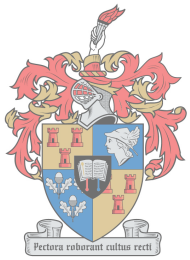
Investigating the influence of a wine yeast consortium on population dynamics, alcoholic and malolactic fermentation

by

Philippe Jacques Janse van Rensburg



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UNIVERSITY

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Supervisor: Dr Mathabatha Evodia Setati
Co-supervisor: Prof Maret du Toit

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Declaration

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Summary

Non-*Saccharomyces* (NS) starter cultures of species such as *Metschnikowia pulcherrima, Torulaspora delbrueckii, Pichia kluyveri* and *Lachancea thermotolerans,* have received attention for their desirable properties and contributions in winemaking. These include the production of volatile compounds or enzymes to modify organoleptic attributes. Most studies have focussed on the use of single NS at a time with *Saccharomyces cerevisiae* to study the above contributions and properties; including effects on malolactic fermentation (MLF). However, there is growing interest in using complex multi-starter cultures to enhance wine aroma. Indeed, commercial products such as Anchor Alchemy II (Anchor Yeast), which comprises of different strains of *S. cerevisiae,* and Melody™ (CHR Hansen), comprising of *S. cerevisiae, L. thermotolerans and T. delbrueckii* in different ratios, are available. To develop such products, the understanding of genetic and phenotypic characteristics of strains and interactions amongst the different organisms is important. Consequently, some studies have investigated yeast-yeast interactions and their mechanisms, such as antagonistic interactions mediated by direct cell contact or through growth inhibitory metabolites. This, results in a decline of certain non-*Saccharomyces* like *Hanseniaspora* and therefore effect the final aroma composition. Conversely, synergistic effects can be observed in which species may be promoted to persist longer and therefore they contribute more to aroma. However, knowledge on population dynamics in multi-species starter cultures and their influence on alcoholic fermentation (AF) and MLF remains limited.

The current study investigated a multi-species yeast consortium during AF and its effects on *Oenococcus oeni* viability during MLF. The consortium comprised of *M. pulcherrima, L. thermotolerans, T. delbrueckii* and *S. cerevisiae.* Fermentations were conducted in Chenin blanc and Pinotage at 15°C and 25°C, respectively. In all trials *M. pulcherrima* declined rapidly, while *L. thermotolerans* persisted until mid-fermentation. The best growth was observed for *T. delbrueckii* and it was able to persist until late fermentation stages. Fermentations that contained *L. thermotolerans* produced L-lactic acid in the Pinotage, but not in the Chenin blanc. There were no negative impacts observed on *O. oeni* populations during MLF for Pinotage and Chenin blanc wines. MLF kinetics were similar in all the Pinotage wines. In the Chenin blanc, the fastest L- malic acid consumption was displayed in wines that were fermented by *L. thermotolerans* and *T. delbrueckii* co-inoculations with *S. cerevisiae*. Different chemical profiles were detected using attenuated total reflection infrared (ATR-IR) spectroscopy. Mostly Chenin blanc wines were found to be significantly different from *S. cerevisiae* controls. Using gas chromatography, fold changes were observed for many volatile compounds.

In conclusion, it is possible to predict a consortium population dynamic based on individual yeast performances in mixed fermentations. The volatile profiles are not additive between treatments and will be unique for each inoculation scheme. MLF seems to not be

detrimentally affected by a consortium so long as each strain is regarded as compatible with lactic acid bacteria. Future work should include the evaluation of more yeast species and at alternate inoculation levels. Additionally, the inclusion of *Lactobacillus plantarum* for MLF needs to be investigated and quantification of detected volatiles should be performed.

Opsomming

Nie-*Saccharomyces* (NS) aanvangskulture van spesies soos *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Pichia kluyveri* en *Lachancea thermotolerans*, het aansienlike aandag ontvang as gevolg van hulle eienskappe vir wynbereiding. Dit sluit die bydrae tot wynaroma in deur die produksie van vlugtige verbindings soos esters, hoër alkohole en vetsure of die vrystelling van terpene tot hul aktiewe sensoriese status. Verder is die verlaging van alkohol of asynsuur asook die toename in gliserol of melksuur vlakke van belang. Die meeste studies het voorheen gefokus op die gebruik van enkel NS, soos *T. delbrueckii* of *M. pulcherrima,* wat agtermekaar of mede ingeënt is met *Saccharomyces cerevisiae* om bogenoemde bydraes en eienskappe te bestudeer; insluitend die effek op appelmelksuurgisting (AMG). Daar is egter toenemende belangstelling in die gebruik van komplekse multi-spesies aanvangskulture om wynaroma te verbeter. Kommersiële produkte soos Anchor Alchemy II (Anchor Yeast), wat bestaan uit verskillende stamme van *S. cerevisiae* of Melody™ (CHR Hansen), wat bestaan uit *S. cerevisiae*, *L. thermotolerans* en *T. delbrueckii* in verskillende verhoudings, is beskikbaar. Om sulke produkte te ontwikkel, is die begrip van genetiese en fenotipiese eienskappe van stamme en interaksies tussen die verskillende organismes, belangrik. Gevolglik het sommige studies gis-gis interaksies en hul meganismes ondersoek, soos antagonistiese interaksies bewerkstellig deur direkte sel kontak of deur groei-inhibitoriese metaboliete. Dit lei gevolglik tot 'n afname van sekere nie-*Saccharomyces* soos *Hanseniaspora* en beïnvloed dus die finale aroma samestelling. Omgekeerd, kan sinergistiese effekte waargeneem word in spesies wat bevorder kan word om langer te oorleef tydens fermentasie en daarom dra hulle meer by tot aroma. Die kennis oor populasie dinamika in kulture met meer spesies, hul invloed op alkoholiese fermentasie (AF) en AMG, bly egter beperk.

Die huidige studie het 'n multi-spesies gis konsortium ondersoek tydens AF en die effek daarvan op *Oenococcus oeni* se lewensvatbaarheid gedurende AMG. Die konsortium bestaan uit *M. pulcherrima*, *L. thermotolerans*, *T. delbrueckii* en *S. cerevisiae*. Fermentasies is onderskeidelik in Chenin blanc en Pinotage by 15 ° C en 25 ° C uitgevoer. In alle proewe het *M. pulcherrima* vinnig gedaal, terwyl *L. thermotolerans* tot mid-fermentasie oorleef het. Die beste groei is waargeneem vir *T. delbrueckii* en dit kon oorleef tot laat in die fermentasie. Fermentasies wat *L. thermotolerans* bevat, het L-melksuur in die Pinotage, maar nie in die Chenin blanc bevat nie. Daar was geen negatiewe effekte op *O. oeni* populasies tydens AMG vir beide Pinotage en Chenin blanc wyne waargeneem nie. AMG-kinetika was soortgelyk in al die Pinotage-wyne. In die Chenin blanc is die vinnigste L-appelsuur verbruik vertoon in wyne wat gefermenteer is deur *L. thermotolerans* of *T. delbrueckii* saam met *S. cerevisiae*. Verskillende chemiese profiele is waargeneem met behulp van verswakte totale refleksie infrarooi (ATR-IR) spektroskopie. Chenin Blanc-wyne is meestal aansienlik anders as die *S. cerevisiae*-kontrole in vergelyking met Pinotage. Met behulp van gaschromatografie is veelvoud veranderinge waargeneem vir baie vlugtige verbindings.

Ten slotte is dit moontlik om 'n konsortia populasie dinamika te voorspel wat gebaseer is op individuele gisprestasies in gemengde fermentasies. Die vlugtige profiele is nie ‘n toevoeging tussen behandelings nie en sal uniek wees vir elke inentingskema. AMG blyk nie nadelig beïnvloed te word deur 'n konsortium nie, solank as wat elke stam verenigbaar met melksuurbakterieë, beskou word. Toekomstige werk moet die evaluering van meer gisspesies en alternatiewe inokulasievlakke insluit. Daarbenewens moet die insluiting van *Lactobacillus plantarum* vir AMG ondersoek word en kwantifisering van vlugtige verbindings moet uitgevoer word.

This thesis is dedicated to my family, loved ones, friends and My Lord Jesus Christ.

Biographical sketch

Philippe Jacques Janse van Rensburg was born in 1992. He matriculated from Grey Boys High School in 2010 and graduated from Stellenbosch University with a Bachelor of Science in Molecular Biology and Biotechnology in 2015. He completed his Honours Bachelor of Science Degree in Wine Biotechnology under the supervision and guidance of Dr Mathabatha Evodia Setati from the Institute of Wine Biotechnology in Stellenbosch University. He was inaugurated into the Golden Key International Honour Society in 2017. He was accepted for a Master of Science in Wine Biotechnology under the supervision of Dr Mathabatha Evodia Setati and Prof Maret du Toit at Stellenbosch University.

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Preface

This thesis is presented as a compilation of four chapters and two appendices. Each chapter and appendix are introduced separately, and referencing was done according to the style of the South African Journal of Enology and Viticulture.

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Chapter 1

Introduction and project aims

Chapter 1 – Introduction and project aims

1.1 Introduction

The conversion of grape must to wine is a complex progression. It involves an important biological process which is alcoholic fermentation (AF) by yeasts (Fleet, 2003), the primary fermentation in winemaking and may include malolactic fermentation (MLF) by lactic acid bacteria (LAB), the secondary process aimed at lowering the malic acid content (Lerm *et al.*, 2010). The two processes can occur spontaneously, through the action of natural microbiota, or they may be induced deliberately by winemakers to protect against sluggish fermentations. The induction of AF is primarily done through inoculation with commercial strains of *Saccharomyces cerevisiae*, while MLF is induced with LAB, such as *Oenococcus oeni* or *Lactobacillus plantarum.*

Non-*Saccharomyces* yeasts, such as *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Hanseniaspora uvarum,* have gained attention for their oenological properties, especially in aiding wine aroma (Oro *et al.*, 2014; Belda *et al.*, 2015; Velázquez *et al.*, 2015; Kántor *et al.*, 2016; Tristezza *et al.*, 2016). This has led to the commercialisation of some non-*Saccharomyces* strains, which have specifically shown robustness in wine fermentation. Most of these strains can ferment to at least the mid stages of fermentation. Additionally, these strains may release enzymes during fermentation to change certain wine parameters. These strains are from species such as, *M. pulcherrima*, *Pichia kluyveri*, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) and *T. delbrueckii,* which are available from different commercial suppliers. However, it is still suggested by the suppliers that a *S. cerevisiae* commercial strain is required to ensure fermentation completion. This is generally employed since final stages of AF can still prove too harsh for the aforementioned non-*Saccharomyces* to survive, due to the high ethanol levels and limited nutrients. Studies have been conducted on some non-*Saccharomyces* yeasts, specifically on *H. uvarum, Starmerella bacillaris* (formerly *Candida zemplinina*) and *M. pulcherrima* (Oro *et al.*, 2014; Zara *et al.*, 2014; Wang *et al.*, 2015) to understand their individual impacts on the fermentation process and resulting wines. The work done on *T. delbrueckii* by Velázquez *et al.* (2015) and Ramírez *et al.* (2016) investigated how *T. delbrueckii* strains interacted with *S. cerevisiae* in white and red wine respectively. The above studies however, mainly focused on mixed cultures containing two different strains at a time and mostly in synthetic grape must. Additionally, the strains used were natural isolates and not commercial strains. Other work on the interaction of *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* with *S. cerevisiae* under oxygenation has also been published (Shekhawat *et al.*, 2017). However, it was done in synthetic grape must, only one-to-one mixed

fermentations were performed and only one of the strains (*T. delbrueckii*) was a commercial strain. Bagheri *et al.* (2017), also studied 7 different non-*Saccharomyces* in a multi-species consortium. The authors described how the different species in the consortium respond to the presence of *S. cerevisiae*. However, the organisms used were not commercial strains and involved *S. cerevisiae* inoculated at low populations (10^3 CFU/mL). Additionally, the above works do not investigate the implications on MLF. Other authors do cover multi-species consortia, though again, no investigation on MLF was performed (Suzzi *et al.*, 2012; Tofalo *et al.*, 2016).

Malolactic fermentation is not a true fermentation but a decarboxylation of L-malic acid to L- lactic acid with the release of CO_2 via the malolactic enzyme (*MLE*) of LAB (Lerm *et al.*, 2010). Very little has been investigated on this secondary process in terms of when wines are produced by mixed yeast cultures during AF. The course that the yeast population dynamics takes during AF may influence how MLF occurs through parameters such as, final amounts of free nitrogen (Guilloux-Benatier *et al.*, 2006) and compounds such as medium chain fatty acids or SO_2 (Lonvaud-Funel *et al.*, 1988; Carreté *et al.*, 2002; Alexandre *et al.*, 2004). Conversely, it is hypothesized that MLF can be mimicked, using specific strains of *Schizosaccharomyces pombe* and *L. thermotolerans.* The consumption of L-malic acid is performed by *Sch. pombe* and the production of L-lactic acid is performed by *L. thermotolerans* during AF separately (Benito *et al.*, 2016). Furthermore, *L. thermotolerans* and *Sch. pombe* are able to perform these processes in isolation from each other as these processes are a result of their individual metabolisms. Occasionally, MLF occurs spontaneously after AF when yeast autolysis begins and this provides some nutrients for LAB to grow. However, some strains of *T. delbrueckii* have been shown to promote MLF indirectly during AF, due to slower fermentation kinetics and therefore allowing LAB to adapt to increasing ethanol concentrations (Ramírez *et al.*, 2016). A recent study has shown how different non-*Saccharomyces,* such as *L. thermotolerans, H. uvarum, M. pulcherrima* and others may either negatively or positively effect MLF in sequential or co-fermentations and therefore effect wine aroma (du Plessis *et al.*, 2017). However, the study was only performed with mixed fermentations of single non-*Saccharomyces* such as *H. uvarum, Starmerella bacillaris* and others as well as *S. cerevisiae* and not in a complex multi-yeast system.

Complex multi-yeast starter cultures already exist, such as Melody™ (CHR. Hansen), which comprises of *S. cerevisiae, L. thermotolerans* and *T. delbrueckii* in different ratios, and Anchor Alchemy II (Oenobrands) comprising of different strains of *S. cerevisiae*. It is therefore important to expand on the works mentioned above (Suzzi *et al.*, 2012; Tofalo *et al.*, 2016; du Plessis *et al.*, 2017) and investigate the implications of complex mixed cultures (consortia) on AF and MLF.

1.2 Rationale and project aims

With the wine industry now having the option to inoculate with many commercialised strains and some products containing multiple species, it is important to investigate how these commercial starter cultures interact with one another. Additionally, investigations on how they might affect MLF and wine chemical properties should also be conducted. Previous studies on mixed–starter fermentations, were conducted on single strains of *S. cerevisiae* and selected strains of non-*Saccharomyces* species such as *T. delbrueckii, M. pulcherrima* and others (Romano, 2003; Comitini *et al.*, 2011; Sadoudi *et al.*, 2012; van Breda *et al.*, 2013; Contreras *et al.*, 2014; Jolly *et al.*, 2014; Maturano *et al.*, 2015; Ciani *et al.*, 2016; Albertin *et al.*, 2017; Shekhawat *et al.*, 2017). Furthermore, these works have mainly been conducted in synthetic grape juice and predominantly with non-commercial strains. Only a few studies have been conducted on multi-yeast systems in real grape juice (Suzzi *et al.*, 2012; Tofalo *et al.*, 2016; Bagheri *et al.*, 2017; Del Fresno *et al.*, 2017). In terms of MLF, little data has been published on the effects of non-*Saccharomyces* on MLF efficacy (Guilloux-Benatier *et al.*, 2006; Ramírez *et al.*, 2016; du Plessis *et al.*, 2017). Additionally, studies have only been done with pure or mixed cultures. Other attempts to "simulate" MLF were made (Benito, *et al.*, 2015; Benito *et al.*, 2016) but were not considered with *O. oeni* in *L. thermotolerans* and *S. cerevisiae* fermented wines.

This project investigated the population dynamics of a consortium of commercial yeast strains in Chenin blanc and Pinotage grape juice, as well as assess the volatile profiles in the resulting wines. This is the first study to investigate the potential influence of a multi-species yeast consortium on MLF in sequential fermentation conditions.

The specific aims are summarized below.

1. Investigate the population dynamics and fermentation kinetics of a multi-yeast consortium containing commercial strains compared to mixed fermentations.
2. Investigate the effect of the consortium on *O. oeni* viability and how MLF proceeds when compared to mixed fermentations.
3. Assess the mid-infrared spectra and volatile profiles following AF and MLF from the different wines.

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Chapter 2

Literature review

The use of commercial *S. cerevisiae* and non-*Saccharomyces* yeast in wine making

Chapter 2 – Literature review: The use of commercial *S. cerevisiae* and non-*Saccharomyces* yeast in winemaking

2.1. Introduction

Originally, the characteristics of wine were mainly associated with its region of origin or terroir. "*Terroir, is a concept which refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environment and applied vitivinicultural practices develops, providing distinctive characteristics for the products originating from this area. Terroir includes specific soil, topography, climate, landscape characteristics and biodiversity features"* (OIV, 2010). This biodiversity includes the microbial ecology in the vineyards ranging from filamentous fungi and yeast to bacteria. It has been found that different *terroirs* over different vintages can result in different communities of fungi and bacteria on grapes that are ready for harvesting (Bokulich *et al.*, 2014). Therefore, this influences the starting populations of microbes in grape must regardless of whether it will be inoculated or naturally fermented. Alcoholic fermentation (AF), unless inoculated, is mainly initiated by yeast genera such as *Candida, Issatchenkia, Hanseniaspora, Metschnikowia, Torulaspora, Lachancea, Pichia* and other non-*Saccharomyces* yeasts (Fleet, 2003; Oro *et al.*, 2014; Maturano *et al.*, 2015; Liu *et al.*, 2016). However, *Saccharomyces cerevisiae* will mostly dominate the latter stage of fermentations as seen in Figure 2.1.

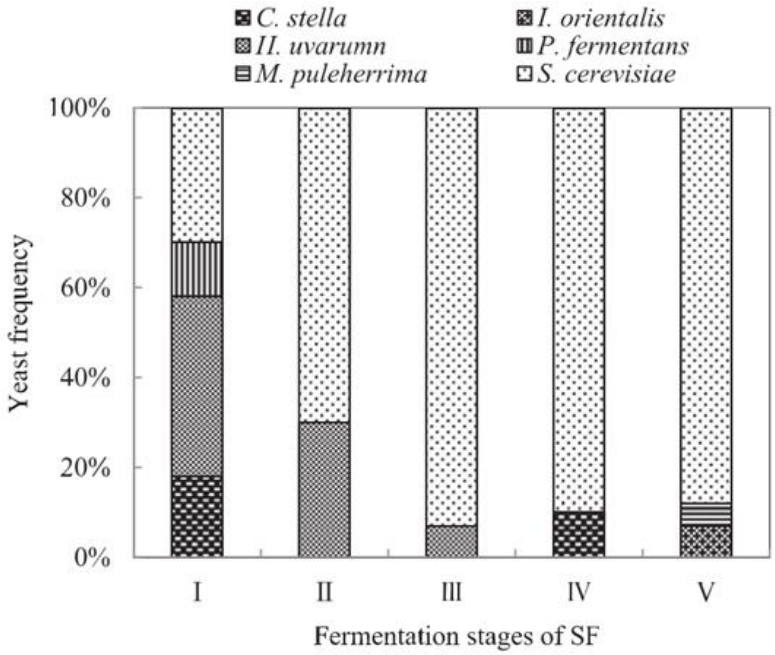

Figure 2.1 Example of population dynamics during a spontaneous fermentation (SF). Stage I : 1.100 specific gravity (SG) (Day 0), Stage II : 1.080 SG (Day 3), Stage III : 1.050 SG (Day 4), Stage IV : 1.020 SG (Day 5) and Stage V : >4 g/L sugar (end of fermentation). Taken from (Liu *et al.*, 2016)

It is important to realise however, that these fermentations are not without problems, as native *S. cerevisiae* may not be able to complete a fermentation. This may be due to biotic interactions and abiotic factors as depicted in Figure 2.2. An example of these biotic interactions is the consumption of nitrogen sources by the initial prominent level of *Hanseniaspora* or *Candida* and other non-*Saccharomyces* yeasts (Bely *et al.*, 2008; Taillandier *et al.*, 2014). If the amount of yeast assimilable nitrogen (YAN) is approximately 150 mg/L or less it will likely lead to stuck fermentations, where residual sugar will result in an incomplete fermentation (Albergaria and Arneborg, 2016). This directly determines the amount of biomass that can be reached (Tronchoni *et al.*, 2017). Spoilage microorganisms can occupy this niche to proliferate and impart undesirable characteristics to the wine. Other possible microbial interactions include cell contact (Nissen *et al.*, 2003; Arneborg *et al.*, 2005) or growth inhibitory compounds (Maturano *et al.*, 2012; Velázquez *et al.*, 2015) that may occur during a fermentation. These factors certainly influence the ability of yeasts to proliferate. Although, these biotic factors are usually strain specific for both *S. cerevisiae* and non-*Saccharomyces* species, like *Torulaspora delbrueckii* or *Lachancea thermotolerans*. Several abiotic factors also influence the fermentation process since different yeasts have different optimal temperatures for growth, different ethanol and SO_2 tolerance levels, as well as oxygen requirements (Fleet, 2003; Salvadó *et al.*, 2011; Brandam *et al.*, 2013).

