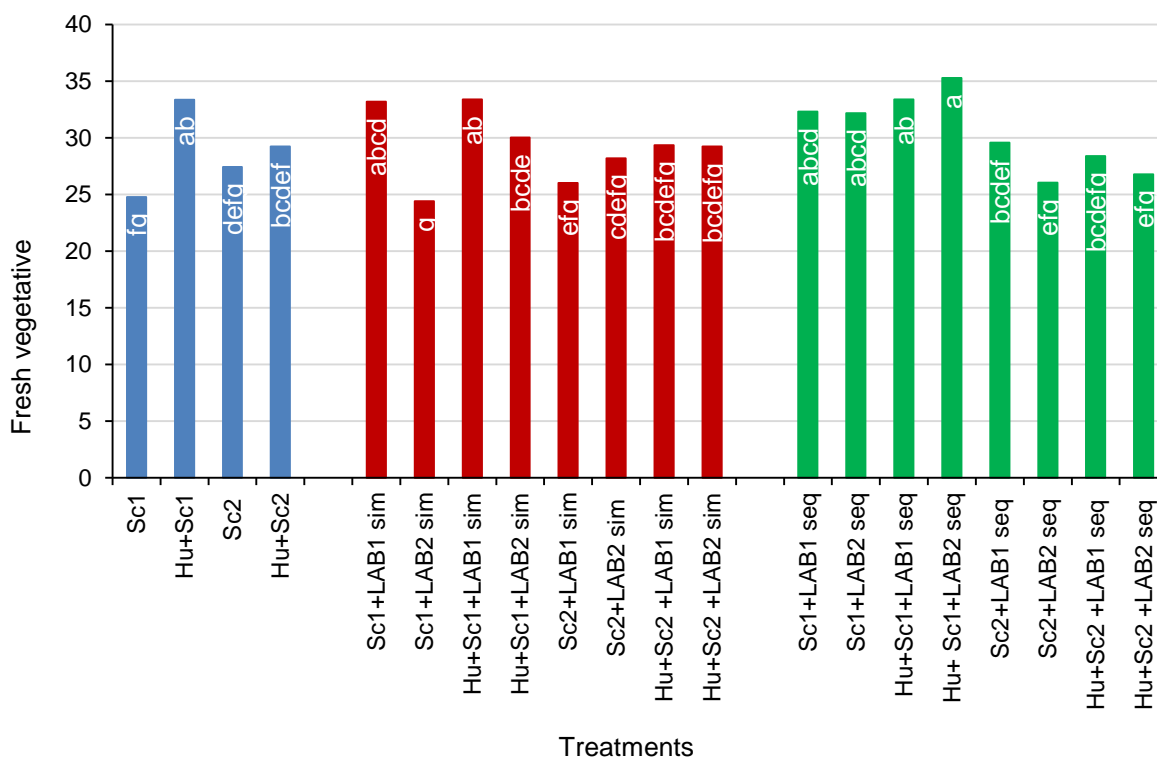


FIGURE 5.5. Percentage fresh vegetative aroma of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc1 and Sc2) in combination with *Hanseniaspora uvarum*, two lactic bacteria strains (LAB1 and LAB2) and three malolactic fermentation (MLF) strategies. Abbreviations: LAB1 = *Oenococcus oeni*, LAB2 = *Lactobacillus plantarum*, sim = simultaneous MLF and seq = sequential MLF.

### 5.3.5.2 Spicy aroma

Wines produced with Sc2 scored the highest for spicy aroma (32.71) amongst the wines that did not undergo MLF (Fig. 5.6 and Table 5.8). Overall, wines that underwent sequential MLF scored higher for spicy aroma than wines that underwent simultaneous MLF and wines that did not undergo MLF (Fig. 5.6). Of all the various treatments, wines produced with Hu+Sc1+LAB2 that underwent sequential MLF, scored the highest for spicy aroma. Differences in spicy aroma scores were observed for wines produced with the two LAB strains, and were affected by yeast combination as well as simultaneous or sequential MLF. Similar to the trend observed for fresh vegetative aroma, wines that underwent sequential MLF, scored higher for spicy aroma than wines that underwent simultaneous MLF.

TABLE 5.8. Sensory descriptors of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc1 or Sc2) only, or in combination with *Hanseniaspora uvarum* (Hu), two lactic acid bacteria (LAB) strains and three MLF strategies (none, simultaneous and sequential).

Treatment <sup>1</sup>	Berry	Fruity	Sweet associated	Fresh vegetative	Cooked vegetative	Spicy	Floral	Acidity	Body	Astringency	Bitterness
Sc1	52.61d <sup>2</sup>	34.33abc	29.29abcde	24.77fg	18.23abcd	29.05bcdefg	14.12ab	53.46a	47.50f	32.60efg	18.08cd
Sc1+LAB1 sim MLF	54.04bcd	33.42bc	26.16cde	33.19abc	17.21abcd	29.26bcdefg	12.30abcd	49.81c	51.52cde	35.39abcde	18.23cd
Sc1+LAB1 seq MLF	58.06abcd	32.21c	27.39abcde	32.30abcd	17.73abcd	33.05ab	11.91abcd	49.41c	51.90bcde	35.77abcde	20.22abcd
Sc1+LAB2 sim MLF	57.99abcd	37.79abc	27.67abcde	24.39g	20.84a	29.47bcdefg	13.55abc	48.15c	55.38ab	36.29abcde	18.52cd
Sc1+LAB2 seq MLF	60.35ab	35.86abc	29.68abcde	32.17abcd	14.65de	32.82ab	11.62abcd	48.03c	53.10abcde	30.72g	22.40ab
Hu+Sc1	56.76abcd	38.42abc	30.42abcd	33.35ab	17.30abcd	28.61cdefg	13.69abc	54.86a	51.88bcde	37.70ab	20.53abcd
Hu+Sc1+LAB1 sim MLF	54.23bcd	31.71c	23.91de	33.38ab	18.94abcd	31.56abcde	15.04a	49.41c	53.05abcde	38.65a	21.18abc
Hu+Sc1+LAB1 seq MLF	58.30abcd	35.49abc	26.79bcde	33.37ab	18.61abcd	31.06abcde	10.84bcd	50.27bc	55.89a	36.97abcd	18.31cd
Hu+Sc1+LAB2 sim MLF	60.43ab	37.64abc	35.00a	30.01bcde	11.50e	25.86g	10.27cd	49.21c	54.64abc	38.24ab	18.68bcd
Hu+ Sc1+LAB2 seq MLF	61.46a	37.70abc	30.31abcd	35.27a	16.59abcd	34.12a	13.30abc	48.17c	56.02a	36.55abcde	18.45cd
Sc2	58.36abcd	33.69abc	25.91cde	27.42defg	17.97abcd	32.71ab	10.39cd	49.27c	50.27ef	35.87abcde	16.89d
Sc2+LAB1 sim MLF	52.55d	33.09bc	22.41e	26.01efg	17.16abcd	32.49abc	9.04d	48.21c	47.41f	33.18defg	20.46abcd
Sc2+LAB1 seq MLF	57.29abcd	35.32abc	25.97cde	29.57bcdef	18.37abcd	31.89abcd	11.21bcd	49.01c	50.53edf	35.06abcdef	20.47abcd
Sc2+LAB2 sim MLF	61.29a	38.41abc	34.63a	28.18cdefg	15.22bcde	25.47g	15.00a	48.57c	51.67bcde	34.36bcdefg	18.59cd
Sc2+LAB2 seq MLF	61.83a	41.12a	34.39ab	26.03efg	14.98cde	30.38abcdef	14.10ab	48.55c	51.50cde	32.84efg	23.53a
Hu+Sc2	57.03abcd	36.63abc	32.71abc	29.24bcdefg	19.46abc	28.41cdefg	11.38bcd	53.02ab	54.36abcd	37.55abc	19.68bcd
Hu+Sc2 +LAB1 sim MLF	53.28cd	31.67c	28.32abcde	29.33bcdefg	19.39abc	28.14defg	11.51abcd	48.79c	53.99abcde	33.53cdefg	19.53bcd
Hu+Sc2 +LAB1 seq MLF	52.46d	35.83abc	28.44abcde	28.38bcdefg	15.49bcde	26.67fg	10.51cd	48.22c	51.74bcde	31.11fg	18.14cd
Hu+Sc2 +LAB2 sim MLF	58.05abcd	35.21abc	31.75abc	29.23bcdefg	16.32abcd	27.58efg	9.00d	48.36c	56.70a	36.60abcde	19.53bcd
Hu+Sc2 +LAB2 seq MLF	59.49abc	40.46ab	32.39abc	26.79efg	19.74ab	29.44bcdefg	11.33bcd	48.65c	54.58abc	34.56bcdefg	18.11cd

<sup>1</sup>*Saccharomyces cerevisiae* (Sc1 or Sc2), *Hanseniaspora uvarum* (Hu) and simultaneous (sim) MLF or sequential (seq) MLF induced with a two different LAB strains (LAB1 or LAB2).<sup>2</sup>Values in the same column followed by the same letter did not differ significantly ( $p \leq 0.05$ ).



