Interaction of multiple yeast species during fermentation

by

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Declaration

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Summary

The use of non-*Saccharomyces* yeasts together with the yeast *S. cerevisiae* in multistarter wine fermentations has emerged as a useful tool to modulate wine aroma and/or to decrease the concentration of undesirable compounds. However, upon inoculation, these yeast species do not co-exist passively, but interact in various ways. While competition for nutrients and the excretion of killer toxins in an antagonistic relationship are obvious and well established types of interactions, some studies have suggested the existence of other forms of cellular or molecular interactions. One of these includes physical cell-cell contact and to our knowledge, only one previous study has confirmed its existence in wine yeasts. Yeast interactions are also influenced by other factors, such as ethanol concentration, however some studies have highlighted the role that dissolved oxygen plays on the survival of non-*Saccharomyces* yeasts and their ability to compete for space with *S. cerevisiae* and little research has focused on this.

This study aimed to investigate the occurrence of a physical cell-cell and/or metabolic interaction between *S. cerevisiae* and *L. thermotolerans* in mixed culture fermentations of synthetic grape must. For this purpose, fermentations in a Double Compartment Bioreactor (DCB) which separates yeast population through the use of a membrane were compared to mixed fermentations in the absence of the membrane, using the same reactor. Furthermore, the impact of oxygen supply on yeast behaviour was also assessed.

Following mixed culture fermentations in a DCB, it was observed that the presence of *S. cerevisiae* led to a significant decline in viability in *L. thermotolerans.* This decline was significantly less prominent in mixed cultures where the cells were in indirect contact. Together, the data provided evidence for both cell-cell and metabolic interactions whereby *S. cerevisiae* had a strong negative influence on the growth of *L. thermotolerans*. However, it was also observed that L. thermotolerans had some negative impact on the growth of S. cerevisiae, leading to a reduction in biomass (when in indirect contact) and a reduced maximum CFU/mL compared to pure cultures. The data also suggest that direct physical contact may increase the production of glycerol and propanol, but this needs further investigation.

By decreasing the frequency at which oxygen pulses were provided, a reduction in biomass and increase in fermentation duration was observed for all fermentations. However, this effect was somewhat reduced in mixed cultures. Here, no impact on fermentation duration was observed and the decrease in biomass was less compared to pure cultures. The impact of these oxygen pulses was also greater on *L. thermotolerans*. In the latter yeast's pure culture a slight increase in glycerol was observed when less oxygen was provided and in general there appeared to be no impact on acetic acid production. Furthermore, there was little or no impact on volatile production, however, more repeats might reveal different results and therefore more research is needed to confirm these results.

To our knowledge, this is the first study of its kind to confirm a physical cell-cell interaction between the yeast pair *S. cerevisiae* and *L. thermotolerans*

Opsomming

Die gebruik van nie-*Saccharomyces* gis saam met die gis *S. cerevisiae* in multi-inokuleringskulture het die afgelope paar jaar as n goeie hulpmiddel na vore gekom om wyn aroma te moduleer en/of om die konsentrasie van ongewensde verbindings te verminder. Sodra inokulasie plaasgevind het, het hierdie gis die potensiaal om op verskeie maniere teenoor mekaar te reageer. Kompetisie vir nutriente en die afskeiding van toksiese verbindings in n antagonistiese verhouding is alreeds goed beskryf in die literatuur. Somige studies het, alhoewel, die bestaan van ander vorme van sellulêre of molekulêre interaksies voorgestel. Een van hierdie sluit in n fisiese sell-sell interaksie en so ver as wat ons kennis strek, het nog net een studie van tevore so ‘n interaksie bevestig tussen wyn giste. Gis interaksies word ook beïnvloed deur ander faktore, soos byvoorbeeld etanol konsentrasie. Terwyl sommige studies die rol wat opgelosde suurstof speel in die oorlewing van nie-*Saccharomyces* gis en hulle vermoë om te kompeteer vir spasie met *S. cerevisiae* alreeds beklemtoon, het min navorsing al hierop gefokus.

Hierdie studie het gestreef om die voorkoms van n fisiese sell-sell en/of metaboliese interaksie tussen *S. cerevisie* en *L. thermotolerans* in gemengde kultuur fermentasies van sintetiese druiwe sap te ondersoek. Vir hierdie doeleinde was fermentasies uitgevoer met behulp van ‘n Dubbel Kompartement Bioreaktor (DKB) wat gis populasies skei deur middel van ‘n membraan en hierdie was vergelyk met gemengde kultuur fermentasies sonder die membraan in dieselfde reaktor sisteem. Verder was die impak van suurstof toevoer op gis gedrag ook geassesseer.

