Evaluating the impact of yeast co-Inoculation on individual yeast metabolism and wine composition

by

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Declaration

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Summary

The use of non-*Saccharomyces* yeasts together with *Saccharomyces cerevisiae* in mixed starter cultures has become an accepted oenological tool to enhance the organoleptic properties of wine. Recent studies have indeed demonstrated the positive contribution that non-*Saccharomyces* yeasts may have on the bouquet of wine. These mixed starter cultures are characterized by high inoculation levels of individual strains into the must, and each strain in turn is characterized by its own specific metabolic activity. These factors lead to a multitude of interactions occurring between the individual populations within the must. The fundamental mechanisms which drive these interactions are still largely unknown, but several studies have been conducted in order to investigate the metabolic outcome of these interactions. In this study, we endeavour to further characterize the interactions which occur between four individual non-*Saccharomyces* yeast strains in mixed culture fermentation with *S. cerevisiae*.

Metschnikowia pulcherrima IWBT Y1337, *Lachancea thermotolerans* IWBT Y1240, *Issatchenkia orientalis* Y1161 and *Torulaspora delbrueckii* CRBO LO544 were used in mixed culture fermentations with a commercial strain of *S. cerevisiae* at an inoculation ratio of 10:1 (non-*Saccharomyces*: *S. cerevisiae*). The biomass evolution and fermentation kinetics of both participating species were affected by the high cell density of the other, with neither population reaching the maximal density attained by the pure culture fermentation. The final wine composition of each individual mixed fermentation showed clear differences, from the pure cultured *S. cerevisiae* and from each other, based on the concentrations of the major volatile compounds found in the wine. Upon further characterization of these specific mixed culture fermentations, it was found that each individual combination of non-*Saccharomyces* and *S. cerevisiae* produced similar increases and decreases of certain major volatile compounds as demonstrated by previous authors, using the same combination of non-*Saccharomyces* species together with *S. cerevisiae*. From a winemaking perspective, the use of these non-*Saccharomyces* yeast strains in combination with *S. cerevisiae* could be a useful strategy to diversify the chemical composition of wine, by increasing the concentration of certain desirable volatile compounds and by modulating the concentration of undesirable metabolites. Furthermore, this research serves as a foundation for further elucidation of the interactions which drive these metabolic outcomes in response to the high cell density of two yeast populations in mixed culture fermentations.

This thesis is dedicated to my family

Biographical sketch

Arlene Mains was born on December 16th in 1988. She attended Blackheath Primary School and thereafter she matriculated from The Settlers High School in Bellville. She enrolled at The University of Stellenbosch in 2007, where she completed a Bachelor of Science degree specializing in Viticulture and Oenology in 2011. In 2012 she enrolled for the MSc in Wine Biotechnology.

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Preface

This thesis is presented as a compilation of four chapters.

Chapter 1 **General Introduction and project aims**

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Chapter 1

Introduction and project aims

Chapter 1: Introduction

1.1 Introduction

Wine flavour and aroma define wine styles. Several factors affect the organoleptic properties of wine, from farming practices in the vineyard to winemaking procedures in the cellar, including the yeast species selected to perform alcoholic fermentation. The latter has been shown to have a significant influence on the final bouquet of the wine, as depicted in Figure 1. Alcoholic fermentation of grape must may proceed through spontaneous or inoculated fermentation. However, in both circumstances, yeasts of the *Saccharomyces* genus play a key role. *Saccharomyces* spp. are indeed ethanol tolerant and have been shown to rapidly outcompete the other yeasts present in grape must (Querol *et al.*, 1990). *Saccharomyces* yeast strains are therefore the obvious choice of microbial starter culture to drive alcoholic fermentation.

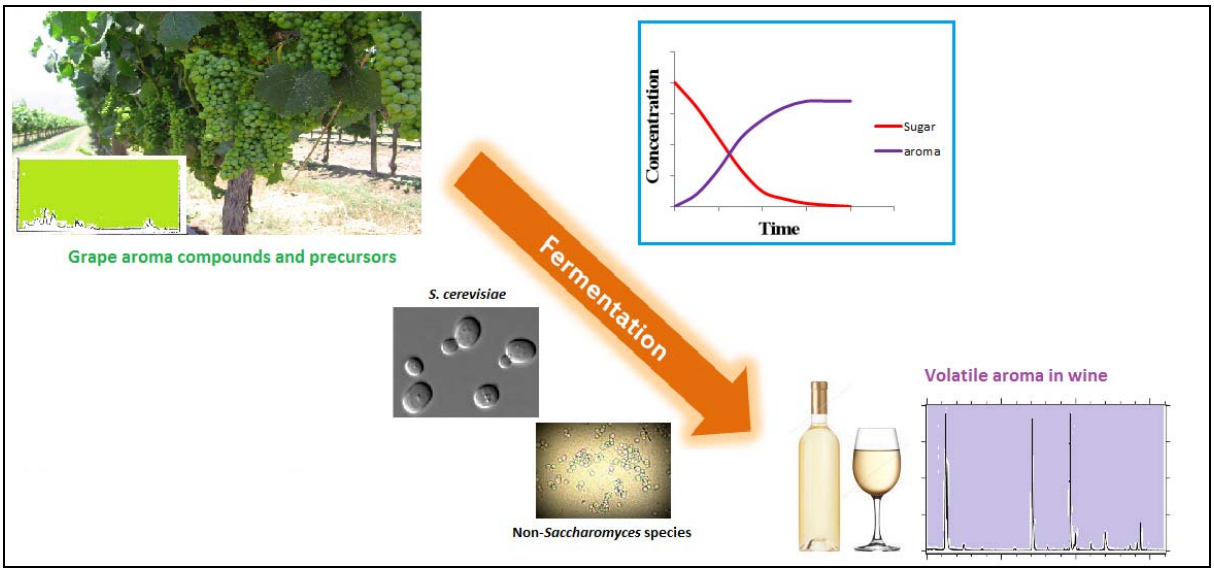

Figure 1: A diagrammatic representation of the microbial modulation of the profile of volatile compounds in wine. Wine yeast can produce desirable volatile aroma compounds by modifying grape-derived molecules and producing flavour active metabolites (Adapted from Swiegers *et al*., 2005)**.**

The implementation of yeast inoculation in winemaking has enhanced the reproducibility and predictability of wine fermentation, but some authors have reported that the practice may lead to a lack of distinctive traits (Ciani *et al.*, 2010). This opinion is also held by many winemakers who consider spontaneously fermented wines superior to wines produced from inoculated musts. Indeed, the former are usually considered to display improved complexity, a more balanced mouth-feel and a better integration of flavour components (Heard & Fleet, 1985; Bisson & Kunkee, 1991; Gil *et al.*, 1996; Lema *et al.*, 1996; Soden *et al.*, 2000). However, Amerine & Cruess (1960), Van Zyl & Du Plessis (1961) Van Kerken (1963), Rankine (1972), and Le Roux *et al.* (1973) had referred to non-*Saccharomyces* yeasts as spoilage microorganisms, an opinion that was based on the fact that non-*Saccharomyces* yeast strains were frequently isolated from stuck or sluggish

fermentations. In addition, it is well established that certain non-*Saccharomyces* yeast species belonging to the genus *Candida*, *Pichia* and *Hansenula* can be responsible for the excessive production of unwanted compounds such as acetic acid, ethyl acetate and acetaldehyde (Grgin, 1999).

This has led to several studies to establish the impact on the chemical composition of wine of the inoculation of selected non-*Saccharomyces* yeasts (Swiegers *et al*, 2005; Domizio *et al.*, 2007; Renouf *et al.*, 2007; Fleet, 2008). The data show that some non-*Saccharomyces* yeast species significantly partake in fermentation and can contribute to aroma complexity and improve other quality parameters of wine (Ciani *et al.*, 2010).

1.2 Rationale

With the mounting perception that wines produced with single inoculated *Saccharomyces* starter cultures are less complex and more standardized (Rainieri and Pretorius, 2000; Mannazzu *et al.*, 2002), the use of carefully selected non-*Saccharomyces* together with *Saccharomyces cerevisiae* has been proposed as a means to produce wines which are more complex whilst averting the risks related to fermentations solely executed by non-*Saccharomyces* yeast strains (Bisson and Kunkee, 1993; Heard 1999; Rojas *et al.*, 2003; Romano, 2003; Ciani *et al.*, 2006).

In this study, we investigated the impact of interactions between *S. cerevisiae* and four non-*Saccharomyces* yeast species which have previously been isolated from South African grape must, and their combined impact on the production of wine-relevant metabolites and final wine composition. The study was aimed to provide increased knowledge pertaining to yeast-yeast interactions. With the existing interest in co-inoculation and associated diversification of metabolite production, the results which were obtained from the study will provide a useful basis to further characterise the metabolic traits related to the non-*Saccharomyces* strains which will be tested.

Specific aims of the study

1. Determine the impact of co-inoculation on the population dynamics of *S. cerevisiae* and selected non-*Saccharomyces* yeast in the mixed fermentation.
2. Evaluate the impact on fermentation kinetics.
3. Assess the final metabolic profile of mixed fermentations in comparison to the fermentation performed by the single strains.

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Chapter 2

Literature review

Yeast Interactions and their impact on wine composition

Chapter 2: Yeast interactions and their impact on wine composition

2.1 Introduction

Wine is a product which has been part and parcel of daily living for millennia and has always been an essential part of the Mediterranean lifestyle (Blanco, 1997). The evolution of grape and wine production methods in Western civilization during this period has mostly coincided with general technological developments (Kennedy *et al.*, 2005). However, despite millennia of grapevine cultivation and winemaking, the science of the transformation process of grape juice to wine has only started to be understood 150 years ago.

A pivotal point in the advancement in winemaking was the ability to inoculate grape juice with selected cultures of *Saccharomyces cerevisiae*, the species that had previously been identified as the main yeast conducting alcoholic fermentation. Owing to the dominance of the inoculated yeast, microbiological control of the fermentation process allows for better management of alcoholic fermentation, in part through the suppression of the other naturally occurring yeasts. Nevertheless, anecdotal evidence suggests that inoculated musts, whose microbial diversity is being restricted, may reduce the organoleptic complexity of the final product (Reed and Nagdawithana, 1988; Grossman *et al.*, 1996). In spontaneous fermentation of grape must, the succession of yeast populations is characterized by the initial development of low alcohol tolerant non-*Saccharomyces* yeast species that are then superseded by *Saccharomyces* species, which continue to persist and complete fermentation (Amerine *et al.*, 1980; Martini, 1993). Despite the fact that *Saccharomyces* species dominate the latter part of spontaneous fermentations, it is well acknowledged that the yeast ecology of wine fermentation is very complex, and that non-*Saccharomyces* yeast species may play relevant roles in the metabolic outcome and consequently the aroma complexity of the final product (Ciani *et al.*, 2009). The inclusion of non-*Saccharomyces* yeast strains with traditional *Saccharomyces* starter cultures has therefore been proposed as a tool to enhance the chemical composition and sensory properties of wine, without running the risk of stuck fermentations (Bisson and Kunkee,1991; Heard, 1999; Rojas *et al.*, 2003; Romano *et al.*, 2003a; Ciani *et al.*, 2006; Jolly *et al.*, 2006).

2.2 The role of non-*Saccharomyces* wine yeast strains in wine-making

Non-*Saccharomyces* yeasts at the commencement of spontaneous fermentation are usually abundant and the nature and variety of species are unpredictable (Fleet, 2003). Species typically belong to genera such as *Hanseniaspora*, *Candida*, *Torulaspora*, *Metschnikowia* and *Kluyveromyces* and originate from the surface of the berry skin and from the winery environment (Fleet *et al.*, 1984; Fleet and Heard, 1993). Alongside these yeast genera, *Issatchenkia* (Van Zyl and du Plessis, 1961) and *Pichia* (Kurtzman and Fell, 1998) may also be found at the early stage

of fermentation. Table 2.1 lists a few examples of non-*Saccharomyces* yeast genera which have been reported to be isolated from spontaneous wine fermentations.

Table 2.1: Examples of the frequently occurring non-*Saccharomyces* yeast in the Ascomycetous genera encountered in wine fermentations (Kurtzman and Fell, 2011)

Anamorphic Form	Teleomorphic form	Former name
Bettanomyces bruxellensis	*Dekkera bruxellensis*	
Candida colliculosa	*Torulaspora delbrueckii*	*Saccharomyces rosei*
Candida famata	*Debaryomyces hansenii*	
Candida globosa	*Citeromyces matritensis*	
Candida carpophila		*Candida guilliermondii*
Candida pelliculosa	*Wickerhamomyces anomalus*	*Pichia anomala*
Candida lambica	*Pichia fermentans*	*Torulopsis pulcherrima*
Candida reukaufii	*Metschnikowia reukaufii*	
Candida pulcherrima	*Metschnikowia pulcherrima*	
Candida valida	*Pichia membranifaciens*	
Candida bombicola	*Starmerella bombicola*	*Candida stellata*
Kloeckera apiculata	*Hanseniaspora uvarum*	
Kloeckera apis	*Hanseniaspora guilliermondii*	
	Hanseniaspora occidentalis	
Kloeckera corticis	*Issatchenkia terricola*	*Pichia terricola*
	Lachancea thermotolerans	*Kluyveromyces thermotolerans*
	Saccharomycodes ludwigii	
	Zygosaccharomyces bailii	*Saccharomyces bailii*
	Millerozyma farinosa	

Considering that the Table 2.1 only shows frequently encountered species, it is likely that the grape must microbiome is highly diverse (Jolly *et al*., 2006). However, the continuous changes in fermenting grape juice have a dramatic impact on the prevailing population. The initial must is characterised by high osmotic pressure, high sugar concentrations, low pH and frequently the presence of sulphur dioxide, all of which impact the survival of the yeasts population (Bisson and Kunkee, 1991; Longo *et al.*, 1991). Furthermore, the yeast population is influenced by the shift from aerobic to anaerobic conditions, as well as the progressively increasing ethanol concentration and decreasing amounts of assimilable carbon and nitrogen sources. These factors negatively impact the survival of many non-*Saccharomyces* yeasts. However, the extent of the initial development of several species suggests that before their decline during fermentation, such species may secrete metabolites that contribute to the final bouquet of the wine (Lambrechts and Pretorius, 2000; Fleet 2003). Furthermore, recent studies using molecular biology techniques have demonstrated that certain non-*Saccharomyces* yeasts may survive until the later stages of fermentation (Zohre and Erten, 2002; Fleet, 2003; Combina *et al.*, 2005) at cell densities as high as

10^6-10^8 cfu.mL^{-1}, suggesting an even stronger impact of these species on wine composition than initially thought.

2.3 The influence of non-*Saccharomyces* on wine composition

The lead role of wine yeasts is to catalyse the rapid, complete and efficient conversion of grape sugars to ethanol, carbon dioxide and other minor, but sensorially important secondary metabolites, without producing off-flavours (Swiegers *et al.*, 2005; Pretorius 2000). These secondary metabolites are produced throughout alcoholic fermentation as a result of wine yeast metabolism and are essential for the wine aroma. A list of some of the principal volatile aroma compounds that influence wine aroma is shown in Table 2.2. These volatile compounds are produced when the fermentable sugars along with long-chained fatty acids, nitrogen and sulphur compounds are degraded during fermentation (Manzanares *et al.*, 2011). Although both spontaneous and inoculated fermentations are completed by alcohol tolerant *S. cerevisiae*, in each circumstance the contribution of non-*Saccharomyces* yeasts cannot be excluded, even though the latter might reduce the effect of non-*Saccharomyces* yeasts more drastically than the former. Indeed, several studies have shown that the use of starter cultures does not totally prevent the development and metabolic activity of naturally occurring strains of *S. cerevisiae*, and of species such as *K. apiculata*, *H. uvarum*, *C. stellata*, or *T. delbrueckii* (Egli *et al.*, 1998; Heard and Fleet, 1986b, 1985; Henick-Kling *et al.*, 1998; Lema *et al.*, 1996).

Table 2.2: Principal volatile fatty acids, higher alcohols, esters and carbonyl compounds produced during alcoholic fermentation (Manzanares *et al.*, 2011)

Volatile Fatty Acids	Higher Alcohols	Esters	Carbonyl compounds
Acetic acid	Propanol	**Ethyl acetate**	**Acetaldehyde**
Butyric acid	Butanol	**2-Phenylethyl acetate**	Benzaldehyde
Formic acid	Isobutyl alcohol	**Isoamyl acetate**	Butanal
Isobutyric acid	Amyl alcohol	**Isobutyl acetate**	Diacetyl
Isovaleric acid	**Isoamyl alcohol**	Hexyl acetate	Propanal
Propionic acid	Hexanol	Ethyl butanoate	Isobutanal
Valeric acid	**Phenylethanol**	Ethyl caprate	Pentanal
Hexanoic acid		Ethyl caprylate	Isovaleraldehyde
Heptanoic acid		Ethyl caproate	2-Acetyl tetrahydropyridine
Octanoic acid		Ethyl isovalerate	
Nonanoic acid		Ethyl 2-methylbutanoate	
Decanoic acid			
Tridecanoic acid			

The most abundant compounds found in wine are shown in boldface

Furthermore, Domizio *et al.* (2011) demonstrated with the use of three different ratios of *Saccharomyces* yeast to non-*Saccharomyces* yeast, namely 1:1; 1:100 and 1:10,000, that while inoculum ratio 1:1 did not impact the fermentation rate or biomass production of *S. cerevisiae,* the higher inoculum ratios resulted in delays of *S. cerevisiae* cell growth and decreases in the rates of fermentation and biomass production, as compared to the control culture of *S. cerevisiae*, and the analytical profiles of the mixed culture fermentations showed inoculum ratio-dependent increases in the production of selected secondary metabolites.

The subsequent paragraphs endeavour to describe the influence that varying yeast species, in single- and mixed-cultured fermentations, may have on the production of the major aromatic compounds which define the secondary aroma of the final wine product.