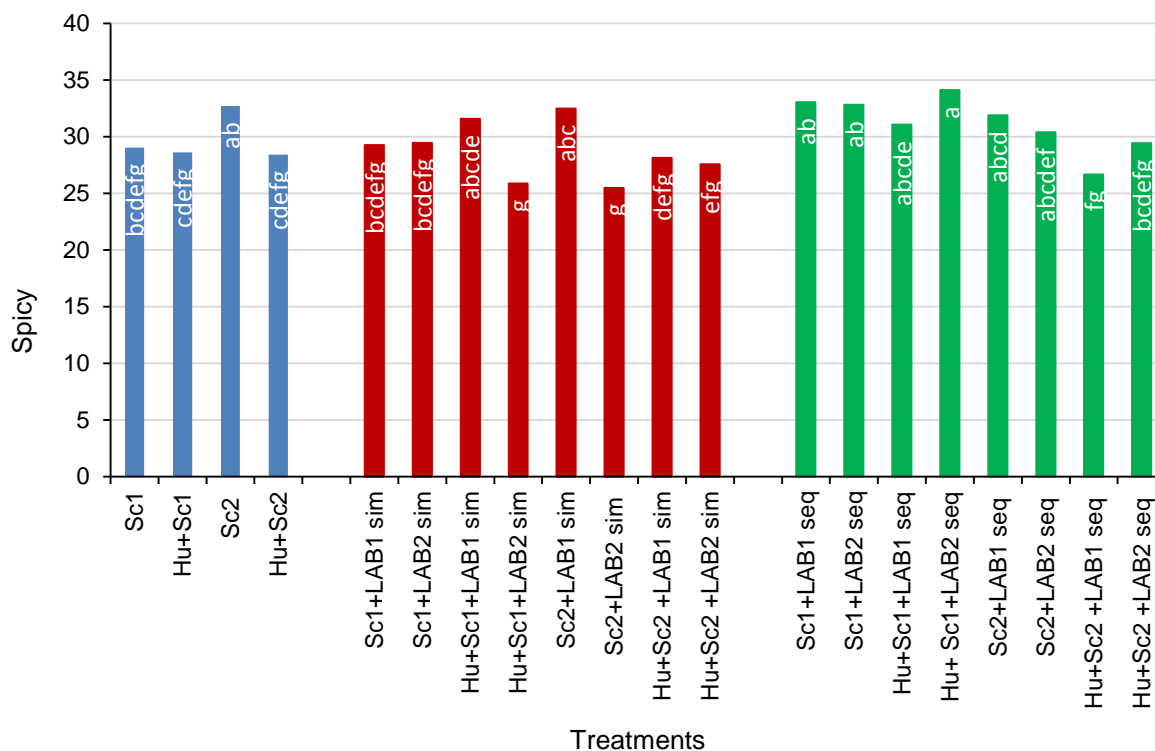


FIGURE 5.6. Percentage spicy aroma of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc1 and Sc2) in combination with *Hanseniaspora uvarum*, two lactic bacteria strains (LAB1 and LAB2) and three malolactic fermentation (MLF) strategies. Abbreviations: LAB1 = *Oenococcus oeni*, LAB2 = *Lactobacillus plantarum*, sim = simultaneous MLF and seq = sequential MLF.

### 5.3.5.3 Body

The wines produced with Sc1 and Sc2 that did not undergo MLF scored lower for the taste descriptor, body (mouthfeel) than wines produced with *H. uvarum* in combination with Sc1 or Sc2 that did not undergo MLF (Fig. 5.7). Wines that underwent MLF scored higher for body than wines that did not undergo MLF. Wines produced with Sc1 that underwent MLF scored higher for body than wines produced with Sc2 that underwent MLF. Wines produced with LAB2 scored higher for body than wines produced with LAB1. It is noteworthy that the relative scores for body varied with the respect to the yeast combination used. Winemakers can manipulate the body (mouthfeel) of wines by applying the aforementioned combinations to achieve the wine style they prefer.

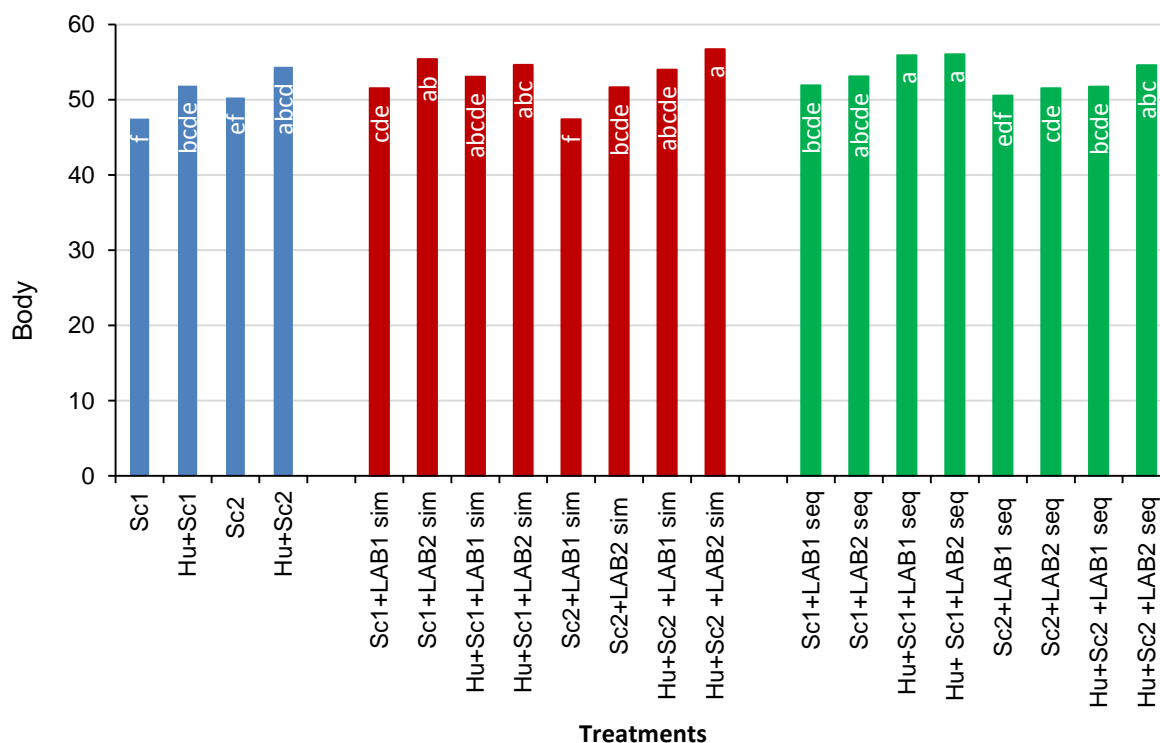


FIGURE 5.7. Percentage body of Shiraz wines produced with *Saccharomyces cerevisiae* (Sc1 and Sc2) in combination with *Hanseniaspora uvarum*, two lactic bacteria strains (LAB1 and LAB2) and three malolactic fermentation (MLF) strategies. Abbreviations: LAB1 = *Oenococcus oeni*, LAB2 = *Lactobacillus plantarum*, sim = simultaneous MLF and seq = sequential MLF.

### 5.3.6 Overall effects

Chemical and sensory results support our opinion that the selected *H. uvarum* strain contributed positively to wine flavour, due to its presence at high numbers for a sufficient period during fermentation. None of the treatment combinations produced off-flavours. Wines produced with *H. uvarum* in combination with Sc1 and Sc2 were different to wines produced with the Sc1 or Sc2 on their own. These results show how *H. uvarum* can be used to reduce the duration of MLF and to change the style or flavour profile of a wine. Wines where yeast and LAB were applied as a simultaneous inoculation reduced the duration of MLF and also produced wines that were different with regard to their flavour profiles than wines that were sequentially inoculated. Notable differences were also observed between wines inoculated with LAB1 and LAB2 with regard to duration of MLF and their flavour profiles. The yeast treatments, LAB strains and MLF strategies had a significant effect on the standard chemical parameters and volatile composition of the wines, and the differences in chemical composition translated to perceivable sensory differences.

## 5.4 CONCLUSIONS

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One of the *S. cerevisiae* yeast strains inhibited LAB growth, which resulted in delayed MLF. The *H. uvarum* strain combination with the selected *S. cerevisiae* yeasts produced wines with a stimulating effect on simultaneous and sequential MLF, which completed in a shorter period. Wine that underwent simultaneous MLF proceeded faster than wines that underwent sequential MLF. The use of *H. uvarum* appears to be beneficial to the growth of the inoculated and naturally occurring LAB, and may be beneficial to winemakers who want to induce spontaneous MLF. Unsuccessful induction can be circumvented through the use of a *S. cerevisiae* strain that is MLF compatible, unlike Sc1. Wines produced with the selected yeast, LAB treatments and MLF strategies differed with regard to fermentation kinetics, chemical and sensory properties. Yeast treatment had a bigger effect on the volatile composition of the wines, but LAB strain and MLF strategy also had a significant impact on volatile composition. The interaction between yeast, LAB strain and MLF strategy had a significant impact on the flavour profile of the wines. The sensory differences between wines that did not undergo MLF, wines that underwent simultaneous or sequential MLF were as significant as wines produced with different yeast strains. Results also showed that LAB strains have a perceivable impact on the sensory properties. The interactions between non-*Saccharomyces*, *Saccharomyces* yeasts and LAB are complex, and each strain has different attributes that can impact on flavour and quality of wines. This study also showed that non-*Saccharomyces* yeast, different LAB treatments as well as timing of MLF induction can be used to modify the flavour profile of Shiraz wines.