Na afloop van gemengde kultuur fermentasies in ‘n DKB, was daar waargeneem dat die teenwoordigheid van *S. cerevisiae* gelei het tot ‘n betekenisvolle afname in lewensvatbaarheid in *L. thermotolerans*. Hierdie afname was aansienlik minder in gemengde kulture waar die gis in indirekte kontak was. Saam verskaf hierdie data bewyse vir n sell-sell asook metaboliese interaksie waardeur *S. cerevisiae* ‘n sterk, negatiewe invloed op die groei van *L. thermotolerans* gehad het. Daar was egter ook waargeneem dat *L. thermotolerans* tot ‘n mindere mate ‘n negatiewe impak op die groei van *S. cerevisiae* gehad het en dat dit gelei het tot ‘n verlaging in biomassa (toe die gis in indirekte kontak was) en ‘n verlaagde maksimum CFU/mL in vergelyking met suiwer kulture. Die data dui ook aan dat fisiese kontak kon gelei het tot ‘n verhoging in gliserol en propanol produksie, maar hierdie kort verdere ondersoek.

Deur die frekwensie te verminder waardeur suurstof pulse aan die fermentasies verskaf was, was ‘n verlaging in biomassa produksie en ‘n verlenging in fermentasie tydperk waargeneem. Hierdie tendense was waargeneem in almal, behalwe die gemengde kultuur fermentasies. Die effek van suurstof puls verlaging was minder op hierdie fermentasies aangesien daar geen impak op fermentasie tydperk was nie en die verlaging in biomassa minder was. Die impak van hierdie suurstof pulse was ook groter op *L. thermotolerans*. ‘n Klein toename in gliserol produksie was waargeneem in laasgenoemde gis se suiwer kultuur toe minder suurstof

beskikbaar was en oor die algemeen was asynsuur onveranderd. Verder was daar ‘n klein of geen impak op vlugtige verbindings nie, alhoewel, meer herhalings mag verskillende resultate lewer en daarom is meer navorsing nodig om hierde resultate te bevestig.

So ver as wat ons kennis strek is hierdie die eerste studie van sy soort om ‘n fisiese sell-sell interaksie tussen die gispaar *S. cerevisiae* en *L. thermotolerans* te bevestig.

Biographical sketch

Natasha Alethea Luyt was born in Worcester in the Western Cape on 8 January 1990. She attended Langebaanweg Primary School, Hopefield High School and matriculated from Strand High School in 2008. In 2009, she enrolled at the University of Stellenbosch and completed a BSc in Molecular Biology and Biotechnology in 2011. In 2012 she obtained a HonsBSc in Microbiology from the University of Stellenbosch. Since the beginning of 2013 she has been working towards obtaining her MSc in Wine Biotechnology at the Institute for Wine Biotechnology at the University of Stellenbosch.

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Preface

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal Applied Microbiology and Biotechnology

Chapter 1	**Introduction and project aims**
Chapter 2	**Literature review:** Mixed culture fermentations of *S. cerevisiae* and non-*Saccharomyces* yeast: ecological interactions and potential benefits
Chapter 3	**Research results** Interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed culture fermentations of synthetic grape must using a double compartment bioreactor
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Chapter 1

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Introduction and project aims