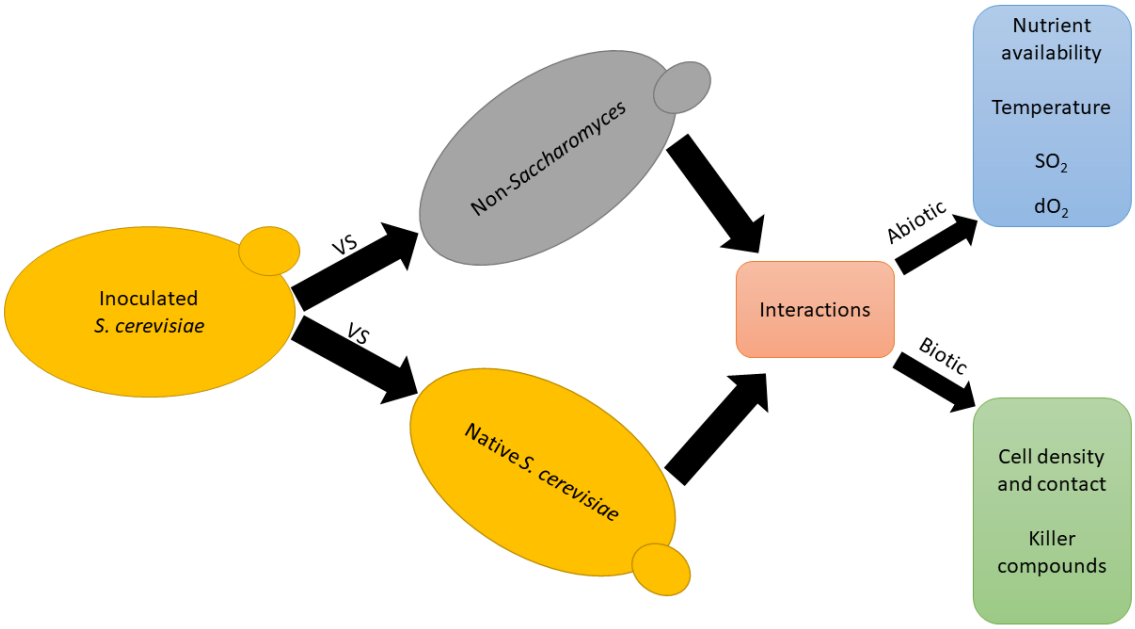

Figure 2.2 Examples of abiotic and biotic interactions that yeasts may encounter with each other in wine (Nissen *et al.*, 2003; Arneborg *et al.*, 2005; Albergaria *et al.*, 2010; Salvadó *et al.*, 2011; Brandam *et al.*, 2013; Branco *et al.*, 2014; Ciani and Comitini, 2015).

To combat the risks associated with spontaneous AF, commercialised products containing strains of *S. cerevisiae* with desirable characteristics were developed. These strains are inoculated at high cell densities (>10^6 CFU/mL), to ensure the dominance of *S. cerevisiae* from the early stages of fermentation and to complete fermentation (Belda *et al.*, 2017; Petruzzi *et al.*, 2017). The practice became immensely popular through the late 20th and early 21st centuries, however, it was not without problems. According to a review done by Capozzi *et al.* (2015), opinions were forming that due to the use of commercial strains, wines were becoming too reproducible, predictable and lacking in complexity between and within cellars when compared to natural fermentations.

An increased interest in understanding the effect that non-*Saccharomyces* yeasts, such as *Metschnikowia pulcherrima, T. delbrueckii, Starmerella bacillaris* and *Hanseniaspora* spp., to name a few, have on wines started to develop to combat this perceived lack of complexity in inoculated *S. cerevisiae* wines. The investigations found that indigenous yeasts can have positive organoleptic properties and mainly contribute during the initial stages of fermentation (Silva *et al.*, 2003; Ciani and Comitini, 2006; Bely *et al.*, 2008; Kurita, 2008; Moreira *et al.*, 2008). Some of the organoleptic properties are summarised in Figure 2.3. Interestingly, in some investigations there were instances where certain species, from *Hanseniaspora, Lachancea, Candida* and *Torulaspora,* could even persist until the middle or late stages of fermentations and contributed to aroma complexity (Mills *et al.*, 2002; Xufre *et al.*, 2006; Nisiotou *et al.*, 2007; Bely *et al.*, 2008)

Figure 2.3 Different effects that can result by using a combination of *S. cerevisiae* and industrialised non-*Saccharomyces* (Ciani and Comitini, 2015; Petruzzi *et al.*, 2017).

Commercial strains of these organisms started to appear in the 21st century and some examples are given in Table 2.1. The products not containing *S. cerevisiae* are generally incapable of completing fermentation on their own and are always suggested to be either sequentially or co-inoculated with a commercial *S. cerevisiae* strain. However, the attributes they impart on wine can be significant (Beckner Whitener *et al.*, 2015, 2016; Benito *et al.*, 2016; Liu *et al.*, 2016). To generate complexity in wines with the use of commercial non-*Saccharomyces* yeasts (Table 2.1) it is important to realise that every organism involved in winemaking can modulate aroma and flavour in some way. This literature review, therefore, addresses the studies performed on *S. cerevisiae* and strains of various non-*Saccharomyces* for their different outcomes in wine aroma or other properties.

Table 2.1 Examples of commercialized non-*Saccharomyces* and mixed starter cultures.

Product Name	Organisms	Use	Manufacturer
Biodiva™	Td	Esters and low VA	Lallemand
Flavia™	Mp	Thiols and Terpenes	Lallemand
Level 2 TD	Td + Sc	Ester and complexity	Lallemand
Concerto™	Lt	Increased lactic acid	CHR. Hansen
Frootzen™	Pk	Esters and low VA	CHR. Hansen
Harmony	Td + Lt + Sc	Varietal aromas	CHR. Hansen
Melody™	Td + Lt + Sc	Floral and fruit esters	CHR. Hansen
Prelude™	Td	Low VA	CHR. Hansen
Rhythm	Lt + Sc	Varietal aromas	CHR. Hansen
Anchor Alchemy II	Different Sc	Thiols	Oenobrands
Alpha	Td	Improved complexity	Laffort
ÉGIDE	Td + Mp	Improved complexity	Laffort

Abbreviations: Td = *T. delbrueckii* | Lt = *L. thermotolerans* | Mp = *M. pulcherrima* | Sc = *S. cerevisiae* | Pk = *P. kluyveri* | VA = Volatile acidity

2.2. Benefits of using *S. cerevisiae*

S. cerevisiae has been used extensively in the past to study cellular functioning and the biochemistry of eukaryotes (Karathia *et al.*, 2011). It has also been widely used in many industries ranging from food and beverages to pharmaceuticals and renewable energy production (Cubillos, 2016). In winemaking, *S. cerevisiae* has indeed played an integral role. This is due to a highly efficient ability to ferment sugars to ethanol and carbon dioxide as part of its primary metabolism in high sugar environments (Fleet, 2003). Apart from this, it also holds the capacity to modify aroma precursors in grape juice to their sensorial active state (Jolly *et al.*, 2014; Belda *et al.*, 2017; Petruzzi *et al.*, 2017). In Figure 2.4, the different environmental stressors that *S. cerevisiae* can survive, as well as the desired properties and processes that *S. cerevisiae* possesses, are shown. *S. cerevisiae* clearly has the means to

dominate its niche in wine. A genetic study by Steensels and Verstrepen (2014) demonstrated how *S. cerevisiae* became so specialized. The authors showed how duplication of genes for alcohol dehydrogenase (*ADH*), hexose transporters and glycolytic enzymes were key factors in fermentative capacity. In addition to this, the transcriptional mechanisms of *S. cerevisiae* have also been found to be optimized for fermentative metabolism (Steensels and Verstrepen, 2014; Petruzzi *et al.*, 2017).

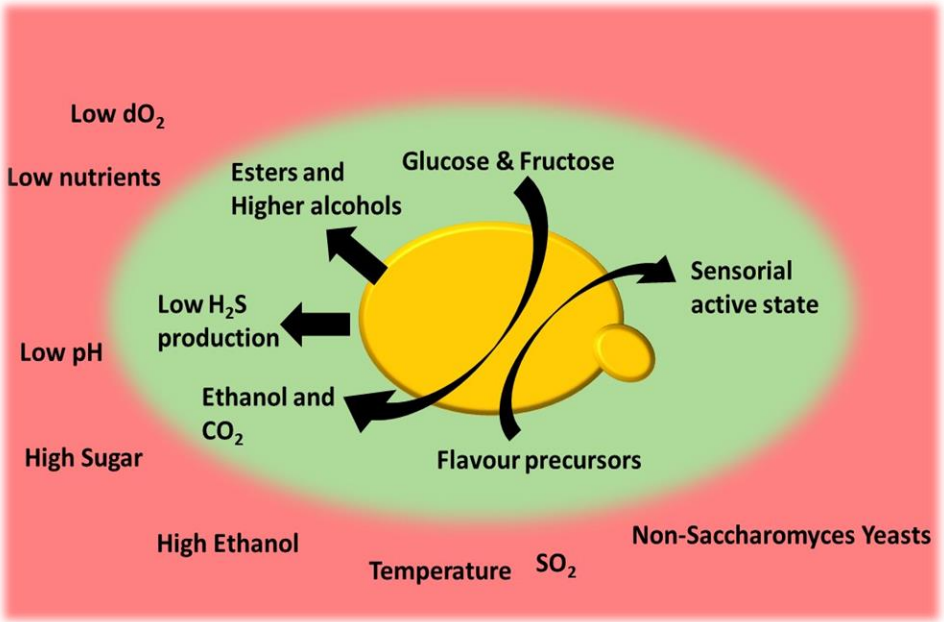

Figure 2.4 Examples of desirable processes (in green) that *S. cerevisiae* carries out during wine fermentation as well as environmental pressures (in red) that *S. cerevisiae* simultaneously withstands (Lambrechts and Pretorius, 2000; Fleet, 2003; Ciani and Comitini, 2015).

2.2.1 Fermentative Robustness

The Oxford English Dictionary defines robustness as, "The ability to withstand or overcome adverse conditions". In terms of fermentation these adverse conditions are: competition for depleting sugars, low nitrogen content, low pH, low temperature and increasing ethanol levels (Albergaria and Arneborg, 2016). The growth rate within the wine matrix naturally plays a critical role, as it has a direct correlation to how fast an organism can take-up and ferment sugars. *S. cerevisiae* under anaerobic conditions and moderate temperature is known to have a higher growth rate than other wine yeasts, such as *T. delbrueckii*, *Hanseniaspora uvarum* and *Candida stellata* as shown in a study performed by Ciani and Picciotti (1995). Moreover, Nissen *et al.* (2004) confirmed this on studies performed with *S. cerevisiae, T. delbrueckii* and *L. thermotolerans*. They determined specific glucose uptake rates (q_s) for the species mentioned and expectedly found *S. cerevisiae* to be the highest.

Apart from growth rate, the regulation of sugar metabolism is also significant. Oxygen plays a crucial role in central metabolism and three classes have been characterized, with respect to yeast. The first class is obligatory aerobic, second is facultative fermentative and finally obligatory fermentative (Jolly *et al.*, 2014), with most wine related yeast falling into the second category. However, except for *S. cerevisiae*, most of them display slow growth rates in strictly anaerobic environments. Since wine is mainly considered to be made under oxygen limiting conditions, yeasts that are facultatively fermentative, but still sensitive to low oxygen levels, will not persist as well as *S. cerevisiae* does. This results in *S. cerevisiae* sometimes being responsible for at least 50% of the sugar fermentation even when oxygen and biodiversity levels are higher in the initial stages (Albergaria and Arneborg, 2016).

S. cerevisiae has another way to survive even in the initial stages. In high sugar environments, this species will induce fermentative metabolism, regardless of the presence of oxygen. This phenomenon is known as the Crabtree effect and is present in other yeasts, such as *Torulaspora globosa, T. delbrueckii, Hanseniaspora vineae, L. thermotolerans, Hanseniaspora occidentalis* and *Zygosaccharomyces bailii* (Merico *et al.*, 2007; Goddard, 2008; Albergaria and Arneborg, 2016). However, they do not exhibit the Crabtree effect as pronounced as *S. cerevisiae.* An advantage of being Crabtree positive might be that competing organisms (if any) for substrates, are more sensitive to ethanol. Ethanol is agreed to be a broad spectrum antimicrobial (Janzen, 1977; Thomson *et al.*, 2005), which will now be produced at a significantly higher rate due to the Crabtree effect (Goddard, 2008). Although, it is important to realise that this behaviour is observed irrespective of the presence of other organisms. Certainly, in the case of *S. cerevisiae,* it has a very high tolerance for ethanol while obligatory aerobic and facultative yeasts are more sensitive. Additional to ethanol production, glycolytic flux is increased and this will limit the amount of substrate available to other organisms (Conant and Wolfe, 2007). The use of *S. cerevisiae* as a principal starter culture in winemaking therefore became of intrinsic value, since the practice led to more predictable outcomes and simultaneously cut risk of spoilage (Chambers and Pretorius, 2010).

2.2.2 Yeast biotic interactions

Apart from its robustness against the abiotic factors of a wine matrix, *S. cerevisiae* has been shown to possess numerous biotic ways of supressing other native yeasts. These mechanisms of yeast-yeast interactions were proposed in the first decade of this century, namely growth inhibiting compounds, killer toxins and cell contact (Ciani and Pepe, 2002; Nissen and Arneborg, 2003; Nissen *et al.*, 2003; Arneborg *et al.*, 2005; Pérez-Nevado *et al.*, 2006). An example of growth inhibition of *Hanseniaspora guilliermondii* and other non-*Saccharomyces* yeasts by *S. cerevisiae* was demonstrated by Albergaria *et al.* (2010). The authors found small proteinaceous compounds (2-10 kDa), termed anti-microbial peptides

(AMPs), that exhibited inhibitory activity against *H. guilliermondii* that normally can persist during winemaking. Studies have also shown how similar mechanisms involving AMPs, such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) protein fragments (Branco *et al.*, 2014), were expressed by *S. cerevisiae* against other yeasts and *Oenococcus oeni* (Albergaria *et al.*, 2010; Branco *et al.*, 2015). The study by Albergaria *et al.* (2010) found that these protein fragments (<10 kDa) were active against *H. guilliermondii, T. delbrueckii, Kluyveromyces marxianus* and *L. thermotolerans.* However, the *GAPDH* fragments were only produced in the late stages of exponential growth and their production was associated with starvation and apoptosis via metacaspase (Delgado *et al.*, 2003; Silva *et al.*, 2011).

Killer toxins are also a mechanism for interactions between yeasts. Killer toxins K1, K2, K28, and Klus are reviewed by Schaffrath *et al.* (2018). These K1, K2 and K28 toxins, unlike the *GAPDH* protein fragment, are dimers of two peptides covalently linked by a di-sulphide bridge (Bostian *et al.*, 1984; Schmitt and Tipper, 1995). They bind to the cell membrane of killer sensitive strains and cause membrane permeability. Two factors need to be realised when considering killer toxin production. Firstly, toxins may only be effective against the same or similar species (Wloch-Salamon *et al.*, 2008). Secondly, the production of toxins syphons off energy and nutrients in metabolism (reduces fitness), that could have been used for growth and therefore hinders their ability to be competitive for resources (Pintar and Starmer, 2003; Wloch-Salamon *et al.*, 2008). An investigation was performed on nutrient availability and dispersion of killer strains of *S. cerevisiae,* by Wloch-Salamon *et al.* (2008). The authors concluded that dispersion causes fitness reduction even when nutrients are abundant. It is important to realise however, that the killer phenotypes and killer sensitive yeasts are strain specific and therefore cannot be applied in a generalised sense. From an industrial perspective, it might be advantageous to use killer active *S. cerevisiae* as the killer toxins might help prevent the proliferation of *Brettanomyces* and other spoilage non-*Saccharomyces* or lactic acid bacteria (LAB) such as *Pediococcus*. These organisms may alter certain white wine styles. Whereas in the case of most red and certain white wines it will be disadvantageous to inhibit LAB such as *O. oeni* or *Lactobacillus plantarum* for obvious reasons where malolactic fermentation (MLF) is required for their respective wine-styles.

Cell-to-cell contact is another example of interaction between *S. cerevisiae* and other yeasts, such as *L. thermotolerans* and *T. delbrueckii.* This interaction was shown by Nissen *et al.*, (2003). The group concluded that the growth arrest of the *L. thermotolerans* and *T. delbrueckii* was not due to nutrient or other abiotic factors, but rather due to the high presence of viable *S. cerevisiae*. The study's findings were somewhat confirmed by Renault *et al.*, (2013), where fermentations were conducted in double-compartment bioreactors with *T. delbrueckii* and *S. cerevisiae*. This setup allowed for the separation of the different species

while enabling the matrix to be shared. Therefore, if growth inhibition of *T. delbrueckii* was still observed the cause would be either nutrient depletion, the production of ethanol, killer toxins, AMPs or SO_2 by *S. cerevisiae*. In agreement with Renault *et al.* (2013), similar findings where observed for *L. thermotolerans* by Luyt (2015). In contrast however, it was found that *L. thermotolerans* was also able to inhibit *S. cerevisiae*, though to a lesser extent. It was also shown that if abiotic factors such as oxygen availability are altered, the phenomenon can be alleviated such as when increased dissolved oxygen is available. Other work done by Shekhawat *et al.* (2016) showed that oxygenation during fermentation, enhanced the growth and persistence of *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* in mixed fermentation with *S. cerevisiae*. The population levels could reach between 10^9 and 10^{10} CFU/mL while *S. cerevisiae* reached lower population levels for different oxygenation strategies. The higher the dissolved oxygen the lower the population level was observed for *S. cerevisiae*. However, one must realise that *S. cerevisiae* was inoculated at a 1:10 lower inoculation level than the aforementioned non-*Saccharomyces* yeasts. Additionally, the strain of *S. cerevisiae* (Cross evolution-285) was also different to the strain used in Luyt (2015) (EC1118) indicating that strain variance and the environment of the fermentation can affect yeast-yeast interactions.

Wang *et al.* (2016) also investigated interactions between *S. cerevisiae* and several strains from species, such as *H. uvarum, Starmerella bacillaris, M. pulcherrima,* and *T. delbrueckii*. The authors found that cell free supernatants, from *S. cerevisiae* fermentations, influenced cellular viability differently for every species tested and strain differences were also observed. Cells of *T. delbrueckii* and *Starm. bacillaris* survived longer with *S. cerevisiae* than *H. uvarum* and *M. pulcherrima.* In a previous investigation between *H. uvarum* and *S. cerevisiae* (Wang *et al.*, 2015), the authors speculated that it might be, in addition to nutrient depletion (carbon and nitrogen sources), the total amount of sugar metabolized rather than the residual sugar level that has an impact on growth. Fermentations with 200 g/L of initial sugar showed a more rapid decline in *H. uvarum* viability than 100 g/L trials. The effect of ethanol was not found to be detrimental to *H. uvarum* growth, confirming previous findings where high tolerance for ethanol was observed in *H. uvarum* (Pina *et al.*, 2004). Wang *et al.* (2015) also suspected the action of AMPs, such as the previously mentioned *GAPDH* protein fragments, and confirmed the presence of such AMPs in their follow up study (Wang *et al.*, 2016).

Yeast interactions clearly vary with strains, environmental parameters and matrix composition as seen in the work of Wang *et al.* (2016) and other studies (Renault *et al.*, 2013; Luyt, 2015; Shekhawat *et al.*, 2017). Certain strains of *S. cerevisiae* clearly possess biotic means of managing its environment under normal conditions of winemaking. Therefore, using strains that have been commercialised for these abilities in preventing growth of spoilage non-

Saccharomyces, such as *Brettanomyces bruxellensis* and bacteria alike can help ensure certain wine styles.

2.3. Benefits of using non-*Saccharomyces* yeasts

The recent trend of sequential or co-inoculation with certain species of non-*Saccharomyces* yeasts (Table 2.1) and *S. cerevisiae,* in order to imbue wine aroma with the complexity of spontaneous ferments, has been investigated (Jolly *et al.*, 2014; Padilla *et al.*, 2016; Whitener *et al.*, 2017). By using non-*Saccharomyces* yeasts, such as *M. pulcherrima, Pichia kluyveri* and other species and knowing how to improve their persistence in wine, one can modulate the aroma profile of resulting wines. For instance, several studies showed how *Starm. bacillaris, L. thermotolerans, M. pulcherrima* and *T. delbrueckii* are able to reduce ethanol and modulate other parameters in wine (Contreras *et al.*, 2014; Quirós *et al.*, 2014; Englezos *et al.*, 2015, 2016; Varela *et al.*, 2016; Wang *et al.*, 2016; Shekhawat *et al.*, 2017, 2018). The findings were that each yeast responded differently to the increased dO_2 levels and distinct species-specific chemical volatile profiles were associated with non-*Saccharomyces* persistence in wine.

Many different organoleptic properties may be altered due to the presence of *L. thermotolerans* or *M. pulcherrima* and other non-*Saccharomyces* yeasts. Additional examples are increased glycerol from mixed cultures with *Starmerella bombicola* (Ciani and Ferraro, 1998; Comitini *et al.*, 2011) and increased thiol levels with *P. kluyveri* (Anfang *et al.*, 2009). Additionally, yeasts such as *T. delbrueckii, L. thermotolerans,* and *M. pulcherrima* might yield reduced acetic acid and ethanol levels when the fermentations are aerated or performed at lower temperatures (García *et al.*, 2010; Gobbi *et al.*, 2013; Morales *et al.*, 2015).