## 5.5 ACKNOWLEDGEMENTS

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# Chapter 6

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## Research results IV

**The use of mid-infrared spectroscopy to discriminate among wines produced with selected yeasts, lactic acid bacteria and malolactic fermentation strategies**

## Chapter 6

The use of mid-infrared spectroscopy to discriminate among wines produced with various yeasts, lactic acid bacteria and malolactic fermentation strategies

### ABSTRACT

The interactions between yeast and bacteria are complex and have a significant impact on the chemical and sensory properties of wine. The aim of this study was to evaluate the usefulness of mid-infrared (MIR) spectroscopy combined with pattern recognition methods as a tool to differentiate among Shiraz wines ( $n = 60$ ) produced with *Hanseniaspora uvarum* in combination with two *Saccharomyces cerevisiae* yeast strains, two lactic acid bacteria treatments (*Oenococcus oeni* and *Lactobacillus plantarum*) and three malolactic fermentation (MLF) strategies. Principal component analysis (PCA) and orthogonal projections to latent structures for discriminant analysis (OPLS-DA) were applied to wine spectra. Results demonstrated that Shiraz wines produced with selected yeast, lactic acid bacteria and MLF strategies had different spectral profiles, and that the MIR spectrum contained valuable information to discriminate among these wines. Results further showed that MIR spectroscopy combined with pattern recognition methods has the necessary information for successful classification of wine samples produced with different wine yeast strains and MLF strategies.

### 6.1 INTRODUCTION

Infrared spectroscopy (IR) can be used to provide information of wine biochemical components, and is a non-destructive, fast and easy to perform analytical technique (Cozzolino *et al.*, 2006; Ricci *et al.*, 2013). Mid-infrared (MIR) spectroscopy is a valuable analytical tool that measures changes in the absorption of energy by different functional groups, for example those comprising carbon-oxygen (C–O), oxygen-hydrogen (O–H) and carbon-carbon (C–C) bonds, due to characteristic vibrational frequencies associated with stretching and/or bending of bonds (Williams & Fleming, 1995). Unique spectral fingerprints can therefore be obtained for different samples, with more absorption bands typically being observed for more complex molecular structures (Bevin *et al.*, 2006; Cozzolino *et al.*, 2011). Near infrared (NIR) and MIR spectroscopy provide information with regard to the near infrared (14,000 to 4000  $\text{cm}^{-1}$ ) and mid-infrared (4000 to 400  $\text{cm}^{-1}$ ) regions, respectively (Smith, 2011). The mid-infrared region contains more spectral peaks, which are better defined and easier to interpret (Smith, 2011; Cozzolino *et al.*, 2012). Information provided by MIR using these fundamental absorption bands can also offer

information regarding the chemical structure of food samples (Bevin *et al.*, 2006; Karoui *et al.*, 2010). The advantages of MIR spectroscopy include the speed of analysis and potential selectivity when coupled with chemometric data analysis techniques (Cozzolino *et al.*, 2012). Commercially useful applications in the food industry have been demonstrated (Bevin *et al.*, 2006; Karoui *et al.*, 2010).

Wine is a complex matrix, which results from the interactions of different microorganisms. The two main groups of microorganisms involved are yeasts and bacteria (Fleet & Heard, 1993; Ribéreau-Gayon *et al.*, 2006). Yeasts are mainly responsible for the alcoholic fermentation, especially *Saccharomyces cerevisiae*, and contribute to the production of major aroma compounds such as esters, higher alcohols, aldehydes and fatty acids (Ribéreau-Gayon *et al.*, 2006; du Toit *et al.*, 2011). Non-*Saccharomyces* yeasts can affect fermentation directly or indirectly, by producing volatile compounds and non-volatile compounds, and indirectly by modifying the growth and metabolism of *Saccharomyces cerevisiae* (Albertin *et al.*, 2017). Malolactic fermentation (MLF) is a secondary process conducted by lactic acid bacteria (LAB) and is important in winemaking as it deacidifies the wine, contributes to microbial stability and enhances wine flavour through the production of metabolites (Lerm *et al.*, 2010; du Toit *et al.*, 2011).

The volatile and non-volatile compounds that occur in wine can range from the low ng/L to the high g/L and make wine chemistry analyses very challenging (Culbert *et al.*, 2015). However, MIR spectroscopy has been used to analyse and differentiate between both juice and wine (Bevin *et al.*, 2008; Cozzolino *et al.*, 2009, 2012; Fudge *et al.*, 2012; Culbert *et al.*, 2015; Aleixandre-Tudo *et al.*, 2018). In Chapter 5, Shiraz wines were produced with two *Saccharomyces cerevisiae* yeast strains in combination *Hanseniaspora uvarum*, two lactic acid bacteria species and three malolactic fermentation strategies. Wines produced with the various yeast treatments could be distinguished from each other using the volatile compound data obtained with a gas chromatograph coupled to a flame ionization detector (GC-FID). The aim of this study was to explore the use of MIR spectroscopy, in combination with pattern recognition methods, as a rapid and inexpensive tool to distinguish between wines produced with the selected *Saccharomyces*, non-*Saccharomyces* yeast, LAB strains and MLF strategies used in Chapter 5.

## 6.2 MATERIALS AND METHODS

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### 6.2.1 Microorganisms and treatments

The same yeast, LAB strains and MLF strategies were used as mentioned in Chapter 5 and are listed in Table 6.1. Two commercial *S. cerevisiae* yeast strains (VIN 13 and NT 202 coded as Sc1 and Sc2, respectively) from Anchor Yeast (South Africa) were used on their own or in combination with a *H. uvarum* (Hu) isolate from the yeast gene bank of ARC Infruitec-Nietvoorbij



(The Fruit, Vine and Wine Institute of the Agricultural Research Council). These yeast combinations were evaluated in combination with two commercial LAB strains (*O. oeni*, Viniflora® oenos, Chr. Hansen A/S, Denmark and *Lb. plantarum*, Enoferm V22, Lallemand Inc., France, coded as LAB1 and LAB2, respectively) and three MLF strategies. The MLF strategies were: (1) the yeast strains (*S. cerevisiae* only or in combination with *H. uvarum*) without MLF (no MLF), (2) yeast strains in combination with LAB1 or LAB2 as a simultaneous inoculation (simultaneous MLF) and (3) yeast strains in combination with LAB1 or LAB2 as a sequential inoculation (sequential MLF). All commercial cultures were inoculated according to the manufacturer's recommendations. Details about the culturing of the *H. uvarum* yeast isolate are discussed in Chapter 4 (Du Plessis *et al.*, 2017).

TABLE 6.1. Yeast, lactic bacteria strains and treatments applied during the Shiraz wine production trials.

Treatment	Description
Sc1	<i>Saccharomyces cerevisiae</i> 1 (Sc1) without malolactic fermentation (MLF)
Sc1+LAB1 sim MLF	Sc1 with <i>Oenococcus oeni</i> inoculated after 24 hours
Sc1+LAB1 seq MLF	Sc1 with <i>O. oeni</i> inoculated after alcoholic fermentation
Sc1+LAB2 sim MLF	Sc1 plan with <i>Lactobacillus plantarum</i> inoculated after 24 hours
Sc1+LAB2 seq MLF	Sc1 with <i>Lb. plantarum</i> inoculated after alcoholic fermentation
Hu+Sc1	<i>Hanseniaspora uvarum</i> + Sc1 inoculated after 24 hours, no MLF
Hu+Sc1+LAB1 sim MLF	<i>H. uvarum</i> + Sc1 and <i>O. oeni</i> inoculated after 24 hours
Hu+Sc1+LAB1 seq MLF	<i>H. uvarum</i> + Sc1 inoculated after 24 hours, <i>O. oeni</i> after alcoholic fermentation
Hu+Sc1+LAB2 sim MLF	<i>H. uvarum</i> + Sc1 and <i>Lb. plantarum</i> inoculated after 24 hours
Hu+Sc1+LAB2 seq MLF	<i>H. uvarum</i> + Sc1 inoculated after 24 hours, <i>Lb. plantarum</i> after alcoholic fermentation
Sc2	Sc2 without MLF
Sc2+LAB1 sim MLF	Sc2 with <i>O. oeni</i> inoculated after 24 hours
Sc2+LAB1 seq MLF	Sc2 with <i>O. oeni</i> inoculated after alcoholic fermentation
Sc2+LAB2 sim MLF	Sc2 plan with <i>Lb. plantarum</i> inoculated after 24 hours
Sc2+LAB2 seq MLF	Sc2 with <i>Lb. plantarum</i> inoculated after alcoholic fermentation
Hu+Sc2	<i>H. uvarum</i> + Sc2 inoculated after 24 hours, no MLF
Hu+Sc2+LAB1 sim MLF	<i>H. uvarum</i> + Sc2 and <i>O. oeni</i> inoculated after 24 hours
Hu+Sc2+LAB1 seq MLF	<i>H. uvarum</i> + Sc2 inoculated after 24 hours, <i>O. oeni</i> after alcoholic fermentation
Hu+Sc2+LAB2 sim MLF	<i>H. uvarum</i> + Sc2 and <i>Lb. plantarum</i> inoculated after 24 hours
Hu+Sc2+LAB2 seq MLF	<i>H. uvarum</i> + Sc2 inoculated after 24 hours, <i>Lb. plantarum</i> after alcoholic fermentation

## 6.2.2 Wine production

Shiraz grapes were obtained from the Nietvoorbij research farm (Stellenbosch, South Africa) and a standardised small-scale (20 L) winemaking procedure was followed as described in Chapter 5. Twenty different treatments were applied and each treatment had three replicates (Table 6.1). The *S. cerevisiae* strains Sc1 and Sc2 were inoculated on day 0 in the reference

treatments. *H. uvarum* was inoculated on day 0 and Sc1 and Sc2 were inoculated after 24 hours (day1) for the mixed yeast fermentation. The LAB in the simultaneous MLF samples were added 25 hours after the initial yeast inoculation on day 0. Fermentations were carried out at ca. 24°C and after completion of the alcoholic fermentation, the sequential MLF treatments were inoculated with LAB1 or LAB2. All treatments were racked, fined, cold stabilized and bottled as described by Minnaar *et al.* (2015). After bottling, all wines were stored at 15°C until needed.