2.3.1 Volatile fatty acids

Acetic acid is the most abundant volatile acid found in wine and accounts for 90% of the volatile acids (Fowles, 1992; Henschke and Jiranek, 1993; Radler, 1993). The concentration at which acetic acid occurs in wine is of significant importance as it has a direct impact on the quality of the product. At levels exceeding 0.7 $g.L^{-1}$, acetic acid may masks the aroma of the wine, and a vinegar-like character dominates, while levels of 0.2 - 0.7 $g.L^{-1}$ are considered as acceptable (Corison *et al.*, 1979; Dubois, 1994) and may in some cases have a positive impact on the overall perception of the wine. The concentration range at which acetic acid is produced during fermentation by different *S. cerevisiae* strains and in different conditions has been shown to vary between 100 $mg.L^{-1}$ and 2 $g.L^{-1}$ (Radler, 1993). Within non-*Saccharomyces* yeasts, acetic acid production is highly variable, with for example, *M. pulcherrima* producing acetic acid concentration varying between 0.1 and 0.15 $g.L^{-1}$ and *K. apiculata* between 1.0 - 2.5 $g.L^{-1}$ (Fleet and Heard, 1993; Renault *et al.*, 2009). However, mixed cultures of selected non-*Saccharomyces* and *S. cerevisiae* have been show to frequently lead to substantially reduced levels of the compound. Co-cultures of *S. cerevisiae* with *L. thermotolerans* and *T. delbrueckii* are for example characterized by such a reduction in total volatile acidity (Ciani and Maccarelli, 1998; Sadoudi *et al.*, 2012; Bely *et al.*, 2008) and certain strains of *M. pulcherrima* have similarly showed a reduction in acetic acid (Comitini *et al.*, 2011; Sadoudi *et al.*, 2012).

The production of acetic acid is closely linked to the production of glycerol during alcoholic fermentation, as they both play a direct role in maintaining the redox balance within the cell. Glycerol does not directly impact wine aroma, but is considered to contribute to the mouth-feel, sweetness and complexity of some wines (Ciani and Maccarelli, 1998). Unfortunately, an increased production of glycerol is generally accompanied by an increased production of acetic acid (Prior *et al*., 2000). The use of non-*Saccharomyces* yeasts to reduce the amount of acetic acid and to simultaneously maintain a desirable glycerol content in the wine has been suggested. Ciani

and Ferraro (1998) showed that the use of immobilised *Candida zemplinina* cells in co-inoculation with *S. cerevisiae* in a high sugar must, increased the production of glycerol and lowered the production of acetic acid, while all the sugars were consumed simultaneously, owing to the fructophilic nature of *C. zemplinina* and the glucophilic nature of *S. cerevisiae*. These results were confirmed by Rantsiou *et al*. (2012) who demonstrated that in wines characterized by increased sugar concentration, the co-cultured fermentation of *C. zemplinina* and *S. cerevisiae,* may contribute to the control of acetic acid production by *S. cerevisiae* while still producing elevated levels of glycerol.

Other, longer chain fatty acids, in particular hexanoic (C6), octanoic (C8) and decanoic (C10) acids are also produced by yeast during fermentation. They are found in low concentrations of 0.5 – 10 $mg.L^{-1}$, and considered to be by-products of fatty acid metabolism (Viegas *et al.*, 1989). These medium chain fatty acids have a toxic effect to *S. cerevisiae* and may result in the arrest of fermentation, but can also form esters which significantly contribute to the pleasant fermentation aroma of wine. Comparatively, these fatty acids and their esters are produced at lower concentrations by non-*Saccharomyces* yeasts than by *S. cerevisiae* (Renault *et al.*, 2009; Rojas *et al.*, 2001; Viana *et al.*, 2008) and the concentrations at which these fatty acids are produced in mixed culture fermentations are mostly substantially below levels that could inhibit the growth of *S. cerevisiae* and halt fermentation (Edwards *et al.*, 1990).

2.3.2 Higher alcohols

Higher alcohols are secondary yeast metabolites which when found in concentrations below 300 $mg.L^{-1}$, are considered to impart aromatic complexity and fruity notes to the wine. However, at levels above 400 $mg.L^{-1}$ (Rapp and Versini, 1991), the wine can be perceived as strong and pungent in smell and taste (Lambrechts and Pretorius, 2000; Swiegers and Pretorius, 2005). These compounds gain further importance as precursors for the formation of mostly desirable esters (Soles et al., 1982). The choice of yeast strain for alcoholic fermentation is known to contribute to the variation in the higher alcohol content Giudici *et al*., 1990; Rankine, 1986b). In monocultures of non-*Saccharomyces* yeasts, it was found that the final concentration of higher alcohols is, more often than not, lower than the concentration found in pure culture *S. cerevisiae* (Moreira *et al.*, 2008; Rojas *et al.*, 2003; Viana *et al.*, 2008, 2009) but the concentrations found in reported mixed fermentations are often similar to those occurring in pure cultured *S. cerevisiae*. There are however exceptions: pure cultures of *C. zemplinina* have indeed been shown to produce more higher alcohols when compared to pure-cultured *S. cerevisiae* (Andorrá *et al.*, 2010). In studies where the objective was to determine if non-*Saccharomyces* yeasts contributed to the increased production of higher alcohols in mixed fermentations, it was found that the concentration of higher alcohols was similar in pure *S. cerevisiae* and mixed fermentations (Gil *et al*., 1996; Longo *et al*., 1992; Mateo et al., 1991).

2.3.3 Esters

Esters produced by yeasts during fermentation have a considerable effect on the fruity aromas in wine. They are some of the most abundant compounds found in wine and are frequently in concentrations above perception threshold values (Salo, 1970a, 1970b). The most predominant esters are ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl caproate and 2-phenylethyl acetate (Thurston *et al.*, 1982) that are associated with fruity, pear-drops, banana, apple and flowery aromas, respectively. The esters produced throughout alcoholic fermentation significantly influence the sought-after fermentation bouquet. Thus, the yeast strain conducting alcoholic fermentation has a direct impact on the fermentation bouquet of the wine. A significant amount of non-*Saccharomyces* yeast strains have been described as being proficient in the production of esters. Table 2.3 shows some of these non-*Saccharomyces* genera that are known to be good producers of esters. It is important to note that there is no standard for ester production by yeast, as with all other metabolites, but rather the production of esters during fermentation is species and strain dependent, among other contributing factors (Lambrechts and Pretorius, 2000). In a study conducted by Rojas *et al.* (2003), *Hanseniaspora guilliermondii* and *Pichia anomala* were used in co-cultures with *S. cerevisiae.* This study revealed an increase in acetate ester concentrations when compared to the pure cultured *S. cerevisiae.* These results were later confirmed by Viani *et al.* (2008). In the latter study, 38 non-*Saccharomyces* yeast strains from the genera *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and *Zygosaccharomyces*, were screened for ester production in synthetic medium. The authors found that the ester production from the genera *Hanseniaspora* and *Pichia* were the most prominent, and specifically that of *H. osmophila* which displayed an increased production of 2-phenylethyl acetate. *Candida pulcherrima* has also been reported to produce increased levels of esters (Bisson and Kunkee, 1991). Furthermore, Comitini *et al.* (2010) showed that in mixed fermentations of *L. thermotolerans*, *M. pulcherrima* and *T. delbrueckii* with *S. cerevisiae,* where the inoculation ratio was (10,000:1), an increase in the concentration of ethyl acetate and ethyl lactate was observed. The concentrations did not exceed the sensory threshold limits which would have led to an undesirable impact, and are thus likely to positively contribute to wine character.

Table 2.3: Non-*Saccharomyces* yeast genera that produce esters (Manzanares *et al.*, 2011)

Genus	Esters produced via Yeast metabolism			
	Ethyl acetate	Isoamyl acetate	2-Phenylethyl acetate	Ethyl caproate
Candida	+			
Hanseniaspora	+	+	+	
Pichia	+	+		
Rhodotorula		+		
Torulaspora				+

2.4 Extracellular enzymes of oenological interest produced by non-*Saccharomyces* yeasts

As mentioned in the previous paragraphs, the selected yeast strain which conducts alcoholic fermentation plays an important role in the final wine aroma and composition and the impact of its metabolism cannot be negated. But in addition to the yeast metabolism derived compounds, grape derivatives and precursors play an equally large role, and the concentration as well as the availability of these precursors contributes to the volatile aroma of the wine. Of these grape derivatives and precursors, terpenes and thiols are amongst the most important. Both are present in grape must in a non-volatile and non-fragrant form. As a part of common winemaking practices, exogenous enzymes are frequently added to the grape must in order to hydrolyse glycosylated precursors and release free terpenes from their sugar moiety. Thiols, on the other hand, are released by yeast during fermentation from odourless S-cysteine-conjugateses (Tominaga et al., 1998).

Data have shown that some non-*Saccharomyces* yeast strains have notable hydrolytic activity which is non-existing in most *Saccharomyces* yeast strains (Charoenchai *et al.*, 1997; Fernández *et al.*, 2000; Gunata *et al.*, 1994; Mendes-Ferreira *et al.*, 2001; Strauss *et al.*, 2001). One example is the production of ß-D-glucosidase enzymes by some non-*Saccharomyces* yeast. This enzyme can enhance the concentration of the aromatic compounds derived from the grape by converting the molecules from non-aromatic precursors to aromatic molecules in the wine. In a prime example, a ß-D-glucosidase enzyme was purified in *Debaryomyces hansenii* (Riccio *et al.*, 1999; Yanai and Sato, 1999) and the enzyme was found to remain active in the presence of 15% (v/v) ethanol concentration and to liberate terpenes from both extracts of glycosylated precursors, originating from the grapes and glycosylated precursors which were added to the must during fermentation. In this case, the concentration of linalool (rose) and nerol (rose-like) increased by 90 and 116%, respectively. The use of this purified enzyme from *D. hansenii* in traditional winemaking might therefore lead to an increase of aroma active free terpenes in the wine. Similarly, the concentration of volatile thiols has been shown to be dependent on the yeast strain which conducts alcoholic fermentation (Dubourdieu *et al.*, 2006; Swiegers *et al.*, 2009). Anfang *et al*. (2009) showed that mixed fermentations of *Pichia kluyevri* and *S. cerevisiae*, and *C. zemplinina* and *S. cerevisiae* resulted in significantly elevated concentrations of 3-mercaptohexyl-acetate and 3-mercaptohexan-1-ol, which are associated with passion fruit or grapefruit (Tominaga *et al*., 1998). In more recent studies, it has been demonstrated that certain non-*Saccharomyces* yeast strains are able to liberate these volatile thiols, as they possess the ß-lyase enzymes which can cleave the precursors in the must (Anfang *et al.*, 2009; Zott *et al.*, 2011). These ß-lyases, or more specifically cysteine-S-conjugate lyases, form part of a large family of enzymes, namely the carbon-sulphur-lyases, in which a carbon bond is cleaved in a ß-elimination reaction. This reaction then yields a free thiol and an intermediate product that spontaneously degrades to pyruvate and ammonia (Davis and Metzler, 1972). The exploitation of these enzymes produced by non-*Saccharomyces*

yeasts may be a useful tool in enhancing the chemical composition of wine, and ultimately wine aroma (Charoenchai et al., 1997) without the use of exogenous enzymes.

2.5 Evidence for yeast-yeast interactions as a result of co-inoculation

As can be seen from the impact on specific compounds synthesized during fermentation, wines produced from mixed starter cultures may be notably more varied in both their chemical make-up and sensorial profiles (Egli *et al.*, 1998). Table 2.4 lists several studies in which non-*Saccharomyces* yeasts were co-inoculated with *S. cerevisiae*, and the impact of these mixed cultures on the final wine composition. Mixed culture fermentations produced a mixture of volatile aroma compounds which were markedly different from the wines which were produced when monocultures of the same yeasts were blended together. For example, *Hanseniaspora uvarum* produces high concentrations of isoamyl acetate in pure cultures, but in mixed fermentation with *S. cerevisiae*, the increase of isoamyl acetate is limited, and the modulating effect of *S. cerevisiae* can be observed (Moreira *et al*., 2008).These results therefore suggest that interactions occur at metabolic level between the individual yeast strains (Sadoudi *et al*., 2012) and the final wine flavour which is produced is partly due to the composite of volatile aroma compounds generated by the co-inoculated strains (Lambrechts and Pretorius, 2000; Fleet, 2003).

Table 2.4: Fermentation behaviour of non-*Saccharomyces* and *Saccharomyces cerevisiae* strains in multistarter inocula (Adapted from Ciani et al., 2010)

Non-*Saccharomyces* yeast species	Characteristic behaviour of pure culture	Effects produced by mixed fermentation with *S. cerevisiae* compared with pure *S .cerevisiae*
Starmerella bombicola (previously known as *Candida stellata*)	Fructophilic yeast	Combined consumption of reduced sugars (improved consumption)
	High glycerol producer	Increase in glycerol production
	High succinic acid producer	Increase in succinic acid production
	High acetaldehyde producer	No increase (combined consumption)
	High acetoin producer	No increase (combined consumption)
	Low ethanol yield	Reduction in final ethanol concentration
Kluyveromyces thermotolerans (now known as *Lachancea thermotolerans*)	Low acetaldehyde producer	Reduction in final acetaldehyde formation
	Lactic acid producer (some strains)	Increase in titratable acidity
Hanseniaspora uvarum	High acetic acid producer	No increase in acetic acid production
	High ethyl acetate producer	Slight increase in ethyl acetate production (strong reduction in comparison with pure culture)
Torulaspora delbrueckii	Low acetic acid producer	Reduction in acetic acid production
Hanseniaspora osmophila	High 2-phenyl ethyl acetate producer	Increase 2-phenyl acetate

Table 2.4 (cont.)

Pichia anomala (now known as *Wickerhamomyces anomalus*)	High producer of isoamyl acetate (EAHase)	Increase in isoamyl acetate production
Pichia kluyveri	High producer of 3- mercaptohexyl acetate	Increase in thiol content
Debaryomyces variji	High levels of ß-glucosidase activity	Increase in terpenols content
Schizosaccharomyces pombe	High rate of malic acid degradation	Reduction in titratable acidity
**Issatchenkia orientalis*	Low producer of malic acid	Reduction in total malic acid

***Addition to original Table**

2.6 Yeast – yeast interactions in wine fermentation

The effect of the co-inoculation of non-*Saccharomyces* together with *S. cerevisiae* on wine composition is undeniable. However, the final composition of the wine product is merely an observation of the yeast metabolism that produces these compounds throughout alcoholic fermentation. It has become gradually more clear that when different species and strains are inoculated together in mixed fermentations, the strains do not inertly co-exist with one another, but rather interact, and unpredictable changes in fermentation behaviour and in the concentrations of aromatic compounds can occur (Howell *et al.*, 2006; Anfang *et al.*, 2009). Indeed, in mixed-culture fermentations, the individual yeast populations in the must will influence the physiological and metabolic activities of each other, which may result in the generation of desirable or undesirable transformations of metabolite and/or the death of some microorganisms (Wood and Hogde, 1985; Leroi and Pidoux, 1993; Geisen et al., 1992; Rossi, 1978).

2.6.1 Interaction mechanisms

The result of the interaction is most frequently assessed on the basis of the effect on the population size (Odum, 1953). Ecological theory describes an array of interactive associations between mixed populations of microorganisms (Boddy and Wimpenny, 1992; Fleet 2003). These interactions may be divided into two distinct categories, namely direct and indirect (Bull and Slater, 1982). Direct interaction refers to predation and parasitism, and implies physical contact, whilst indirect interaction consists of neutralism, commensalism, mutualism, antagonism and competition. Table 2.5 lists the various interactions occurring.

Table 2.5: Definitions and descriptions of interaction between two species populations
(Adapted from Boddy and Wimpenny, 1992; *Rayner and Webber 1984)

Type of Interaction	The effect described between two interacting species		Nature of Interactions	Occurrence	*Classification of Interactions
Competition	**-**	**-**	Both populations are restricted, because of their common dependence on a limiting factor e.g. nutrients or O_2	Arguably the most common interaction type. Often results in the dominance of one species in laboratory systems, although does not preclude co-existence in more natural situations	**Competitive**
Amensalism	**-**	**0**	Growth of one population is restricted by the presence of the other, although the latter is not affected. e.g. the restricted growth of non-*Saccharomyces* yeast by *S. cerevisiae* in the early stages of fermentation.	Leads to one species predominating	
Parasitism/ Predation	**+**	**-**	One organism benefits at the expense of the other. With parasitism the organism gaining benefit is initially smaller whereas with predation the organism gaining benefit is the larger	Occurs frequently in nature, but probably infrequently if at all in food beverage.	
Neutralism	**0**	**0**	Neither species affects the other	Probably does not occur outside the laboratory	**Neutralistic**
Commensalism	**+**	**0**	One population benefits, but the other is unaffected	Probably occurs infrequently, but apparently occurs commonly in mixed cultures for single cell protein production	
Mutualism	**+**	**+**	Populations receive reciprocal benefit	Ranges between 'loose' interactions and dependency. Crucial in nature	**Mutualistic**

(-) detrimental effect; (+) positive effect; (0) no effect

The most predominant parameters which modulate the growth of yeast populations during alcoholic fermentation are competition for limited nutritional resources within the grape must (i.e. the efficiency of the yeast species at utilizing resources, to the detriment of the other species present in the medium, thus enabling it to survive) and the liberation of toxic compounds into the medium (Renault et al., 2012). Many secondary metabolites besides ethanol indeed play a role in the inhibition of yeast species in mixed culture fermentation; these include short-chain fatty acids, acetic acid and acetaldehyde (Bisson, 1999; Ciani et al., 2010; Eschenbruch, 1974; Fleet, 2003). Other inhibition factors include proteins and glycoproteins such as killer toxins. Moreover, whilst particular compounds might have an inhibitory effect on yeast development, their combinatory effect might also contribute to other inhibition mechanisms (Bisson, 1999; Edwards et al., 1990; Fleet, 2003; Ludovico et al., 2001).

Examples demonstrated within mixed cultures of *S. cerevisiae* and non-*Saccharomyces* include the early death of *H. guilliermondii* caused by the secretion of toxic metabolites produced by *S. cerevisiae* (Pérez-Nevado *et al.*, 2005) and the early death of *L. thermotolerans* and *T. delbrueckii* in mixed fermentation with *S. cerevisiae*, which will be described in more detail below.