2.3.1 Volatile acidity

Volatile acidity (VA) relates to the acetic acid (and sometimes other acid) levels in wine (Zoecklein *et al.*, 1995). It is considered to be a sign of spoilage in wine when out of balance with other parameters such as ethanol levels, residual sugar and glycerol (Zoecklein *et al.*, 1995). Other acids that may contribute to volatile acidity are also lactic, formic, butyric and propionic acids. Acetic acid arises from the sugar metabolism of acetic acid bacteria, lactic acid bacteria and all yeasts found in wine (Fleet, 2003). Additionally, certain strains of *S. cerevisiae* have been shown to be inhibited by high levels of acetic acid (Ludovico *et al.*, 2001).

Yeasts such as *T. delbrueckii* and *M. pulcherrima* have been shown through recent years to lower acetic acid levels in wines, as well as modifying other parameters such as glycerol and ester concentrations (Ciani *et al.*, 2006; Bely *et al.*, 2008; Renault *et al.*, 2009; García *et*

al., 2010; González-Royo *et al.*, 2014). Indeed, products (containing *T. delbrueckii),* such as ones listed earlier in Table 2.1, are advertised to be low producers of volatile acidity and improve aroma complexity. A recent study by Puertas *et al.* (2017) performed an investigation on industrial strains of *T. delbrueckii* (TD291) and *S. cerevisiae* (QA23). The experiments were in a semi industrial scale to assess acetic acid production in Chardonnay and Palomino cultivars over different vintages. The authors noted lower levels of acetic acid in fermentations sequentially inoculated; as opposed to control fermentations for the 2011 Chardonnay and Palomino vintages. However, higher amounts were found in the sequential fermentations when compared to controls in the 2012 Palomino vintage. No significant differences were observed between sequential and control fermentations for the Chardonnay 2012 vintage. In Whitener *et al.* (2017) *T. delbrueckii* (BIODIVA) and *S. cerevisiae* (Enoferm M2) and several other yeasts were evaluated in Shiraz fermentations. Specifically, no significant differences were noted for *T. delbrueckii* paired with *S. cerevisiae* in volatile acidity concentrations when compared to the controls. This was also observed alongside *M. pulcherrima* (FLAVIA) and *P. kluyveri* (FROOTZEN) ferments. However, the volatile acidity was significantly higher in the other non-*Saccharomyces* yeast fermentations, particularly in the fermentations with *Kazachstania aerobia* (IWBT Y845). These above studies demonstrate the variance between species, strain, vintage and cultivar. Additionally, certain strains of non-*Saccharomyces* may produce more VA such as *Hanseniaspora vinea (reference??).*

2.3.2 Acid modulation

Acid modulation is a topic of interest in winemaking (Fleet, 2008). *Lachancea thermotolerans and Schizosaccharomyces pombe* shows potential in acid modulation as these organisms may possess the ability to produce and consume acids in wine (Gobbi *et al.*, 2013; Benito *et al.*, 2016).

A study done by Gobbi *et al.* (2013) shows comparisons between *L. thermotolerans* 101 and *S. cerevisiae* (EC1118) in mixed fermentations. Different setups of simultaneous and sequential inoculums were used. The findings illustrated increases in D,L-lactic acid and glycerol, while lower volatile acidity, ethanol and pH was observed when compared to *S. cerevisiae* controls. Benito *et al.* (2016) had similar findings where fermentations containing *L. thermotolerans* (CONCERTO) showed trends of increasing lactic acid during fermentation. The authors also demonstrate how *L. thermotolerans*, combined with *Schizosaccharomyces pombe* 4.5, can mimic the outcome of MLF when compared to *S. cerevisiae* 88 and *Oenococcus oeni* 217 controls. Interestingly, it was demonstrated that in the trials, malic acid was degraded by *Sch. pombe* (regardless of the presence of *L. thermotolerans*), and lactic acid was produced by *L. thermotolerans* (regardless of the presence of *Sch. pombe*). The production of lactic acid and degradation of malic acid was not

directly linked as in the case with MLF carried out by *O. oeni.* Rather, it was as result of the individual metabolisms of *L. thermotolerans* and *Sch. pombe* respectively. Figure 2.5 illustrates how yeast such as *L. thermotolerans* can produce lactic acid compared to *O. oeni*.

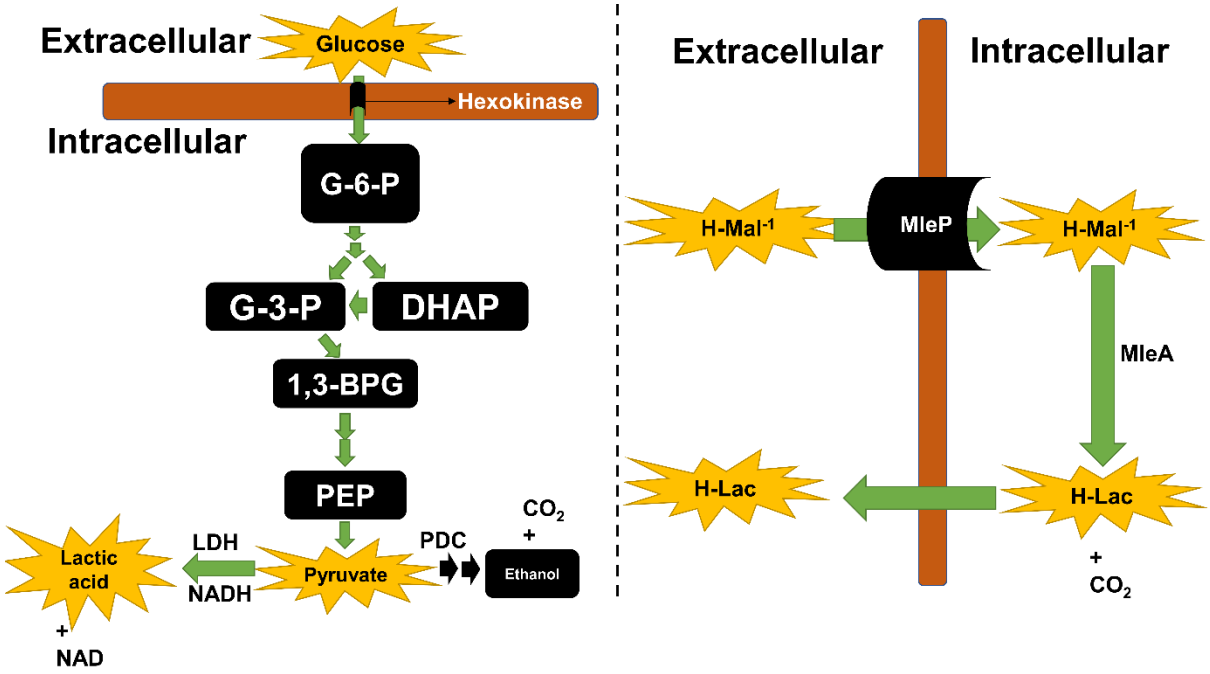

Figure 2.5 Simplified biochemical pathways for lactic acid production by yeast (Left) and lactic acid bacteria (Right).

Yeast like *L. thermotolerans* use sugar metabolism with lactate dehydrogenase (*LDH*) and NADH as the final catalytic step (Witte *et al.*, 1989) to maintain redox balance. Lactic acid bacteria like *O. oeni* use a malolactic permease (*mleP*) to import malic acid with a proton (≤pH 4 environment) and de-carboxylate to lactic acid using the malolactic enzyme (*mleA*) (Fugelsang and Edwards, 2007). In *O. oeni* the lactic acid is then exported out to maintain a pH gradient for ATP production. Pathways were adapted from Fialho *et al.* (2016) and Fugelsang and Edwards (2007).

Figure 2.6 depicts the consumption of malic acid in yeast such as *Sch. pombe* versus lactic acid bacteria. It is important to note, that in comparison to control fermentations produced with *S. cerevisiae* and *O. oeni,* significantly higher amounts of lactic acid were found in wines that contained *L. thermotolerans*.

The above findings illustrate that the combination of *L. thermotolerans* and *Sch. pombe* has an added benefit of enhancing total acidity on top of degrading malic acid without having to subject the wines to sequential MLF. This may be applied to areas where lower amounts of initial malic acid may occur due to climatic conditions.

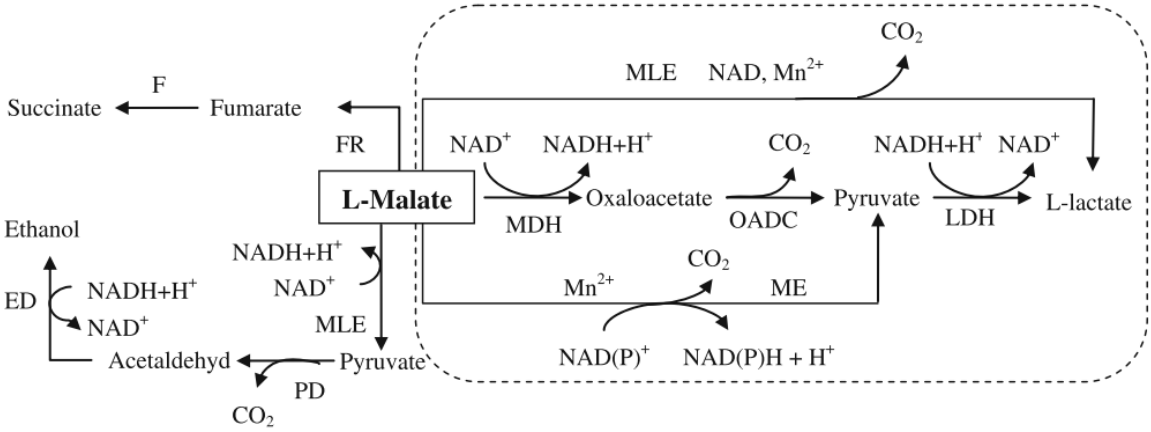

Figure 2.6 Detailed malic acid metabolism in yeast (non-dashed section) versus malic acid metabolism in lactic acid bacteria (dashed section). Enzymes involved are malate dehydrogenase (*MDH*), malic enzyme (*ME)*, malolactic enzyme (*MLE*), oxaloacetate decarboxylase (*OADC*), lactate dehydrogenase (*LDH)*, pyruvate decarboxylase (*PD)*, ethanol dehydrogenase (*ED*), fumarase (*F*) and fumarate reductase (*FR*). Taken from (Su *et al.*, 2014).

2.3.3 Aroma compounds

Many studies have involved different non-*Saccharomyces* yeasts, such as *P. kluyveri, M. pulcherrima* and *Hanseniaspora* species in the venture to improve wine aroma and quality (Rojas *et al.*, 2003; González-Royo *et al.*, 2014; Jolly *et al.*, 2014). A review by Mateo and Maicas (2016), comprehensively covers the contributions that several non-*Saccharomyces* yeasts may give. These include glycerol production, reducing ethanol or acetic acid, de novo volatile compound synthesis and enzyme activities such as β-glucosidase. *M. pulcherrima* has received a lot of attention for its β-glucosidase production in varietal aroma enhancement, as well as modification of wine parameters such as, ethanol, medium chain fatty acids, fusel alcohols, esters and glycerol (Rodríguez *et al.*, 2007, 2010; Contreras *et al.*, 2014; Morales *et al.*, 2015; Shekhawat *et al.*, 2017).

Varietal aroma is linked to certain classes of compounds based on the cultivar such as monoterpenes, C13-norisoprenoides and aromatic alcohols that are either in their volatile or non-active mono- or disaccharide glycosidic forms (Maicas and Mateo, 2005; Swiegers *et al.*, 2005). The non-active forms of these compounds are more abundant in the grape juice matrix and require hydrolysis to be rendered active. Yeasts such as *M. pulcherrima* (Rodríguez *et al.*, 2010) and *P. kluyveri* (Steensels and Verstrepen, 2014) are examples of producers of β-D-glucosidase which is responsible for the hydrolysis of the above compounds. Although, it is important to note that the reaction requires the preliminary step carried out by α-L-arabinofuranosidase, α-L-rhamnosidase and β-D-apiosidase (Bayonove, 1988; Rodríguez *et al.*, 2007; Villena *et al.*, 2007) as seen in Figure 2.7. It is hypothesized that *M. pulcherrima* and

P. kluyveri are some of the many yeasts that are therefore able to enhance varietal aroma. Studies indeed show an increase of terpenols in mono-cultures and the expression of β-glucosidase (Steensels and Verstrepen, 2014). However, these trends were not to the same extent and with some differences in results. The differences between terpenol trends occur in the studies with co-cultures of *S. cerevisiae* and *M. pulcherrima* (Rodríguez *et al.*, 2010; Sadoudi *et al.*, 2012). In Rodríguez *et al.* (2010), the authors hypothesized that the *S. cerevisiae* strain used was capable of transforming nerol and geraniol into α-terpineol at must pH, since similar trends were seen in *S. cerevisiae* mono-cultures. Therefore, no significant differences were seen in terpenol concentrations for co-culture fermentations. It is important to remember that in the case of mono-cultures, *M. pulcherrima* is not very fermentative and will struggle to complete fermentation. Therefore, a sequential or co-inoculation is still required. Sadoudi *et al.* (2012), showed however, a synergistic effect between *S. cerevisiae* and *M. pulcherrima* in co-culture for terpenol compounds. Bučková *et al.* (2018) also showed that not all strains of *M. pulcherrima* express β-glucosidase.

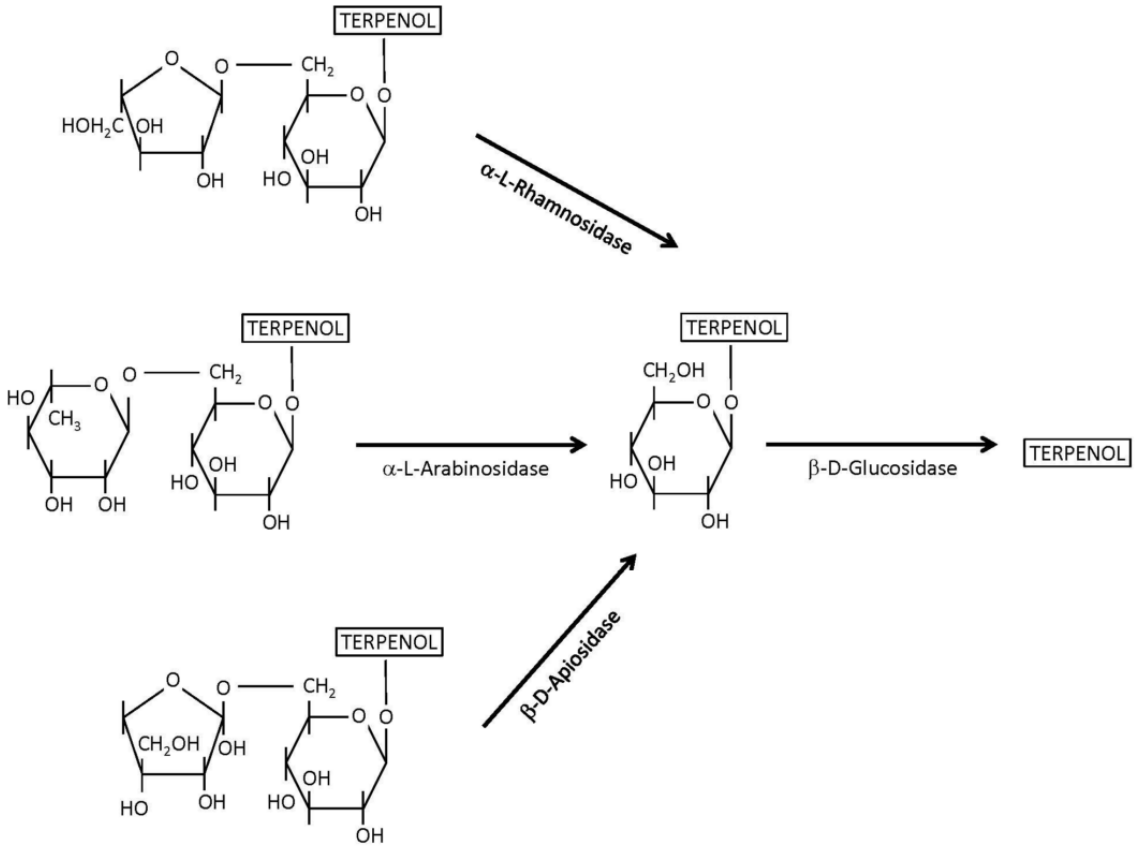

Figure 2.7 Enzymatic conversion of disaccharide precursors to terpenol. Taken from (Mateo and Maicas, 2016).

Fermentative aroma is derived from classes like higher alcohols and esters are also a matter of interest that non-*Saccharomyces* like *M. pulcherrima* and others have shown potential in producing. González-Royo *et al*. (2015) observed significantly higher levels of fusel

alcohols. However, it should be noted that only 3-Methyl-1-butanol appeared to be contributing to the difference as shown in Table 2.2. Levels of higher alcohols should be retained at relatively low levels as they can contribute negatively to wine aroma.

Table 2.2 Examples of higher alcohols detected in *M. pulcherrima* co-cultures and *S. cerevisiae* control

Higher Alcohols (mg/L)	***S. cerevisiae* (control)**	***M. pulcherrima* co-culture**
3-Methyl-1-butanol	178.6 ± 11.3	231.0 ±23.8
β-Phenylethanol	53.6 ± 3.3	48.2 ± 1.8
2-Methylpropanol	22.2 ± 2.1	29.9 ± 1.5
1-Hexanol	0.92 ± 0.06	0.92 ± 0.05
cis-3-hexen-1-ol	0.55 ± 0.03	0.54 ± 0.02
Benzyl alcohol	0.01 ± 0.00	0.01 ± 0.01
Methionol	1.04 ± 0.13	0.64 ± 0.03
1-Butanol	0.51 ± 0.04	0.52 ± 0.03
Total	257.4 ± 16.8	311.7 ± 22.6

With regards to esters, it was found in Shiraz and Chardonnay fermentations investigated by Varela *et al.* (2016), that higher levels of ethyl acetate were found in treatments that involved *M. pulcherrima* (AWRI1149) than in controls with *S. cerevisiae*. It should be noted that the total esters, excluding ethyl acetate, show lower levels in all treatments when compared to *S. cerevisiae* fermentations as a control in Chardonnay. This also shows the effect of cultivar choice and demonstrates that aroma quantitative changes are a factor of both yeast strain and cultivar. Though, the trend is evident in other studies for the yeast strain choice regardless of cultivar (González-Royo *et al.*, 2014; Benito *et al.*, 2015; Varela *et al.*, 2017). The authors also show the effect that inoculation dosage may have at the start of fermentation. However, it was only tested for *Saccharomyces uvarum* at 10^6, 10^5 and 10^4 CFU/mL. Additionally, when *L. thermotolerans* has been used, lower concentrations of certain major volatiles such as 2-methyl-1-proponol, 3-methyl-1-butanol and acetate/ethylic esters were observed (barring ethyl acetate). Conversely, higher amounts of 2-phenylethanol were observed for *L. thermotolerans* (Benito *et al.*, 2016). It should be considered that elevated levels of esters may impart negative effects on wine (Varela *et al.*, 2016). Other parameters that can be altered by *T. delbrueckii* include higher varietal thiols, 2-phenylethanol and fatty acid production (Comitini *et al.*, 2011; Azzolini *et al.*, 2012; van Breda *et al.*, 2013), however, other findings have reported that no changes or significant differences in these parameters can occur (Petruzzi *et al.*, 2017; Whitener *et al.*, 2017). This may be due to strain variance as well as vintage and varietal differences.

Considering the above, it is important to carefully select yeasts like *M. pulcherrima, L. thermotolerans* or *P. kluyveri* based on their previous performances and pair it with a

suitable cultivar to bring about an aromatic profile that is desirable since both factors play a major role.

2.3.4 Low ethanol production

Technology has improved to include winemaking practices centred around physical alcohol stripping after AF was performed (Belisario-Sánchez *et al.*, 2009; Catarino and Mendes, 2011; Schmidtke *et al.*, 2012). Unripe berries are also often used in order to produce low ethanol wines (Canals *et al.*, 2008; Kontoudakis *et al.*, 2011). Additionally, attempts have been made to alter *S. cerevisiae* via directed metabolism and evolution in order to reduce ethanol production during AF. However, these attempts have been difficult due to the tight regulation of pyruvate metabolism under anaerobic conditions (Quirós *et al.*, 2013; Rossouw *et al.*, 2013).

Lower ethanol production in wine has therefore been a consistent theme in the studies performed on various non-*Saccharomyces* yeasts, like *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima.* Mainly, the methods involve increasing the available oxygen in the matrix (Morales *et al.*, 2015; Shekhawat *et al.*, 2017, 2018). The concept involves increasing the dO_2 levels, at specific intervals, to induce a respirative metabolism of sugar instead of fermentative. The advantage of taking this biological approach apart from the consolidation of fermentation and lowering alcohol is that other parameters, such as glycerol and acetic acid, are also modified versus using the physical methods above. The study performed by Morales *et al*. (2015), using *S. cerevisiae* (EC1118) and *M. pulcherrima* (CECT12841), showed that ethanol, glycerol and acetic acid were affected by oxygenation. Specifically, mixed cultures exhibited lower ethanol levels when dO_2 was increased (Figure 2.9). The study exhibited a trend that when *S. cerevisiae* is used, in conjunction with *M. pulcherrima,* lower levels of ethanol are reached. Other studies have also found lower levels of ethanol when sparging with O_2 using *M. pulcherrima, T. delbrueckii* and *L. thermotolerans* (Shekhawat *et al.*, 2017, 2018). It is important to realise that final ethanol levels are also dependent on starting sugars, so it is imperative to monitor the sugars in the berries before harvest. Indeed, other strains such as *T. delbrueckii, H. uvarum* and *L. thermotolerans* have also been investigated for lowering ethanol levels and altering other standard parameters of wine (García *et al.*, 2010; Gobbi *et al.*, 2013; Contreras *et al.*, 2014; Varela *et al.*, 2016, 2017).