### 6.2.3 Fourier transform mid-infrared spectroscopy

Fourier transform mid-infrared (FTMIR) spectra of the bottled wines were generated using a WineScan™ FT120 instrument (FOSS Analytical A/S, Denmark) at the Institute for Wine Biotechnology (Stellenbosch University, South Africa) as described by Louw *et al.* (2009) and Malherbe (2011). FTIR and GC-FID analyses were performed from the same bottles.

### 6.2.4 Data analysis

Principal Component Analysis (PCA) and orthogonal projections to latent structures for discriminant analysis (OPLS-DA) was performed with the use of SIMCA® software (version 14, MKS Data Analytics Solutions, San José, CA, USA) to evaluate relationships between treatments and MIR spectral profiles of the wines. The averaged wine spectra were mean-centred. The wavenumbers at which the wine absorbance was measured were used as input X-variables and a dummy variable denoting treatment class membership as input Y-variable. The actual absorbance values of each wine were considered as the observations (samples). Separate models were built for each of the various treatments. OPLS-DA has been described (Wiklund *et al.*, 2008) and the model is rotated so that the variation correlating to class separation is projected on the first predictive component [t1]. Variation that is uncorrelated with class separation is projected on one or more orthogonal components [to]. The outputs of the discriminant analysis in this study were visualised in scores scatter plots. In the 2-D scores scatter plots, the between-class variation was projected on the predictive component (horizontal direction), and the within-class variation on the orthogonal component(s) (vertical direction). Furthermore, since it was of interest to interpret the effect of the X-variables on the between-class (yeast treatment or MLF strategy) and within-class variation. These plots combine covariance and correlation loadings obtained from the predictive component, [t1], of the OPLS-DA models (Wiklund *et al.*, 2008) and thereby identifying the wavelengths that are important for the separation between classes. The quality of the models are described by  $R^2$  and  $Q^2$  values, where  $R^2X$  is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and  $Q^2$  is defined as the proportion of variance in the data predicted by the model and indicates predictability (Trygg & Wold, 2002). A  $Q^2$  value of > 0.5 indicates good predictability, and a value of >0.9 shows excellent predictability (Ali *et al.*, 2012; Chang *et al.*, 2014).

## 6.3 RESULTS AND DISCUSSION

### 6.3.1 Multivariate data analysis of treatments

Principal component analyses (PCA) was performed to determine the correlation between the spectral data of Shiraz wines produced with *H. uvarum* in combination with two *S. cerevisiae* (Sc1 and Sc2), two LAB (LAB1 and LAB2) strains and three MLF strategies (none, simultaneous and sequential). The data set included all 60 Shiraz wines and the first two predictive components of the PCA score plot explained 68.3% (PC1 = 39.9% and PC2 = 28.4%) of the variance in the data set (Fig. 6.1). Two clusters were observed, *i.e.* the wines that did not undergo MLF (left side of PC1) and the wines that underwent MLF (middle and right side of PC1). Some separation between the wine samples was observed, but no clear trends were observed with regard to yeast or LAB treatments. No separation between wines that were inoculated for simultaneous and sequential MLF was observed. The aforementioned results indicate that MLF strategy had greater effect than yeast combination with regard to the separation of wines using MIR.

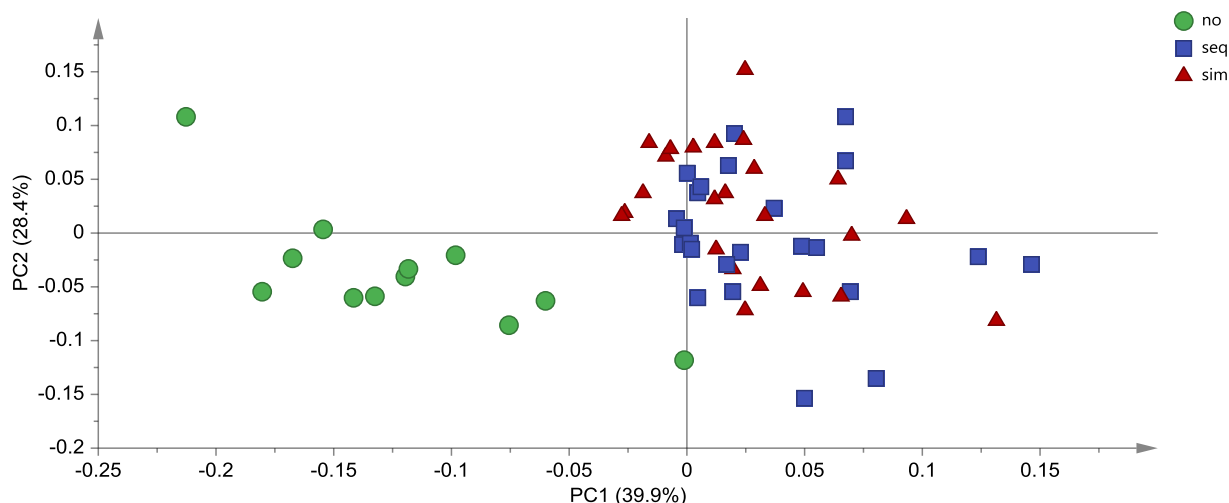


FIGURE 6.1. Score scatter plot generated with principal component analysis (PCA) of infrared spectroscopy data of Shiraz wines produced with *Hanseniaspora uvarum* (Hu) in combination with *Saccharomyces cerevisiae* 1 (Sc1) or *Saccharomyces cerevisiae* 2 (Sc2) and three malolactic fermentation strategies (none [No], simultaneous [sim] and sequential [seq]).

#### 6.3.1.1 OPLS-DA of MLF strategies

With the aim of further investigating the differences between the three MLF strategies, OPLS-DA was applied to the spectral data (Fig. 6.2). For this model, all 60 wines were subjected to OPLS-DA and the groups obtained for MLF strategies are based on the MIR spectra (variables) that were analysed. No outliers were observed. The model resulted in two predictive components (0.643), which explained 64.3% of the variation within the data set (Table S6.1). As

observed with the PCA plot of the wines (Fig. 6.1) separation was observed between wines that did not undergo MLF and wines that underwent MLF (Fig. 6.2). Some differences can be observed between wines that underwent simultaneous MLF and wines that underwent sequential MLF, but no clear trends could be observed. Variation within the aforementioned cluster can be due to the yeast (*S. cerevisiae* only or *H. uvarum* in combination with *S. cerevisiae*) or LAB (LAB1 or LAB2) treatment that was applied. Variation within the cluster of wines that did not undergo MLF (no MLF) was due to the yeast combination used.

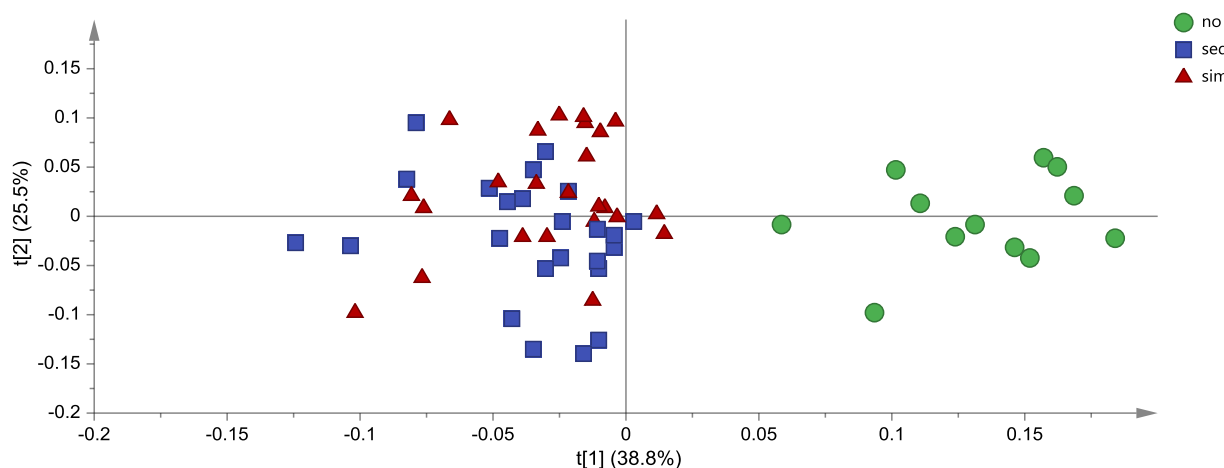


FIGURE 6.2. Score scatter plot generated with orthogonal projections to latent structures for discriminant analysis (OPLS-DA) of mid-infrared spectroscopy data of Shiraz wines produced with three malolactic fermentation (MLF) strategies (none [No], simultaneous [sim] and sequential [seq]).