2.6.1.1 Physical Interaction

Cell proximity stimuli are acknowledged as being influential in the competitive interaction between yeast species and strains in a mixed cultured wine fermentation (Yap *et al.*, 2000; Fleet, 2003; Nissen *et al.*, 2003; Hogan, 2006; Perez-Nevado *et al.*, 2006). Granchi *et al.* (1998) observed that the increase in population of *Kloeckera apiculata* ceased when *S. cerevisiae* reached high cell densities and that the arrest in growth was not attributed to ethanol or temperature. A study by Nissen *et al.* (2003) further strengthened this hypothesis by conducting an experiment in which mixed culture fermentations were performed, using two strains of non-*Saccharomyces* yeasts (i.e. *L. thermotolerans* and *T. delbrueckii*) in combination with *S. cerevisiae*. Physical contact between the species was prevented by the implementation of a dialysis membrane to separate them into two compartments. The metabolites were allowed free passage between the compartments without cell-to-cell physical interaction. The results demonstrated that *S. cerevisiae* reached a cell concentration of 7.3-7.4 log CFU.mL^{-1} in the compartmentalized fermentation which was similar to the concentrations achieved in the mixed fermentations. Conversely, the two non-*Saccharomyces* yeast strains reached a cell concentration of 7.4-7.6 log CFU.mL^{-1} in mono-cultured and in the compartmentalized fermentation. However, in the mixed cultured fermentation, both *L. thermotolerans* and *T. delbrueckii* only reached cell densities of 6.9 and 6.7 log CFU.mL^{-1}, respectively. Recently, using a similar apparatus, Renault *et al.* (2012) also demonstrated that the physical contact between *S. cerevisiae* and *T. delbrueckii* induced the rapid death of *T. delbrueckii*. This phenomenon was attributed to the cell-to-cell contact mechanism. Additionally, Arneborg *et al.* (2005) showed, by utilizing interactive optical tapping, that a non-*Saccharomyces* yeast

(*Hanseniaspora uvarum*), when in close proximity to viable *S. cerevisiae*, displayed delayed growth which also suggested the phenomenon of cell-to-cell contact mechanisms. Following this experiment, Nissen *et al.* (2003) strongly suggested that the arrest in early growth of the non-*Saccharomyces* yeast strains in mixed fermentations was due to cell-to-cell contact, but the explanation thereof at a molecular level and its dependency on *S. cerevisiae* population remain to be established.

2.6.1.2 Metabolic Interaction

To put the production of major volatiles by individual yeast species into perspective, the driving force behind their production needs to be considered. Amino acids originating from the medium are the main source of substrates that are converted to aroma compounds (Lambrechts and Pretorius, 2000) During the course of fermentation, amino acids, for example, valine, leucine, isoleucine, methionine and phenylalanine are gradually acquired by the yeast cells and may be assimilated by the Ehrlich pathway leading to the release of a variety of aroma compounds. The Ehrlich pathway has been elucidated nearly a century ago, as reviewed by Hazelwood *et al.* (2008) but the metabolic regulation of the pathway, the physiological roles of individual parts of the pathway, as well as the enzymes which take part in specific reactions are yet to be fully established (Styger *et al.*, 2010). A simplified diagram representing the Ehrlich pathway is shown in Figure 2.1. In relation to mixed cultured fermentation, inoculated populations of non-*Saccharomyces* and *Saccharomyces* into grape must will result in competition for assimilable nitrogen (amongst others), as yeast assimilable nitrogen (YAN), comprising ammonium and amino acids, is essential for biomass production, and in turn the aroma profile of the final wine product will be determined by the efficiency of the individual yeast strain to consume amino acids.

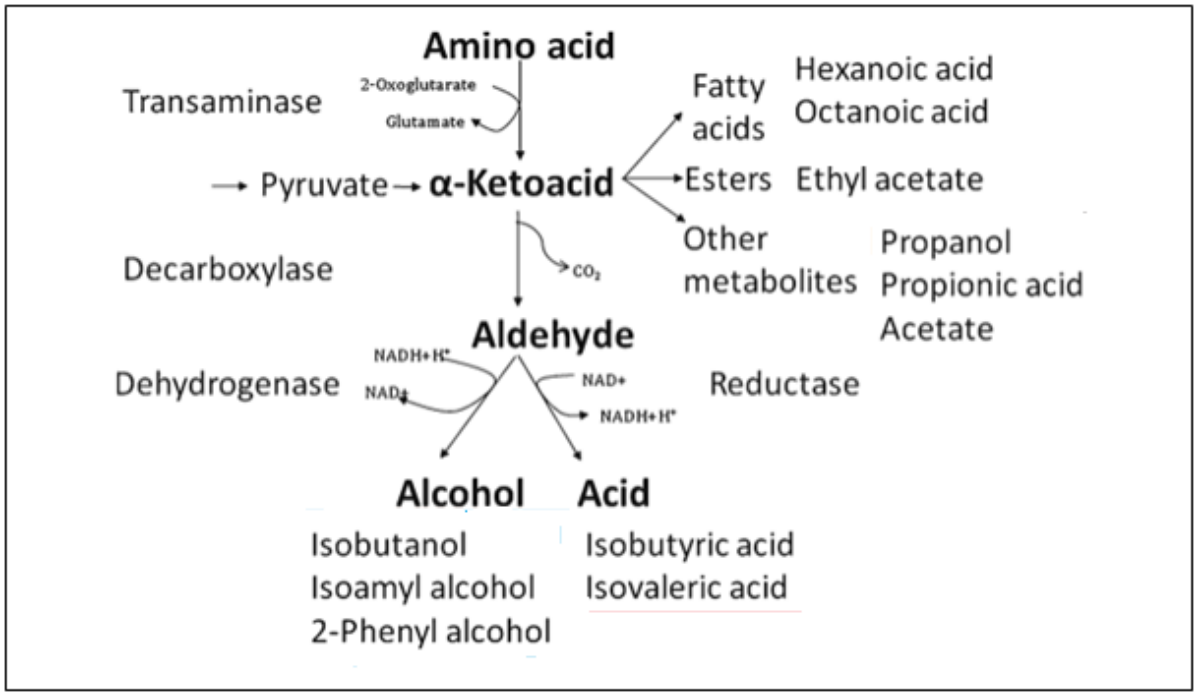

Figure 2.1: A simplified metabolic map of certain aroma compounds produced by *Saccharomyces cerevisiae* via the Ehrlich pathway and related pathways (Styger *et al.*, 2013)

Andorra *et al.* (2012) showed that non-*Saccharomyces* species substantially escalate their use of amino acids for biomass production in comparison to the amount of amino acids used for biomass production by *S. cerevisiae*. Of the non-*Saccharomyces* yeast species which were tested in the study, *H. uvarum* was the least efficient at producing biomass, since it needed to consume the most nitrogen in order to produce equivalent biomass. In co-cultured fermentations of *H. uvarum* and *S. cerevisiae*, *S. cerevisiae* dominated the fermentation. Similarly, in an experiment conducted by Ciani *et al.* (2006), the yeast assimilable nitrogen (YAN) consumption was monitored in mixed fermentations of *L. thermotolerans* and *S. cerevisiae*, and the results showed an increase in the nitrogen consumption, compared to *S. cerevisiae* alone, with the subsequent wine containing a concentration of ethyl acetate which could be considered to be desirable, and could possibly contribute to the fruitiness and overall complexity of the wine. These findings imply that there is a clear competition for nutritional resources that are available in the media.

2.7 Conclusion

The diversity of non-*Saccharomyces* species present during the winemaking process has been shown to be broad, and their concentration ranges from 10^1 CFU.mL^{-1} to 10^6 CFU.mL^{-1}. However, little is truly known about the impact that most of these species might have on the complexity of the final wine. Several studies (as reviewed by Ciani et al., 2010) have described the potential impact of a limited number of inoculated non-*Saccharomyces* yeast on a wine's composition, and the results in many cases suggested promising aromatic enhancement. These findings consequently led to the commercialisation of non-*Saccharomyces* yeast strains to be used in mixed starter cultures together with *S. cerevisiae*, and is considered a strategy to improve wine complexity.

Table 2.6 lists non-*Saccharomyces* yeasts which have become commercially available in the past few years.

Table 2.6: Commercially available non-*Saccharomyces* wine dry yeast products

Yeast Company	Product	Non-*Saccharomyces* yeast strain(s)	Manufacturers' Recommendations
Lallemand	Level^2 Td™	*Torulaspora delbrueckii* + *Saccharomyces cerevisiae*	Promote aromatic complexity in white wine with low aromatic potential
	Flavia™ Mp346	*Metschnikowia pulcherrima**	Overexpress aromatic flavours of varietal white and rosé wines
	Biodiva™TD291	*Torulaspora delbrueckii**	Control the development of wines aromatic complexity by favouring the perception of certain esters without overwhelming the wines
	ProMalic®	*Schizosaccharomyces pombe*	Alternative to acid reduction without the production of lactic acid or chemical deacidification
Chr. Hansen	Prelude™	*Torulaspora delbrueckii**	Used in white, red or rosé wines; softer palate, rounder mouth feel, increases the wine aroma spectrum and longevity
	Viniflora®Concerto™	*Lachancea thermotolerans**	Ideal for red and rosé wines from warm/hot climates, as it produces lactic acid, wine freshness is improved
	Frootzen™	*Pichia kluyveri* *	A radical and natural fruit flavour enhancer
	Melody™	*L. thermotolerans* + *T. delbrueckii* + *S. cerevisiae*	Best product available on the market to manage fermentations in high end Chardonnay wines
Laffort	Zymaflore®Alpha TD n. Sacch	*Torulaspora delbrueckii**	Making of wines with high organoleptic complexity.

* Non-*Saccharomyces* to be used in combination with *S. cerevisiae* (the choice of strain is left to the winemaker)

Thus it is evident that the use of non-*Saccharomyces* yeasts together with *S. cerevisiae* may be a useful strategy to enhance the organoleptic properties of the wine, The positive impacts include the suppression of negatively perceived volatile compounds (e.g. acetic acid), the production of desirable esters and the production of enzymes (esterases, glycosidases, lipases, ß-glucosidases, proteases, cellulases etc.), which may be useful for colour extraction and wine quality (Charoenchai *et al.*, 1997).

To gain further insight into the many potential benefits that may come from the co-inoculation of non-*Saccharomyces* and *S. cerevisiae*, more investigations need to be conducted to establish which selected cultures in mixed fermentation may be suitable for a specific wine style. Moreover, the interactions that occur between the species should be investigated from several approaches,

ranging from the biochemistry behind the relevant pathways which the yeast employs to the physical environment in which fermentation occurs. In this manner, a more holistic interpretation of what occurs in mixed fermentations can be made.

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Chapter 3

Research results

Evaluating the Impact of Yeast Co-Inoculation on Individual Yeast Metabolism and Wine Composition

Chapter 3: Research results

Abstract

The yeast population present in grape juice at the beginning of inoculated and spontaneous fermentations is diverse, but ultimately alcoholic fermentation is mostly carried out by *Saccharomyces cerevisiae*. The rapid dominance of *S. cerevisiae*, in particular in inoculated grape must indeed diminishes the potential positive impact indigenous non-*Saccharomyces* yeasts may have on the organoleptic properties of the wine. Although non-*Saccharomyces* yeasts were commonly associated with negative wine properties in the past, recent evidence has indeed shown that some species can contribute positively to the character of wine. In this study, we investigate the possible contributions of 4 selected non-*Saccharomyces* species to wine fermentation and quality. For this purpose, single strains of *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, *Issatchenkia orientalis* and *Torulaspora delbrueckii* were individually co-inoculated at a 10:1 ratio with *S. cerevisiae* in synthetic grape juice. In order to better qualify the complex interactions which occur between the mixed populations, fermentation kinetics and population dynamics were monitored and the concentration of 32 major volatiles were measured in the resultant wine. In all cases, a decrease in the rate of fermentation and in the maximum population of *S. cerevisiae* was observed in mixed fermentations. Moreover, the analysis of the major volatile compounds suggested that metabolic interactions occur between these species. The role of *S. cerevisiae* in completing alcoholic fermentation was clearly demonstrated, as all single culture non-*Saccharomyces* strains resulted in stuck fermentations, and *S. cerevisiae* was able to outcompete all the non-*Saccharomyces* in mixed fermentations, with the exception of *L. thermotolerans*. Data of the mixed culture of *L. thermotolerans* with *S. cerevisiae* suggested a possible increase in the production of higher alcohols. *M. pulcherrima* combined with *S. cerevisiae* resulted in a significant increase in the concentration of medium chain fatty acids, which could possibly be indication of an antagonistic response between these two yeast populations. Furthermore, *I. orientalis*: *S. cerevisiae* exhibited a similar wine composition to single-cultured *S. cerevisiae*, while the mixed culture of *T. delbrueckii*: *S. cerevisiae* showed a decrease in all measured major volatiles, in comparison to single culture *T. delbrueckii* and *S. cerevisiae*. The results obtained in this study demonstrate that wines produced from mono- and mixed-culture fermentations are markedly different, and that multistarter cultures may be a beneficial strategy to increase the organoleptic properties of wine.

Keywords: non-*Saccharomyces* yeasts, major volatiles, wine, yeast-yeast interactions, yeast population dynamics.

Abbreviations: MpSc, *M. pulcherrima* and *S. cerevisiae* mixed culture; LtSc, *L. thermotolerans* and *S. cerevisiae* mixed culture; IoSc, *I. orientalis* and *S. cerevisiae* mixed culture; TdSc, *T. delbrueckii* and *S. cerevisiae* mixed culture

3.1 Introduction

In recent years, more attention has been paid to the role of non-*Saccharomyces* yeasts in wine alcoholic fermentation, and in particular to their impact on the composition of the final product. These yeasts, which are usually found in the beginning stages of spontaneous fermentation, are metabolically active and their metabolites impact on wine quality. Problems such as unpredictability, inconsistency and incomplete fermentation have nevertheless frequently been associated with non-*Saccharomyces* yeasts. However, a substantial amount of evidence suggests that non-*Saccharomyces* yeasts may positively affect wine quality (Jolly *et al*., 2006). As a means to combat these problems associated with non-*Saccharomyces* yeasts, multistarter cultures, consisting of non-*Saccharomyces* yeast strains together with *S. cerevisiae*, have been developed. Using this strategy, a more controlled fermentation can proceed, as known species and cell densities are introduced into the medium. The aromatic impact of non-*Saccharomyces* yeasts participating in the fermentation can thereby be exploited whilst preventing incomplete alcoholic fermentation (Ciani and Ferraro, 1998; Ferraro *et al*., 2000; Jolly *et al*., 2003b).

Co-inoculation of non-*Saccharomyces* and *Saccharomyces* yeasts leads to interactions between these populations. Many of these interactions are poorly understood and may result in both positive and negative outcomes in the resulting wine composition.

The development of each individual species is characterized by particular metabolic activities that impact on the concentrations of flavour compounds in the resulting wine (Romano *et al*., 2003; Tosi *et al*., 2009). In conventional single strain inoculations, many factors may hinder fermentation and growth of the yeast. In a mixed culture medium, the environmental factors which contribute to the inhibition of an individual yeast strain may be amplified by the presence of a competing yeast population. Factors such as spatial proximity (physical interaction) (Nissen *et al*., 2003), competition for nutrients and the production of potentially toxic metabolites, such as medium chain fatty acids (Bisson, 1999) and killer toxins (van Vuuren and Jacobs, 1992) all affect the metabolism of the yeast. These microbial interactions impact the formation of biomass of the individual species, the rate of fermentation and the concentrations of metabolites which are generated throughout the course of fermentation. Indeed, the coexisting species may affect the redox status of the individual cells, implying that an interspecies exchange of metabolites occurs (Cheraiti et al., 2005). Several studies (Ciani *et al*., 2006; Moreira *et al*., 2005; Viana *et al*., 2009) have demonstrated how this exchange of metabolites between species may be beneficial to the final wine composition, for example the decrease of volatile acidity and the increase of concentrations of higher alcohols and esters.

However, the nature of these interactions is complex (Fleet, 2003; Alexandre et al., 2004) and not well understood.

Twelve non-*Saccharomyces* yeasts were previously screened (Fairbairn, 2011, unpublished) for viability in synthetic media with 5% v/v alcohol level, high osmolarity (tested via the addition of varying concentrations of NaCl) and varying levels of oxygen. Of this yeast, four strains, each from a different species namely; *M. pulcherrima* IWBT Y1337, *L. thermotolerans* IWBT Y1240, *I. orientalis* IWBT Y1161 and *T. delbrueckii* CRBO L0544, were selected based on their ability to multiply and survive under these conditions. These non-*Saccharomyces* strains have previously been studied and described, in particular *L. thermotolerans* and *T. delbrueckii* (Castelli, 1969; Mora *et al*., 1990; Ciani *et al*., 2006), *M. pulcherrima* (Jolly *et al*., 2001; Comitini *et al.*, 2010), and to a lesser degree *I. orientalis* (Kim *et al*., 2008).

In this study, we describe the impact of mixed-culture inoculations in synthetic grape juice, of *S. cerevisiae* together with individual non-*Saccharomyces* yeast strains, on fermentation kinetics and population dynamics, as well as on the metabolic profiles of the resulting synthetic wine. The main objective is to evaluate the potential positive impact that controlled mixed starter cultures of individual species may have on the fermentation of the wine and ultimately on the final wine composition. This will be done by establishing which metabolites are produced and in what concentration they are produced in, during mixed fermentation using these specific non-*Saccharomyces* yeast strains thus providing more definitive basis for selecting yeast to use during mixed fermentation.

3.2 Materials and Methods

3.2.1 Yeast strains

Table 3.1 lists the wine yeast strains used in this study along with their origin. A commercial strain of *S. cerevisiae*, Cross Evolution® from Lallemand (Toulouse, France), was used in all experiments. Non-*Saccharomyces* yeast strains, *M. pulcherrima*, *L. thermotolerans* and *I. orientalis* were taken from the culture collection of the Institute for Wine Biotechnology (Stellenbosch, South Africa). *T. delbrueckii* LO544 was provided by the Collection de Ressources Biologiques Œnologique (Villenave d'Ornon, France). Yeasts were maintained on YPD agar (BioLab Diagnostics, Wadenville, South Africa) plates and stored at 4°C.

Table 3.1 Wine yeast strains used in this study

Yeast Species	Strain	Origin	Collection/Reference
Saccharomyces cerevisiae	Cross Evolution®	Hybrid strain generated at the IWBT[b]	Lallemand [a]
Metschnikowia pulcherrima	Y1337	Isolated form Chardonnay juice (Somerset West, South Africa, 2009)	IWBT [b]
Issatchenkia orientalis	Y1161	Isolated from Cabernet sauvignon juice (Welgevallen farm, Stellenbosch, South Africa, 2009)	IWBT [b]

Table 3.1 (cont.)