In short, the use of non-*Saccharomyces* like *M. pulcherrima* or *T. delbrueckii* and others with the combined use of increasing dO_2, through the means of punch downs or pump overs (du Toit *et al.*, 2006; Moenne *et al.*, 2014), allows the reduction of ethanol (although by smaller amounts compared to physical means), modification of standard parameters and modulation of volatile compounds (García *et al.*, 2010; Gobbi *et al.*, 2013; Contreras *et al.*, 2014; Varela *et al.*, 2016, 2017; Shekhawat *et al.*, 2017, 2018). However, physical methods such as

distillation under vacuum, evaporation and others can even achieve alcohol free wines whereas using biological means can result in a relatively small change (Figure 2.9) . By using physical means one can then control the amount of ethanol to blend back. However, the methods will naturally require extra energy and therefore costs to employ.

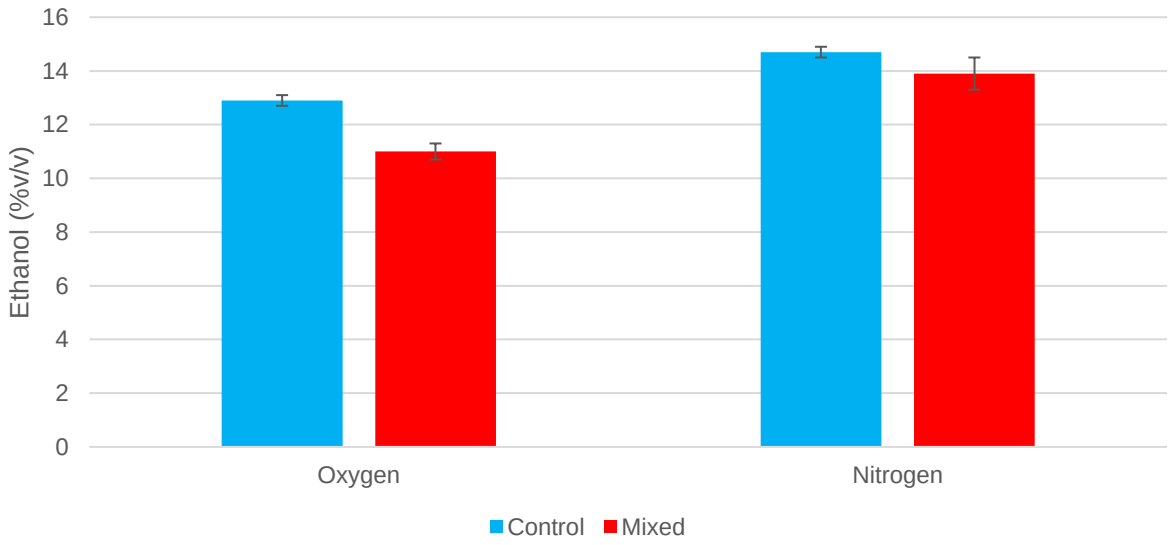

Figure 2.9 Ethanol changes due to oxygen (O_2) sparging when compared to inert gas (N_2) between *S. cerevisiae* (Control) and *M. pulcherrima* + 10% *S. cerevisiae* mixed cultures (Mixed). Data adapted from Morales *et al*. (2015).

2.3.5 Multi-yeast consortia

The use of many of the non-*Saccharomyces* yeasts mentioned so far has mainly involved one-to-one mixed fermentations with *S. cerevisiae.* Attention is also being focussed on the use of multi-species consortia (Xufre *et al.*, 2006; Wang *et al.*, 2014; Bagheri *et al.*, 2017). Indeed, there are now multi species starter cultures from yeast manufacturers on the market (Table 2.1). It is imperative that the same investigations involving environmental parameters of wine and strain combinations are investigated in consortia setups. This is to gain better knowledge on the topic and to be able to exploit the notion to its fullest.

Wang *et al.* (2014) studied mixed fermentations of equal inoculums of *H. uvarum, Starm. bacillaris* and *S. cerevisiae*. The data shows that between one-to-one mixed fermentations and the multi-yeast fermentations, the populations dynamics of each yeast was similar. Indicating that *S. cerevisiae* (when high in populations at the beginning) can have an impact on the growth of multiple non-*Saccharomyces* such as *H. uvarum* or *Starm. bacillaris*. Bagheri *et al*. (2017) however, investigated 8 species (Table 2.3) in a consortium. A mutualism was found between *H. vineae* and *S. cerevisiae*, while antagonism existed between *Wickerhamomyces anomalus* and *S. cerevisiae*. The same consortium was used in Bagheri's

PhD dissertation (2018) to evaluate the environmental parameters, such as temperature and SO_2 on the consortium.

To summarise, each organism will produce their response similarly in one-to-one fermentations and consortia provided that the fermentation parameters were similar between the two setups. From the data that is available, it is apparent that different temperatures, SO_2 levels and the presence of *S. cerevisiae* promote the growth of different non-*Saccharomyces* species. Additionally, aroma profiles can be different for each setup and mainly attributed to the different non-*Saccharomyces* that survives (Suzzi *et al.*, 2012; Tofalo *et al.*, 2016; Bagheri *et al.*, 2017; Del Fresno *et al.*, 2017).

Table 2.3 Yeast species used in the consortium of (Bagheri *et al.*, 2017)

Yeast Species	Strain number
M. pulcherrima	Y981
Pichia terricola	Y974
Starm. Bacillaris	Y975
Candida parapsilosis	Y842
W. anomalus	Y934
L. thermotolerans	Y973
H. vinea	Y980
S. cerevisiae	EC1118

2.4. Conclusion

The use of both *S. cerevisiae* and non-*Saccharomyces* yeasts such as *M. pulcherrima, T. delbrueckii, L. thermotolerans* and the others mentioned in this chapter, can be an advantage to the wine-maker. If one modulates certain properties in wine while wishing to maintain the insurance of complete fermentations; one needs to think carefully of strain compatibility and inoculation techniques such as sequential or co-inoculation or higher doses of non-*Saccharomyces* relative to *S. cerevisiae.* Some characteristics are common between most non-*Saccharomyces.* For example, *M. pulcherrima* and *L. thermotolerans* when exposed to a parameter, like increased dissolved oxygen, will grow better, modify aroma profile, albeit each profile will be different, and standard parameters, such as decreasing ethanol or acetic acid. However, some changes are more specific to a group of organisms belonging to the same species, like lactic acid production in *L. thermotolerans*. A lot of the findings in studies showed trends for specific strains and fermentation setups, indicating that different outcomes may occur if any other factors such as types of strains used, number of strains used, timing of inoculation and fermentation parameters are changed.

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Chapter 3

Research Chapter

Multi-species wine yeast consortium population dynamics and its effects on malolactic fermentation

Chapter 3 Multi-species wine yeast consortium population dynamics and its effects on malolactic fermentation

3.1 Introduction

Winemaking primarily involves yeast (*Saccharomyces* and non-*Saccharomyces* species) for the conversion of monomeric sugar to ethanol during alcoholic fermentation (AF). However, certain wine styles require the use of lactic acid bacteria (LAB) such as *Oenococcus oeni* or *Lactobacillus plantarum* for a secondary fermentation, known as malolactic fermentation (MLF), which entails the decarboxylation of L-malic acid to L-lactic acid and carbon dioxide. This is done to lower perceived acidity and achieve microbial stability (du Toit *et al.*, 2011; Bartowsky *et al.*, 2015).

The use of non-*Saccharomyces* yeast in co-cultures with *Saccharomyces cerevisiae* in one-to-one pairings, has been extensively studied and reviewed (Romano, 2003; Comitini *et al.*, 2011; Sadoudi *et al.*, 2012; van Breda *et al.*, 2013; Contreras *et al.*, 2014; Jolly *et al.*, 2014; Maturano *et al.*, 2015; Ciani *et al.*, 2016; Albertin *et al.*, 2017; Shekhawat *et al.*, 2017). In general, non-*Saccharomyces* yeasts are either weak fermenters (such as *Metschnikowia* and *Pichia*) or moderate fermenters (*Lachancea, Candida, Hanseniaspora* or *Torulaspora*) compared to *S. cerevisiae* and their contribution to fermentation is influenced by the onset of *S. cerevisiae* domination. However, their presence during fermentation has been shown to yield altered wine chemical profiles compared to *S. cerevisiae* monoculture fermentations under different conditions, such as temperature or *S. cerevisiae* lag phase (Maturano *et al.*, 2015; Albertin *et al.*, 2017). Limited studies on inoculated consortia have been conducted to investigate the population dynamics of the yeasts and resulting chemical compositions of wines (Xufre *et al.*, 2006; Suzzi *et al.*, 2012; Wang *et al.*, 2014; Bagheri *et al.*, 2017). Different doses of strains, temperature and SO_2 concentrations were investigated to evaluate the population dynamics of the consortium and the chemical compositions resulting from the treatments (Bagheri , 2018). It was found that *Wickerhamomyces anomalus* and *Hanseniaspora vinea* exhibited suppressed and promoted growth respectively when in the presence of *S. cerevisiae*. Additionally, *Starmerella bacillaris* was found to be synergistic with *S. cerevisiae* but only when inoculated at higher population levels. Lastly the findings confirmed other studies where it has been observed that a signature of a yeast is detected in the aromatic profile if the yeast is able to persist longer in a fermentation (Sadoudi *et al.*, 2012; Gobbi *et al.*, 2013; Jood *et al.*, 2017). However, standard parameters such as glycerol, ethanol and lactic acid were not measured. Other authors do cover these parameters with regards to certain non-*Saccharomyces* species such as *L. thermotolerans, M. pulcherrima, H. uvarum*

and *Starm. bacillaris* (Moreira *et al.*, 2008; Sadineni *et al.*, 2012; Englezos *et al.*, 2015; Morales *et al.*, 2015; Benito *et al.*, 2016; Shekhawat *et al.*, 2017).

Most of the focus in the studies involving the aforementioned non-*Saccharomyces* yeasts, is on AF alone. A few studies and reviews looked at the effect of these yeasts on MLF (Lerm *et al.*, 2010; du Plessis *et al.*, 2017; Balmaseda *et al.*, 2018; Gammacurta *et al.*, 2018). In the review of Balmaseda *et al.* (2018), it is stated that MLF is influenced by yeast metabolism. This can range from being inhibitory to stimulatory depending mainly on factors such as, starting parameters of the juice, competition for nutrients and the production of inhibitory compounds by yeast. Firstly, if the initial sugars are too high in grape juice, the resulting ethanol levels of the wine might be too high for most LAB strains to survive. Using non-*Saccharomyces* species, such as *M. pulcherrima* and *L. thermotolerans,* may help with this since they have been shown to result in lower ethanol levels in wine under aeration (Morales *et al.*, 2015; Shekhawat *et al.*, 2018). Testing the effects of these strains on LAB in anaerobic setups should be investigated too. Secondly, since LAB have complex nutrient requirements, the consumption of certain nutrients, such as nitrogen sources and malic acid are also of importance during AF by yeast. Using yeast that have high nitrogen demands or that consume malic acid such as *T. delbrueckii* (Belda *et al.*, 2015), *Starm. bacillaris* (Tofalo *et al.*, 2012; du Plessis *et al.*, 2017) or *Schizosaccharomyces pombe* (Benito *et al.*, 2016) may deplete the matrix and prevent LAB from growing. Lastly, the production of metabolites such as medium chain fatty acids or sulphur dioxide, in addition to ethanol (Nehme *et al.*, 2008) by yeast, may affect LAB. In du Plessis *et al.* (2017), the effects of one-to-one co-inoculations for several non-*Saccharomyces* yeasts with *S. cerevisiae* (during AF) on LAB growth and MLF kinetics were assessed. The findings showed that, if yeast strains were compatible with LAB, MLF can occur in mixed fermentations. Additionally, different aroma profiles were detected between wines that went through MLF when compared to their respective *S. cerevisiae* controls.

The lack of studies using multi yeast species in a consortium motivated this study to assess a multi-species yeast consortium population dynamic and its potential influence on *O. oeni* viability using classical microbiological techniques. Additionally, AF and MLF kinetics were assessed using enzymatic analysis and (ATR) infrared spectroscopy. Lastly, the final wine chemical compositions were investigated using gas chromatography coupled to mass spectrometry using an untargeted approach.

3.2 Methods and Materials

3.2.1 Method optimization for 2018 experiments

In the first year (2017) micro-fermentations were done in Cabernet Sauvignon obtained from Uva Mira Mountain Vineyards (Stellenbosch, South Africa) to optimize several parameters. These include thermo-vinification conditions, inoculum preparation, yeast enumeration methods and analytical methods. The data that was generated is presented in Appendix B. Based on the generated results, inoculation of yeast was chosen to be done via wet cultures, while inoculation of *O. oeni* was kept as dry active culture. Additionally, mid-infrared spectroscopy was performed as an analytical method and it was decided that for 2018 gas chromatography coupled to mass spectrometry would be performed to investigate individual volatile compounds.

3.2.2 Grape Juice Preparation

For the main experiment (2018) two cultivars were used, namely Pinotage and Chenin blanc. The juices were obtained from Wellington Cellars (Wellington, South Africa). The Chenin blanc was thermo-vinified, by heating with a copper coil steam boiler at 75°C for 15 min in the experimental cellar, at the Department of Viticulture and Oenology (Stellenbosch University), while the Pinotage juice had already been thermo-vinified at Wellington Cellars using their standard cellar equipment and procedures. The pH of the cooled juices was measured by means of a pH probe (702 SM pH meter, Metrohm, Herisau, Switzerland). Total SO_2 was measured using a 702 SM Titrino and the manufacturers specifications (Metrohm, Herisau, Switzerland). Yeast Assimilable Nitrogen (YAN) was measured by an adapted formol titration method as follows. Fifty millilitres of wine samples were adjusted with 1N NaOH to pH 8.5. Afterwards, 20 mL of formaldehyde (pH 8.5) was then added. After the pH was stabilized, the samples were titrated with 0.1 N NaOH back to pH 8.5. The volume titrated multiplied by 28 mg/L yielded the YAN values in mg N/L. Table 3.2 summarises the above parameters in the juices. Enzymatic analysis by an Arena 20XT (Thermo Scientific, Waltham, Massachusetts, United States) was used to determine the initial sugars and malic acid parameters (Table 3.1).

Table 3.1 Juice parameters

Cultivar	pH	Malic Acid (g/L)	Total Sugar (g/L)	Total SO_2 (ppm)	YAN (mg N/L)
Pinotage	3.75	1.69	270	45	286
Chenin blanc	3.52	1.61	230	45	226

3.2.3 Media and microorganisms

Wallerstein Nutrient (WL) agar (Sigma-Aldrich, St. Louis, Missouri, United States) was used for yeast enumeration. The WL agar was supplemented with 200 mg/L biphenyl (Riedel-de Haën, Seelze, Germany) and 30 mg/L chloramphenicol (Sigma Aldrich), to exclude filamentous fungi and bacteria respectively. Incubation times were for 4 days at 30°C. Man de Rosa and Sharpe (MRS) broth (Biolab, Merk, Modderfontein, South Africa) was made with 15 g/L bacteriological agar (Biolab) for bacterial enumeration. The MRS agar was supplemented with 10% (v/v) preservative free tomato juice for *O. oeni* enumeration. For the exclusion of filamentous fungi and acetic acid bacteria, 100 mg/L and 25 mg/L Delvo®Cid Instant (DSM Food Specialists, Heerlen, Netherlands) and Kanamycin disulfate salt, from *Streptomyces kanamyceticus* (Sigma Aldrich) were used respectively to supplement the MRS agar. Plates were incubated for 8 days at 30°C in anaerobic chambers using Anaerocult® A strips (Biolab). Yeast extract peptone dextrose (YPD) broth (Biolab) was used for pre-culturing yeasts under agitation at 30°C. The organisms used are summarized in Table 3.2. All yeasts were streaked out and maintained as plate cultures on WL agar. *O. oeni* was kept at -20°C as a dry active culture.

Table 3.2 Organisms used for this study

Organism	Product Name	Manufacturer	Bench Code
Saccharomyces cerevisiae	EC1118	Lallemand (Montreal, Canada)	Sc
Torulaspora delbrueckii	BIODIVA	Lallemand (Montreal, Canada)	Td
Metschnikowia pulcherrima	FLAVIA	Lallemand (Montreal, Canada)	Mp
Lachancea thermotolerans	CONCERTO	Chr Hansen (Hørsholm, Denmark)	Lt
Oenococcus oeni	CH16	Chr Hansen (Hørsholm, Denmark)	CH16

3.2.4 Small-scale fermentations

For small-scale fermentations, Pinotage and Chenin blanc juice was dispensed in 4.5 L glass fermentation bottles for alcoholic fermentations. The Pinotage was adjusted to 240 g/L sugar with dH_2O to achieve final volumes of 4 L, while the Chenin blanc was left as is (4 L). In total five different inoculation types were performed for alcoholic fermentation. A pure Sc fermentation serving as the control, combinations of Sc with Mp (Mp/Sc), Sc with Td (Td/Sc), Sc with Lt (Lt/Sc) co-inoculations and a final combination of a consortium of all four strains together (CON). All strains were grown in YPD broth as previously mentioned before inoculation. For malolactic fermentation, the wines were racked off the lees into 2 L fermentation bottles and inoculated with 0.1 mg of fresh dry active culture. All alcoholic fermentations were static and performed at approximately 15°C for the Chenin blanc and at approximately 25°C for the Pinotage. Malolactic fermentations (MLF) were conducted at

approximately 22.5°C in an experimental cellar. All inoculation combinations of yeast for AF and the subsequent inoculations of CH16 for MLF were performed in triplicate to serve as biological repeats.

3.2.5 Sampling

Initial samples from each biological repeat were taken at inoculation for alcoholic fermentation, while for malolactic fermentation, samples were taken after an hour following inoculation. Interval sampling was done after the first 24 hours and thereafter as little as possible to not disturb the fermentations. Bottles were thoroughly mixed before 2 mL samples were taken for fermentation monitoring. The 2 mL samples were vortexed for 5 secs before a 100 µL fraction was used for yeast or bacterial enumeration. After the enumeration of yeast or bacteria, the remaining 1.9 mL samples were centrifuged at 10 000×*g* and 900 µL of supernatant was stored at -20°C to be used later for chemical kinetic analyses. The remaining 1 mL supernatant was kept as a backup for chemical kinetic analyses (-20°C), except for the end-points of alcoholic fermentation and malolactic fermentation where mid-infrared spectroscopy was immediately performed after yeast or bacterial enumeration. At the end of alcoholic fermentation and malolactic fermentation, 25 mL of wine was sampled, centrifuged at 10 000×*g* and the supernatants stored at -20°C for gas chromatography.

3.2.6 Yeast and bacterial enumeration

The 100 µL fractions taken for yeast and bacterial enumerations from each biological repeat were serially diluted to 10^{-6}-fold and were spread on WL agar for yeast population dynamics during alcoholic fermentation and on MRS agar for bacterial viability during alcoholic fermentation, using 100 µL of appropriate dilutions. Plates were then stored at 30°C for 4 and 8 days for yeast and bacteria respectively. Colony counts were determined per organism and the respective CFU/mL was calculated.

3.2.7 Enzymatic analysis

For chemical determination, the 900 µL supernatants were thawed and centrifuged for 1 min at 10 000×*g* for each biological repeat. Thereafter, 400 µL supernatants were used to determine trends, for L-malic acid and L-lactic acid, using enzymatic analyses via the ARENA 20XT (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Additionally, juices were measured for starting sugars prior to alcoholic fermentation. The kits used are summarized in Table 3.3 and were obtained from R- Biopharm (Darmstadt, Germany). All kits were used per the manufacturer's specifications.

Table 3.3 Enzymatic kits used to measure L-malic acid, L-lactic acid, D-fructose and D-glucose.

Parameter	Kit name	Experiment	ID/Ref No
L-malic acid	Enzytec Liquid	Fermentation monitoring	E8280
L-lactic acid	Enzytec Fluid	Fermentation monitoring	E5260
L-lactic acid	Enzytec Liquid	End of MLF for Chenin blanc	E8260
D-glucose	Enzytec Fluid	Juice characterisation	E5140
D-fructose	Enzytec Fluid	Juice characterisation	E5120

3.2.9 Mid Infrared spectroscopy

Mid-infrared spectra, for alcoholic fermentation and malolactic fermentation, were obtained for statistical analysis using the end-point 1 mL supernatants retained from sampling for each biological repeat. The spectra were obtained for fermentations immediately after yeast or bacterial enumeration, with 1 mL of centrifuged samples at 10 000×*g* for 1 min, using attenuated total reflection infrared spectroscopy (ATR-IR) on the Alpha II instrument (Bruker, Billerica, Massachusetts, United States). The Alpha II instrument was blanked with ddH_2O. For fermentation monitoring, in terms of total sugar for the fermentations, ATR-IR was used to predict values. Absolute values of sugar consumption rates (g/L per day) were calculated using the average linear gradient between the end of lag phase and start of stationary phase in sugar consumption.