Figure 6.3 shows the wavenumbers and peaks important for differentiation among the wines that were separated on MLF strategy. Most of the notable variation amongst the wines occurred in the regions of  $949$  to  $1277\text{ cm}^{-1}$ ,  $1562$  to  $1763\text{ cm}^{-1}$  and  $2326$  to  $2353\text{ cm}^{-1}$ , which contained a lot of useful information. For grape and wine samples, the region between  $900$  and  $1500\text{ cm}^{-1}$  is known as the “fingerprint” region (Shah *et al.*, 2010; Culbert *et al.*, 2015) and is known to contain absorbance bands attributable to water, sugars, acids (e.g. malic acid) and phenolic compounds (Shah *et al.*, 2010; Cozzolino *et al.*, 2012). The MIR region between  $1000$  and  $1100\text{ cm}^{-1}$  has been attributed to C–O vibrations of sugars, such as glucose and fructose, organic acids and alcohols, phenols, esters and lactones (Williams & Fleming, 1995). The peaks associated with water at  $3300$  and  $1640\text{ cm}^{-1}$  (Hashimoto & Kameoka, 2000; Patz *et al.*, 2004), and also background noise, were excluded from the analysis.

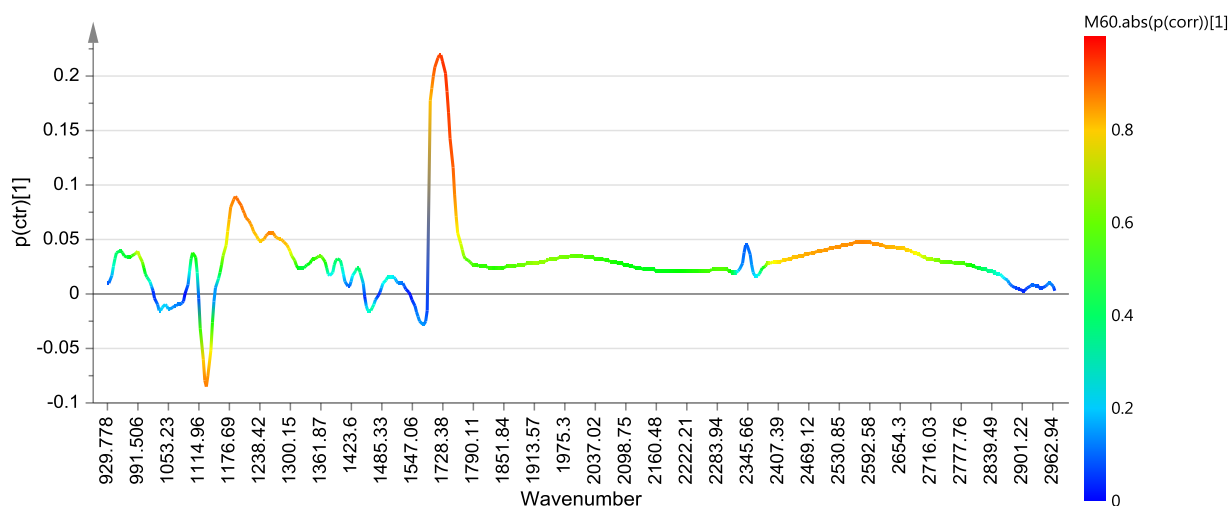


FIGURE 6.3. Infrared spectrum showing the important wavenumbers ( $\text{cm}^{-1}$ ) for differentiation between Shiraz wines produced with different malolactic fermentation strategies.

### 6.3.1.2 OPLS-DA of yeast treatments

With the aim of further investigating the differences between the yeast treatments, OPLS-DA was applied to the spectral data of the wines produced with the selected yeast combinations and the results are shown in Fig. 6.4. From a preliminary data plotting one outlier was removed from the data set, which resulted in 59 Shiraz wines being used in this model. This model generated three predictive (29.6%) and seven orthogonal components (70%), which explained 99.6% of the variation within the data set (Table S6.2). The first two predictive components only explain 28.9% of the variation in the data set (Fig. 6.4). Good separation of the yeast treatments was observed on the OPLS-DA predictive score scatter plot and four distinct clusters were obtained. Clear differences were observed between the wines produced with *H. uvarum* in combination with the two *S. cerevisiae* yeast strains and wines produced with *S. cerevisiae* only. Greater differences were observed between wines produced with Sc2 and Hu+Sc2 than wines produced with Sc1 and Hu+Sc1. The orthogonal components explain the variation within a group and in this case variations within the groups can be explained by the MLF strategy that was applied and LAB strain used.

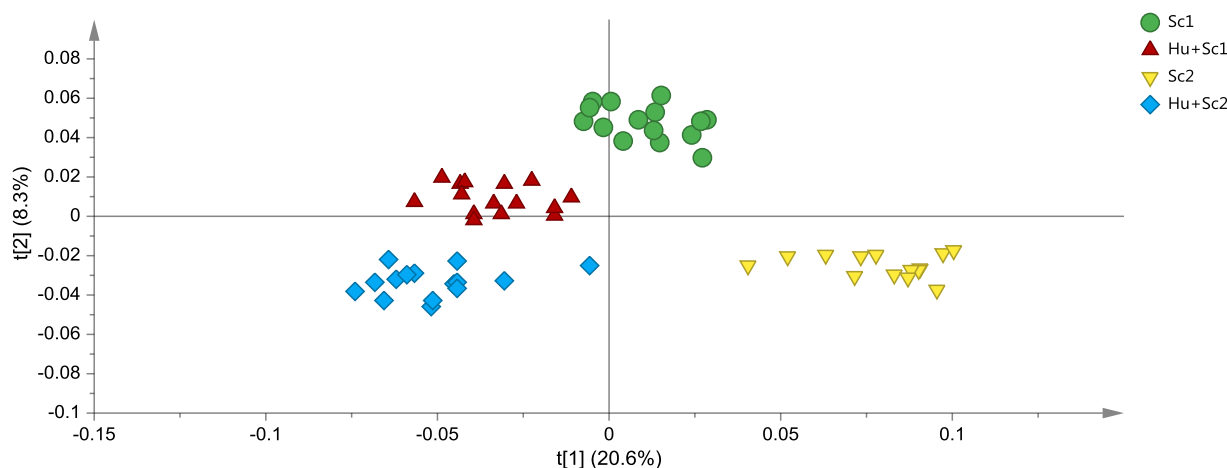


FIGURE 6.4. Score scatter plot generated with orthogonal projections to latent structures for discriminant analysis (OPLS-DA) of mid-infrared spectroscopy data of Shiraz wines produced with various yeast combinations. Abbreviations: Sc1 = *Saccharomyces cerevisiae* 1, Sc2 = *S. cerevisiae* 2, Hu = *Hanseniaspora uvarum*.

Figure 6.5 shows the wavenumbers and peaks important for differentiation between the wines produced with the selected yeast treatments. The most important variation amongst the wines occurred in the regions of 929 to 1211  $\text{cm}^{-1}$ , 1381 to 1419  $\text{cm}^{-1}$ , 1512 to 1747  $\text{cm}^{-1}$ , 2330  $\text{cm}^{-1}$  and 2858 to 2935  $\text{cm}^{-1}$ . As previously mentioned, the MIR region between 1000 and 1100  $\text{cm}^{-1}$  linked with sugars, such as glucose and fructose, organic acids and alcohols, phenols, esters and lactones (Williams & Fleming, 1995). In particular, absorbance in the region of 1045 to 1080  $\text{cm}^{-1}$  has been associated with C–OH bonds present in primary alcohols (e.g., ethanol), glycerol and sugars (glucose and fructose) (Cozzolino *et al.*, 2009, 2011; Riovanto *et al.*, 2011), which are important metabolites of wine.

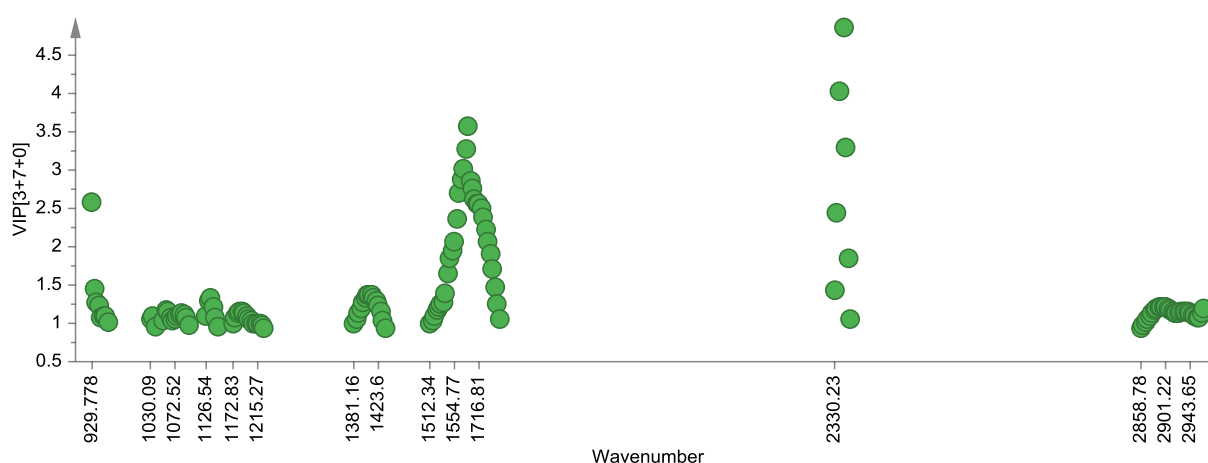


FIGURE 6.5. Important wavenumbers ( $\text{cm}^{-1}$ ) and VIP (variable importance for the projection) values of mid-infrared spectroscopy data of Shiraz wines produced with different yeast combinations grouped according to yeast treatment.