Lachancea thermotolerans	Y1240	Isolated from Muscat d'Alexandre (Jason's Hill, Rawsonville, South Africa, 2009)	IWBT [b]
Torulaspora delbrueckii	L0544	Isolated in French wine (2007)	CRBO[c]
Saccharomyces paradoxus	RO88	Croatian wine	Redžepović et al. (2003)
Schwanniomyces polymorphus var. africanus	CBS 8047	Soil, Graskop, South Africa	CBS [d]

[a]Lallemand: Lallemand SAS (Blagnac, France)
[b]IWBT: Institute for Wine Biotechnology (Stellenbosch, South Africa)
[c]CRBO: Collection de Ressources Biologiques Œnologique (Villenave d'Ornon, France)
[d]CBS: Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands)

3.2.2 Culture conditions

A single yeast colony was inoculated into 5 mL YPD broth (BioLab Diagnostics) and incubated at 30˚C overnight with agitation. One millilitre of this pre-culture was then inoculated into 100 mL YPD broth and incubated at 30˚C, with agitation. When cultures reached a concentration of 10^6 CFU.mL^{-1}, as determined by absorbance readings at a wavelength of 600 nm, the cells were washed and re-suspended in synthetic medium whose composition was adapted from Henschke and Jiranek (1993) and Bely *et al*. (1990) and is described in Table 3.2.Thereafter, yeasts were inoculated into 250 mL Erlenmeyer flasks, with a total volume of 100 mL, and sealed with fermentation caps. The fermentations were performed at 25˚C under autogenously anaerobic conditions without shaking using four different strains of non-*Saccharomyces* yeasts, which were inoculated individually in combination with *S. cerevisiae* Cross Evolution®, in triplicate. All yeast strains were also inoculated on their own as single cultures. The synthetic medium was used to exclude the influence that grape derived metabolites and precursors might have on individual yeast metabolism. In the mixed cultures, the yeasts were co-inoculated at a ratio of 10 non-*Saccharomyces*: 1 *S. cerevisiae,* with a concentration of 1 x 10^7 CFU.mL^{-1} and of 1 x 10^6 CFU.mL^{-1}, respectively. The controls for the individual strains were inoculated at the same concentration to that found in the mixed culture fermentations, i.e. 1 x 10^7 CFU.mL^{-1} for non-*Saccharomyces* and 1 x 10^6 CFU.mL^{-1} for *S. cerevisiae.*

Table 3.2: Synthetic grape must medium amended from Henschke and Jiranek (1993) and Bely *et al*. (1990) adjusted pH of 3.5 with 10M KOH

Carbon source		**Trace elements**	
Glucose	*115 g	Manganese Chloride ($MnCl_2.4H_2O$)	200 µg
Fructose	*115 g	Zinc Chloride ($ZnCl_2$)	135 µg
		Ferric Chloride ($FeCl_2$)	30 µg
Acids		Cupric Chloride ($CuCl_2$)	15 µg
KH tartrate	2.5 g	Boric Acid (H_3BO_3)	5 µg
L-Malic acid	3.0 g	Cobalt Nitrate ($Co(NO_3)_2.6H_20$)	30 µg
Citric acid	0.2 g	Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	25 µg
		Potassium Iodate (KIO_2)	10 µg
Salts			
Potassium hydrogen phosphate (K_2HPO_4)	1.14 g	Magnesium sulphate heptahydrate ($MgSO_4.7H_20$)	1.23 g
Calcium Chloride dihydrate ($CaCl_2.2H_2O$)	0.44 g		
Nitrogen sources (Made in 1L Stock solution)		**Vitamins** (Made in 1L Stock solution)	
Tyrosine	1.4 g	Myo-Inositol	100 mg
Tryptophane	13.7 g	Pyridoxine	2 mg
Isoleucine	2.5 g	Nicotinic Acid	2 mg
Aspartic acid	3.4 g	Ca Panthothenate	1 mg
Glutamic acid	9.2 g	Thiamin.HCl	0.5 mg
Arginine	28.6 g	PABA.K	0.2 mg
Leucine	3.7 g	Riboflavin	0.2 mg
Threonine	5.8 g	Biotin	0.125 mg
Glycine	1.4 g	Folic Acid	0.2 mg
Glutamine	38.6 g		
Alanine	11.1 g	**Lipids/oxygen**	
Valine	3.4 g	Ergosterol	10 mg
Methionine	2.4 g	Tween 80	0.5 mL
Phenylalanine	2.9 g		
Serine	6.0 g		
Histidine	2.5 g		
Lysine	1.3 g		
Cysteine	1.0 g		
Proline	46.8 g		

*Amended value

3.2.3 Monitoring of fermentation kinetics

The kinetics of fermentation was monitored by means of accumulated weight loss, analysis of sugar consumption and yeast population. Fermentation flasks were weighed daily until weight loss ceased, corresponding to three consecutive days of less than 0.5 g weight loss over a 24-h period. Glucose and fructose concentrations were determined by means of an enzymatic assay using the Arena 20XT (Thermo Electron Oy, Finland) automated enzymatic kit robot. One millilitre samples were extracted aseptically throughout fermentation. One hundred microlitres of the samples was then used immediately in the determination of yeast populations, and the remainder was centrifuged at 13,000 rpm for 5 min (Hermle Z233 M-2) and stored at 4˚C until analysis. Yeast viability was monitored by surface plating on Wallerstein Laboratory (WL) nutrient agar (Fluka Analytical, Sigma-Aldrich) and YPD agar, for mixed cultured fermentations and pure culture fermentations, respectively, with the exception of the mixed culture fermentation of *T. delbrueckii* and *S. cerevisiae* which was also plated on YPD agar. An appropriate serial dilution of each sample was made, to achieve a viable cell count of 30 – 300 CFU.mL^{-1} and 100 µL was subsequently plated out. Plates were then incubated at 30˚C for three days and colony counts were performed. Yeast strains in mixed culture fermentations were differentiated on the basis of colony morphology as shown in Figure 1.

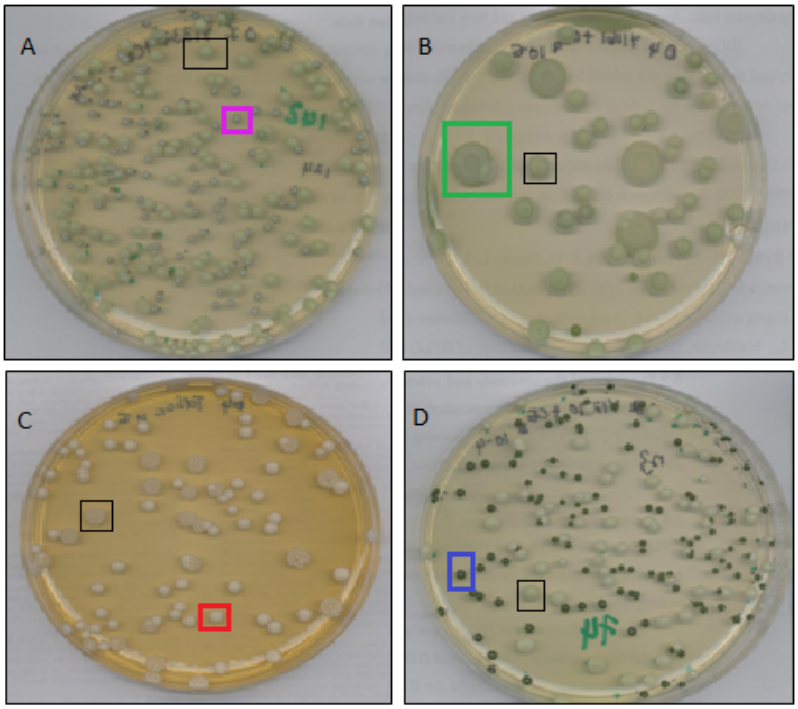

Figure 3.1: Colony Morphology of non-*Saccharomyces* and *S. cerevisiae* in mixed culture fermentations: A- *M. pulcherrima:* *S. cerevisiae* ; **B-** *I. orientalis:* *S. cerevisiae*; **C -** *T. delbrueckii:* *S. cerevisiae* ; **D-** *L. thermotolerans:* *S. cerevisiae* **(A, B and D: WL-Agar; C: YPD-Agar)**

Once fermentation had ceased, 50 mL of the synthetic wine was filter sterilized through a 0.22 µm membrane filter and stored at -20˚C until gas chromatographic analysis could be completed, in order to determine the aromatic profile of the single- and mixed-cultured fermentations.

3.2.4 Species identity confirmation

The species identity of the strains utilized in this study was verified by sequencing of the 5.8S-ITS rDNA region. The PCR was performed using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers described by White *et al*. (1990). The final volume of the PCR reaction was 50 µL. The reaction mixture consisted of 1X reaction buffer, 5 µL dNTPs from TaKaRa (Separations, Randburg, South Africa), 2.5 mM $MgCl_2$, 0.5 µM ITS1 and 0.5 µM ITS4 and, 1 U Phusion Taq Polymerase from Thermo Scientific (Inqaba Biotec, Johannesburg, South Africa). The mixture was subjected to an initial denaturation of 5 min at 94°C; thereafter, 40 cycles consisting of a denaturation of 30 s at 94°C, annealing of 30 s at 51°C, extension of 45 s at 72°C and a final extension of 7 min at 72°C. Five microliters of the PCR products were visualized on a 1% agarose gel containing ethidium bromide. Sequence results were compared against the NCBI nucleotide database using BLAST algorithm and identifications were confirmed when the sequence coverage and maximum percentage of identification were higher than 98% (Query cover > 98%, Max ID % > 98%).

3.2.5 Screening for enzyme activities

For each screening, the yeast cultures were grown aerobically in 5 mL YPD broth for 12 h and 10 µL of the overnight culture were then spotted on the selected agar plates.

3.2.5.1 β-Glucosidase activity

β-Glucosidase activity was determined by spotting the yeasts onto a selective medium as described by Strauss *et al.* (2001) with some modifications. The selective medium contained 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 5 $g.L^{-1}$ arbutin (Sigma) and the pH was adjusted to 3.5. After autoclaving, 20 mL of a 1% filter sterilized ammonium ferric citrate solution and 20 $g.L^{-1}$ previously prepared bacteriological agar (BioLab) was added. Overnight cultures were spotted on the plates together with the positive control *Schwanniomyces polymorphus* var. *africanus* CBS 8047 and incubated at 30°C for 3 days and then observed for a dark brown halo which indicates that the yeast isolate produces extracellular β-glucosidase active against arbutin (Cordero Otero *et al*., 2003).

3.2.5.2 Pectinase Activity

Pectinase activity was determined by spotting the yeasts strains, with *Saccharomyces paradoxus* RO88 as positive control, onto agarose plates containing 0.5% (w/v) polygalacturonic acid, 0.8% (w/v) Type II Agarose (Sigma-Aldrich, Germany) and 40 mM ammonium acetate (pH 4.0), and

incubated at 30°C for three days. The colonies were washed off the surface of the medium and the plates flooded with 6 M HCl. Observations of a clear halo around the colony revealed positive activity (Mocke, 2005).

3.2.6 Gas chromatographic analysis

The concentration of 32 of the major volatile compounds commonly found in wine was determined by means of a gas chromatography equipped with a flame ionization detector as described by Styger *et al*. (2011). The volatiles were extracted through a liquid-liquid extraction technique. A 100 µL 4-methyl-2-pentanol (500 $mg.L^{-1}$ in 12% (v/v) ethanol) internal standard as well as 1 mL diethyl ether was added to 5 mL of the sterilized synthetic wine.

3.2.7 Data analysis

Multivariate data analysis techniques, including principal component analysis (PCA) were used for statistical analysis using Statistica version 10 (Statsoft Inc.) and The Unscrambler software (version 9.2, Camo ASA, Norway).

3.3 Results and discussion

3.3.1 Strain identity confirmation

The identity of all the strains used in this study was confirmed by PCR-RFLP as described in the Materials and Methods section. All of them achieved the sequence coverage and maximum percentage of identification higher than 98% (Query cover > 98%, Max ID % > 98%) when compared against NCBI nucleotide database.

3.3.2 Enzymatic activity of non-*Saccharomyces* yeasts

The screening for extracellular enzymatic activity was part of the broad characterization of the yeast strains used in this study, as these specific enzymes would not influence the outcome of the fermentations in synthetic grape medium, because this medium does not contain the substrates occurring in real grape juice. In this study, glucosidase and pectinase activities were investigated. . *M. pulcherrima* displayed the only positive result for the expression of extracellular β-glucosidase enzyme activity (data not shown). None of the other non-*Saccharomyces* yeast strains exhibited any of the extracellular enzymatic activities tested.

3.3.3 Fermentation kinetics and biomass evolution

Table 3.3 and Figure 3.2 show the maximum and final populations of each individual strain in both pure- and mix culture fermentations, as well as the time to complete fermentation

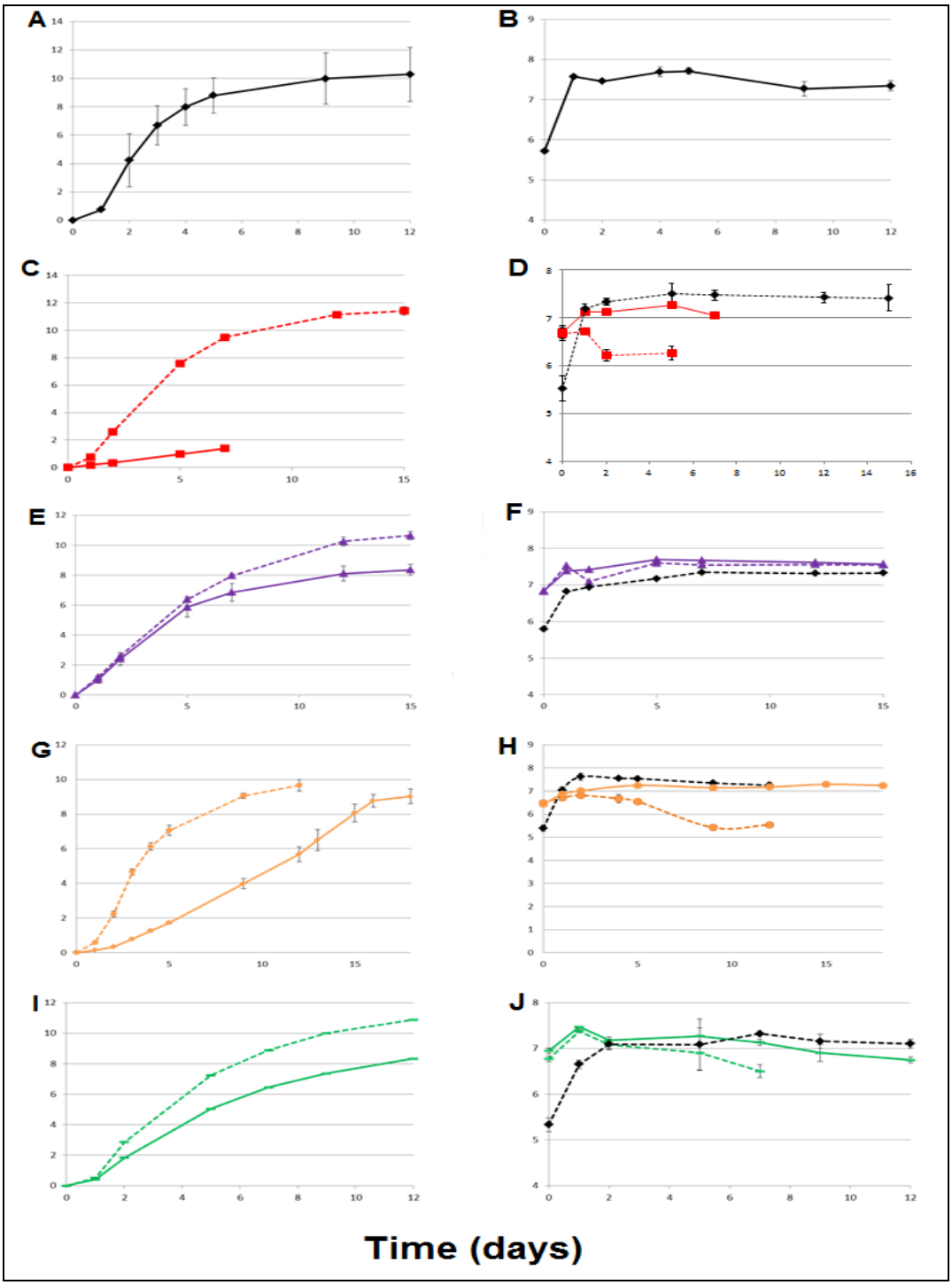

Figure 3.2: Fermentation kinetics, represented as accumulated weight loss (in g) [A; C; E; G; I] and biomass in cfu.mL^{-1} [B; D; F; H; J] of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast strains in pure (indicated with continuous line)- and mixed-culture fermentations (indicated with dashed lines)

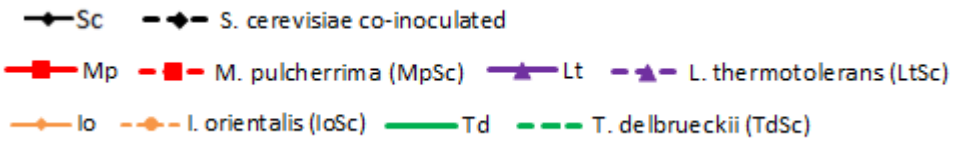

Table 3.3 Populations of single and mixed-fermentations and duration of fermentation until weight loss ceased. Data are shown as the averages of three biological repeats ±Standard deviations.

Fermentation	Strain (s)	Days until fermentation ceases	Max population (10^7 cfu.mL^{-1})	Std deviation	Population at ceased fermentation (10^7 cfu.mL^{-1})	Std deviation	Total accumulated weight loss (g)	Std deviation
Pure-culture	***S. cerevisiae***	12	7.71	±0,078	7,34	±0,124	10,23	±1,9
Pure-culture	***M. pulcherrima***	7	7.27	±0,034	7,04	±0,055	1,38	±0,021
Pure-culture	***L. thermotolerans***	15	7,69	±0,032	7,56	±0,436	8,35	±0,417
Pure-culture	***I. orientalis***	18	7.24	±0,032	7,23	±0,034	9,67	±0,417
Pure-culture	***T. delbrueckii***	12	7.4	±0,058	6.7	±0,065	8,32	±0,176
Mixed culture	*S. cerevisiae*	15	7.518	±0,204	7,42	±0,266	11,42	±0,310
	M. pulcherrima	15	6,7	±0,060	**undetectable**			
Mixed culture	*S. cerevisiae*	15	7.34	±0,020	7,32	±0,065	10,63	±0,269
	L. thermotolerans	15	7.61	±0,110	7,55	±0,104		
Mixed culture	*S. cerevisiae*	12	7.6	±0,163	7.25	±0,113	9,69	±0,331
	I. orientalis	12	6.81	±0,116	5.41	±0,088		
Mixed culture	*S. cerevisiae*	12	7,3	±0,042	7,1	±0,100	10,88	±0,775
	T. delbrueckii	12	7,3	±0,036	**undetectable**			

The pure culture fermentation of *S. cerevisiae* reached its maximum population after one day (Figure 3.2 B) and remained at that cell density until the completion of fermentation.

The population of *M. pulcherrima* was undetectable after 5 days of fermentation (Figure 3.2 D). This result is in agreement with those of Comitini *et al.* (2010), who showed that *M. pulcherrima* could no longer be detected after 3 days of co-fermentation and Sadoudi *et al.* (2012) who reported the persistence of *M. pulcherrima* in a mixed fermentation until day 8.