Chapter 1 – Introduction and project aims

1.1. Introduction

Traditional winemaking practices make use of appropriate starter cultures of *S. cerevisiae* and addition of SO_2 to eliminate spoilage yeasts (Moreno-Arribas and Polo 2005). In recent years, there has been an increasing demand for different styles of wine, and new oenological practices have emerged which deviate from the standard method mentioned above (Fleet 2008). Such practices aim at producing wines with a lower ethanol content, a more complex aromatic profile or with unique characters (Ciani and Comitini 2011; Fleet 2008). This has led to the re-evaluation of the role that non-*Saccharomyces* yeasts play during winemaking and their potential use in multistarter fermentations together with *S. cerevisiae* as a method for creating more complex wines or wines with a different or improved aroma profile (Ciani et al. 2010; Ciani and Comitini 2011; Jolly et al. 2003). Although most non-*Saccharomyces* yeasts are limited in their ability to fully ferment sugars anaerobically and to produce ethanol, some species have been identified as contributing positively to certain wines (Ciani and Ferraro 1996, 1998; Clemente-Jimenez et al. 2005; Domizio et al. 2011; Garcia et al. 2010; Gobbi et al. 2013; Jolly et al. 2003; Medina et al. 2013; Moreira et al. 2008; Soden et al. 2000). For example, it has been found that the glycerol content of a wine can be enhanced through mixed cultures of *S. cerevisiae* and *Starmerella bombicola* (Ciani and Ferraro 1996, 1998), while *Candida pulcherrima*, *Hanseniaspora uvarum*, *Hanseniaspora vineae*, *Pichia fermentans* and *Lachancea thermotolerans* have been used to improve the aromatic profiles or to produce unique flavours in certain wines (Clemente-Jimenez et al. 2005; Domizio et al. 2011; Garcia et al. 2010; Gobbi et al. 2013; Jolly et al. 2003; Medina et al. 2013; Moreira et al. 2008; Soden et al. 2000). Other studies have shown that some non-*Saccharomyces* yeasts can reduce the production of certain undesired compounds such as acetic acid and acetaldehyde (Bely et al. 2008; Ciani et al. 2006; Garcia et al. 2010; Rantsiou et al. 2012). Although these studies are promising, a number of important aspects remain unclear. In particular, in the fermentation ecosystem, these non-*Saccharomyces* yeasts interact with the principal wine yeast *S. cerevisiae* in various ways.

Yeast interactions can either be direct (through physical cell-cell contact) or indirect (through a response to certain metabolites or other compounds, such as killer toxins, produced by one or more of the yeast populations or through competition for nutrients). Few studies have focused on differentiating between the impacts of direct physical and more indirect metabolic interactions. Nevertheless, Nissen et al. (2003, 2004) and Renault et al. (2013) have reported on such interactions, and the latter authors made use of a unique bioreactor system which

physically separates two yeast populations through a membrane that is permeable for metabolites thereby eliminating the effect of a physical interaction. By using such a bioreactor system, Renault et al. (2013) confirmed that physical contact impacts on the interactions between *S. cerevisiae* and *T. delbrueckii*. However, this type of system is still relatively new and has not been standardised across different institutions/laboratories. Furthermore, many factors may influence the ability of non-*Saccharomyces* yeasts to survive throughout fermentation and ultimately, impact on the way in which they interact with *S. cerevisiae*. Some of these factors include the composition of the grape juice, ethanol concentration and fermentation temperature, concentration of SO_2 added (Fleet 2003) and the rapid depletion of dissolved oxygen concentration in the grape must (Hansen et al. 2001). Of these factors, ethanol is believed to play the most important role in the survival of non-*Saccharomyces* yeast. However, recent studies indicate that dissolved oxygen may play an equally relevant role. Indeed, wine-related non-*Saccharomyces* yeasts are globally known for higher oxygen requirements than *S. cerevisiae* and oxygen availability may increase their ability to compete with *S. cerevisiae* (Hansen et al. 2001; Nissen et al. 2004).

1.2. Rationale and project aims

L. thermotolerans is a good candidate for mixed culture wine fermentations with *S. cerevisiae* since it has been shown to increase the glycerol content, reduce acetic acid and ethanol levels, reduce the pH and improve the aroma profile (through the production of certain esters) in certain wines (Ciani et al. 2006; Comitini et al. 2011; Gobbi et al. 2013; Kapsopoulou et al. 2005, 2007; Mora et al. 1990). One strain is already commercialised to the wine industry. Physical interaction between *L. thermotolerans* and *S. cerevisiae* has been hypothesized (Nissen et al. 2003), but has not been demonstrated. Furthermore, the impact of oxygen availability on interactions between these species and on the survival of the non-*Saccharomyces* yeasts in mixed culture fermentations has not been elucidated.

Therefore, the specific aims of this project were:

1. To investigate whether physical interactions impact on fermentation dynamics in mixed cultures of *S. cerevisiae* and *L. thermotolerans*, and
2. To elucidate the role of oxygen availability on these fermentation dynamics.

To achieve these aims, the experimental plan made use of a DCB and a Single Compartment Bioreactor (SCB) system with varying levels of oxygen.