### 6.3.1.3 OPLS-DA of LAB treatments

OPLS-DA of the spectral data of wines produced with the two selected LAB treatments (LAB1 and LAB2) are shown in Fig. 6.6. This model resulted in one predictive (3.4%) and eight orthogonal components (95.5%), which explained 98.9% of the variation within the data set (Table S6.3). Good separation was obtained between wines that were produced with LAB1 and LAB2. However, the LAB strains only explained about 3% of the variation in the data set. The variations explained by the orthogonal components are due to the MLF strategy applied and yeast combination used. The results show that the two LAB strains used in this study had very little impact on the wine composition.

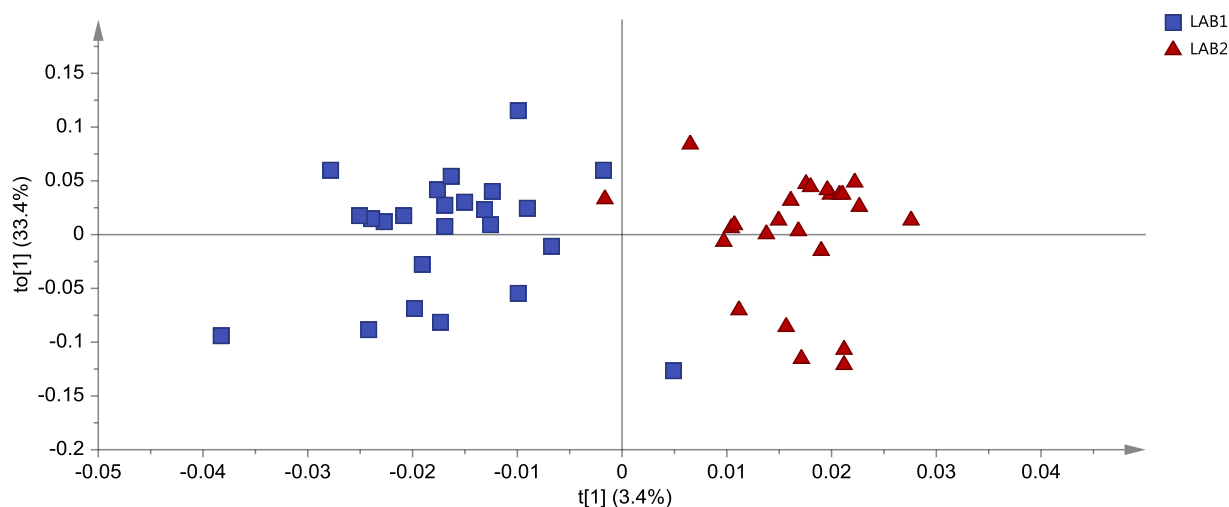


FIGURE 6.6. Score scatter plot generated with orthogonal projections to latent structures for discriminant analysis (OPLS-DA) of mid-infrared spectroscopy data of Shiraz wines produced with two lactic acid bacteria treatments (LAB1 and LAB2).

## 6.4 CONCLUSIONS

Results demonstrated that differences exist between spectral profiles of Shiraz wines produced with different yeast, LAB strains and MLF strategies and showed that the infrared spectral information could be used to discriminate between wines. MLF strategy had the greatest impact on the chemical composition of the wines, followed by yeast treatment. Differences were observed for wines produced with *S. cerevisiae* only and for wines produced with *H. uvarum* in combination with *S. cerevisiae*. The possible compounds responsible for differentiation between wines included sugars, acids, alcohols, phenols, esters and lactones. This was an explorative study, but results show that MIR spectroscopy in combination with pattern recognition methods can be used to distinguish between wines produced with different yeasts, LAB strains and MLF strategies.

## 6.5 ACKNOWLEDGEMENTS

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TABLE S6.1. Model quality, basic statistics and correlations of the OPLS-DA model of Shiraz wines separated by malolactic fermentation strategy.

Component	R <sup>2</sup> X	R <sup>2</sup> X(cum)	Eigenvalue	R <sup>2</sup>	R <sup>2</sup> (cum)	Q <sup>2</sup>	Limit	Q <sup>2</sup> (cum)	R <sup>2</sup> Y	R <sup>2</sup> Y(cum)	EigenvalueY	Significance
Model		0.643			0.426			0.363		1		
Predictive		0.643			0.426			0.363		1		
P1	0.388	0.388	23.3	0.362	0.362	0.302	0.01	0.302	0.445	0.445	1.33	R1
P2	0.255	0.643	15.3	0.0638	0.426	0.0612	0.01	0.363	0.555	1	1.67	R1

TABLE S6.2. Model quality, basic statistics and correlations of the OPLS-DA model of Shiraz wines separated by yeast treatment.

Component	R <sup>2</sup> X	R <sup>2</sup> X(cum)	Eigenvalue	R <sup>2</sup>	R <sup>2</sup> (cum)	Q <sup>2</sup>	Limit	Q <sup>2</sup> (cum)	R <sup>2</sup> Y	R <sup>2</sup> Y(cum)	EigenvalueY	Significance
Model		0.995			0.868			0.789		1		
Predictive		0.296			0.868			0.789		1		
P1	0.206	0.206	12.2	0.306	0.306	0.283	0.01	0.283	0.331	0.331	1.32	R1
P2	0.0831	0.289	4.9	0.318	0.625	0.294	0.01	0.577	0.334	0.665	1.34	R1
P3	0.00677	0.296	0.399	0.243	0.868	0.212	0.01	0.789	0.335	1	1.34	R1
Orthogonal in X (OPLS)		0.7			0							
O1	0.328	0.328	19.4	0	0							R1
O2	0.254	0.583	15	0	0							R1
O3	0.0466	0.629	2.75	0	0							R1
O4	0.0273	0.657	1.61	0	0							R1
O5	0.0274	0.684	1.62	0	0							R1
O6	0.00873	0.693	0.515	0	0							R1
O7	0.00678	0.7	0.4	0	0							R1

TABLE S6.3. Model quality, basic statistics and correlations of the OPLS-DA model of spectral data of Shiraz wines separated by lactic acid bacteria treatment.

Component	R <sup>2</sup> X	R <sup>2</sup> X(cum)	Eigenvalue	R <sup>2</sup>	R <sup>2</sup> (cum)	Q <sup>2</sup>	Limit	Q <sup>2</sup> (cum)	R <sup>2</sup> Y	R <sup>2</sup> Y(cum)	EigenvalueY	Significance
Model		0.989			0.825			0.242		1		
Predictive		0.0338			0.825			0.242		1		
P1	0.0338	0.0338	1.62	0.825	0.825	0.242	0.01	0.242	1	1	2	R1
Orthogonal in X (OPLS)		0.955			0							
O1	0.334	0.334	16	0	0							NS
O2	0.25	0.583	12	0	0							NS
O3	0.182	0.766	8.75	0	0							R1
O4	0.121	0.887	5.83	0	0							NS
O5	0.0352	0.922	1.69	0	0							R1
O6	0.0152	0.937	0.73	0	0							R1
O7	0.0109	0.948	0.521	0	0							R1
O8	0.00678	0.955	0.325	0	0							R1

# Chapter 7

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## General discussion and conclusions

## CHAPTER 7

### 7.1 GENERAL DISCUSSION

The chemical composition of wine, which include non-volatile and volatile compounds all contribute to the appearance, aroma and taste properties (Swiegers *et al.*, 2005). The chemical composition is determined by many factors, such as the grape variety, the geographical and viticultural conditions of grape cultivation, the microbial ecology of the grape and fermentation processes, and winemaking practices (Cole & Noble, 1997). Winemakers employ a variety of techniques and tools to produce wines with specific flavour profiles (Swiegers *et al.*, 2005). The choice of which yeast to use to conduct the alcoholic fermentation is one of those tools and is very important, as the yeast converts grape sugars to ethanol, carbon dioxide and also a range of sensorially important volatile metabolites. The yeast is also responsible for modifying mouth-feel and colour (Swiegers *et al.*, 2005). Usually *Saccharomyces cerevisiae* is used to ensure a rapid fermentation and standardization of wine quality (Capozzi *et al.*, 2015), but non-*Saccharomyces* yeasts, which have different oenological properties to *S. cerevisiae*, can also be used to modulate flavour and add complexity (Swiegers *et al.*, 2005; Ciani *et al.*, 2010; Gobbi *et al.*, 2013, Jolly *et al.*, 2014; Whitener *et al.*, 2016, 2017). The wine lactic acid bacteria (LAB) also contribute to aroma and taste properties of wine by performing the malolactic fermentation (MLF). Malolactic fermentation is important in winemaking because it results in deacidification of the wine, contributes to microbial stability and lastly enhances wine flavour through the production of metabolites (Lerm *et al.*, 2010; Sumby *et al.*, 2014). The aforementioned microorganisms do not act independently from each other and these interactions have an impact on the flavour profile and final quality of wine. To understand these interactions better, this project had the specific aim to investigate the interactions between *Saccharomyces*, non-*Saccharomyces* yeasts and LAB, and the effect these interactions had on MLF and wine flavour. The aim of this study and all the objectives were successfully achieved and the results are discussed in Chapters 3, 4, 5 and 6. In alignment with the long term research programme mentioned in Chapter 1, which included characterisation, evaluation, and utilisation of the natural yeast biodiversity, 37 non-*Saccharomyces* yeast strains were characterised and five isolates were evaluated in wine production trials.