The population dynamics of the co-cultured fermentation of *L. thermotolerans* and *S. cerevisiae* yielded surprising results. The population of *L. thermotolerans* remained at a higher concentration than that of *S. cerevisiae* throughout fermentation. In previous reports by Ciani *et al.* (2006) and Comitini *et al*. (2011) where different ratios of *L. thermotolerans* and *S. cerevisiae* were co-inoculated, the population of *L. thermotolerans* consistently declined after the first few days, even when the initial inoculation ratio was 100:1 (*L. thermotolerans: S. cerevisiae*) which is ten times greater than the initial inoculum applied here. Moreover, the maximum viable population of *L. thermotolerans* was greater when in mixed culture with *S. cerevisiae* than when it was singularly inoculated into synthetic grape media. But, the high cell density of *L. thermotolerans* within the fermentation did not significantly impede the growth of *S. cerevisiae*, as the population of the latter yeast still increased to a concentration of 7.34 x 10^7 cfu.mL^{-1}, which is ten times greater than its initial inoculation density (Table 3.3).

I. orientalis and *S. cerevisiae* mixed fermentation population dynamics occurred in an expected manner, where *S. cerevisiae* superseded the population of *I. orientalis*, and consequently dominated the fermentation until it came to a halt, which was also demonstrated by Kim *et al*. (2008), when *S. cerevisiae* was used in co-inoculation with *I. orientalis* at a 1:4 ratio. *I. orientalis*

persisted throughout fermentation and remained viable until the end of fermentation, albeit at a low cell density.

The population dynamics that were observed for the mixed fermentation of *T. delbrueckii* and *S. cerevisiae* concurred with those described by Ciani *et al*. (2006). *T. delbrueckii* remained at high levels until mid-fermentation, where after the population declined to undetectable levels.
In addition, the accumulated weight loss pattern demonstrated by the individual mixed fermentations showed similar patterns to that of pure *S. cerevisiae*, however LtSc and MpSc mixed fermentations fermented over a longer period (15 days) in comparison to pure *S. cerevisiae* and TdSc and IoSc mixed fermentations (12 days).

3.3.4 Glucose and Fructose degradation

In the pure- and mixed-culture fermentations, glucose and fructose were monitored throughout fermentation to determine the rate of sugar consumption (Figure 3.3).

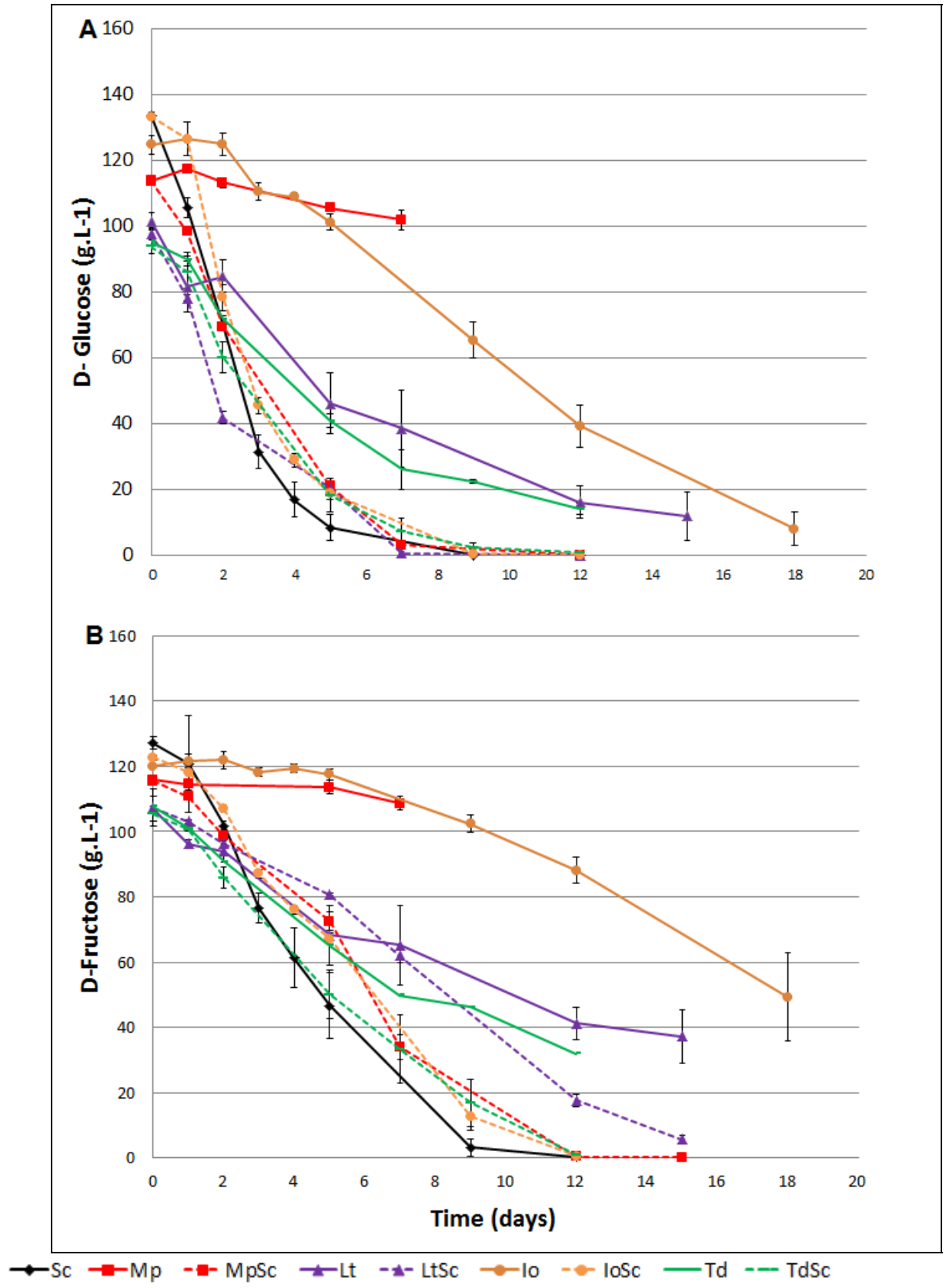

Figure 3.3: (A) Glucose and (B) Fructose consumption of mono- and mixed-cultured fermentations

S. cerevisiae completed fermentation in 12 days, with glucose being consumed at a faster rate than fructose. *S. cerevisiae* is known to show preference to glucose and this sugar is thus always

consumed first. As a consequence, the concentration of fructose is typically higher than that of glucose in the residual sugar of fermented must (Berthels *et al*., 2004). The single-culture of the non-*Saccharomyces* yeast strains assessed here all resulted in stuck fermentations, with *M. pulcherrima* having the highest concentration of total residual sugar, followed by *I. orientalis*, *L. thermotolerans* and *T. delbrueckii* (Figure 3.3 A and B). These results correlate with the fermentation kinetics of each strain, as *T. debrueckii* had the fastest fermentation rate. In all the mixed fermentations, all sugar was consumed. However, the rate of consumption differed between each yeast combination. It has been suggested that the selective consumption of fructose by non-*Saccharomyces* wine yeast strains might have a positive effect on the fermentation behaviour of *S. cerevisiae* in mixed culture fermentations (Ciani and Fatichenti, 1999).

In the mixed fermentation of *M. pulcherrima* and *S. cerevisiae* the total sugar consumption was similar to the pure *S. cerevisiae*. No impact of the difference between glucose and fructose consumption was apparent. This result may be attributed to the fact that *M. pulcherrima* had a short contribution to the fermentation, as it reached relatively low levels and was outcompeted by *S. cerevisiae*. In the IoSc mixed culture fermentation a similar trend was observed as with MpSc. Total sugar consumption was achieved over the same time period as with Sc alone. This result may similarly be due to the fact that the population of *I. orientalis* did not increase significantly, and remained at a relatively low level throughout the fermentation, and *S. cerevisiae* could ferment the remaining sugars at a similar rate which was shown by the pure culture of *S. cerevisiae*. The LtSc mixed fermentation demonstrated the largest difference of total sugar consumption. Glucose consumption was rapid and completed in 12 days. Fructose consumption was noticeably slower and was not completed, with a residual sugar of 5.6 $g.L^{-1}$. The population of *L. thermotolerans* in the mixed fermentation with *S. cerevisiae* was higher than that of *S. cerevisiae* throughout the fermentation, and both yeast populations remained at high concentrations throughout fermentation (Figure 3.2 F). The high presence of each of these strains could suggest a high level of competition for fermentable sugars, therefore slowing down the consumption rate, as neither yeast strain dominates the fermentation. Ciani *et al*. (2006) also showed that in sequential inoculations with *L. thermotolerans* and *S. cerevisiae* where the non-*Saccharomyces* strain persisted for prolonged periods in the fermentation, the total consumption of fermentable sugars was incomplete.

The TdSc mixed fermentation displayed a similar rate of total sugar consumption as single-cultured Sc, with no significant differences observed between the rates of glucose and fructose consumption. Interestingly, the rate of sugar consumption in TdSc was not affected by the high cell density of *T. delbrueckii* which persisted for half of the fermentation, and dryness was achieved in same period of time taken by pure *S. cerevisiae*.

3.3.5 Aromatic profile of the final wine product

GC-FID was utilized for aromatic compound analysis. A total of 32 major volatile compounds were quantified. The subsequent subsections will discuss these results per class of chemical compounds and Table 3.4 which shows the concentration of the quantified major volatiles at the end of fermentation will be referred to throughout.

3.3.5.1 Higher alcohols

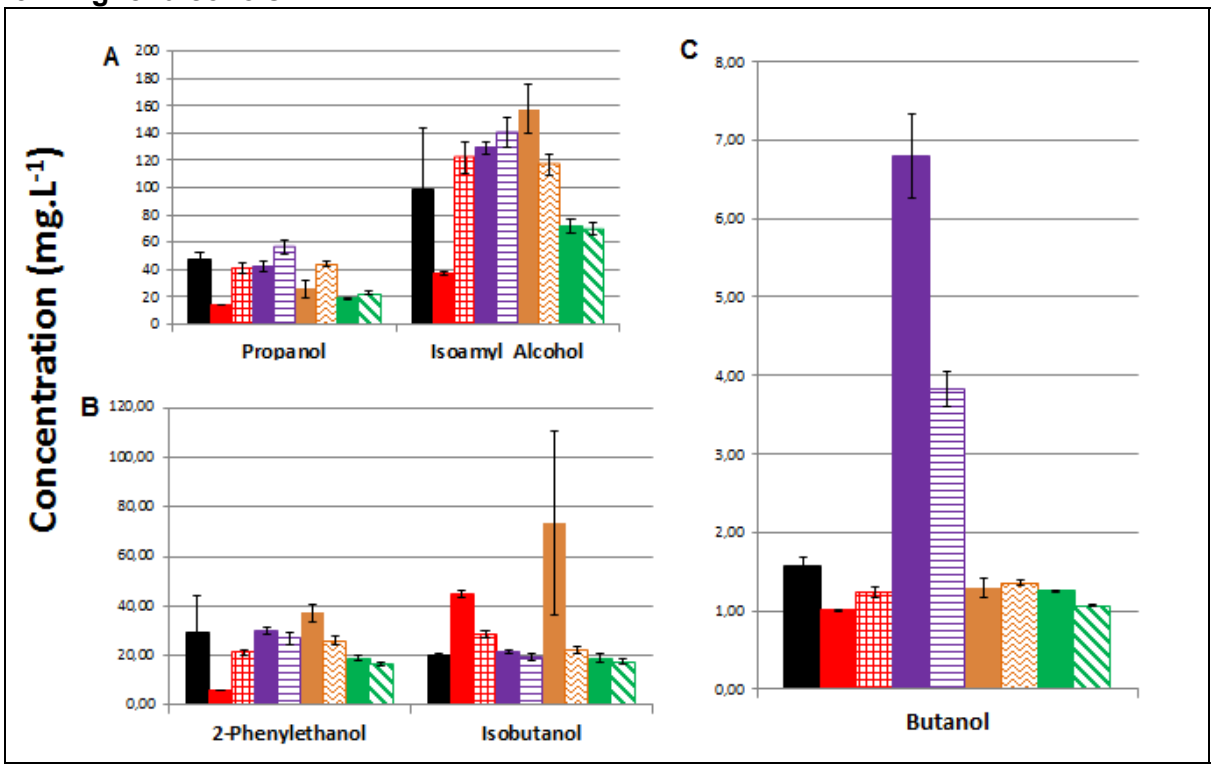

Figure 3.5: Higher alcohols (mg.L^{-1}) measured at completion of fermentation for pure and mixed cultures to determine the impact of co-inoculation on the production of higher alcohols during fermentation Sc Mp MpSc Lt LtSc Io IoSc Td TdSc

Figure 3.5 shows the substantial increases in the production of higher alcohols, produced from pure- and mixed-cultured fermentations. The total higher alcohol concentrations produced by the individual mono-culture of the non-*Saccharomyces* yeasts showed great differences between species. *I. orientalis* produced the highest concentration of total higher alcohols, 297.54 mg.L^{-1}, followed by *L. thermotolerans* 231.30 mg.L^{-1}, *T. delbrueckii* 142.78 mg.L^{-1} and finally the lowest concentration of higher alcohols produced by *M. pulcherrima* 103.49 mg.L^{-1}, which could be attributed to the fact that *M. pulcherrima* did not ferment much, thus its global metabolic activity was low. An interesting observation made amongst the different co-inoculated fermentations is the fact that the LtSc mixed fermentation actually produced an increased higher alcohol concentration than compared to the IoSc mixed fermentation. Considering the fact that the pure culture of *I. orientalis* produced a substantially higher concentration of higher alcohols (Figure 3.5 A and B), the expectation would have been that the mixed fermentation would produce an increased concentration. With the exception of butanol (Figure 3.5 C), which was considerably larger in *L.*

thermotolerans than *S. cerevisiae,* the concentrations at which the compounds were found are similar between the pure cultures of *S. cerevisiae* and *L. thermotolerans*.

The MpSc and IoSc mixed fermentations showed similar concentrations of higher alcohol when compared to pure *S. cerevisiae*. TdSc mixed fermentation demonstrated a notable decrease in the concentration of higher alcohols (203.02 mg.L^{-1} to 134.95 mg.L^{-1}, Table 3.4). Remarkably, even though the concentration of higher alcohols produced by *T. delbrueckii* was the second lowest concentration of higher alcohols, the combined effect of *T. delbrueckii* and *S. cerevisiae* mixed fermentation produced a synthetic wine with a further decline in the concentration of higher alcohols. These results correspond to those obtained by Barrajón *et al.* (2011) who observed a reduction in the concentration of propanol, butanol and Isobutanol, when *T. delbrueckii* was used together with *S. cerevisiae* in mixed culture fermentation.

3.3.5.2 Volatile Fatty Acids

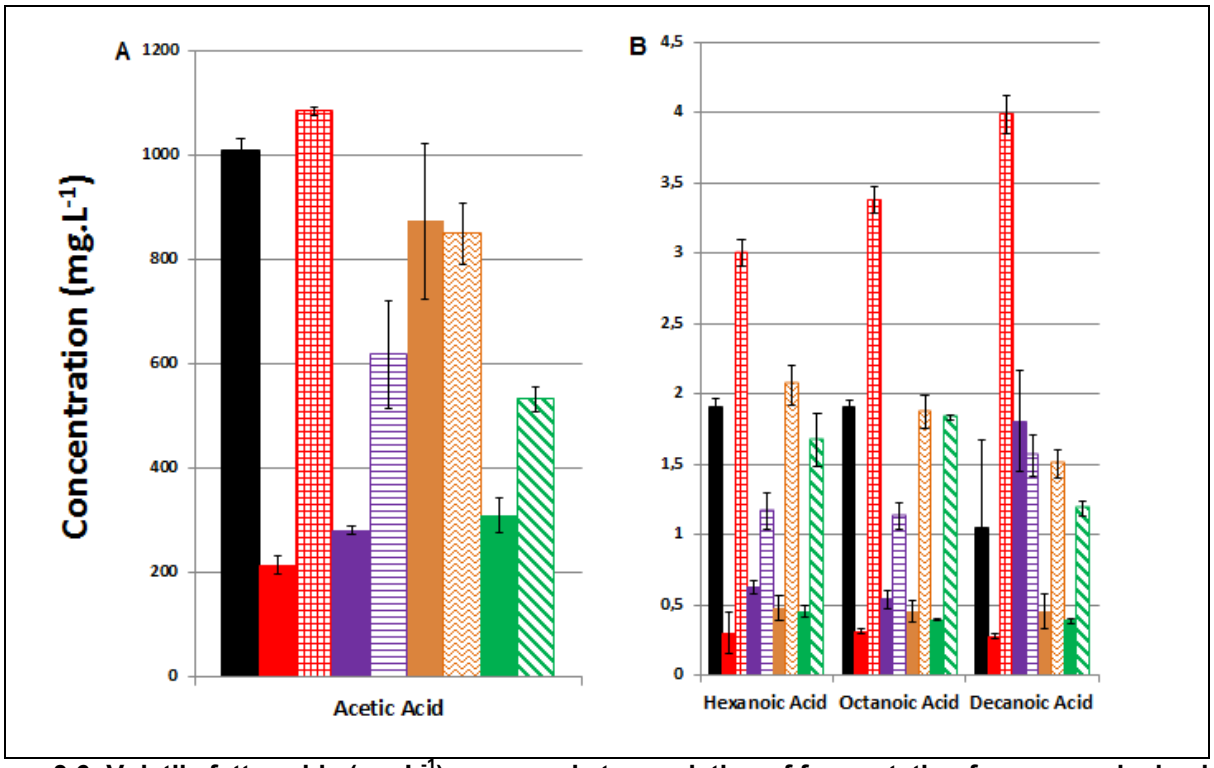

Figure 3.6: Volatile fatty acids (mg.L^{-1}) measured at completion of fermentation for pure and mixed cultures to determine the impact of co-inoculation on the production of volatile fatty acids during fermentation

The quantity of volatile acidity (largely dominated by acetate) contributes significantly to wine aroma and in large concentrations is highly detrimental to wine quality. The amount of acetic acid produced by pure culture of non-*Saccharomyces* yeast strains ranged from 215.54 mg.L^{-1} to 880.35 mg.L^{-1} (Table 3.4). This relatively low production of acetic acid may be in part attributed to the fact that none of the non-*Saccharomyces* yeast strains were individually able to complete fermentation. The acceptable range of acetic acid in wine is 0.2 - 0.7 g.L^{-1} (Corison *et al.,* 1997;

Dubois, 1994). LtSc and TdSc co-culture fermentations reduced the concentration of acetic acid by 38.87% and 47.34% respectively, when compared to the pure strain of *S. cerevisiae*, as shown in Figure 3.6 A. *L. thermotolerans* persisted until the completion of fermentation and *T. delbrueckii* persisted until midway, and the impact that these populations had on the chemical composition of the final product is significant. The synthetic grape-like medium in which the fermentations were conducted was amended from an original total sugar concentration of 200 $g.L^{-1}$ to 230 $g.L^{-1}$, with the intended purpose of determining the response of the co-culture fermentations to the production of acetic acid. Yeasts respond to increased external osmolarity by increasing their production and accumulation of intracellular glycerol in order to create equilibrium between the internal and external osmotic pressure (Blomberg and Alder, 1992; Myers *et al.*, 1997). The intracellular redox balance is maintained by yeast cells regenerating an equimolar amount of cytoplasmic NAD^+, which seems to be in part met by the reduction of acetaldehyde to ethanol and an increased oxidation to acetate (Blomberg and Alder., 1989). Therefore, the increased production of glycerol and acetate is inevitable in high-sugar fermentations by *S. cerevisiae* (Caridi *et al.*, 1999). In the LtSc and TdSc mixed fermentations, the reduction of volatile acidity has been shown to be achieved through different pathways. Mora *et al.* (1990) used *L. thermotolerans* as a natural deacidification agent, as it was found that it reduces volatile acidity and increases the total acidity by producing L-lactic acid (non-volatile) and *T. delbrueckii* has been shown to be unaffected by high osmotic stress, with no increased production of glycerol or acetaldehyde. However, the underlying metabolic mechanisms that reduce the acetic acid concentration within mixed fermentations between *T. delbrueckii* and *S. cerevisiae* are yet to be fully understood (Bely *et al.*, 2008). The IoSc mixed fermentation also showed a decrease in the concentration of acetic acid formed during fermentation compared to the pure *S. cerevisiae* fermentation, but the reduction was not as significant as compared to the decrease shown with the mixed cultures of *T. delbrueckii*- *and L. thermotolerans- S.cerevisiae*. MpSc co-culture fermentation displayed the only increase in the production of acetic acid. This finding concurs with results obtained by Zohre *et al*. (2002) and Rodríguez *et al*. (2010) where pure cultures of *S. cerevisiae* produced a lower concentration of acetic acid, in comparison to the concentration of acetic acid produced by the mixed fermentation of *M. pulcherrima* and *S. cerevisiae*.