3.2.10 Gas chromatography

For solid-phase microextraction (SPME), the 25 mL samples from each biological repeat were thawed and centrifuged at 5000×*g* and 5 mL were aliquoted into separate 20 mL glass vials with magnetic screw caps. An internal standard of 50 µL of 0.2 ppm Anisol-D8 (Sigma Aldrich) and 1 g of NaCl were added to the vials. An Agilent PAL3 autosampler (Agilent technologies, Santa Clara, California, United States) equipped with the standard sample agitator and SPME fibre conditioning station was used to extract the volatiles from the sample vial head-space. Gas chromatography/mass spectrometry (GC/MS) analysis of the extracts was performed using an Agilent HP-5MS column and an Agilent 5977B series MSD detector. The sample incubation was adapted from Beckner Whitener *et al.* (2016). Samples were incubated for 5 min at 35°C under 250 rpm rotation for 3 secs on and 2 secs rest. Extraction took place 30 min prior to desorption in the inlet for 12 secs at 250°C. Quality control (QC) vials containing an equal mix of all wines were spaced at the beginning and every fifteenth sample thereafter within each time batch. The GC oven parameters and MS protocols were taken from Beckner Whitener *et al.* (2016)

3.2.11 Gas chromatography data analysis

Analyte peak areas were divided by the peak areas of the internal standard to normalise the data. Chemical compounds were preliminarily identified using the m/z spectra of each peak and cross referencing with the National Institute of Standard and Technology (NIST) library based on the m/z spectra. Kováts Retention indices (RI values) were calculated using an alkane standard curve shown in Figure 3.1 and Equation 1 taken from Kováts (1958).

$$RI = 100\left[(a) + \frac{\log\left(\frac{RT_x}{RT_a}\right)}{\log\left(\frac{RT_b}{RT_a}\right)}\right]$$

Equation 1

Where a refers to the lower carbon number and b refers to the higher carbon number for analyte x in question. The terms RT_x, RT_a and RT_b refer to the retention times of analyte x, the lower carbon number and higher carbon number respectively. The RI values were used in conjunction with preliminary identifications based on m/z spectra to confirm compound identities using the NIST Chemistry WebBook search tool.

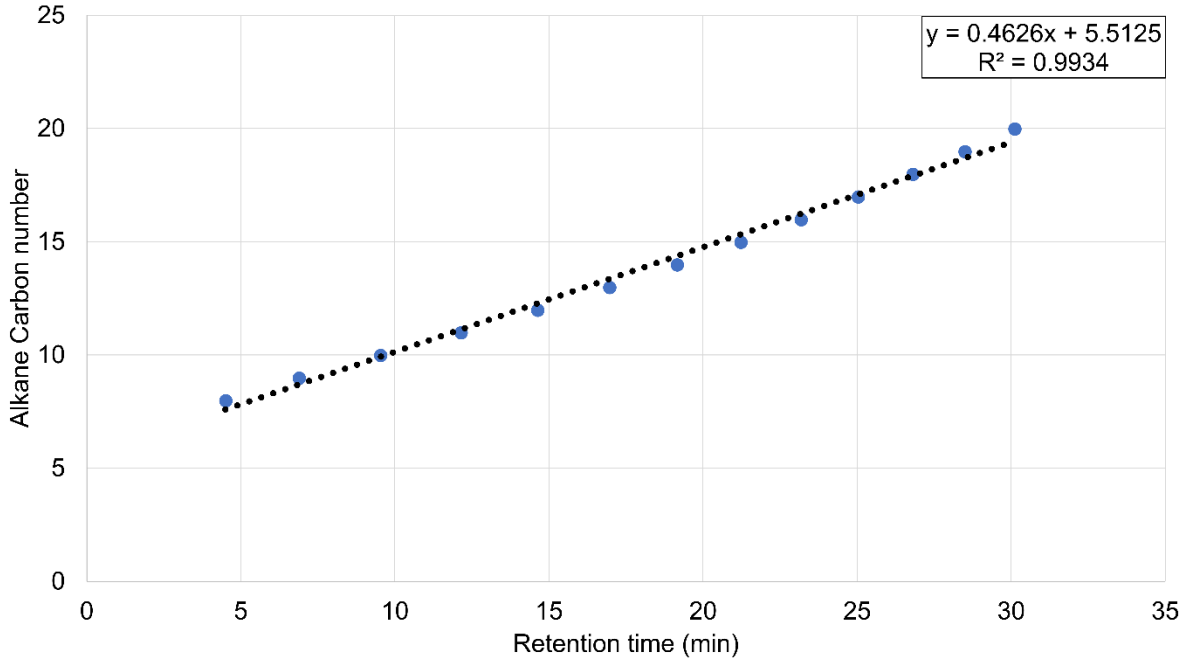

Figure 3.1 Standard curve for retention index calculation based on carbon number.

3.2.12 Statistical analysis

Means and standard deviations from biological repeats were calculated for each treatment and represented on graphs using Microsoft Excel 2016 (Redmond, Washington, United States). Multivariate data analysis and Analysis of Variance of the cross-validated residuals

(ANOVA-CV) tests were performed using SIMCA 14.1, (Umetrics, Umeå, Sweden) software to visualise and summarise the data on ATR-IR spectra. Models were built using Partial Least Squares Discriminant Analysis (PLS-DA). Microsoft Excel 2016 was used with XLSTAT premium 2016.1 (Paris, France) to generate heatmaps.

Spectroscopy data was scaled using CTR scaling for visualisation of the data set and for ANOVA-CV tests. Significant differences were individually tested (p-value below 0.05) between final wines of every different inoculation type at the same fermentation phase and their respective control (pure Sc) wines of each phase respectively. For example, Td/Sc and Sc were chosen as two classes to compare in the Pinotage-AF group were separately tested from the Pinotage-MLF, Chenin blanc-AF and Chenin blanc-MLF groups. The same scheme was performed for every comparison between an inoculation type and Sc for each group. Significant differences were also tested between alcoholic fermentation and malolactic fermentation phases for each cultivar using all the treatments. Cultivars were kept as separate datasets.

For SPME GC-MS data, the treatments were grouped together based on fermentation phase and cultivar. Group A comprised of Pinotage after AF, group B comprised of Pinotage after MLF, group C comprised Chenin blanc after AF and group D comprised of Chenin blanc after MLF. Fold changes in chemical compounds for each group were calculated by dividing the average normalised peak areas from the biological repeats of each treatment in a specific group with the average normalised peak area of Sc biological repeats in the specific group. These fold changes were then used to generate heatmaps using XLSTAT. The results were clustered by feature and coloured from blue to red through white on a 0 to 2 scale. Each group was kept separate when interpreting the data due to the nature of how fold changes were derived by using their specific Sc controls.

3.3 Results

3.3.1 Fermentation kinetics

Generally, all the Pinotage fermentations were able to reach dryness by day 6 (Figure 3.2A and Figure 3.2B), while the Chenin blanc took 33 days (Figure 3.2C and Figure 3.2D). For Pinotage, the fructose consumption for Sc (49.68 g/L per day) Mp/Sc (49.37 g/L per day) and Td/Sc (46.60 g/L per day) fermentations were the fastest, while the CON (39.20 g/L per day) and Lt/Sc (36.48 g/L per day) fermentations were the slower (Figure 3.2A). The D-glucose kinetics were faster for Td/Sc (53.83 g/L per day), Sc (53.03 g/L per day), Lt/Sc (49.45 g/L per day) and Mp/Sc (48,97 g/L per day) fermentations. The CON (42.60 g/L per day) fermentations were slower (Figure 3.2B).

For Chenin blanc, the consumption rate for Td/Sc fermentations were the slowest for D-fructose (3.79 g/L per day), while Lt/Sc (4.54 g/L per day), CON (4.58 g/L per day), Mp/Sc (4.97 g/L per day) and Sc (5.135 g/L per day) showed faster consumption rates (Figure 3.2C). For D-glucose consumption, Sc fermentations had the fastest rate (13.65 g/L per day), while Lt/Sc (11.94 g/L per day), CON (11.23 g/L per day), Mp/Sc (10.43 g/L per day) and Td/Sc (8.47 g/L per day) exhibited slower rates. Only the Td/Sc ferments were not able to achieve levels below 4 g/L total sugar, but instead levels of 9.43 ± 2.91 g/L and 2.6 ± 0.21 g/L of D-fructose and D-glucose respectively.

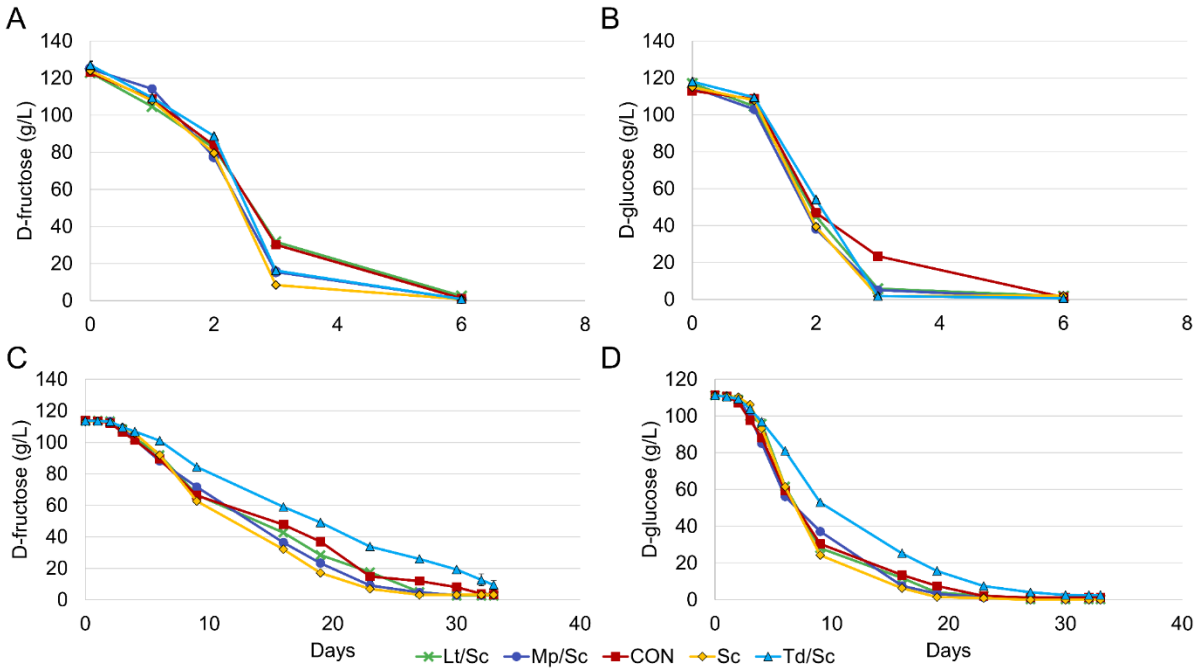

Figure 3.2 Chemical kinetics during 25°C fermentations in Pinotage (In A and B for D-fructose and D-glucose, respectively) and 15°C in Chenin blanc (In C and D for D-fructose and D-glucose, respectively). Error bars were on average <0.01% of the maximum values.

3.3.2 Yeast population dynamics for fermentations

Overall in the Pinotage, the data exhibited that the non-*Saccharomyces* species populations declined rapidly and was below detection by the middle of fermentation, albeit with variable rates as seen in Figure 3.3A and Figure 3.3B. For instance, Mp declined below detection after 1 day while Lt persisted until 2 days and Td showed persistence until 4 days in both pairings and CON fermentations. Populations of Sc were able to reach 7.77 log(CFU/mL) and 7.74 log(CFU/mL), in monoculture and CON fermentations respectively. Populations of Td reached 6.88 log(CFU/mL) and 6.90 log(CFU/mL), in Td/Sc and CON fermentations respectively. Populations of 6.29 log(CFU/mL) and 6.31 log(CFU/mL) were reached for Lt in Lt/Sc and CON fermentations respectively. The population of Mp did not increase.

The Chenin blanc displayed different population dynamics compared to the Pinotage as seen in Figure 3.3C and Figure 3.3D. Generally, the non-*Saccharomyces* species populations declined slower than in the Pinotage, albeit that the relative fermentation stages of falling below detectable levels were similar. The most abundant population was Td in both the Td/Sc. and CON fermentations during the early stages of AF (4 days), reaching 7.18 log(CFU/mL) and 6.91 log(CFU/mL), respectively. A population of 7.05 log(CFU/mL) and 7.00 log(CFU/mL)

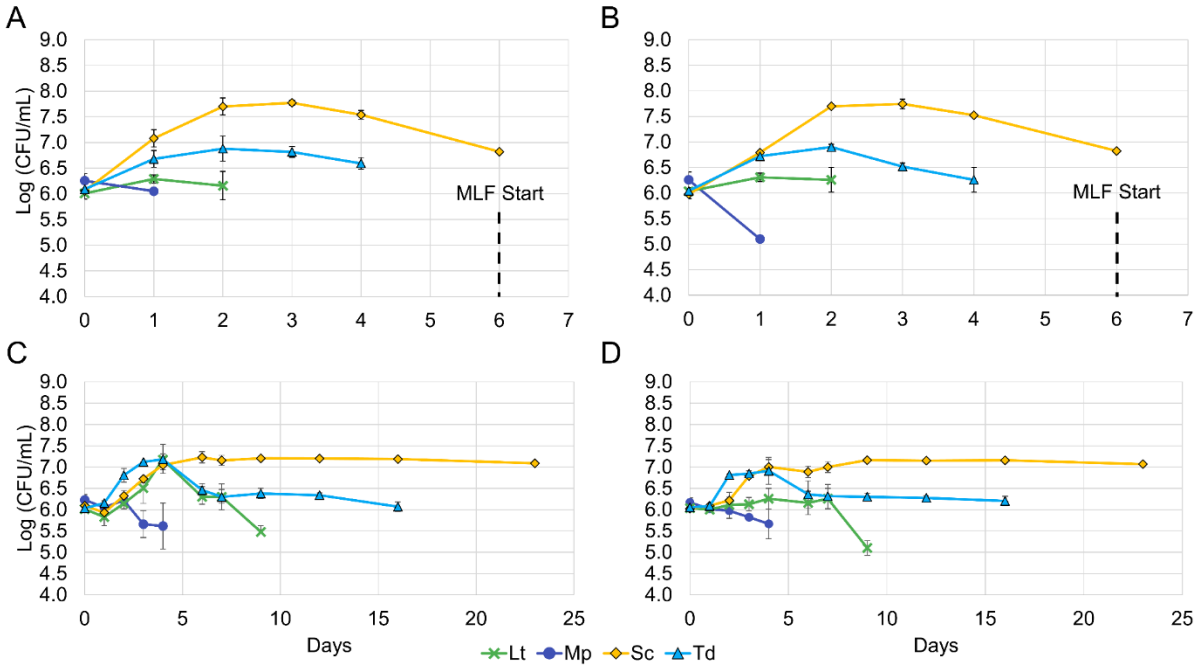

Figure 3.3 Population dynamics, of Lt, Mp and Td in pairings with Sc (A,C) and in the consortium (B,D), in Pinotage (A,B) Chenin blanc (C,D). In A the averages of Sc in all the pairing ferments is represented.

3.3.3 Malolactic fermentation kinetics

The inoculation of CH16 was successful, in all ferments in the Pinotage and Chenin blanc, as seen in Figure 3.4A and Figure 3.4B respectively. Generally, a slow decline in population was observed as MLF proceeded.

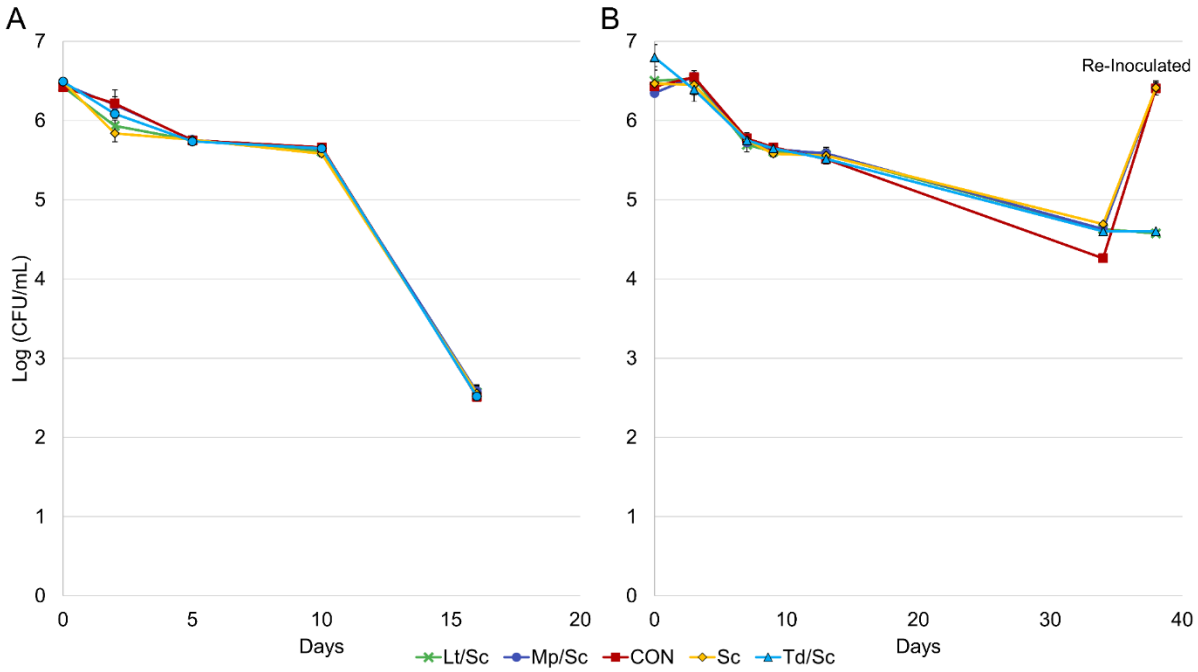

Figure 3.4 Population trends of CH16 during MLF in the Pinotage (A) and Chenin blanc (B) small-scale fermentations.

The Pinotage took 16 days to complete MLF and the kinetics for L-malic acid consumption appeared to be relatively similar between treatments (Figure 3.5A). However, Lt/Sc and CON fermentations had higher levels of L-lactic acid at the end of MLF (1.326 ± 0.090 g/L and 1.304 ± 0.083 g/L, respectively from Figure 3.5B).

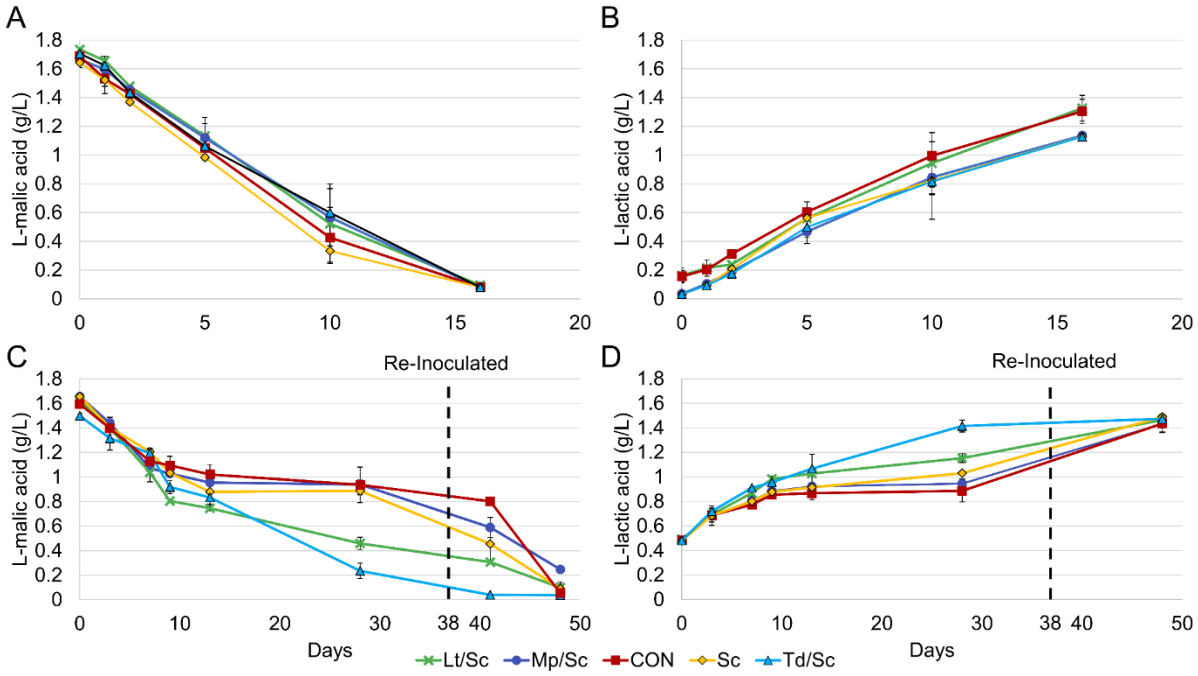

Figure 3.5 Chemical kinetics of MLF in the Pinotage (In A and B for L-malic and L-lactic acid respectively) and the Chenin blanc (In C and D for L-malic and L-lactic acid respectively).