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Chapter 2

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Literature review

Mixed culture fermentations of *S. cerevisiae* and non-*Saccharomyces* yeast: ecological interactions and potential benefits

Chapter 2 – Mixed culture fermentations of *S. cerevisiae* and non-*Saccharomyces* yeast: Ecological interactions and potential benefits

2.1. Introduction

Technical and methodological developments in oenology have enhanced the ability of winemakers to control the wine making process. Such methodologies include the inoculation of the grape must with single, specialised strains of *Saccharomyces cerevisiae* and the addition of sulphur dioxide (SO_2) to eliminate or minimize the effect of other yeasts that are present in the must (Moreno-Arribas & Polo 2005). However, the increasing demand for new and different styles of wine or for wines expressing regional typicality has led to the search for other strategies (Fleet 2008; Moreno-Arribas & Polo 2005), including the use of non-*Saccharomyces* yeasts in conjunction with *Saccharomyces.* Such yeasts may contribute to wines with different and more complex aromatic profiles and/or with unique character (Ciani and Comitini 2011; Fleet 2008; Gobbi et al. 2013; Jolly et al. 2003). This method of wine making has attracted great interest because of its potential to enhance the quality, improve the complexity and modify undesired compounds in the wine and also because wine makers have become more knowledgeable regarding the ecology and biochemistry of wine fermentation and how to manage the process (Ciani and Maccarelli 1998). Although most non-*Saccharomyces* yeasts are limited in their ability to fully ferment the grape juice sugars and to produce sufficient concentrations of ethanol, some have been found to confer positive characteristics to the final wine product (Anfang et al. 2009; Bely et al. 2008; Capece et al. 2005; Ciani and Comitini 2006; Moreira et al. 2008)

In such mixed cultures, yeasts do not co-exist passively, but interact with one another in various ways. Some of these interactions have been well established. These include competition (for nutrients) and antagonism (e.g. via the production of killer toxins). Others that have been hypothesized include physical cell-cell and metabolic interactions. The effect of these cannot be ignored since they might lead to less predictable outcomes. For this reason, studies have also focussed on how exactly these yeasts may interact with one another in mixed cultures (Nissen et al. 2003, 2004; Renault et al. 2013). While early studies have attempted to demonstrate these interactions, they have largely been unsuccessful due to the inability to directly study the effect of cell-cell contact or metabolites (Nissen et al. 2003). However, a new tool for studying yeast interactions has emerged in the last ten years: double compartment bioreactors (Albasi et al. 2001; Salgado-Manjarrez et al. 2000; Renault et al. 2013). This system physically separates two co-fermenting microbiological populations with the use of a membrane, so that the medium is still shared and the effect of physical and metabolic interactions can be monitored effectively.

However, it is still relatively new and has not been standardized across different institutions/laboratories.

Although it has become clear that there are many ways through which wine yeasts interact during vinification, these interactions will also be influenced by factors such as the chemical composition of the grape juice, ethanol concentration and fermentation temperature, concentration of added SO_2 (Fleet 2003) and dissolved oxygen concentration (Hansen et al. 2001). The latter has been shown to play an important role in the survival of non-*Saccharomyces* yeasts throughout the fermentation (Hansen et al. 2001; Nissen et al. 2004), but has never been fully assessed.

This review will focus on the use of non-*Saccharomyces* yeasts in mixed culture fermentations with *S. cerevisiae* and the potential benefits on wine composition. Furthermore, it will investigate potential interactions between these yeasts and other factors that may influence the survival of non-*Saccharomyces* yeasts and how it may impact on wine fermentation.

2.2. The use of non-*Saccharomyces* yeasts in mixed cultures with *S. cerevisiae*

Traditional wine making practices have made use of *S. cerevisiae* starter cultures and the addition of SO_2 to eliminate spoilage yeasts and bacteria, ensure that all sugars are fermented and that wines with specific characters can be reproduced (Moreno-Arribas & Polo 2005). Non-*Saccharomyces* yeasts are present in the grape must and initiate spontaneous fermentation, but they usually die off after 2-3 days, after which *S. cerevisiae* takes over and completes the fermentation (Fleet 2008). For this reason, it was generally accepted that they would not impact significantly on the character of a wine. In recent years, this assumption has been re-evaluated and now there is sufficient data to support the fact that non-*Saccharomyces* yeasts can contribute to wine flavour and aroma to create wines with more complex and unique characters or potentially eliminate certain undesired flavours (Anfang et al. 2009; Bely et al. 2008; Ciani et al. 2006; Ciani and Ferraro 1996, 1998; Comitini et al. 2011; Clemente-Jimenez et al. 2005; Domizio et al. 2011; Garcia et al. 2010; Gobbi et al. 2013; Jolly et al. 2003, 2006; Kapsopoulou et al. 2005, 2007; Medina et al. 2013; Moreira et al. 2008; Soden et al. 2000; Rantsiou et al. 2012). Table 2.1 lists the most recent contributions to our knowledge on how these yeasts can contribute to multistarter wine fermentations. While most of these yeasts are limited in their ability to ferment grape juice to dryness, to produce sufficient ethanol levels and may produce undesirable compounds such as acetic acid and acetaldehyde in pure cultures, they contribute differently in mixed culture fermentations (Ciani et al. 2010; Ciani and Comitini 2011). Here, some undesired characteristics (such as the production of high levels of acetic acid) may remain