The residual volatile fatty acid concentration was generally lower in all pure cultures of non-*Saccharomyces* strains and mixed fermentations, with the exception of *M. pulcherrima* and *S. cerevisiae*. In the mixed fermentation of *M. pulcherrima* and *S. cerevisiae*, an overall increase in the concentration of volatile fatty acids was observed, and in particular that of hexanoic, octanoic and decanoic acids, as can be seen in Figure 3.6 B. In the pure cultures of *S. cerevisiae* and *M. pulcherrima*, the concentrations of these three compounds are relatively low, with *S. cerevisiae* exhibiting an accumulative concentration of 4.83 $mg.L^{-1}$ and *M. pulcherrima*, 0.89 $mg.L^{-1}$. However, when these two yeast strains are in co-culture fermentation the concentration of these fatty acids

increased to 10.37 $mg.L^{-1}$. The opposite effect was observed in the LtSc mixed fermentation, where a reduction in the total concentration of medium chained fatty acids (3.85 $mg.L^{-1}$) was observed. In yeast metabolism, medium chained fatty acids (C6 - C12) are synthesized during fermentation and are accumulated as self-toxic mechanism to yeast development. The medium chain fatty acids are not derivatives of fermentation as such but rather the remaining fragments of long chain lipid acid synthesis, required for the cell's membrane. The hydrophobic nature of the fatty acids allows them to enter the yeast cell membrane and disrupt transport systems between the intracellular- and the extracellular-medium (Margalit *et al.,* 2004). The inhibitory mechanism of fatty acids is that they are activated by acyl-CoA-compounds, which might interfere with fundamental metabolic activities requiring acyl-CoA-compounds (Nordstrom, 1964). For this reason, the release of medium chained fatty acids into the medium has been associated with antagonistic inter-species interaction. It has been hypothesized (Fleet, 2003) that a yeast species co-existing in a mixed fermentation increases its production or release of medium chained fatty acids into the fermentation medium, with the purpose of acting detrimentally towards the opposing yeast population. Antagonistic interactions have been reported by Bisson (1999) where medium chained fatty acids were produced to inhibit *S. cerevisiae.* Other authors (Viegas *et al.*, 1989; Edwards *et al.*, 1990) have also reported the inhibitory effect that hexanoic, octanoic and decanoic acids exceeding certain thresholds have on *S. cerevisiae.* The results obtained for the co-culture fermentation of *M. pulcherrima* and *S. cerevisiae* concur with those obtained in a study conducted by Sadoudi *et al.* (2012), where the concentrations of octanoic and decanoic acids were substantially lower in the pure culture fermentations of *S. cerevisiae* and *M. pulcherrima* than in the mixed fermentation thereof, however the increased levels of these compounds did not negatively impact the fermentation performance of the mixed fermentation. In the case of *L. thermotolerans* and *S. cerevisiae* mixed fermentation, similar results were observed in a study by Comitini *et al.* (2011). A significant reduction in the concentration of octanoic acid and to a lesser extent of hexanoic acid was indeed observed. However, decanoic acid levels remained as a similar concentration.

3.3.5.3 Esters

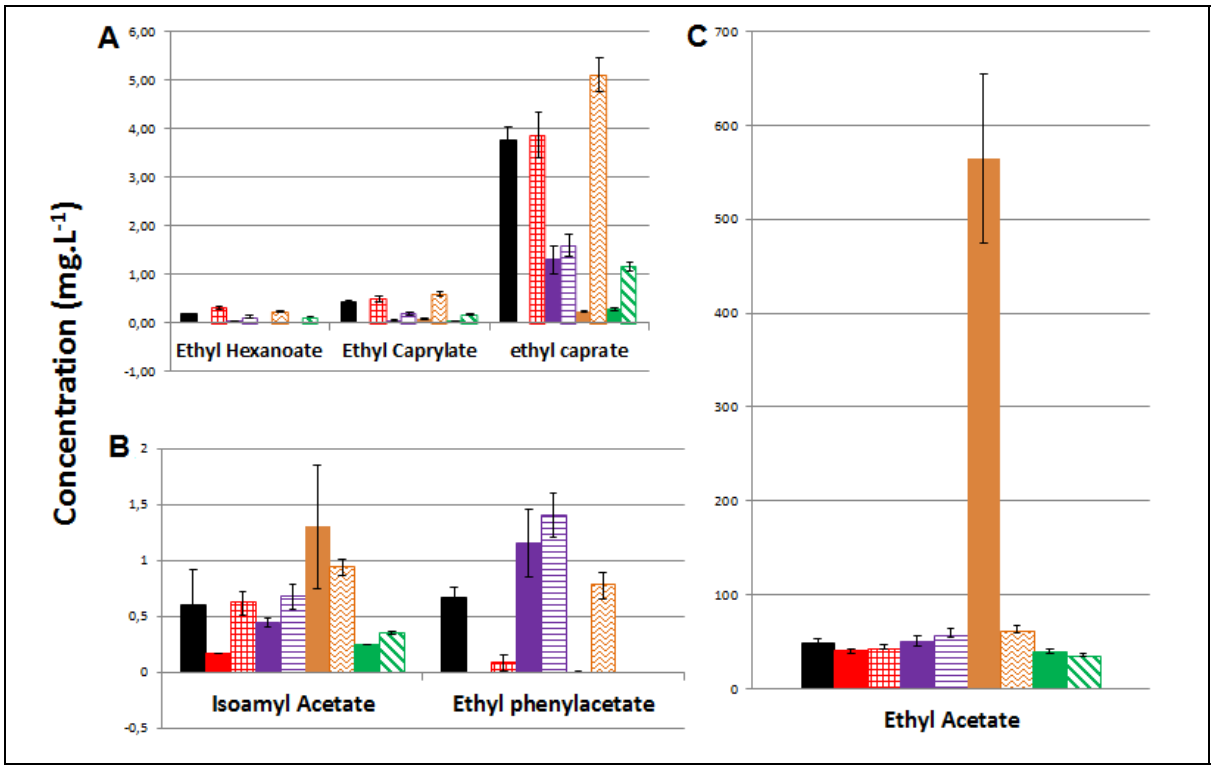

Figure 3.7: Esters (mg.L-1) measured at completion of fermentation for pure and mixed cultures to determine the impact of co-inoculation on the production of esters during fermentation

Sc Mp MpSc Lt LtSc Io IoSc Td TdSc

Various esters might be formed during alcoholic fermentation, and the most abundant are derivatives of acetic acid and higher alcohols (ethyl acetate, isoamyl acetate, isobutyl acetate and 2-phenylethyl acetate) and to lesser degree ethyl esters of saturated fatty acids (ethyl butanoate, ethyl caproate, ethyl hexanoate, ethyl caprylate). The total concentration of esters produced by *S. cerevisiae* was 66.16 mg.L^{-1}. The pure cultures of non-*Saccharomyces* yeast strains produced varying total ester concentrations, with *M. pulcherrima* producing the lowest concentration of 40.32 mg.L^{-1}, *T. delbrueckii* 50.25 mg.L^{-1}, *L. thermotolerans* 64.12 mg.L^{-1} and *I. orientalis* producing a very high concentration of 576.23 mg.L^{-1}. The high total concentration of esters produced by *I. orientalis* is mainly attributed to the high production of ethyl acetate. Ethyl acetate is one of the most significant esters produced during alcoholic fermentation. An aroma descriptor of ethyl acetate is VA (volatile acidity), nail polish or fruity and in wine it is usually found at a concentration range of between 22.5 - 63.6 mg.L^{-1}(Swiegers *et al.*, 2005). At concentrations exceeding 150 - 200 mg.L^{-1}, ethyl acetate can impart spoilage character to the wine. In this context, it can be observed that the use of pure cultured *I. orientalis* in alcoholic fermentation produces a wine which would be rejected (Figure 3.7 C). However, in the IoSc mixed fermentation, the greatest increase in ethyl acetate (62.95 mg.L^{-1}) was achieved when compared to pure culture *S. cerevisiae* (48.80 mg.L^{-1}) and the other combinations of non-*Saccharomyces* yeast strains and *S. cerevisiae*. At this concentration of ethyl acetate, it might contribute to the fruitiness and overall complexity of the wine (Gil *et al.*, 1996; Ciani, 1997). In addition, IoSc mixed fermentation showed an increase in the

production of isoamyl acetate (Figure 3.7 B), banana and pear aroma descriptors (Swiegers *et al.*, 2005), of 0.33 mg.L^{-1} when compared to the control of *S. cerevisiae* (0.61 mg.L^{-1}) and an increase in ethyl caprate (Figure 3.7 A). The LtSc mixed fermentation showed an increase in the concentration of ethyl-phenyl acetate (Figure 3.6 B) which is in agreement with results shown by Ciani *et al*. (2006). Esters originating from medium fatty acid metabolism were also observed at greater concentration in the IoSc mixed fermentation, with the exception of ethyl hexanoate, which was greater in the MpSc mixed culture fermentation, which was also shown by Sadoudi *et al*. (2012). However, the MpSc mixed fermentation resulted in an increase in the total amount of esters that were produced, which is contradictory to these results. In terms of total ester production, the mixed culture fermentations which had the most significant increases were that of LtSc and IoSc producing a total concentration of 73.93 and 82.35 mg.L^{-1}, respectively. In contrast, the TdSc mixed fermentation showed a decrease in concentration of all the esters produced during the course of fermentation, with a total ester concentration of 48.55 mg.L^{-1}. These results are similar to the findings by Barrajón *et al.* (2011) where mixed fermentations of *T. delbrueckii* and *S. cerevisiae* produced lower amounts of ethyl acetate. Sadoudi *et al*. (2012) also showed a decrease in the concentration of total esters produced in the mixed fermentation of *T. delbrueckii* and *S. cerevisiae*. The other quantified esters were not found to be significantly different from the pure cultured *S. cerevisiae*.

Table 3.4: Concentrations of the quantified volatile aroma compounds in wine produced from mono-culture and co-culture fermentations of *S. cerevisiae* Cross Evolution® 285 (Sc), *M. pulcherrima* IWBT Y1337 (Mp), *L. thermotolerans* IWBT Y1240 (Lt), *I. orientalis* (Io) and *T. delbrueckii* CRBO L0544 (Td). Data is the average of three biological repeats. ± Standard deviation

	Sc	*Mp*	*Mp-Sc*	*Lt*	*Lt-Sc*	*Io*	*Io-Sc*	*Td*	*Td-Sc*
Esters									
Ethyl Acetate	48,80 ± 4,06	40,19 ± 2,66	44,93 ± 2,41	50,60 ± 5,11	58,83 ± 4,66	564,12 ± 89,86	62,95 ± 3,79	39,81 ± 2,75	36,21 ± 1,54
Ethyl Butyrate	0,78 ± 0,014	nd	0,83 ± 0,008	nd	0,77 ± 0,02	0,48 ± 0,37	0,80 ± 0,012	nd	0,73 ± 0,003
Isoamyl Acetate	0,61 ± 0,31	0,17 ± 0,199	0,62 ± 0,105	0,45 ± 0,04	0,68 ± 0,11	1,30 ± 0,55	0,94 ± 0,07	0,25 ± 0,004	0,36 ± 0,014
Ethyl Hexanoate	0,17 ± 0,016	nd	0,31 ± 0,029	0,03 ± 0,003	0,12 ± 0,03	0,00 ± 0,006	0,23 ± 0,02	nd	0,12 ± 0,005
Ethyl Lactate	9,95 ± 0,063	nd	9,66 ± 0,39	10,06 ± 0,41	9,86 ± 0,08	9,52 ± 0,08	9,98 ± 0,10	9,38 ± 0,30	9,34 ± 0,28
Ethyl Caprylate	0,43 ± 0,025	0,03 ± 0,023	0,49 ± 0,058	0,05 ± 0,005	0,19 ± 0,03	0,07 ± 0,02	0,60 ± 0,04	0,04 ± 0,005	0,18 ± 0,018
ethyl caprate	3,77 ± 0,26	nd	3,88 ± 0,47	1,30 ± 0,29	1,59 ± 0,22	0,22 ± 0,012	5,11 ± 0,35	0,27 ± 0,026	1,16 ± 0,09
Diethyl succinate	0,33 ± 0,005	nd	nd	nd	0,11 ± 0,16	0,05 ± 0,13	0,33 ± 0,006	0,32 ± 0,004	nd
Ethyl phenylacetate	0,68 ± 0,09	nd	0,08 ± 0,073	1,16 ± 0,30	1,41 ± 0,20	0,00 ± 0,007	0,78 ± 0,12	nd	nd
2-Phenylethyl Acetate	0,64 ± 0,043	nd	0,47 ± 0,011	0,34 ± 0,002	0,38 ± 0,01	0,45 ± 0,03	0,64 ± 0,03	0,36 ± 0,007	0,57 ± 0,11
Σ Esters	**66,16 ± 4,59**	**40,32 ± 2,77**	**61,27 ± 2,80**	**64,12 ± 6,04**	**73,93 ± 5,38**	**576,23 ± 89,85**	**82,35 ± 4,53**	**50,25 ± 2,77**	**48,55 ± 1,45**
Higher Alcohols									
Propanol	47,40 ± 5,39	14,04 ± 0,17	41,07 ± 4,34	42,37 ± 4,25	56,36 ± 5,76	25,97 ± 6,23	44,11 ± 2,38	18,85 ± 0,66	22,79 ± 1,01
Isobutanol	19,92 ± 1,25	44,72 ± 1,47	28,70 ± 1,55	21,49 ± 0,53	19,62 ± 1,46	73,26 ± 37,16	22,39 ± 1,32	18,92 ± 1,62	17,60 ± 1,29
Butanol	1,57 ± 0,10	1,00 ± 0,01	1,24 ± 0,06	6,79 ± 0,53	3,83 ± 0,22	1,30 ± 0,12	1,36 ± 0,038	1,25 ± 0,014	1,07 ± 0,01
Hexanol	0,60 ± 0,0007	0,60 ± 0,01	0,61 ± 0,006	0,65 ± 0,017	0,63 ± 0,023	0,60 ± 0,0007	0,60 ± 0,0005	0,61 ± 0,002	0,60 ± 0,0006
3-ethoxy-1-propanol	5,55 ± 0,74	nd	2,51 ± 0,18	1,25 ± 0,37	3,12 ± 0,51	1,26 ± 0,25	3,53 ± 0,28	12,64 ± 1,37	6,26 ± 0,58
Isoamyl Alcohol	98,52 ± 45,17	37,21 ± 1,24	122,09 ± 11,36	128,90 ± 5,13	140,78 ± 11,22	157,78 ± 18,01	116,64 ± 7,68	71,51 ± 4,89	70,02 ± 4,95
2 Phenylethanol	29,46 ± 14,38	5,92 ± 0,23	21,15 ± 1,10	29,90 ± 1,37	26,82 ± 2,38	37,37 ± 3,53	25,93 ± 1,84	19,00 ± 1,32	16,60 ± 0,97
Σ Higher Alcohols	**203,02 ± 65,05**	**103,49 ± 2,91**	**217,36 ± 18,45**	**231,30 ± 6,32**	**251,16 ± 21,07**	**297,54 ± 53,11**	**214,56 ± 13,52**	**142,78 ± 9,82**	**134,95 ± 8,76**
Volatile Fatty Acids									
Acetic Acid	1009,02 ± 21,74	213,05 ± 16,62	1084,59 ± 23,2	280,43 ± 7,80	616,83 ±102,86	872,73 ± 150,28	849,24 ± 58,21	308,59 ± 33,70,	531,39 ±23,44
Propionic Acid	1,49 ± 0,062	0,38 ± 0,16	1,46 ± 0,039	0,58 ± 0,042	1,16 ± 0,16	0,87 ± 0,15	1,15 ± 0,10	0,52 ± 0,11	0,62 ± 0,027
Isobutyric Acid	0,71 ± 0,043	0,39 ± 0,017	0,67 ± 0,02	1,06 ± 0,032	0,96 ± 0,053	3,55 ± 0,42	1,12 ± 0,06	1,88 ± 0,16	1,48 ± 0,055
Butyric acid	0,15 ± 0,024	0,08 ± 0,002	0,17 ± 0,003	0,09 ± 0,011	0,12 ± 0,001	0,10 ± 0,009	0,14 ± 0,08	0,08 ± 0,002	0,12 ± 0,012
isovaleric acid	0,71 ± 0,032	0,42 ± 0,003	0,67 ± 0,029	0,49 ± 0,009	0,66 ± 0,023	1,40 ± 0,10	0,91 ± 0,036	0,56 ± 0,02	0,61 ± 0,001
Valeric Acid	0,63 ± 0,044	0,34 ± 0,003	0,42 ± 0,017	0,64 ± 0,05	0,63 ± 0,045	0,31 ± 0,023	0,50 ± 0,018	0,33 ± 0,009	0,33 ± 0,006
Hexanoic Acid	1,91 ± 0,058	0,30 ± 0,15	3,00 ± 0,090	0,62 ± 0,051	1,16 ± 0,13	0,48 ± 0,084	2,07 ± 0,14	0,45 ± 0,04	1,67 ± 0,19
Octanoic Acid	1,90 ± 0,05	0,31 ± 0,02	3,38 ± 0,09	0,54 ± 0,064	1,13 ± 0,094	0,45 ± 0,08	1,87 ± 0,12	0,39 ± 0,009	1,83 ± 0,015
Decanoic Acid	1,05 ± 0,63	0,28 ± 0,02	3,99 ± 0,13	1,81 ± 0,36	1,56 ± 0,15	0,46 ± 0,13	1,50 ± 0,10	0,38 ± 0,016	1,19 ±0,051
Σ Volatile fatty acids	**1017,58 ± 21,30**	**215,54 ± 16,46**	**1098,36 ± 23,46**	**286,26 ± 8,18**	**624,20 ± 103,27**	**880,35 ± 150,50**	**858,50 ± 58,79**	**313,19 ± 33,90**	**539,25 ±23,29**

3.3.6 Multivariate data analysis

The fermentations of single and mixed inoculations demonstrated significant differences for all of the major volatile compounds which were analysed. A PCA was conducted to acquire a visual depiction of what differentiates the individual mixed fermentations, using the multivariate data to assess the biological interaction.