In Chapter 3 (du Plessis *et al.*, 2017a), the genetic diversity and variation between non-*Saccharomyces* yeast species and strains was investigated with contour-clamped homogeneous electric field gel electrophoresis (CHEF) karyotyping and matrix-assisted laser desorption ionization using a time-of flight (MALDI-TOF) biotyping. The aforementioned techniques were used to verify non-*Saccharomyces* species identity to determine inter-species as well intra-species variation among 37 yeast strains (*Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Candida zemplinina*, *C. stellata*, *Metschnikowia pulcherrima*, *Torulaspora*

*delbrueckii* and *Schizosaccharomyces pombe*). This study is the first to report on the use of MALDI-TOF MS for typing of non-*Saccharomyces* wine yeasts. Most published MALDI-TOF MS research focused on identifying and typing of clinical yeast and bacteria and the research relevant to the wine industry focused more on identifying *Saccharomyces* and non-*Saccharomyces* yeasts (Moothoo-Padayachie *et al.*, 2013; Usbeck *et al.*, 2014; Kántor & Kačániová, 2015). Both techniques could distinguish between the different non-*Saccharomyces* yeast species and more importantly could distinguish between different *H. uvarum*, *L. thermotolerans* and *T. delbrueckii* strains. Unfortunately, both techniques showed limited ability to differentiate between *C. zemplinina* and *M. pulcherrima* strains. This could be due to the high level of conserved genetic material within the aforementioned species. The limited differentiation between *C. zemplinina* and *M. pulcherrima* strains was addressed by using an extended 72-hour CHEF programme. This resulted in better differentiation between strains from both species. The *C. zemplinina* strains showed a higher level of heterogeneity than the *M. pulcherrima* strains with the 72-hour programme. The 72-hour programme could also distinguish between the *C. zemplinina* and *C. stellata* type strains. These two species are closely related and were only reclassified as two different species when Sipiczki (2003, 2004) revealed the differences between them. The 72-hour programme was an improvement on the over 99 and 96 hrs electrophoretic karyotyping procedure of Sipiczki (2004) and Csoma and Sipiczki (2008), respectively. For the typing of species with high genetic similarity, *i.e.* *M. pulcherrima* strains, alternative PCR methods, such as amplified fragment length polymorphism (Spadaro *et al.*, 2008) or tandem repeat-tRNA PCR (Barquet *et al.*, 2012), could be considered.

The 37 non-*Saccharomyces* yeast strains were further characterised by determining their acid protease, polygalacturonase/pectinase and  $\beta$ -glucosidase enzyme activities as well as their ability to degrade malic acid (Chapter 3; du Plessis *et al.*, 2017a).  $\beta$ -Glucosidase activity plays an important role in releasing volatile compounds from non-volatile precursors, which can enhance wine flavour (Hernández-Orte *et al.*, 2008). Extracellular proteolytic and pectinolytic enzymes of non-*Saccharomyces* yeasts might also be beneficial by improving wine processing through the facilitation of juice extraction and clarification, wine filtration and colour extraction (Van Rensburg & Pretorius, 2000; Reid, 2012). None of the *S. cerevisiae*, *L. thermotolerans*, *C. zemplinina*, *T. delbrueckii* and *Sc. pombe* strains showed any enzyme activity. The lack of enzyme activity for the strains from aforementioned species is contrary to findings from other reports (Charoenchai *et al.*, 1997; Maturano *et al.*, 2012; Mostert, 2013). The differences in findings can be attributed to the use of a different set of strains and the large biodiversity found amongst yeasts. According to Ganga and Martínez (2004), secretion of enzymes is not characteristic of a particular genus or species, but depends on the specific yeast strain analysed. Only *H. uvarum* and *M. pulcherrima* strains were found to have  $\beta$ -glucosidase activity. Additionally, *M. pulcherrima* strains also showed protease activity. These findings are in

agreement with other studies (Charoenchai *et al.*, 1997; Mostert, 2013). *H. uvarum* and *M. pulcherrima* are frequently found on grapes and in grape must and the naturally occurring strains from these species could also have the same enzymatic activities, which could explain why some spontaneous fermentations produce more complex wines.

Most of the strains showed limited malic acid degradation, and only *Sc. pombe* and the *C. zemplinina* strains showed mentionable degradation. The ability of *Sc. pombe* to utilise malic acid is well known (Baranowski & Radler, 1984; Rodriguez & Thornton, 1989, Benito *et al.*, 2013; 2014), but partial malic acid utilisation by *C. zemplinina* has not previously been reported. The low malic acid utilisation by *S. cerevisiae*, *C. stellata*, *T. delbrueckii* and *H. uvarum* is in agreement with other studies (Gao & Fleet, 1995; Volschenk *et al.*, 2003; Ribéreau-Gayon *et al.*, 2006; Saayman & Viljoen-Bloom, 2006). Comparison of a plate and broth assay for rapid screening of strains that can degrade malic acid showed that the plate assay was not very reliable and gave a lot of negative results as well as false positives. It is therefore recommended that a liquid medium should be used when screening yeast strains for their ability to utilise malic acid. In a liquid medium malic acid degradation can be measured using enzyme kits, high-performance liquid chromatography (HPLC), or other techniques.

The ability of the non-*Saccharomyces* yeast strains to conduct the alcoholic fermentation in a synthetic grape juice was also investigated (Chapter 3, du Plessis *et al.*, 2017a). *C. stellata*, *C. zemplinina*, *H. uvarum*, *M. pulcherrima* and *Sc. pombe* strains were shown to be slow to medium fermenters, while *L. thermotolerans* and *T. delbrueckii* strains were found to be medium to strong fermenters. These findings are in agreement with literature (Ciani & Maccarelli, 1998; Jolly *et al.*, 2003a; Comitini *et al.*, 2011; Van Breda *et al.*, 2013; Mostert & Divol, 2014; Renault *et al.*, 2015). While *L. thermotolerans* and *T. delbrueckii* strains can be used as single starter cultures, the other non-*Saccharomyces* strains should only be used in mixed culture fermentations with a strong fermenter, such as *S. cerevisiae*.

The effect of the aforementioned non-*Saccharomyces* yeast strains on MLF in the synthetic wine was also investigated and all *M. pulcherrima* and *L. thermotolerans* strains were compatible with the commercial *Oenococcus oeni* strain used and MLF completed quickly. The compatibility with MLF varied among the other yeast species, but inhibition was strain dependent. Three of the five *S. cerevisiae* strains studied, delayed MLF. Two of these *S. cerevisiae* strains had a higher nutrient demand or uptake, which depleted the nutrients available for LAB growth and resulted in slower progression of MLF. Nutrient supplementation (carbon source, amino acids, vitamins and trace elements) of synthetic wines produced by the aforementioned yeast allowed MLF to complete quickly and without any delays. However, nutrient supplementation did not work for one of the antagonistic *S. cerevisiae* strains and in this case, delayed MLF was possibly due to the production of inhibitory compounds. The antagonistic effect of some *S. cerevisiae* on MLF has been reported, and yeast and LAB compatibility is an important factor to consider for successful MLF (Henick-Kling & Park, 1994;

Costello *et al.*, 2003). The *C. stellata* strain had an inhibitory effect on MLF and the delayed MLF could be partially alleviated by nutrient supplementation. However, inhibition by *C. stellata* is also probably due to the production of inhibitory compounds. One *C. zemplinina* (C7), five *H. uvarum* and two *T. delbrueckii* strains caused delayed MLF. Inhibition by the *C. zemplinina* strain, three of the five *H. uvarum* and one of the two *T. delbrueckii* strains was alleviated by nutrient supplementation. The cause of inhibition in the remaining *T. delbrueckii* and two *H. uvarum* strains was most likely the production of inhibitory compounds.

In Chapter 4 (du Plessis *et al.*, 2017b), the use of one *H. uvarum*, two *L. thermotolerans*, one *C. zemplinina*, one *M. pulcherrima* and two *T. delbrueckii* strains, in combination with a commercial *S. cerevisiae* yeast strain for Shiraz wine production are discussed. The non-*Saccharomyces* yeast strains were selected for their fermentation characteristics and the potential to contribute to aroma or flavour complexity. Three MLF strategies (none, simultaneous and sequential inoculation) were also applied and the effect that the selected *Saccharomyces* and non-*Saccharomyces* yeast combinations had on *O. oeni* growth and MLF was investigated. The impact of the yeast and LAB interactions on the volatile composition and wine flavour was also determined. This is the first report on the use of the non-*Saccharomyces* yeast strains *C. zemplinina* C7, *H. uvarum* H4 and *L. thermotolerans* L2 in wine production and first report on the use of strains, *M. pulcherrima* M2 and *T. delbrueckii* T6 for the production Shiraz wines. The last mentioned two yeast strains had previously been used in Chardonnay, Chenin blanc and Sauvignon blanc wine production, but without MLF (Jolly *et al.*, 2003a, b). Strains *C. zemplinina* C7 and *L. thermotolerans* L2 had a negative effect on LAB growth and the progress of MLF when the commercial *O. oeni* culture was applied as a simultaneous inoculation, but the same effect was not observed when *O. oeni* inoculated sequentially. Except for C7 and L2, the other non-*Saccharomyces* yeast strains had a beneficial effect on the progress of MLF and MLF completed in a shorter time in these wines than wines produced with only *S. cerevisiae*. This finding could be of benefit to wine producers who rely on spontaneous MLF and those that want to prevent spontaneous MLF should also take this into consideration. Wines produced with non-*Saccharomyces* yeasts had lower alcohol levels than wines produced with *S. cerevisiae* only. These findings are in agreement with those of other studies (Comitini *et al.*, 2011; Benito *et al.*, 2015; Contreras *et al.*, 2015).