In the Chenin blanc, there were differences in the kinetics. The kinetics for L-malic acid consumption appears to be similar for all treatments for the first 9 days. Thereafter, Td/Sc fermentations superseded the other trials, followed by Lt/Sc fermentations. A delay in MLF was observed for Mp/Sc, CON and Sc fermentations (Figure 3.5C). However, after re-inoculation for these trials, MLF was able to complete for these fermentations with the slowest kinetic being CON fermentations. Similar starting and final levels of L-lactic acid were observed for all fermentations (Figure 3.5D).

3.3.4 Mid infrared spectrum results

In general, significant differences were observed between the chemical spectra of the different wines after AF and after MLF in both the Pinotage and Chenin blanc (Table 3.4). The wines clearly separated according to fermentation phase, although the wines could not clearly be resolved based on inoculation type (Figure 3.6). However, when comparing pairings with the Sc mono-culture fermentations in the Pinotage, only the Mp/Sc wine was significantly different from Sc wines after AF.

Table 3.4 ANOVA-CV generated p-values for tests between AF and MLF wines for Pinotage. Tests were performed on individual PLS-DA models between pairing inoculations and Sc.

Matrix	Phase	Test	p-Value	Significant
Pinotage	AF and MLF	All Wines (AF vs MLF)	3.95e-18	Yes
	AF	Lt/Sc vs Sc	0.07	No
	AF	Mp/Sc vs Sc	0.01	Yes
	AF	CON vs Sc	0.10	No
	AF	Td/Sc vs Sc	0.27	No
	MLF	Lt/Sc vs Sc	0.07	No
	MLF	Mp/Sc vs Sc	0.06	No
	MLF	CON vs Sc	1.00	No
	MLF	Td/Sc vs Sc	0.29	No
Chenin Blanc	AF and MLF	All Wines (AF vs MLF)	2.18e-13	Yes
	AF	Lt/Sc vs Sc	0.004	Yes
	AF	Mp/Sc vs Sc	0.006	Yes
	AF	CON vs Sc	0.12	No
	AF	Td/Sc vs Sc	0.019	Yes
	MLF	Lt/Sc vs Sc	0.17	No
	MLF	Mp/Sc vs Sc	0.30	No
	MLF	CON vs Sc	1.00	No
	MLF	Td/Sc vs Sc	0.62	No

This difference is observed to be diminished following MLF (Table 3.4). In contrast, the Chenin blanc wines from Lt/Sc, Mp/Sc and Td/Sc were significantly different to Sc wines after AF. However, this significance is diminished following MLF like in the Pinotage (Table 3.4).

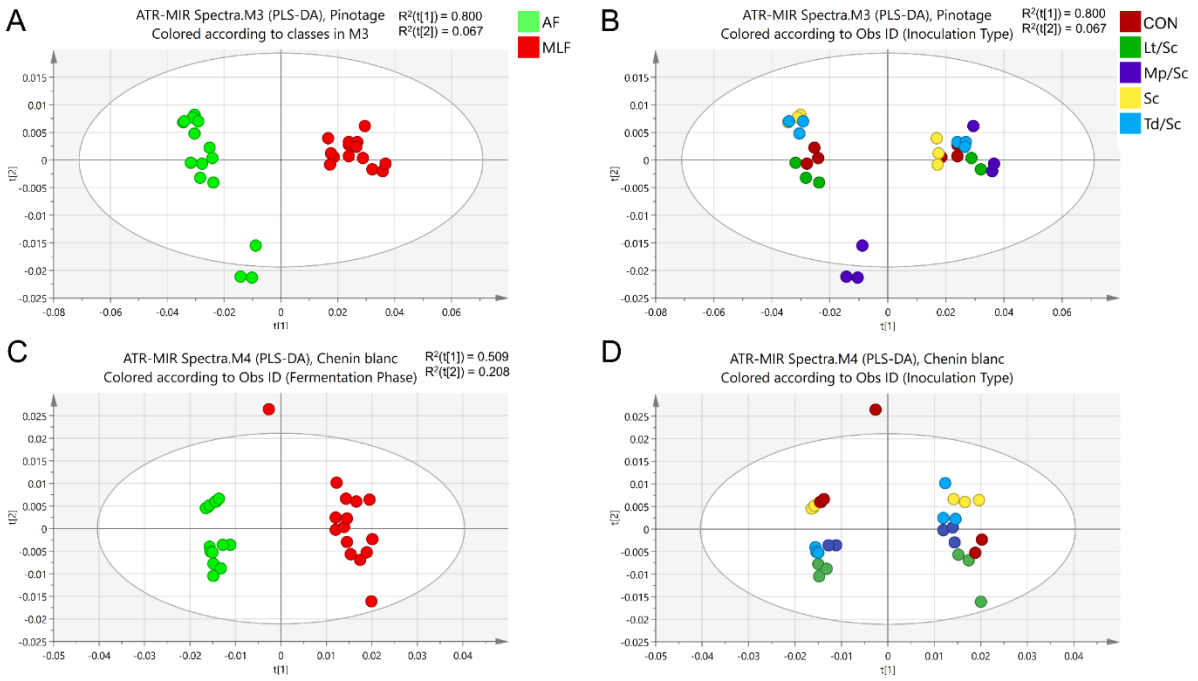

Figure 3.6 PLS-DA models of infrared spectrum on Pinotage (A and B) and Chenin blanc (C and D) small-scale fermentations after AF and MLF. The model is coloured according to fermentation phase (A and C), and inoculation type (B and D). Each point represents a biological repeat for each treatment.

3.3.5 Gas chromatography results

In total 68 compounds were detected, and 53 identities were confirmed based on m/z spectra and retention indices. The 15 compounds not confirmed by retention indices were preliminarily identified by m/z spectra but labelled by their respective calculated retention indices. Fold changes were detected for certain compounds when compared to Sc fermentations of Pinotage and Chenin blanc wines after AF and MLF as seen in Figure 3.8. There is a shift in the intensity of compounds from AF to MLF for both Pinotage and Chenin blanc, however the compounds affected depended on the type of inoculum.

For the Pinotage after AF, the Lt/Sc treatments showed that methyl hexanoic acid, methyl octanoate and methyl caprate were detected to be at least 2-fold greater than in Sc fermentations. In CON treatments only 1-dodecanol was detected to be at least 2-fold greater than in Sc treatments. In Td/Sc fermentations, ethyl propionate, propyl acetate, methyl hexanoic acid and methyl caprate were found to be at 2.35, 2.13, 2.55, and 2.16-fold more respectively. No compounds were detected to be more than 2-fold greater in Mp/Sc treatments than in Sc treatments. Methyl octanoate, 1-dodecanol and compound 1130 were however detected to be at 1.67, 1.80 and 1.88-fold more in Mp/Sc treatments than in Sc treatments, respectively.

For MLF treatments in the Pinotage more compounds were detected to be higher than 2-fold greater than Sc treatments. For Lt/Sc treatments, isobutyl alcohol and methyl caprate were detected at 3.38 and 5.87-fold greater than in Sc treatments, respectively. Another eleven compounds were detected to be between 2 and 3-fold greater than in Sc treatments as seen in Figure 3.9. In Mp/Sc treatments 1-dodecanol was detected to be 9.28-fold greater than in Sc treatments, while five other compounds where detected to be between 2 and 3-fold greater than in Sc treatments. Only 1- dodecanal was detected to be more than 2-fold greater (4.95-fold) in CON treatments than in Sc treatments. Twelve other compounds were found to be between 1.5 and 2-fold greater in CON treatments than in Sc treatments. For Td/Sc treatments ethyl pelargnate and methyl caprate were detected to be 3.29 and 5.11-fold greater than in Sc treatments. An additional ten compounds were found to be between 2 and 3-fold greater than in Sc treatments.

For the AF treatments in the Chenin blanc, the Lt/Sc treatments had 3-octanol, methyl caprate and compound 1030 at 7.19, 2.47 and 2.33-fold greater than in Sc treatments. Additionally, 3-methylbutyl hexanoate and isoamyl caprylate were found to be between 1.5 and 2-fold greater than in Sc treatments. For Mp/Sc treatments, compound 1130 was found to be 3.63-fold greater than in Sc treatments while an additional five compounds were observed to be between 1.5 and 2-fold greater. Table 3.6 summarises the fold changes in CON treatments. No compounds were found to be greater than 1.5-fold in Td/Sc treatments than in Sc treatments.

For MLF treatments in the Chenin blanc, Lt/Sc treatments had citronellyl acetate and compound 1566 at 6.58 and 5.20-fold greater than in Sc treatments. An additional twenty-five compounds were detected to be between 2 and 4-fold greater including decanoic acid at 4.04-fold greater than in Sc treatments. In Mp/Sc treatments, citronellyl acetate and decanoic acid were detected to be 3.56 and 3.82-fold greater than in Sc treatments. Additionally, nineteen compounds were found to be between 2 and 3.5-fold greater. For CON treatments, methyl caprate and citronellyl acetate were found to be 5.22 and 5.42-fold greater respectively than in Sc treatments. Additionally, twenty-two compounds were detected to be between 2 and 5-fold greater including decanoic acid at 4.84-fold greater than in Sc treatments. Finally, in Td/Sc trials, compound 1443 and decanoic acid was found to be 4.95 and 5-fold greater respectively than in Sc treatments. Additionally, fourteen compounds were found to be between 2 and 4.5-fold greater. Exact fold changes of all additional compounds in the different treatments can be found in Table ii in Appendix A.

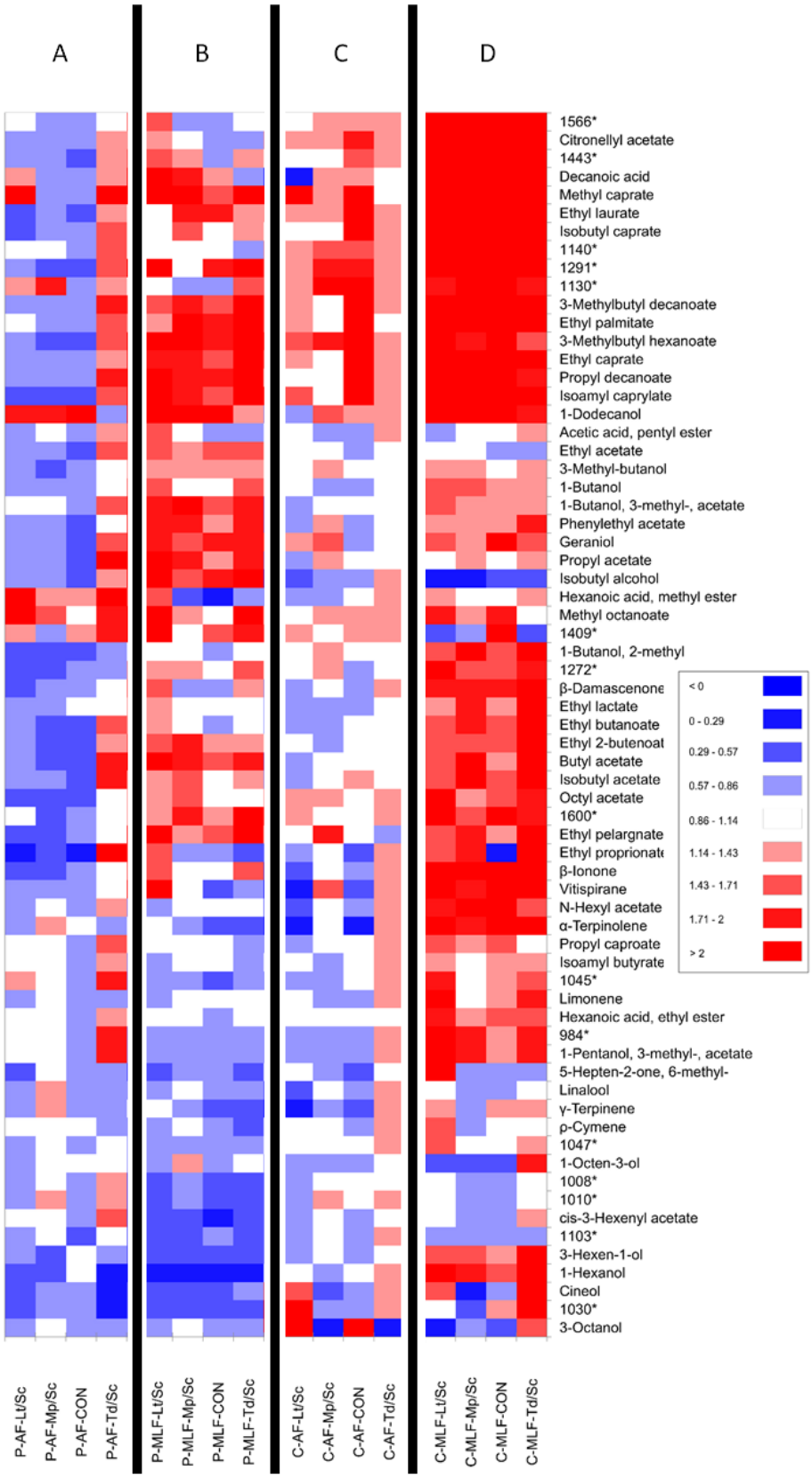

Figure 3.8 Relative fold changes coloured from 0 (blue) through 1 (white) to 2 (red) of all the treatments when compared to their respective Sc fermentations in groups A (Pinotage-AF), B (Pinotage-MLF), C (Chenin blanc-AF) and D (Chenin blanc-MLF). Compound names indicated by an * are the calculated RI vales. Preliminary identifications can be found in Table i (Appendix A) based on m/z spectra and exact fold changes can be bound in Table ii (Appendix A).

Table 3.6 Volatile compounds detected above 2-fold for CON treatments in Chenin blanc after AF (Group 2) using GC/MS and their respective fold changes across other treatments in Chenin blanc after AF when compared to Sc trials.

Label	C-AF-Lt/Sc	C-AF-Mp/Sc	C-AF-CON	C-AF-Td/Sc
1130*	1.25	3.63	2.08	1.21
3-Methylbutyl decanoate	1.22	0.95	2.20	1.15
3-Methylbutyl hexanoate	1.71	1.98	2.10	1.18
3-Octanol	7.19	0.00	3.06	0.01
Ethyl caprate	1.38	1.07	2.26	1.18
Ethyl laurate	1.29	1.14	2.75	1.26
Ethyl palmitate	1.29	1.05	2.29	1.02
Isoamyl caprylate	1.54	0.98	2.14	1.16
Isobutyl caprate	1.00	0.87	2.44	1.18
Methyl caprate	2.47	1.27	2.17	1.09
Propyl decanoate	1.06	0.96	2.26	1.18

Compound names indicated by an * are the calculated RI vales. Preliminary identifications can be found in Table i (Appendix A) based on m/z spectra

3.4 Discussion

Since the knowledge on how yeasts grow and survive in multi-species setups during AF and how MLF might be affected by this, is limited, this study aimed at providing a better understanding on yeast population dynamics and downstream effects on MLF.

Based on the population dynamics it was evident that the behaviour of the Lt, Mp and Td remained similar in mixed fermentations with Sc and in the multi-species consortium. The population trends were similar within cultivars but not between the Pinotage and Chenin blanc. This could be because of temperature on yeast populations and growth. It is apparent that these strains grow and fall below detectable limits based on their respective fermentative robustness and response to anaerobic environments. According to the population dynamics, Td persisted longer, followed by Lt while Mp always declined rapidly. The Mp trends agree with a previous study done by Wang *et al.* (2016) where *M. pulcherrima* did not persist after 48 hours. Studies suggest that oxygen availability, nitrogen availability and glucose consumption rate are factors as to why certain strains persist longer than others (Holm Hansen *et al.*, 2001; Nissen *et al.*, 2004; Andorrà *et al.*, 2012; Brandam *et al.*, 2013; Wang *et al.*, 2016). This effect appears to be consistent on a species level though exhibited at different extents between strains.

Interestingly, in the Chenin blanc Td was able to grow better in the initial stages of the fermentations for both the mixed and consortium fermentations. The rates of D-fructose and D-glucose consumption were also slower. Similar findings have been reported in a study done by Taillandier *et al.*(2014), where *T. delbrueckii* NSC123 was investigated under different

nitrogen levels, with *S. cerevisiae* QA23 at 20°C. The sugar consumption was slower in mixed fermentations with QA23 when compared to mono-culture QA23 fermentations and the population of NSC123 was similar to the population of QA23 during mixed fermentations. Though, in mono-cultures of NSC123 the populations reached 8.63 log(CFU/mL) as opposed to 8.47 log(CFU/mL) of QA23. The reason for *T. delbrueckii* being able to grow better and alter fermentation rates could be attributed to temperature. It is well known that non-*Saccharomyces* species, like *T. delbrueckii* and *M. pulcherrima,* persist better at lower temperatures since ethanol effects membrane permeability less at colder temperatures (Salvadó *et al.*, 2011). At 25°C however, in this study, Td was not able to exceed Sc population levels and did not slow down the fermentations.

Gobbi *et al.* (2013) contrasts this study's results regarding *L. thermotolerans*, since the strain used in their trial was able to persist to the end of fermentation in co-inoculations. This could however be strain dependent. The production of L-lactic acid in 25°C treatments does, however, agree with the findings of the current study, in that an increase was observed during AF. Though the amounts for L-lactic acid differ from this study's observation at 365 mg/L, this could also be strain and juice parameter dependent as seen in other studies (Benito *et al.*, 2015, 2016). One should note, that the production of L- lactic acid is due to primary metabolism in *L. thermotolerans* and is dependent on the time that *S. cerevisiae* is inoculated during fermentation (Kapsopoulou *et al.*, 2007; Gobbi *et al.*, 2013; Benito *et al.*, 2016). This might explain why very little production of L-lactic acid was observed during AF, since Sc was co-inoculated at the same population as Lt in this study and in Benito *et al.* (2016) sequential inoculation was used. The slower kinetics in 15°C during AF could be the reason why the levels of L-lactic acid were relatively the same after AF, as primary metabolism is generally retarded at lower temperature for yeasts. It is apparent in this case that, since mono-cultures of Sc treatments had the same level as Lt/Sc and CON trials, Lt wasn't active enough for L-lactic acid production at 15°C. This is confirmed, since little differences were present in the concentrations of L-lactic acid after MLF. This contrasts the 25°C fermented wines that still showed higher levels of L-lactic acid for Lt/Sc and CON treatments after MLF. As for why the levels were higher after AF for 15°C as opposed to 25°C treatments, this could be an error in the enzymatic kit used as it was close to expiry date and could have cause errors in measurement. Therefore, a new one was used for the other time points for the Chenin blanc wines.

The infrared spectra obtained provided a fast means to compare treatments with each other. The method, however, does not allow us to compare specifically for aroma compounds and rather gives a response spectrum on all the chemical compounds that can give an IR signal in the wines. The treatments do show differences in chemical profiles and it is well

known that the use of various non-*Saccharomyces* species such as *T. delbrueckii*, *L thermotolerans* and *M. pulcherrima,* depending on the strain used, can either decrease or increase concentrations of esters, fusel alcohols and terpenes (Comitini *et al.*, 2011; Azzolini *et al.*, 2012; Sadoudi *et al.*, 2012; Whitener *et al.*, 2017). Standard parameters, such as residual sugar and ethanol, are more dependent on *S. cerevisiae*. Considering that *S. cerevisiae* was the main driver in all the fermentations and present from the start of fermentation, this perhaps explains why the CV-ANOVA tests yielded very few comparisons that were significantly different from each other. The phenolics in the red wine would have also masked the differences greatly in the spectrum and is perhaps a reason why the differences in Chenin blanc were significant and not in the Pinotage. This is exacerbated by the fact that the Chenin blanc was fermented at 15°C and non-*Saccharomyces* are known to perform better at lower temperatures (Erten, 2002; Maturano *et al.*, 2015), therefore allowing greater levels of aroma modulation to occur. This gave reason that any differences observed (significant or not) in the PLS-DA models needed to be investigated with a method that would target aroma compounds specifically, such as chromatography techniques.

The GC/MS data showed how the use of these non-*Saccharomyces* species during AF can yield aroma profiles that contain different amounts of volatile compounds. It is apparent here that, indeed, higher levels in the 68 volatile compounds where observed. Chenin blanc AF wines were found to have greater increases (when compared to Chenin blanc AF Sc wines) than the Pinotage AF wines (when compared to Pinotage AF Sc wines), possibly due to the cooler fermentation temperature (15°C) which leads to greater persistence in all the non-*Saccharomyces* used. This has been suggested in previous studies since temperature effects yeast metabolism (Torija, 2003; Maturano *et al.*, 2016; Alonso-del-Real *et al.*, 2017). Although, a repeat of this study involving 15°C fermentations of Pinotage and 25°C should be repeated to confirm this hypothesis. The CON fermentations were also found to be higher in ester concentrations than in pairings for Chenin blanc AF wines, although decreases were observed mainly for the Pinotage AF wines, except for 1-dodecenal and methyl esters of hexanoic and octanoic acid. The increase in esters could be attributed to the presence of all the non-*Saccharomyces* used and their increased persistence in colder fermentations. However, the aromatic profiles of the CON wines are not a sum of the individual profiles from each pairing.