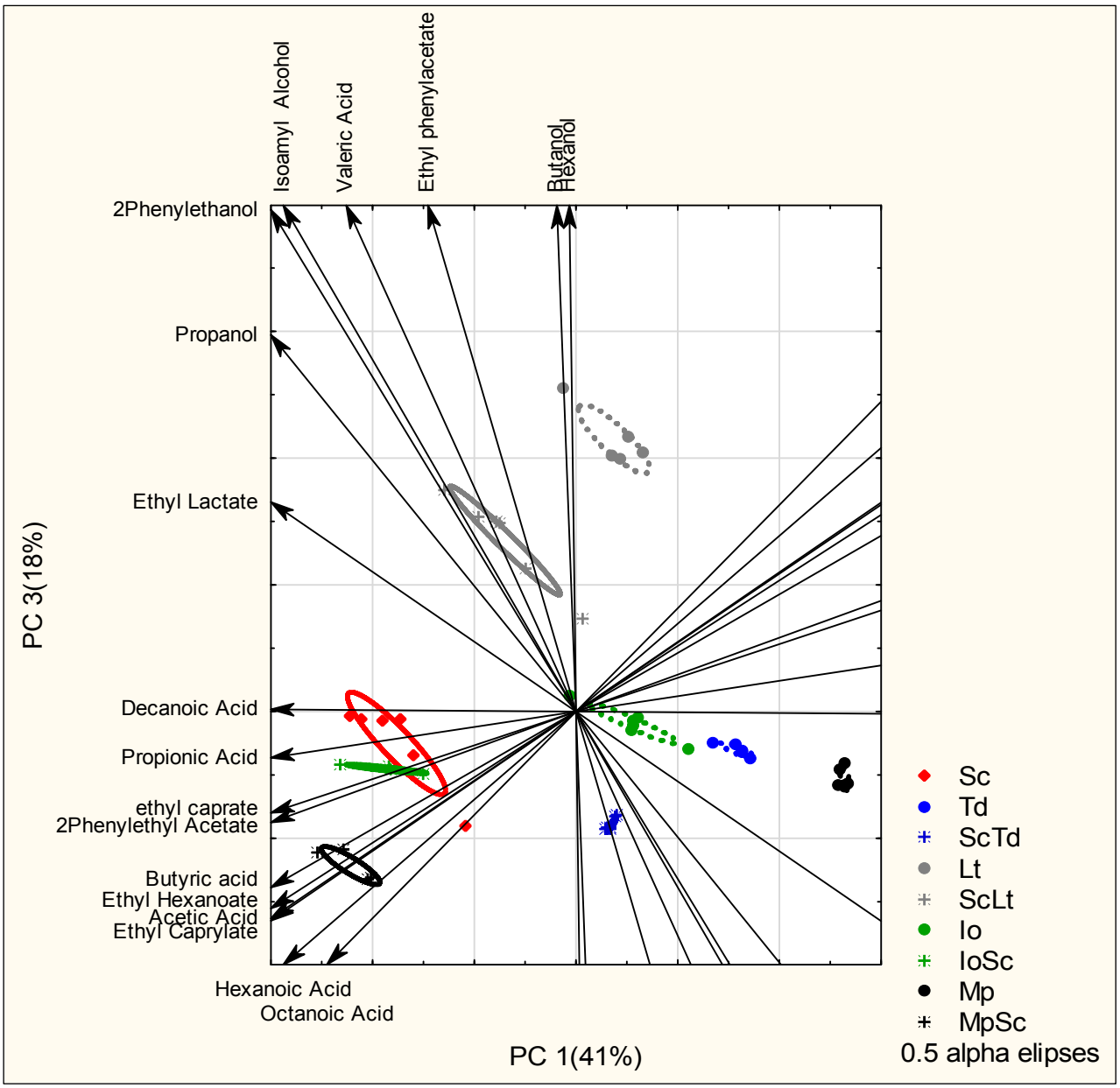

Figure 3.8: Biplot of the principle component analysis (PC1 vs. PC3) of the metabolite profiles from single- and mixed-culture fermentations

The PCA plot (Figure 3.8), described by the first and third principle component, accounts for 59% of the total variance. The biological repeats clustered well together, which indicated high reproducibility of the experimental procedure. As can be seen in Figure 3.8, the differences between pure and mixed culture fermentation can clearly be seen based on their metabolic

differences. The contribution of the non-*Saccharomyces* yeast strains in the production of aromatic compounds is also evident from the PCA plot.

All the non-*Saccharomyces* yeast strains, with the exception of *L. thermotolerans*, are clustered in the area which represents low concentrations of the quantified compounds shown on the graph. *L. thermotolerans* was the only non-*Saccharomyces* yeast that produced a significantly higher amount of a single compound, namely butanol, and therefore was not positioned on the same area on the graph as the other non-*Saccharomyces* yeasts. Conversely, all the mixed fermentations, besides *T. delbrueckii*: *S. cerevisiae*, produced significantly greater concentrations of the quantified volatiles in comparison to their corresponding pure-culture fermentations.

The IoSc co-culture shows a similar aroma profile as pure culture *S. cerevisiae*, with a tendency to increased production of esters such as ethyl acetate, isoamyl acetate, ethyl caprylate and ethyl caprate, which respectively measured 62.95 $mg.L^{-1}$, 0.94 $mg.L^{-1,}$ 0.60$mg.L^{-1}$ and 5.11 $mg.L^{-1}$ in the mixed culture, in comparison to the lower concentrations of 48.80 $mg.L^{-1}$, 0.61 $mg.L^{-1}$, 0.43 $mg.L^{-1}$ and 3.77 $mg.L^{-1}$, achieved by pure cultured *S. cerevisiae*.

The MpSc mixed fermentation was characterised by high concentrations of fatty acids and ethyl esters, which is different from the aroma profile achieved with pure culture *S. cerevisiae* and *M. pulcherrima*. This finding is interesting because even though the population of *M. pulcherrima* in the mixed culture with *S. cerevisiae* only persisted for 5 days, with a cell density substantially lower than that of *S. cerevisiae* (Figure 3.2 D), the impact of its contribution to the final wine profile was quite significant, with a high production of ethyl hexanoate, 0.31 $mg.L^{-1}$, ethyl caprylate, 0.49 $mg.L^{-1}$, along with an increased production of the hexanoic, octanoic and decanoic medium chain fatty acids, in comparison to the other pure- and mixed-cultured fermentations. These ethyl esters are all within threshold levels of being described as banana, pineapple and pear, respectively. The medium chain fatty acids are below threshold values which could impart a rancid character to the wine (Lambrechts and Pretorius, 2000). From a winemaking perspective, these compounds in the range at which they are found in the wine could be viewed as a positive interaction between *M. pulcherrima* and *S. cerevisiae*, as cumulatively they increase the production of these metabolites in desirable ranges.

The TdSc co-culture fermentation led to an unexpected outcome. The mixed fermentation of these two strains resulted in an aroma profile which showed a dramatic decline in the concentrations of all major volatiles. The influence of the population of *T. delbrueckii* in the wine fermentation is evident from the resulting wine composition. The TdSc mixed fermentation displayed on the PCA-plot, shows that the concentration of all the major volatiles are intermediately situated between the

profiles of pure cultured *T. delbrueckii* and *S. cerevisiae*, which shows that both *T. delbrueckii* and *S. cerevisiae* contributed to the profile of the mixed fermentation.

L. thermotolerans had a significant influence on the aroma profile demonstrated in its mixed fermentation with *S. cerevisiae*. The cluster of LtSc has a greater pull towards the volatile compounds which characterize the pure culture of *L. thermotolerans*, and there was a considerable increase in the concentration of higher alcohols when compared to the pure culture of *S. cerevisiae*.

3.4 The influence of mixed culture fermentations on the formation of medium chain fatty acids

The total concentration of the medium chain fatty acids found in the MpSc mixed fermentation just exceeded 10.5 $mg.L^{-1}$, which was more than double the total concentration of medium chain fatty acids produced by mono-cultured *S. cerevisiae*. Conversely, the total concentration of the medium chain fatty acids found in the LtSc mixed fermentation showed an overall decrease. It was therefore decided to investigate this aspect further, by monitoring the production of medium chain fatty acids over time in order to assess whether it correlated with shifts in population dynamics.

The same experimental procedure was followed as before, with a 10:1 of non-*Saccharomyces* to *S. cerevisiae* inoculation ratio and fermented in AWRI medium (as described in Table 3.2). The only variation was the temperature at which the fermentation was performed, which had been changed from 25˚C to 20˚C. Gao and Fleet (1988) and Erten (2002) indeed demonstrated that in mixed cultures of *K. apiculata* and *C. stellata*, and *K. apiculata* and *S. cerevisiae*, the presence and persistence of non-*Saccharomyces* yeast strains are increased, and their sensitivity to ethanol is decreased when the temperature is decreased. Therefore, the temperature was decreased in the experimental procedure, as a prolonged fermentation would allow for a prolonged period of monitoring the evolution of medium chain fatty acids.

3.4.1 Fermentation kinetics and yeast population dynamics

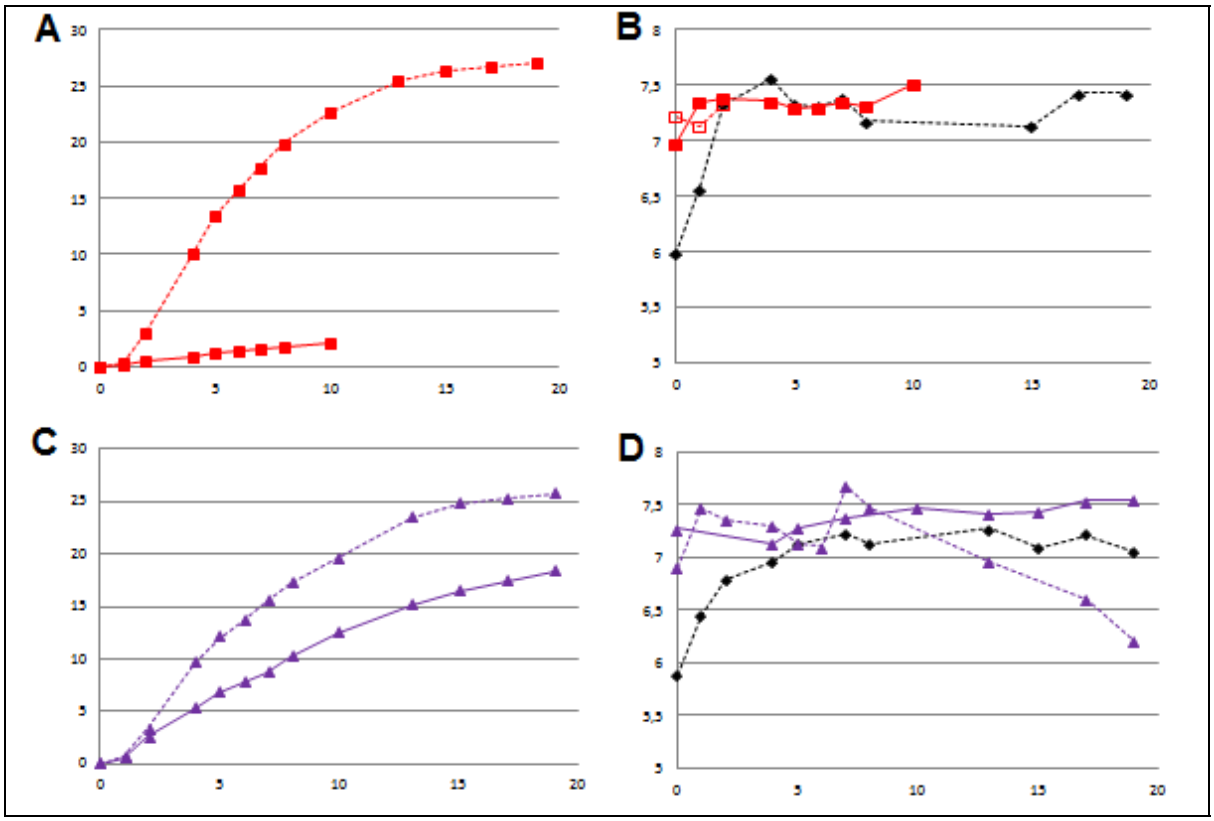

Figure 3.9: Fermentation kinetics, released CO_2 (g) [A; C] and yeast population dynamics, Log cfu.mL^{-1} [B; D] of *S. cerevisiae* (control) and non-*Saccharomyces* yeast strains in mono- and mixed-culture fermentations

The fermentation kinetics and biomass evolution were monitored over time for the individual pure and co-culture fermentations until fermentation completion (Figure 3.9). The control strain *S. cerevisiae* completed fermentation in 19 days, and demonstrated similar population dynamics to what was shown in the previous set of results (Figure 3.2 B). The pure culture of *M. pulcherrima* stopped fermenting after 10 days and the pure culture of *L. thermotolerans* after 19 days (Figure 3.9 A and C). The fermentation rate of the co-culture fermentation *M. pulcherrima*: *S. cerevisiae* was consistently greater than that of *S. cerevisiae*, *and L. thermotolerans: S. cerevisiae* co-culture fermentation was slower than pure *S. cerevisiae* until day 13, after which it fermented at a faster rate. The population of *M. pulcherrima* in the mixed fermentation declined to undetectable levels after day two (Figure 3.9 B) whereas that of *L. thermotolerans* remained viable until the completion of fermentation, declining slowly but continuously after day 7, ending with a final population of 6.2 log cfu.mL^{-1}. In comparison to the previous set of results (Figure 3.2), the fermentation persisted for a longer period at 20˚C. The viability period of *M. pulcherrima* was substantially lower than previously, only surviving for two day as opposed to five days however the overall trend of the population dynamics of pure- and mixed-cultured *M. pulcherrima* was similar. In contrast, LtSc mixed fermentation displayed a different population dynamic in comparison to the previous set of

results. The population of *L. thermotolerans* was greater than *S. cerevisiae,* as beforehand, but at mid-fermentation *L. thermotolerans* population declined below *S. cerevisiae*, which allowed *S. cerevisiae* to complete the fermentation.

3.4.2 The evolution of medium chain fatty acids in mixed culture fermentations of *M. pulcherrima* IWBT Y1337 and *S. cerevisiae* Cross Evolution® 285 and *L. thermotolerans* IWBT Y1220 and *S. cerevisiae* Cross Evolution® 285

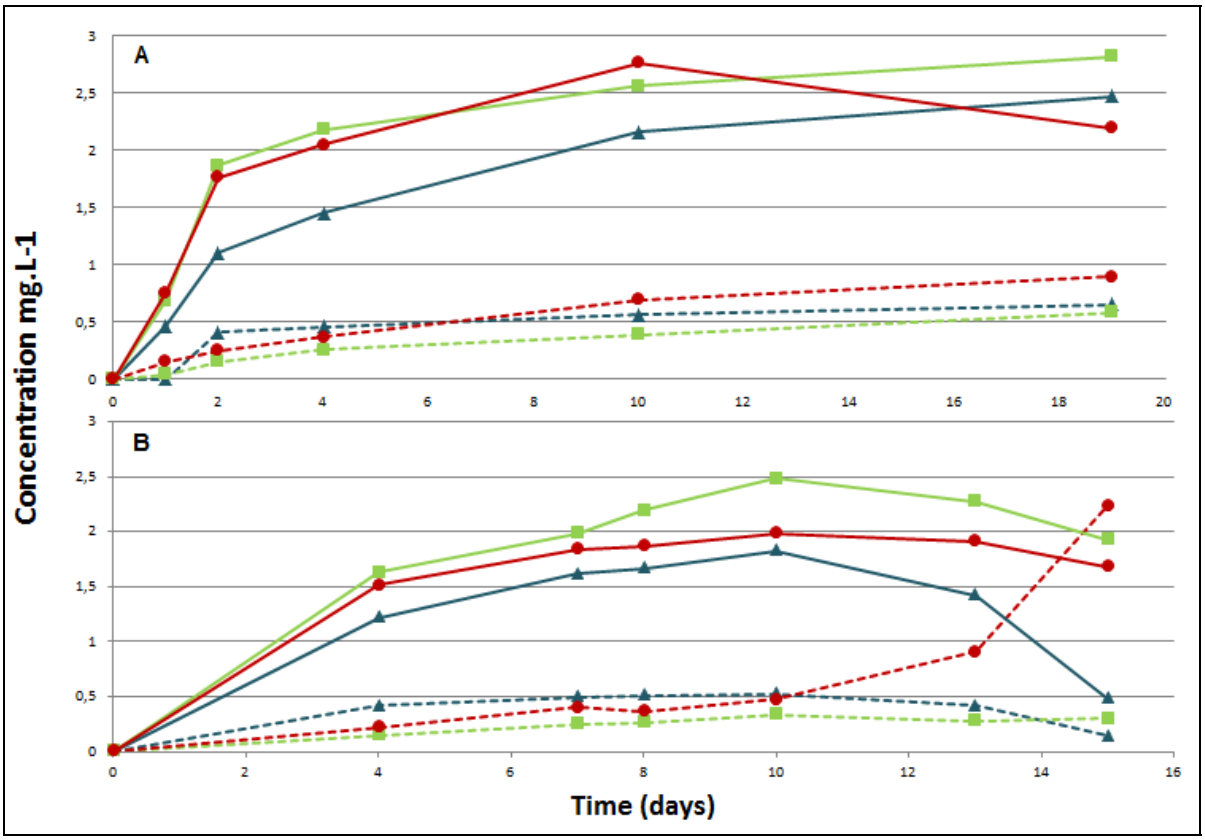

Figure 3.9: The evolution of medium chained fatty acids and their respective ethyl esters in synthetic wine medium: Mixed fermentation (A) *M. pulcherrima* IWBT Y1337: *S. cerevisiae* Cross Evolution® 285 and (B) *L. thermotolerans* IWBT Y1240: *S. cerevisiae* Cross Evolution® 285

The production of hexanoic, octanoic and decanoic acids in the MpSc mixed culture was very rapid over the first two days of fermentation, which coincides with the early death of *M. pulcherrima* on day 2 (Figure 3.9 B). The concentration of hexanoic and octanoic acids gradually increased until the completion of fermentation, and decanoic acid steadily increased until day 10, where after it decreased to its final concentration (Figure 3.9 A), with the end-point concentrations, of the respective medium chain fatty acids (MCFAs), being comparable to those shown in Figure 3.5 C. The corresponding ethyl esters of MCFAs progressively increased throughout the course of fermentation, with an inverse proportion being observed between the concentration of the medium chained fatty acid and its corresponding ethyl ester. The concentration of MCFAs in the LtSc co-culture was not as high as the concentration seen in MpSc over the first four days of fermentation, after which the concentration of each MCFA increased gradually until it peaked at day 10.