Wines produced with various yeast combinations were significantly different with regard to chemical composition (Chapter 4, du Plessis *et al.*, 2017b). Wines that did not undergo MLF were significantly different to wines that underwent MLF in terms of chemical composition. Differences between strains from the same non-*Saccharomyces* species were shown to be as significant as the variation between different non-*Saccharomyces* species, or as significant as the differences between non-*Saccharomyces* and *Saccharomyces* yeasts. Multivariate data analysis showed that the most important compounds for differentiating among wines produced with the selected yeast combinations and MLF strategies were volatile acidity, acetic acid, ethyl



acetate, isoamyl alcohol, 3-methyl-1-pentanol, ethyl octanoate, diethyl succinate, 2-phenyl ethanol and octanoic acid.

Evaluation of the sensory properties of the aforementioned wines only revealed significant differences for berry aroma, acid balance and astringency (Chapter 4, du Plessis *et al.*, 2017b). Both yeast selection and MLF strategy had a significant effect on berry aroma, but MLF strategy also had a significant effect on acid balance and astringency of wines. Malolactic fermentation strategy had a significant effect on the chemical and sensory properties of the wines, and had a greater effect on the sensory properties than the yeast combinations used. Wines that underwent simultaneous MLF scored slightly higher for berry aroma than wines that did not undergo MLF, but both treatments scored significantly higher than wines that underwent sequential MLF. Even though wines that underwent sequential MLF contained higher concentrations of most esters than wines that underwent simultaneous MLF and wines that did not undergo MLF, it did not contribute to more perceivable berry aroma in those wines. The study showed that significant variation in chemical composition does not always translate to perceivable sensory differences. High concentrations of volatile compounds with high odour threshold values do not always contribute to sensory differences. In general, wines that underwent sequential MLF were perceived to be less balanced and scored lower for acid balance than wines that underwent simultaneous MLF, and wines that did not undergo MLF. The lack of balance did not affect the overall quality. Wines that underwent simultaneous MLF were perceived to be more astringent than wines that did not undergo MLF and significantly more astringent than wines undergoing sequential MLF. Simultaneous MLF might be beneficial for wines that are made to be aged for a long period, because astringency decreases over time and may contribute to the ageing potential of such wines. Overall, wines that underwent simultaneous MLF scored higher for overall quality than wines that underwent sequential MLF and wines that did not undergo MLF, even though the differences were not significant. The optimal MLF strategy for each yeast strain or yeast combination to improve wine flavour and quality appears to be strain dependent.

In a follow up Shiraz wine production trial (Chapter 5), the interaction between *H. uvarum*, two commercial *Saccharomyces cerevisiae* yeast strains, two LAB species (*Lactobacillus plantarum* and *O. oeni*) in combination with three malolactic strategies were investigated. One of the *S. cerevisiae* strains (Sc1) had an inhibitory effect on LAB growth and progression of MLF, while wines produced in combination with *H. uvarum* had a stimulatory effect on LAB growth and completed MLF in a shorter period than wines produced with *S. cerevisiae* only. The use of the selected *H. uvarum* strain had a beneficial effect on the growth of the inoculated and naturally occurring LAB. Wine that underwent simultaneous MLF proceeded faster than wines that underwent sequential MLF. Implantation of the *H. uvarum* strain was 100% successful and was verified by performing random amplified polymorphic DNA PCR. Wines produced with *H. uvarum* in combination with *S. cerevisiae* contained lower alcohol levels and higher glycerol

levels than wines produced with *S. cerevisiae* only. These results were in agreement with findings of Mendoza *et al.* (2011). Yeast treatment had a bigger effect on the volatile composition of the wines, but LAB strain and MLF strategy also had a significant impact on volatile composition. The interaction between yeast, LAB strain and MLF strategy had a significant impact on the flavour profile of wines. The following compounds: isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, 2-phenyl acetate, isobutanol, 3-methyl-1-pentanol, hexanoic acid and octanoic acid, and the concentrations at which they occurred, were important in discriminating between the different wines.

In Chapter 5, it was shown that the yeast treatment had a significant effect on fresh vegetative and spicy aroma, as well as body and astringency of the wines. *H. uvarum* in combination with the suitable *S. cerevisiae* strain can be used to enhance vegetative aroma and body of wine. The LAB strain and MLF strategy had a significant effect on berry, fruity, sweet associated and spicy aroma, as well as acidity and body of the wines. The sensory differences between wines that did not undergo MLF, wines that underwent simultaneous or sequential MLF were as significant as the differences between wines produced with the selected yeast strains. This study confirmed reports about the sensory differences between wines that underwent simultaneous or sequential MLF and is in agreement with literature (Antalick *et al.*, 2014; Bartowsky *et al.*, 2015; Guzzon *et al.*, 2015). However, no definitive answer can be given with regard to which MLF inoculation strategy (simultaneous or sequential) should be applied to improve wine flavour and quality. This study showed that interactions between yeast and LAB are complex and in most cases inoculation strategy was yeast strain dependent. Therefore, winemakers should select yeast strains and a MLF inoculation strategy according to the wine style they want to produce. Results also showed that different LAB strains have a perceivable impact on the sensory properties and correlates with other studies (Izquierdo-Cañas *et al.*, 2013; Malherbe *et al.*, 2013). In general, wines produced with *O. oeni* completed MLF in less time than wines where *Lb. plantarum* was used. However, no definitive answer can be provided as to which LAB species should be used to guarantee improvements in wine flavour. The impact of the LAB strains on wine flavour varied with regard to the yeast combination and MLF inoculation strategy that was applied. Results showed that the optimal LAB combination is different for each yeast strain and MLF inoculation strategy.

In Chapter 6, the use of mid-infrared (MIR) spectroscopy, in combination with pattern recognition methods, as a rapid and inexpensive tool to distinguish between wines produced with various combinations of *H. uvarum*, *S. cerevisiae*, LAB strains (*O. oeni* and *Lb. plantarum*) and MLF strategies were explored. Principal component analysis (PCA) and orthogonal projections to latent structures for discriminant analysis (OPLS-DA) were applied to wine spectra. Results demonstrated that Shiraz wines produced with *H. uvarum* in combination with *S. cerevisiae* and the three MLF strategies had different spectral profiles, and that the infrared

(IR) spectrum contained valuable information to discriminate among these wines. MLF strategy had a greater impact on the chemical compounds relevant to the spectral profiles than yeast treatment. These results are similar to trends reported in Chapter 4. The compounds linked to the differences observed in the spectra include sugars, acids, alcohols, phenols, esters and lactones.

## 7.2 CONCLUDING REMARKS

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The effect of non-*Saccharomyces* yeast on LAB growth and MLF has not received as much attention as *S. cerevisiae* and LAB interactions. This dissertation therefore contributes to the knowledge of the impact of non-*Saccharomyces* yeast on LAB growth and MLF. Most non-*Saccharomyces* yeast strains had a positive effect on LAB growth and MLF. The specific contribution that different non-*Saccharomyces* yeasts in combination with *S. cerevisiae*, LAB strains and MLF strategies had on wine flavour was supported by the changes observed in a large variety of chemical compounds. This study showed that the interactions between non-*Saccharomyces*, *Saccharomyces* yeasts and LAB are complex, and that each yeast and LAB strain has different attributes that can impact flavour and quality of wines. This study also showed that non-*Saccharomyces* yeasts, LAB treatments, as well as timing of MLF induction can be used to modify the flavour profile of wines and are important tools that can be used by wine producers. In conclusion, the comprehensive investigation of fermentation kinetics, chemical, spectral and sensory data led to several definitive results, such as the inhibitory activity of specific yeast strains, significant differences in volatile composition does not guarantee perceivable sensory differences, and MLF strategy can have a greater impact on sensory properties of certain wines than the yeast combination used. This research provides information, which is both of fundamental and industrial importance, and confirms the complex nature of the interactions between *Saccharomyces*, non-*Saccharomyces* yeasts and LAB.

## 7.3 FUTURE RESEARCH

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This study showed that there is great variability among the strains investigated. One of the limitations of this study was the number of non-*Saccharomyces* species, but also strains within the species that was screened. Screening a larger number of non-*Saccharomyces* yeast strains, from different non-*Saccharomyces* species, for potential enzyme activity, malic acid degradation and even antimicrobial activity could be beneficial to the wine industry. Finding one or more non-*Saccharomyces* yeast strains that are able to modify flavour, while reducing alcohol concentration and stimulating MLF could be one of the objectives of such a study. With the consumers looking for wines that contain lower sulphites and other preservatives, finding a non-*Saccharomyces* yeast that is able to produce antimicrobial compounds to inhibit the growth of spoilage microorganisms could be of great value.

The interactions between a few commercial *S. cerevisiae*, non-*Saccharomyces* yeast and LAB strains were investigated. Future research should investigate more commercial yeast and LAB strains to better understand the interactions between different yeast and LAB strains.

Although more is now known about the interactions between non-*Saccharomyces* yeasts and LAB, more research is needed to elucidate the inhibitory action of certain non-*Saccharomyces*. This study showed that some strains had high nutrient demands, which caused delayed MLF due to the depletion of nutrients the growth medium. However, other strains were shown to produce inhibitory compounds. The type of compound(s) and the mode of action need to be investigated.

This study showed that the limited number of volatile compounds measured could not always explain the differences in the sensory profiles that were obtained. Follow up studies should investigate untargeted metabolome and volatolome analyses.

Only one wine grape cultivar/variety was investigated in this study, but the results and trends observed might not be same for other cultivars. Therefore, further research is needed to investigate the effect of different *Saccharomyces*, non-*Saccharomyces* yeast and LAB interactions in a variety of cultivars.

#### 7.4 LITERATURE CITED

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