In terms of MLF (22.5°C), the viability of CH16 might have been different if other yeast strains were employed that are incompatible with LAB. Certain strains could have caused inhibition on their growth and therefore influence MLF negatively. Examples of inhibition are through fatty acid, SO_2 and high ethanol production (Alexandre *et al.*, 2004; Arnink and Henick-Kling, 2005). Since all the strains used were compatible with LAB, it is not surprising that MLF was able to proceed in all the treatments. However, it is still interesting to note that MLF was

delayed in treatments (CON, Mp/Sc and Sc) that were at 15°C during AF and that the treatments could resume fermentation after re-inoculation of CH16. This could be due to the presence of decanoic acid, however elevated levels of decanoic acid (when compared to Sc ferments) was only detected after MLF.

Decanoic has been known to retard the malolactic enzyme and affect *O. oeni* viability as reported in a study done by Carreté *et al.*(2002). Their study showed that (in the presence of ethanol) decanoic acid, lauric acid, Cu^{2+} and SO_2 retard ATPase activity in *O. oeni*. Before MLF however, the decanoic acid levels were not higher than in Sc treatments. This may be due to the early sampling of the wines as soon as AF was complete and MLF only being induced once AF in Td/Sc fermentations were completed in the Chenin blanc. The decanoic acid might have been released due to yeast autolysis before the wines were racked off into the MLF vessels and thus have caused the MLF delays in the treatments. The amounts of decanoic acid needs to be quantified to determine if the decanoic acid is indeed responsible for the delay in MLF. Other inhibitory compounds or residual nutrient after AF should also be investigated.

Wines that undergo MLF can also be modified aromatically due to the presence of *O. oeni* (Swiegers *et al.*, 2005; Gammacurta *et al.*, 2018). According to the infrared data, it is interesting to observe that MLF might cause a loss in chemical composition differences in wines fermented at moderate temperatures (25°C) as opposed to when fermented at cooler temperatures (15°C). It is important to remember that this may be specific to grape cultivar as volatile precursors might be different in the juices (Ghaste *et al.*, 2015) and resulting wines before MLF. These precursors can then be modulated by *O. oeni* (Pérez-Martín *et al.*, 2014; Gammacurta *et al.*, 2018).

The GC/MS results confirm that the Chenin blanc wines where more distinct from their Sc treatments and significant in the ATR-IR findings for MLF. It should also be noted that a similar trend was observed in Pinotage MLF wines, indicating that indeed the background of the phenolics of red wine causes some masking effect when using ATR-IR spectroscopy. To test for significance in these differences the detected compounds will need to be quantified and subjected to ANOVA tests against their Sc treatments to obtain *p*-values. Though it is expected that considering the observed trends for moderate temperature fermentations and timing of *S. cerevisiae* inoculations, the degree of significance might be less than what could be seen in cooler temperature fermentations (Erten, 2002; Kapsopoulou *et al.*, 2007; Maturano *et al.*, 2015). It is apparent from this dataset that MLF has a greater effect on wine chemical composition than yeast treatment, this confirms what has been found in a previous study by du Plessis *et al.* (2017).

3.5 Conclusion

Non-*Saccharomyces* yeast, such as *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima*, have been studied extensively in one-to-one pairings with *S. cerevisiae* and therefor strains have been commercialised. However, it is important to understand how these species behave in a multi-species consortium, as well as what the resulting effects on MLF and wine chemical composition might be. There were little differences in the persistence and growth of each strain between pairings and the consortium for both the Pinotage at 25°C and Chenin blanc at 15°C.

No effect on *O. oeni* populations was observed but a delay in MLF was seen in Chenin blanc fermentations. One still needs to be aware of strain compatibility with LAB and under what conditions yeast could become inhibitory to MLF.

The wine chemical composition is not only directly related to the population dynamics of the yeast, but it is also dependent on whether MLF occurred or not, especially for moderate temperature wines. Consortium fermentations resulted in higher concentrations of esters in Chenin blanc AF wines. In general, MLF had a greater effect on wine chemical composition than AF leading to increases in ester concentrations when compared to *S. cerevisiae* trials.

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Chapter 4

General Discussion and Conclusion

Chapter 4 General discussion and conclusion

4.1 Discussion

Starter cultures such as Melody™ or Harmony (CHR. Hansen) comprising multiple species of yeast are already available to the winemaker as active dry yeast preparations. However, the production of such cultures is still in infancy. To develop such cultures, it is important to understand how the individual species will perform within such a system and therefore affect fermentation kinetics and final wine parameters. Constituent species of such mixtures should be chosen individually based on their individual MLF compatibility, however, whether this trait is maintained even in complex consortia has not been the focus of research.

The current study was aimed assessing the growth and fermentation performance of commercialised *Torulaspora delbrueckii, Lachancea thermotolerans, Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* strains within a consortium. The population dynamics, chemical kinetics and volatile profile, during AF was investigated. Additionally, the effects of the resulting wines on *Oenococcus oeni* viability, its ability to perform MLF as well as the resulting wine volatile profiles, was investigated. In general, species dynamics were similar in fermentations performed in Pinotage (25°C) and Chenin blanc (15°C). Different growth and persistence patterns were apparent for *M. pulcherrima, T. delbrueckii* and *L. thermotolerans.* The trends for *M. pulcherrima, T. delbrueckii* and *S. cerevisiae* were consistent with literature (Taillandier *et al.*, 2014; Wang *et al.*, 2016). However, *L. thermotolerans* in our study showed less persistence than in a previous study (Gobbi *et al.*, 2013), though this could be due to different strains tested. The least persistence was seen in *M. pulcherrima,* followed by intermediate persistence in *L. thermotolerans* while the longest was observed in *T. delbrueckii.* The chemical kinetics showed little differences in sugar consumption during AF due to the presence of *S. cerevisiae*, except a slower kinetic was observed for 15°C fermentations containing *T. delbrueckii*. This may be due to increased competition for sugar since *T. delbrueckii* may perform better in cooler temperatures than moderate ones (Taillandier *et al.*, 2014).

Elevated levels of L-lactic acid were also observed in fermentations containing *L. thermotolerans* compared to other setups at 25°C, but not at 15°C. This could be indicative of a retarded central metabolism in *L. thermotolerans* due to the cooler temperature since L-lactic acid production results from the reduction of pyruvate to lactate in the presence of lactate dehydrogenase (*LDH*). Previously, *LDH* has been described to have increased activity with an increase in temperature (Birkbeck and Stewart, 1961), therefore the 15°C fermentations might have inhibited *LDH*. However, this was determined with *LDH* extracted from blood

samples. A study on *LDH* from *L. thermotolerans* should be performed in order to better understand the optimum conditions of yeast-derived *LDH*. For volatiles, greater increases were observed in the Chenin blanc than in Pinotage when comparing them to their respective controls. The effects of each treatment on volatile compounds levels were not additive in the consortium (CON) wines, but rather generated its own volatile fingerprint in both wines. Eleven different compounds were found to be increased in consortium wines when compared to the other treatments after AF in Chenin blanc, while consortium treatments in the Pinotage yielded a diminished aroma modulation. This could be due to the increased performance of *S. cerevisiae* at 25°C, since it is well known that the populations and aroma effects of non-*Saccharomyces* (such as the ones used in the consortium) are heavily dependent on *S. cerevisiae* presence and performance (Nissen *et al.*, 2003; Kapsopoulou *et al.*, 2007; Albertin *et al.*, 2017). It is apparent that the use of the consortium at a cooler temperature has the potential to modify aroma better than the other treatments.

With regards to consortium design, the construction of this consortium with all strains at equivalent initial populations indicates that a lower initial population of *S. cerevisiae* might be an advantage in these setups. Certain commercial products such as Melody™ and Harmony from Chr. Hansen however have *S. cerevisiae* at a higher population (60% in Melody™ and 80% in Harmony) in the starter cultures than *L. thermotolerans* (20% in Melody™ and 10% in Harmony) and *T. delbrueckii* (20% in Melody™ and 10% in Harmony). In situations such as these it might be better to increase the levels of the non-*Saccharomyces* (perhaps to 20% *S. cerevisiae,* 40% for *T. delbruecki* and 40% for *L. thermotolerans* or starting both non-*Saccharomyces* with a 10-fold advantage). This should lead to a better performance of the non-*Saccharomyces* in a consortium and therefore aid in aroma modulation as found in studies who used lower initial levels of *S. cerevisiae* with higher levels of non-*Saccharomyces* such as *Pichia kluyveri, Starmerella bacillaris* (formerly *Candida zemplinina*) and *Hanseniaspora uvarum* (Anfang *et al.*, 2009; Andorrà *et al.*, 2012). Conversely, the opposite might be true where higher initial levels of non-*Saccharomyces* might deplete the environment of nitrogen or vitamins and prevent *S. cerevisiae* from driving the fermentation (Bisson and Butzke, 2000; Wang *et al.*, 2003; Medina *et al.*, 2012). Therefore, when considering the use of higher levels of non-*Saccharomyces,* as previously suggested, one needs to consider the total amount of nitrogen available in the juice before inoculation. Additionally, yeasts should also perhaps be investigated for the amino acids preferences as they have different uptake rates for different amino acids (Barrajón-Simancas *et al.*, 2011) when considering the design of a consortium. Furthermore, fermentation temperature is also a factor, since products prescribed for Chardonnay and other white wine ferments are usually performed at lower temperatures which will aid the non-*Saccharomyces* found in Melody™ and Harmony to

perform better. In terms of *M. pulcherrima,* the use of this organism in red winemaking could be of benefit, since it yielded significantly different results in the Pinotage (which was not aerated). Using aerating methods commonly employed for anthocyanin extraction in red wine, such as pump overs or punch downs, could aid *M. pulcherrima* to persist longer since oxygen helps negate the effects of ethanol and improve cell physiology (Varela *et al.*, 2012; Brandam *et al.*, 2013) and *M. pulcherrima* is known to be more oxygen dependant than other wine related yeasts (Morales *et al.*, 2015; Shekhawat *et al.*, 2017). This practise should also benefit yeasts such as *T. delbrueckii, H. uvarum* and *Hanseniaspora guilliermondii* (Hanl *et al.*, 2005; Pérez-Nevado *et al.*, 2006), due to their oxygen requirements.

During MLF (22°C), no hinderances to *O. oeni* populations and chemical kinetics were observed for Pinotage wines. However, for the Chenin blanc, slower kinetics were observed for CON, Mp/Sc and Sc wines, but the *O. oeni* populations were similar between all treatments. It should be noted that the initial levels of L-malic acid were slightly lower in the Chenin blanc, though a delay still occurred in some of the trials. It was difficult to establish why MLF was delayed in Chenin blanc fermentations, as levels of known inhibitors of MLF, such as decanoic acid, could not be quantified. Parameters such as nitrogen sources and SO_2 in addition to fatty acid quantification should be analysed throughout fermentation. Kinetics of L-lactic acid were found to be similar in all treatments, although higher concentrations of L-lactic acid was found in Pinotage wines that contained *L. thermotolerans* (CON and Lt/Sc), due to its production during AF. In terms of volatiles, the differences in fingerprints (compared to MLF Sc wines) were greater than previously observed in AF wines. This has confirmed what was found in a recent study were MLF had a greater impact than yeast treatment on aroma profiles (du Plessis *et al.*, 2017). The volatile fingerprints of each treatment were again not additive in the consortium. This is likely due to the diversity in ester metabolism found in yeast that varies on a strain level and coupled to the external factors (temperature, nutrient availability, pH, unsaturated fatty acid levels, and oxygen) which influence ester formation in wine (Styger *et al.*, 2011). However, higher relative levels of citronellyl acetate, decanoic acid, methyl caprate, ethyl pelargnate, ethyl palmitate, butyl acetate and ethyl butanoate and other esters and fatty acids were observed in consortium wines after MLF.

This study, however, did not evaluate yeasts inoculated at different ratios or at different sequential times. These are important aspects to consider, since the persistence and metabolic processes of non-*Saccharomyces* such as *L. thermotolerans* and others are directly related to the presence of *S. cerevisiae* at high populations (Nissen *et al.*, 2003; Kapsopoulou *et al.*, 2007; Gobbi *et al.*, 2013; Albertin *et al.*, 2017). Different combinations of the yeast (i.e. rotating the exclusion of one or two species at a time) would also be of benefit since the volatile profiles might be different resulting from each setup or perhaps signatures might be

established. All of these investigations (including the findings in this study) can be of use to industry when designing products as some effects by other species may be negated due to the pre-mature domination of *S. cerevisiae* or inclusion of incompatible strains. Additionally, the use of different lactic acid bacteria strains from *Lactobacillus plantarum* and *O. oeni* should be investigated and the effect of co-inoculation versus sequential MLF.

4.2 Conclusion

Overall, the data has shown that a multi-yeast consortium can follow predictable population dynamics and chemical kinetics based on each individual yeast's ability to survive the winemaking process and operating conditions. Additionally, if each strain was previously determined to be compatible on its own with *O. oeni* for MLF, it is likely that a consortium of these strains would also have little effect on *O. oeni* populations. However, one should keep in mind strain differences and the initial parameters of the juice before fermentation. High initial sugars, low nitrogen and the presence of SO_2 will affect the ability of *O. oeni* to perform MLF. It is interesting however, that the volatile fingerprint (particularly the esters) of the consortium was not additive of the one-to-one mixed fermentations even though chemical kinetics and population dynamics were similar. This therefore makes it difficult to predict an outcome for volatiles in new combinations of yeast starter cultures. In general, each combination of strains at 15°C and 25°C, generated a unique volatile fingerprint after AF. Following MLF, the volatile fingerprints of treatments where more different to the mono-cultures *S. cerevisiae* fermented wines than in the AF wines. This indicates that *O. oeni* has a great role to play in wine aroma in addition to yeast strain combination during AF.

The use of multi-starter cultures can be of great use to enhance wine complexity This is apparent since each different combination of yeasts (in number of strains used and different species used) in the cultures have the potential to generate unique profiles especially when combined with different cultivars and fermentation techniques.

4.3 Future Work

From these findings it has been shown that given previously characterised yeast strains in specific parameters (temperature, initial sugar, pH etc) fermentation kinetics and population dynamics can be reliably predicted. However, the final volatile profiles proof difficult to predict as the effects of each strain are not additive in a consortium. Each new starter combination should be investigated for their own resulting volatile profiles. This is, however, an early investigation into these systems and it needs to be repeated with different combinations of strains and parameters. Trials in real juice should perhaps be conducted alongside synthetic

media fermentations to establish a baseline for trends in LAB and yeast-derived volatile compounds. Studies on gene expression should also be conducted in the future to get an understanding on the functional genes in the system contributing to volatile profile modulation. Additionally, other factors such as aeration, dispersion, cell segregation and the presence of killer strains should be used to follow up these findings. Comparison studies between native yeasts (resulting from the *terroir*) in a spontaneous fermentation and in constructed consortia should also be performed to assess differences in population performances, acid modulation, volatile profile modulation and MLF compatibility.

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Appendix A

Appendix A

i. Compounds detected using SPME-GC/MS

The compounds detected in the different wines for Chapter 3 were identified based on their m/z spectra and retention index. Table I lists the identified compounds based on m/z spectra, their calculated retention indices from Chapter 3 and their reference retention indices. Compounds that did not fit in the standard curve in Figure 3.1 (Chapter 3) and identified based on m/z spectra were treated as confirmed identities due to their relatively lower carbon numbers. Higher carbon compounds that could not be matched to a reference retention index were named by their calculated retention index instead of their identification based on m/z for data analysis in Chapter 3. Figure I and Figure II are PCA and PLS-DA models of the Pinotage and Chenin blanc wines respectively. According to CV-ANOVA tests, no comparisons between non-*Saccharomyces* mixed fermentations and consortium wines were significantly different from Sc controls in each group respectively. Only wines that underwent MLF were significantly different to wines that only went through AF in both cultivars.

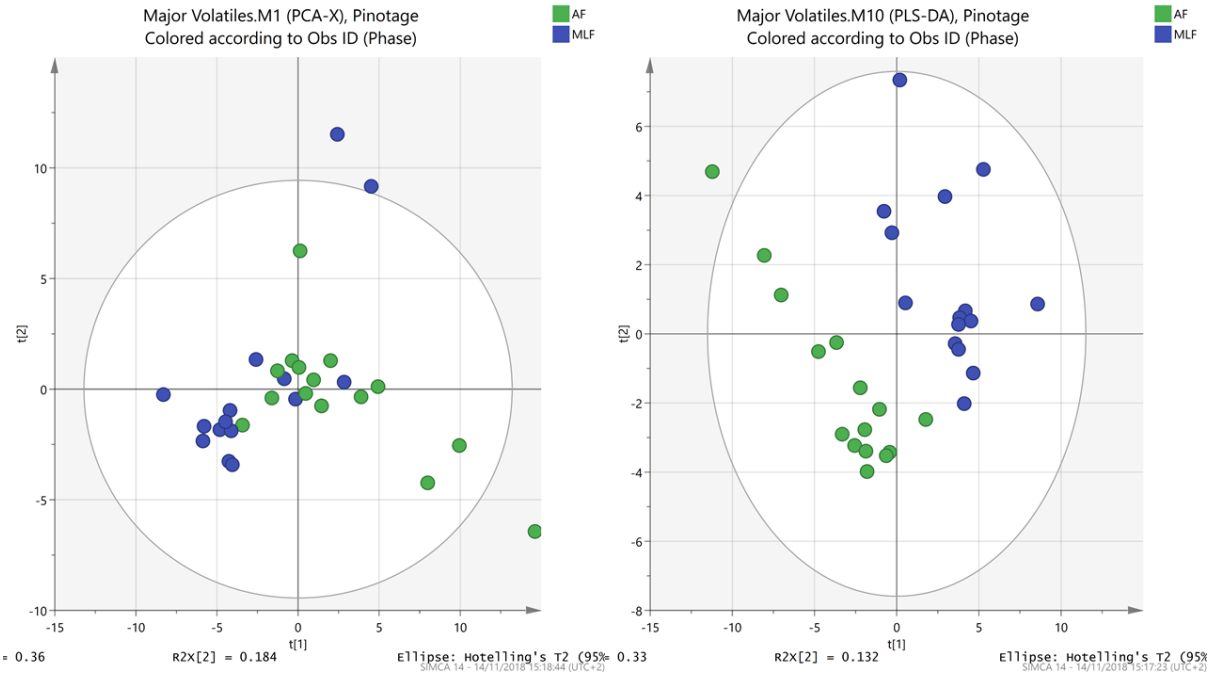

Figure I PCA (Left) and PLS-DA (Right) models of GC/MS data on Pinotage wines after AF (green) and MLF (blue).

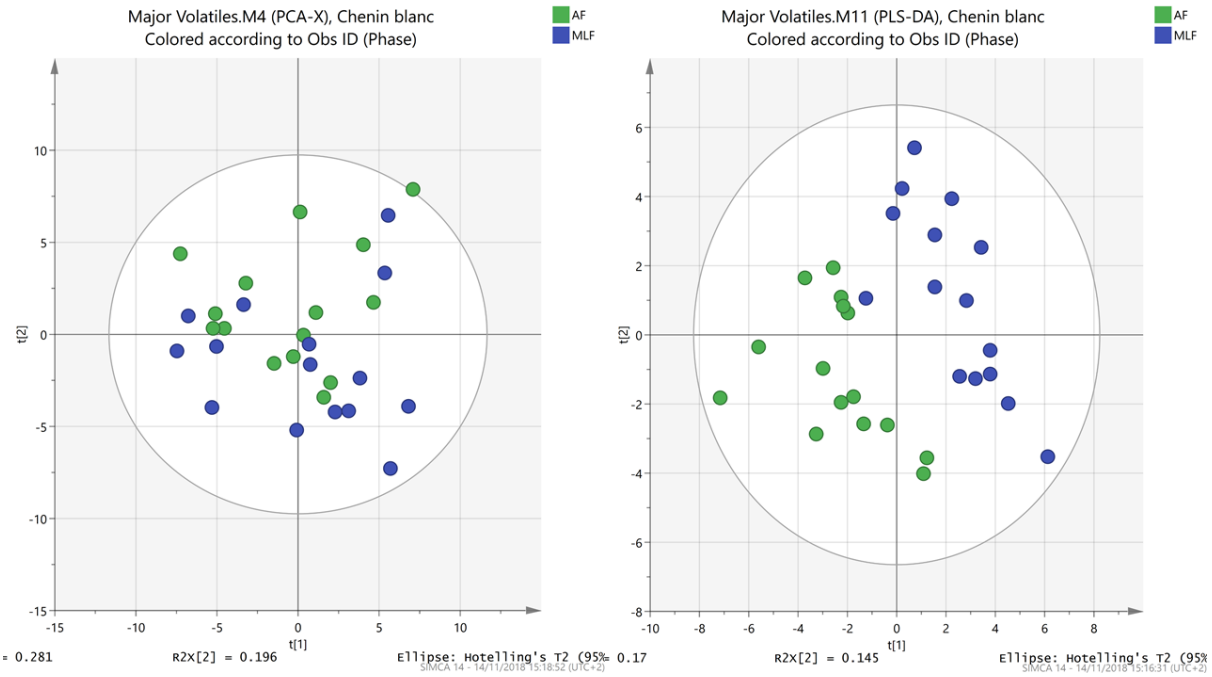

Figure II PCA (Left) and PLS-DA (Right) models of GC/MS data on Chenin blanc wines after AF (green) and MLF (blue).

Table I Detected compounds accompanied by their calculated and reference retention indices from the GC/MS data in Chapter 3.