Thereafter, the concentration of the MCFA declined until the end of fermentation (Figure 3.9 B). An interesting observation was that the concentrations of hexanoic acid and ethyl hexanoate were proportional to each other, showing the same evolution trend. Decanoic acid and its corresponding ethyl ester showed an inversely proportionate relationship after day 10, where decanoic acid concentration had declined, and ethyl caprate concentration increased.

The concentration of MFCAs that is produced during fermentation and released into the extracellular medium is highly dependent on the medium composition, fermentation conditions (pH, temperature and aeration) and the yeast strain which conducts alcoholic fermentation (Aries *et al.*, 1977; Jones *et al.*, 1981; Krauss *et al.*, 1975; Lafon-Lafourcade *et al.*, 1984; MacDonald *et al.*, 1984; Nordstrom 1964). In a study conducted by Viegas *et al.* (1988), it was found that the maximum specific growth rate of *S. cerevisiae* and *Kluyveromyces marxianus* decreased when 16 and 8 $mg.L^{-1}$ of octanoic and decanoic acid, respectively, were added to the medium. In addition, it has been well described that MCFAs can act as antimicrobial agents (Nordstrom 1964; Freese *et al.*, 1973). Therefore, this investigation was done in order to draw a better correlation between the evolution of medium chain fatty acids and the population dynamics which exist between the two yeast strains in the mixed fermentation.

The accumulation of MCFA normally occurs when the synthesis thereof is inhibited due to anaerobic conditions (Dufour *et al.*, 2003). The MCFA which are then accumulated are toxic for the developing or prevailing yeast population (Alexandre *et al.*, 2006), and it has been suggested that MCFAs are transformed into less toxic ethyl esters and could diffuse more easily through the plasma membrane (Saerens *et al.*, 2010). Of the three MCFAs that were quantified, the order of toxicity based on molarity is decanoic acid >> octanoic acid > hexanoic acid corresponding to their liposolubilities (Sá Correia and van Uden, 1983; Viegas *et al.*, 1985). After day 8, the population of *L. thermotolerans* started to decline (Figure 3.3.1 D) and shortly after (Day 10, Figure 3.9 B), the concentration of decanoic acid declined and the production of ethyl caprate increased. Ethyl caprate is almost always bound in the plasma membrane of the yeast cell (Nordstrom, 1964). A possible explanation in the surge of ethyl caprate starting from the decline of *L. thermotolerans* could suggest that *L. thermotolerans* had a detoxifying effect on the medium, and that its autolysis released the less toxic ethyl ester of decanoic acid, however this cannot be said definitely as the origin of the MCFAs is unknown.

It is well recognised that the production of MCFAs by a yeast species is linked to an antagonistic interaction between populations in a mixed fermentation, due to their inhibition mechanism. In the MpSc mixed culture, the concentrations of the MCFA were significantly higher than the other mixed fermentations. It was therefore suggested that an antagonistic interaction between *M. pulcherrima* and *S. cerevisiae* occurs. Conversely, LtSc mixed fermentation showed a very low concentration of

these compounds. However, results obtained from the investigation into the evolution of MCFA and its correlation to population dynamics did not reveal the origin or the precise impact that the MCFAs had on the existing populations, as *M. pulcherrima* became undetectable before the MCFAs reached their maximum concentrations and *L. thermotolerans* declined along with the concentrations of MCFAs. Moreover, the concentrations at which the MFCAs together with their corresponding ethyl esters were found in concentrations which could be perceived as desirable from an aromatic perspective.

3.5 General conclusion

The results of this study confirm that mixed-cultured fermentations of non-*Saccharomyces* yeasts with *S. cerevisiae* in most cases do not negatively influence the evolution of fermentation, as the co-cultured fermentations went to dryness. The population dynamics of the mixed cultured fermentations clearly demonstrated the competition that occurred between the populations, as neither population achieved the maximum population reached in its respective pure-cultured fermentation. In this context, the production of certain compounds which were found to characterize the individual profiles of the mixed fermentations could be attributed to the interactions that occur between the two mixed populations, and changes in the concentrations of certain compounds could possibly suggest competition or antagonism. However, these interactions are largely unknown and the mechanisms behind them are yet to be elucidated.

Taken together with the results of other studies, the data confirm that non-*Saccharomyces* yeasts strains can be used to achieve specific desirable aromatic profiles. For example, it is evident that these strains of *L. thermotolerans* and *T. delbrueckii* may be used in the management of volatile acidity through the reduction of acetic acid. In addition, the increased production in the concentration of medium chain fatty acids and esters in the mixed fermentation of *M. pulcherrima* and *S. cerevisiae* might lead to the production of wines with increased mouth feel and aroma.

Moreover, the results show that the choice of species to complete fermentation definitely impacted on the concentration of compounds that were formed. This agrees with results demonstrated by other authors (Tosi *et al.*, 2009; Swiegers *et al.*, 2009), that the production of particular secondary metabolites influencing wine aroma is not only species dependant but also strain dependent. In addition, co-inoculated fermentations showed chemical profiles markedly different from those produced by pure *S. cerevisiae*, which ranged from increases in ester and higher alcohol concentrations to a decrease in all major volatiles.

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Chapter 4

General discussion and conclusions

Chapter 4: General discussion and conclusion

4.1 General Discussion

Yeasts employ numerous mechanisms for the liberation of compounds which contribute to wine flavour and aroma. These mechanisms, in particular those involved in the production of secondary metabolites, differ at both species and strain level (Fleet, 2003). The some of the main contributing factors to wine composition include organic acids, esters and higher alcohols.

The yeast population dynamics that occurs during alcoholic fermentation of grape must has been studied by many authors. It is recognised that *S. cerevisiae* has very strong fermentative abilities (Querol et al., 1990), owing to its physiological attributes which make it suitable for the process of alcoholic fermentation, allowing it to surpass the population of other species, even if it was the minor species initially. However, the fundamental mechanisms that makes *S. cerevisiae* the foremost competitive species in the fermenting must is yet to be elucidated. Indeed, biological systems are difficult to characterize based on the fact that each biological system is multi-layered and a shift in one factor could result in a cascade of changes occurring throughout the system. In this context, the use of two high density populations to ferment wine may result in a number of varying results owing to the unique metabolic properties of a single population and external factors such as oxygen availability, temperature, growth medium, etc.

The use of non-*Saccharomyces* yeasts together with *S. cerevisiae* in mixed fermentations has been proposed as a means of simulating spontaneous fermentation, thereby enhancing the wine complexity (Ciani *et al.*, 2010). The multistarter fermentations are characterized by predetermined inoculation concentrations of the individual yeast strains to be added to the must. Studies (Andorra *et al.*, 2012; Ciani *et al.*, 2006) which employed varying inoculation ratios of *Saccharomyces* yeast strains and non-*Saccharomyces* yeast, demonstrated the importance of selecting the appropriate concentration levels for the desired effect. The main objective of this study was to establish what impact the co-inoculation of various non-*Saccharomyces* strains and *Saccharomyces cerevisiae* had on the metabolic changes which occurred within the wine, in comparison to wine produced from single cultures of *S. cerevisiae.*

The inoculum ratio of 1:10 (*S. cerevisiae*: *non-Saccharomyces*) employed in this study proved to be suitable to yield significant differences between the wine composition of each single and mixed fermentation. The fermentation kinetics and biomass evolution of *S. cerevisiae* in mixed cultures was indeed affected by the presence and persistence of the non-*Saccharomyces* yeast strain which was co-inoculated.

The influence of non-*Saccharomyces* yeasts was observed from the onset of fermentation, where it was clearly seen that mixed culture fermentations had a slower rate of fermentation in comparison

to the pure strain of *S. cerevisiae*. The decrease could be attributed to the competition which occurred between the two large populations within the medium. The presence of a large population of non-*Saccharomyces* yeasts has been reported to affect both the rate of fermentation and the metabolism of *S. cerevisiae* (Lema et al., 1996) since they remain at a relatively high cell density of between 10^6 - 10^7 cfu.mL^{-1}. *L. thermotolerans* and *I. orientalis* remained viable until the end of fermentation. Unexpectedly, *L. thermotolerans* had a higher cell density than that of *S. cerevisiae* in their mixed culture fermentation. Ciani *et al*. (2006) and Comitini *et al*. (2011) both showed that the population of *L. thermotolerans* was always superseded by *S. cerevisiae* in co-inoculated fermentations, even when the initial population was one hundred times greater than *S. cerevisiae* (Comitini et al., 2011). A possible hypothesis as to why *L. thermotolerans* reached a greater concentration than *S. cerevisiae* could be due to increased oxygen availability. Indeed, Holm Hansen *et al*. (2001) did not attribute the early death of *L. thermotolerans* to toxic compounds, in the mixed-cultured fermentation with *S. cerevisiae*, but rather to the low available oxygen conditions. In our study, the assumption of complete anaerobic conditions cannot be confirmed or negated, as oxygen levels were not measured. Therefore, it is possible that there was an increased level of oxygen available and *L. thermotolerans* could dominate the fermentation under these circumstances.

The fermentation rate of the mixed fermentations was inversely correlated with the concentrations of the non-*Saccharomyces* yeast population in the medium, thereby demonstrating the influence of the non-*Saccharomyces* on the fermentation kinetics of *S. cerevisiae.* Indeed, the impact of the non-*Saccharomyces* on the biomass evolution of *S. cerevisiae* also showed that in mixed fermentations, *S. cerevisiae* did not reach the same levels as that of pure-culture *S. cerevisiae*, which is in agreement with results found by Mendoza *et al*. (2007), Ciani *et al*. (2006) and Comitini *et al.* (2011). In fact, Comitini *et al*. (2011) showed that with an increase in the ratio of non-*Saccharomyces* to *S. cerevisiae* being used in the multistarter fermentation, the biomass evolution of *S. cerevisiae* was delayed by several days before reaching similar concentration levels to what was found in the pure culture fermentation thereof.

The use of synthetic grape-like medium allowed the study to purely investigate the metabolism of the yeast species involved in the fermentation, and to ascertain that the aroma compounds produced were only as a result of their metabolic interactions. The metabolic interspecies interaction was clearly evident from the significant aroma profile variations achieved through the specific mixed fermentations (Table 3.4 and Figure 3.4). In addition, the PCA-biplot depicts the low concentration of secondary volatile compounds produced by the non-*Saccharomyces* yeast, concurring with the results which demonstrate incomplete fermentation, by this yeast. Therefore, in order to take advantage of the positive attributes which non-*Saccharomyces* yeast strains could have on the final wine product, *S. cerevisiae* has to be used in combination with non-*Saccharomyces* yeast so that *S. cerevisiae* provides the fermentative power required for the

completion of alcoholic fermentation. Furthermore, the production of high concentrations of undesirable secondary metabolites can be modulated by the presence of *S. cerevisiae* in the mixed fermentation e.g. decrease in the production of ethyl acetate in the mixed culture of IoSc in comparison to the undesirable concentration produced by the pure culture of *I. orientalis.* This result is in accordance to what has been shown by other authors (Anfang *et al*., 2008; Bely *et al*., 2008), where the expression of negative oenological traits from non-*Saccharomyces* yeast were modulated by *S. cerevisiae*. Moreover, the aroma profiles which characterize the individual mixed fermentations are significantly different from one another and influence of non-*Saccharomyces* yeasts, even at substantially lower levels in comparison to *S. cerevisiae*, can undoubtedly be seen.

The nature of the inter-species interactions which occur are still largely unknown. However, from a winemaking perspective, more knowledge has been provided to better establish which non-*Saccharomyces* yeasts will most likely produce a certain aroma profile in alcoholic fermentation, purely based on their metabolism. Positive (synergistic) interaction was observed between *L. thermotolerans* and *S. cerevisiae*, where there was a substantial increased production of propanol, butanol and isoamyl alcohol, which when present at a concentration below 300 $mg.L^{-1}$, can contribute to the aromatic complexity of the final wine product (Manzanares *et al.*, 2011). However, when regarding the survival of these two species within mixed culture fermentation, with *L. thermotolerans* and *S. cerevisiae* populations both prevailing until the completion of fermentation, competition between the two species most likely arose. It has been hypothesized that the formation of higher alcohols and volatiles, in part, help to maintain the $NADH/NAD^+$ ratio and redox balance of the cell (Van Dijken and Scheffers 1986). However, other authors believe that the cell produces enough acetaldehyde to fulfil this function (Boulton *et al.*, 1995). Other possible hypotheses for higher alcohol formation include the elimination of toxic aldehyde compounds or as a substitute nitrogen source for the cell (Boulton *et al.* 1995; Volbrecht and Radler 1973). Indeed in a study conducted by Ciani *et al.* (2006), it was shown that there was a significant decrease in the amount of acetaldehyde formed (99.7 $mg.L^{-1}$ in *S. cerevisiae* to 63.7 $mg.L^{-1}$ in the mixed fermentation of *Kluyeromyces thermotolerans* (now known as *L.* thermotolerans) and *S. cerevisiae*), and there was an increase in consumption of assimilable nitrogenous compounds, demonstrating that there was competition between the two species within the mixed fermentation. However, the final wine product achieved through this mixed fermentation displayed a similar wine profile to that of pure *S. cerevisiae,* which differs from the results obtain in this study.

Amensalism was identified between *I. orientalis* and *S. cerevisiae*, as *S. cerevisiae* restricted the growth of *I. orientalis* but it was not affected itself. The pure culture of *I. orientalis* produced high concentrations of esters and higher alcohols, but due to the restriction of its growth in the mixed fermentation, the concentration of these compounds were reduced, which then produced an almost identical analytical profile to pure cultured *S. cerevisiae*. An antagonistic interaction was also observed in the mixed fermentation of *T. delbrueckii* and *S. cerevisiae*, demonstrated by the

dramatic decline in the production of all major volatiles, which are essential for wine composition. Lastly, the compounds which characterized the chemical composition of the single cultured of *M. pulcherrima* and *S. cerevisiae* was notably different from the mixed fermentation of these two species. It would seem that the presence of *M. pulcherrima* in the mixed fermentation shifted the chemical composition towards increased volatile fatty acids and their associated esters.

The great increase in the concentration of medium chain fatty acids (MCFA), which are known to be toxic to yeasts, would indicate an antagonistic interaction. MCFA toxicity is due to their unsaturated nature, the prevailing pH in wine and the escalation in ethanol levels, which increases their diffusion over the plasma membrane. Other authors (Jolly *et al.*, 2003; Comitini *et al.*, 2011) also noted the antagonism that occurs in mixed cultures of MpSc but they also have not specifically attributed the early death of *M. pulcherrima* to the high concentrations of MCFA. *T. delbrueckii*: *S. cerevisiae* mixed fermentation yielded unanticipated results with a significant decline in its total concentrations of all major volatiles. Ciani *et al.* (2006) showed that the aroma profile of *T. delbrueckii* in mixed fermentation with *S. cerevisiae* was not drastically altered from the profile demonstrated by single cultured *S. cerevisiae,* with the exception of the substantial decrease in acetic acid, which was also shown in this study.

The observations made of the interactions which prevail amongst the yeast species involved in these mixed fermentations, demonstrate that far more intricate underlying mechanisms occur, and are yet to be elucidated.

4.2 Future prospects

The results and observations made from this study demonstrate that the inoculation of synthetic must with mixed starter cultures, results in wines which are characterized by distinctive secondary compounds, arising solely from yeast metabolism, compared to fermentations performed by single-cultured wines. The interactions which occur between the yeast species are confirmed by the differences between the aromatic profiles achieved through mono- and mixed-culture fermentation. In addition, the impact of non-*Saccharomyces* yeasts to the mixed fermentation, even when the final cell density was considerably lower than that of *S. cerevisiae*, demonstrated the influence they can have even at low levels.

To gain further insight into these specific mixed fermentations, additional ratios of non-*Saccharomyces*: *S. cerevisiae* mixed fermentations should be performed, to assess whether the fermentation kinetics and biomass evolution would occur in a similar pattern or if the further increase of the initial cell density of the non-*Saccharomyces* population would cause *S. cerevisiae* to behave differently, and ultimately to evaluate the chemical composition of these varying ratios. In addition, would the higher incidence of non-*Saccharomyces* population at the beginning of fermentation cause a higher concentration in the compounds which they are characterized by, or would *S. cerevisiae* still out-compete them and modulate the effect of the non-*Saccharomyces*

population? The outcome of some of the aroma profiles which were achieved was overall in agreement with studies conducted by other authors. However, in the instance with *I. orientalis* and *S. cerevisiae*, little is known about the mixed interactions between these two strains and the aroma profile was closely related to that of pure cultured *S. cerevisiae*. Finally, these inoculation strategies should be assessed in real grape must to determine the impact of grape precursors on the metabolism of the individual yeast and to establish if a similar profile will be achieved.

4.3 References

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