Compound based on m/z spectra	Calculated RI	Reference RI	CAS Number	References
Ethyl acetate	609	N/A	141-78-6	N/A
Isobutyl alcohol	618	N/A	78-83-1	N/A
1-Butanol	653	N/A	71-36-3	N/A
Ethyl propionate	704	N/A	105-37-3	N/A
Propyl acetate	706	N/A	109-60-4	N/A
3-Methyl-butanol	734	N/A	123-51-3	N/A
1-Butanol, 2-methyl	736	N/A	137-32-6	N/A
Isobutyl acetate	774	N/A	110-19-0	N/A
Ethyl butanoate	804	N/A	105-54-4	N/A
Ethyl lactate	818	N/A	97-64-3	N/A
Butyl acetate	818	N/A	123-86-4	N/A
2-Butanoic acid, ethyl ester	849	844	10544-63-5	Miyazaki *et al.*, (2011)
3-Hexen-1-ol	859	858	544-12-7	Zhao, Liu, *et al.*, (2006)

1-Hexanol	877	876	111-27-3	Kim *et al.*, (2001)
Isoamyl acetate	884	883	123-92-2	Shalit *et al.*, (2001)
Amyl acetate	917	916	628-63-7	Weissbecker *et al.*, (2004)
Methyl caproate	928	927	106-70-7	Tešević *et al.*, (2005)
1-Octen-3-ol	981	981	3391-86-4	Liu *et al.*, (2006)
3-Methylpentyl acetate	984	980	35897-13-3	Andriamaharavo, (2014)
1-Hexyl-acetate	984	N/A	N/A	N/A
6-Methyl-5-hepten-2-one	989	988	110-93-0	Jalali-Heravi *et al.*, (2006)
3-Octanol	998	998	589-98-0	Sartoratto *et al.*, (2004)
Ethyl caproate	1002	1002	123-66-0	Mahattanatawee *et al.*, (2005)
cis-3-Hexenyl-acetate	1006	1007	3681-71-8	Zhao, Wang, *et al.*, (2006)
cis-3-hexenyl-acetate	1008	N/A	N/A	N/A
2-Hexenyl acetate	1010	N/A	N/A	N/A
N-Hexyl Acetate	1016	1015	142-92-7	Ruther, (2000)
ρ-Cymene	1026	1025	99-87-6	Akpulat *et al.*, (2005)
1-Octanol	1030	N/A	N/A	N/A
Limonene	1030	1030	138-86-3	Harzallah-Skhiri *et al.*, (2006)
Cineol	1032	1032	470-82-6	Ramos *et al.*, (2000)
Heptyl acetate	1045	N/A	N/A	N/A
Ethyl E-2-hexanoate	1047	N/A	N/A	N/A
Isoamyl butyrate	1058	1058	106-27-4	Forero *et al.*, (2009)
γ-Terpinene	1061	1061	99-85-4	Siani *et al.*, (1999)

α-terpinolene	1088	1088	586-62-9	Asuming *et al.*, (2005)
Propyl caproate	1094	1094	626-77-7	Pino *et al.*, (2010)
Linalool	1099	1099	78-70-6	Vagionas *et al.*, (2007)
3-methyl-1-cyclohexene	1103	N/A	N/A	N/A
Phenylethyl alcohol	1116	1116	60-12-8	Zhao, Li, *et al.*, (2006)
Methyl-octanoate	1125	1125	111-11-5	Tešević *et al.*, (2005)
Cyclopentane, 1,2-dimethyl-3-methylene	1130	N/A	N/A	N/A
Isobutyl caprate	1140	N/A	N/A	N/A
Octyl acetate	1213	1213	112-14-1	Saroglou *et al.*, (2006)
Isoamyl hexanoate	1251	1250.9	2198-61-0	Andriamaharavo, (2014)
Geraniol	1256	1256	106-24-1	Hennig & Engewald, (1994)
3-Methylbutyl hexanoate	1254	1254	2198-61-0	Forero *et al.*, (2009)
Phenylethyl acetate	1259	1258	103-45-7	Oliveira *et al.*, (2007)
1-Decene	1272	N/A	N/A	N/A
Vitispirane	1282	1281	65416-59-3	Demyttenaere *et al.*, (2003)
Propyl caprylate	1291	N/A	N/A	N/A
Ethyl pelargnate	1295	1294	123-29-5	Pino *et al.*, (2005)
Methyl caprate	1324	1325	110-42-9	Saroglou *et al.*, (2006)
Citronellyl acetate	1353	1354	150-84-5	Jalali-Heravi *et al.*, (2006)

β-Damascene	1386	1386	23726-93-4	Nickavar *et al.*, (2002)
Decanoic acid	1385	1387	334-48-5	Alissandrakis *et al.*, (2007)
Ethyl caprate	1399	1399	110-38-3	Pino *et al.*, (2005)
Tetra decanal	1409	N/A	N/A	N/A
2-Phenylethyl ester	1443	N/A	N/A	N/A
Isoamyl octanoate	1447	1446	2035-99-6	Tešević *et al.*, (2005)
Isoamyl caprylate	1450	1450	2035-99-6 (2)	Andriamaharavo, (2014)
1-dodecanol	1474	1474	112-53-8	David *et al.*, (2002)
β-Ionone	1485	1488	14901-07-6	Zhao, Li, *et al.*, (2006)
Propyl decanoate	1490	1493	30673-60-0	Zhao *et al.*, (2008)
Isobutyl Caprate	1547	1549	30673-38-2	Andriamaharavo, (2014)
Nerolidol	1566	N/A	N/A	N/A
Ethyl laurate	1595	1596	106-33-2	Flamini *et al.*, (2006)
Hexadecane	1600	N/A	N/A	N/A
3-Methylbutyl-decanoate	1647	1646	2306-91-4	Zhao *et al.*, (2008)
Ethyl palmitate	2001	1999	628-97-7	Isidorov *et al.*, (2001)

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Appendix B

Appendix B

i. Trial micro-fermentations performed on Cabernet Sauvignon for method optimization

This appendix contains data from Cabernet Sauvignon trial runs, performed in 200 mL Erlenmeyer flasks, that was used to optimise the methods used in Chapter 3. Methods and materials were the same as found in Chapter 3 for fermentation monitoring and juice characterisation. The grapes were obtained from Uva Mira mountain vineyards, were crushed and thermo-vinified with the skins, as done in Chapter 3 with the Chenin blanc juice, before pressing. Only ATR-IR was used for chemical profiling and no GC/MS was performed. Juice parameters were as follows: pH 3.6, 1.82 g/L malic acid, 250 g/L total sugar (adjusted to 230 g/L with dH_2O), 30 ppm total SO_2 and 243 mg/mL YAN and determined the same as in Chapter 3.

ii. Fermentation kinetics

The different fermentations all showed similar behaviour with regards to the consumption of D-glucose, D-fructose and L-malic acid (Figure i-A, B and C). Only Lt/Sc and CON fermentations showed L-lactic acid production (0.171 ± 0.004 g/L and 0.162 ± 0.005 g/L, respectively) in the first day (Figure i-D). The fermentations lasted 6 days and all of them were able to achieve dryness (<4 g/L total sugar). After, MLF was induced by inoculating CH16.

The inoculation of CH16 was successful in all fermentations. Overall the populations for CH16 did not differ between the fermentations (data not shown) and MLF took 3 days to complete. Generally, the kinetics were similar (Figure i-C) though a faster kinetic was observed after 2 days for Mp/Sc treatments (0.250 ± 0.013 g/L of L-malic acid) as opposed to the other treatments (an average L-malic acid level of 0.740 ± 0.051 g/L). The kinetic for L-lactic production is however similar.

iii. Population dynamics

Generally, the non-*Saccharomyces* could not persist past mid fermentation and fell below detectable limits at different times (Figure ii). The weakest persistence was exhibited by Mp, in both the Mp/Sc and CON fermentations. Mp persisted until 2 days before falling below detectable limits. However, the rate of decline was greater in the CON fermentations than in the Mp/Sc fermentations. Intermediate persistence was exhibited by Lt in both the Lt/Sc and CON fermentations . It was able to reach a population density of 7.21 and 7.30 log(CFU/mL) in 1 day, for Lt/Sc and CON fermentations respectively. Thereafter, the population gradually

declined until falling below detectable limits by 3 days in Lt/Sc fermentations and 2 days in CON fermentations. The greatest persistence was seen by Td in Td/Sc and CON fermentations. Like Lt, it showed a similar growth rate for the first day, reaching a population of 7.21 log(CFU/mL). Thereafter, Td reached a population of 7.28 log(CFU/mL) in Td/Sc fermentations and 7.25 log(CFU/mL) in CON fermentations. After 5 days, Td fell below detectable limits. The population dynamics for Sc exhibited similar trends between the different pairing fermentations. It should be noted that on average a higher number of Sc was found in Mp/Sc, Lt/Sc and Td/Sc pairings of 8 log(CFU/mL) by 2 days when compared to CON fermentations. Gradual decreases in numbers were then observed for all the trials until the end of fermentation (6 days).

iv. Trial run mid-infrared spectrum results

The PLS-DA score plots, generated for the wines using the infrared spectrum, are displayed in Figure iii and coloured according to fermentation phase (Figure 3.iiiA) and inoculation type (Figure 3.iiiB). It was observed that the sequential induction of MLF (red) after AF (green) affects the different wines. A shift in both dimensions of t[1] and t[2] occurs from AF to MLF. It appears that the MLF wines are more like each other than wines that only went through AF. The data shows that each replicate of the different inoculation treatments for AF are different but more similar for MLF in t[1] and t[2].

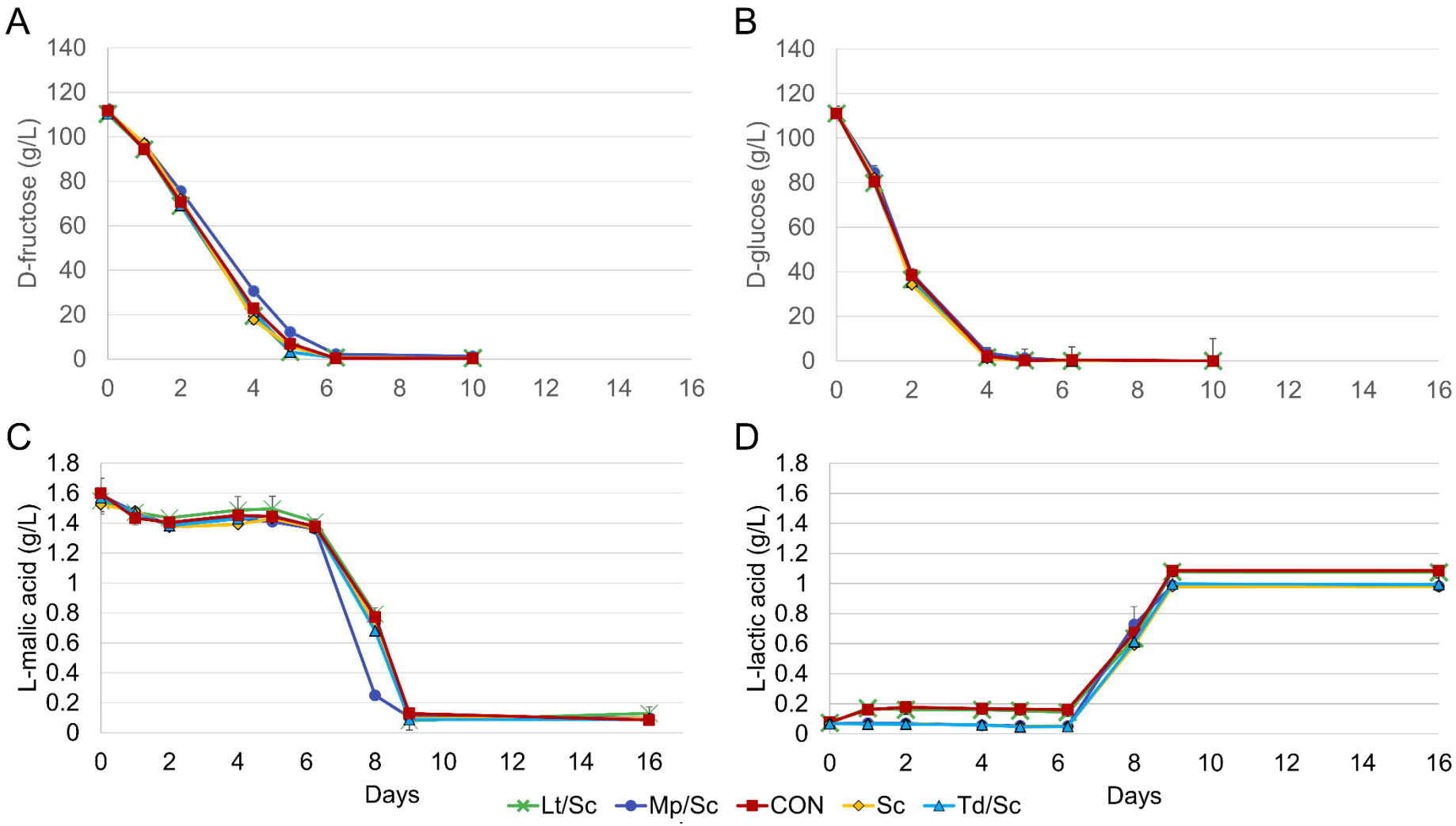

Figure i Chemical kinetics of D-fructose, D-glucose, L-malic acid and L-lactic acid during 25°C fermentations in Cabernet Sauvignon (In A, B, C and D for D-fructose, D-glucose, L-malic acid and L-lactic acid respectively). AF is depicted as the first 6 days before MLF was induced after day 6.

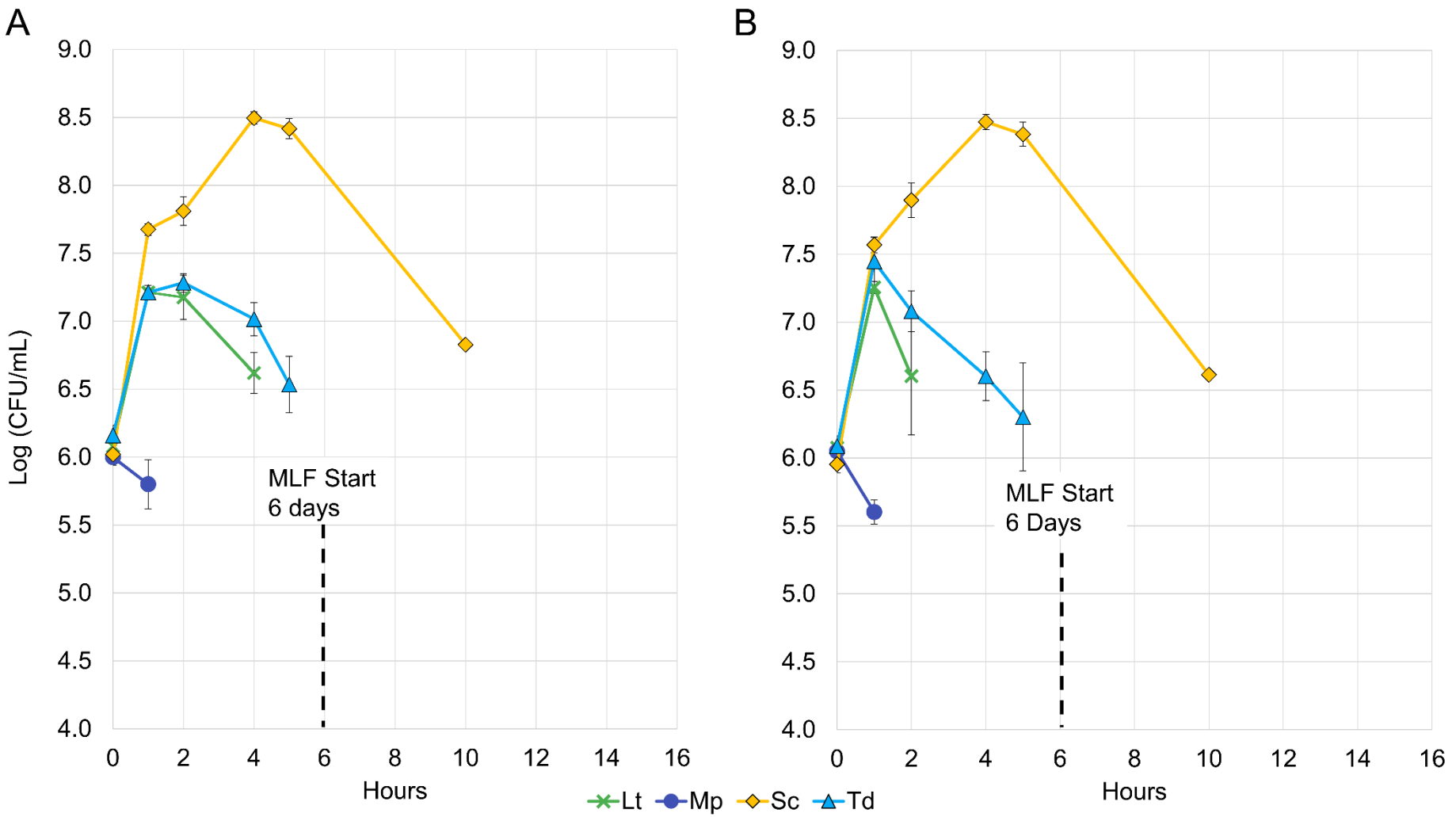

Figure ii Culture based yeast population dynamics of Lt, Mp and Td in pairings with Sc (A) and population dynamics of Lt, Mp, Sc and Td in the consortium (B) for 25°C in Cabernet Sauvignon (In A and B for pairings and the consortium respectively).

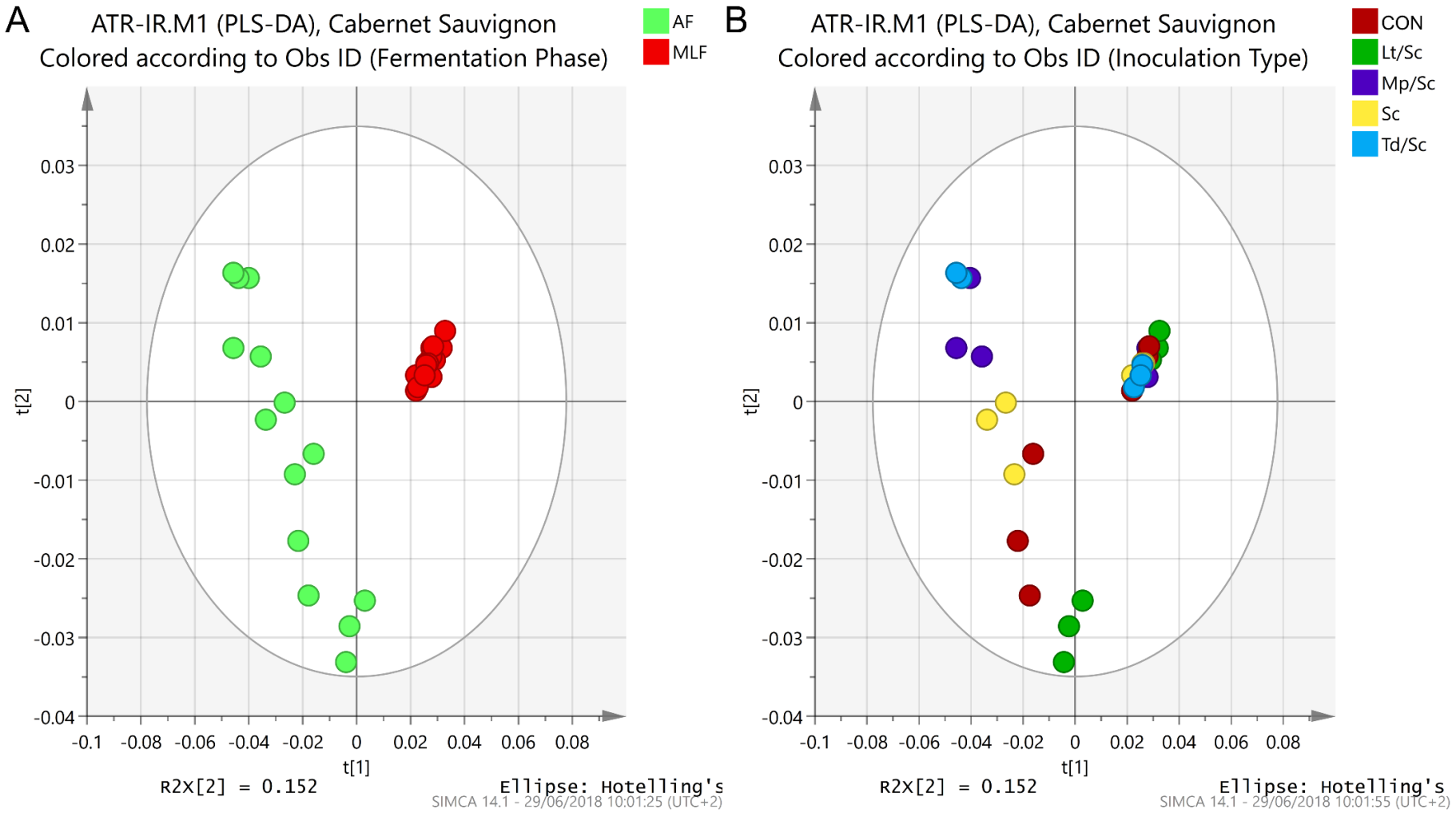

Figure iii PLS-DA model of infrared spectrum on Cabernet Sauvignon trial runs after AF and MLF. The model is coloured according to fermentation phase (A), and inoculation type (B).