unexpressed or be modified by the metabolic activity of *S. cerevisiae* (Ciani and Comitini 2011). Furthermore, because they are not able to dominate the fermentation, but still contribute to a certain extent, the outcome of their inoculation may be a reduced production of certain undesired compounds compared to what would be observed in pure cultures. As mentioned above, these positive contributions to mixed culture fermentations have been studied extensively and studies have found positive contributions to glycerol content, wine aroma and complexity, reduced levels of acetic acid and ethanol and the increased production of varietal thiols (Table 2.1).

Table 2.1: Recent studies related to the positive contributions that non-*Saccharomyces* yeasts may bring to mixed culture or sequential wine fermentations with *S. cerevisiae*

Non-*Saccharomyces* yeasts co-fermented with *S. cerevisiae*	Method		Positive contribution	References
Starmerella bombicola (formerly known as *Candida stellata*)	Synthetic grape must	Immobilized cells (sequential or pretreatment)	Enhanced glycerol content	Ciani and Ferraro 1996; Ciani and Ferraro 1998
	Grape must			
	Grape must	Sequential cultures	Improved aroma profile	Soden et al. 2000
Pichia kluyveri	Grape must	Mixed cultures	Increases in varietal thiols	Anfang et al. 2009
Candida pulcherrima (also known as ***Metschnikowia pulcherrima***)	Grape must	Mixed cultures	Higher quality Chenin blanc wines	Jolly et al. 2003
Candida membranifaciens	Grape must	Mixed cultures	Reduced ethanol levels Reduced acetic acid Improved aroma profile	Garcia et al. 2010
Starmerella bacillaris (formerly known as ***Candida zemplinina***)	Grape must	Sequential and Mixed cultures	Reduced acetic acid	Rantsiou et al. 2012
Hanseniaspora uvarum	Grape must	Sequential and mixed cultures	More complex aroma profile	Moreira et al. 2008;
Hanseniaspora vineae	Grape must	Sequential cultures	More complex aroma profile Enhanced glycerol content	Medina et al. 2013
Toluraspora delbrueckii	Grape must	Sequential and Mixed cultures	Reduced acetic acid and acetaldehyde	Bely et al. 2008; Ciani et al. 2006
Pichia fermentans	Grape must	Sequential cultures	Improved flavour and aroma profile	Clemente-Jimenez et al. 2005; Domizio et al. 2011
Lachancea thermotolerans (formerly known as *Kluyveromyces thermotolerans*)	Grape must	Sequential and mixed cultures	Increased titratable acidity Enhanced glycerol content Reduced acetic acid and acetaldehyde Improved aroma profile Reduced pH	Ciani et al. 2006; Comitini et al. 2011; Gobbi et al. 2013; Kapsopoulou et al. 2005, 2007; Mora et al. 1990

2.2.1. Enhanced glycerol content

For the purpose of enhancing the glycerol content of wines, it has been proposed to make use of *Starmerella bombicola* (formerly known as *Candida stellata*) in mixed cultures with *S. cerevisiae* (Ciani and Ferraro 1996, 1998). High levels of acetaldehyde and acetoin were observed in *S. bombicola* pure cultures, but following the co-fermentation of grape must using *S. cerevisiae* and immobilized cells of *S. bombicola*, these levels dropped significantly. This could be attributed to

the fact that *S. cerevisiae* had metabolised acetaldehyde and converted acetoin into 2,3-butanediol, ethanol or other secondary compounds (Ciani and Ferraro 1998). Furthermore, a significant increase in glycerol and succinic acid was observed. Sequential wine fermentations using this yeast pair, have also produced wines with certain aroma scores similar to the control fermentations (Soden et al. 2000). Therefore, this co-culture could also improve the wine aromatic profile.

2.2.2. Improved wine aroma and complexity

Other multistarter combinations have been proposed to improve wine aroma and complexity. Some of these include the use of *Candida membranifaciens*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, *Hanseniaspora vineae*, *Pichia fermentans* and *Lachancea thermotolerans* (Clemente-Jimenez et al. 2005; Domizio et al. 2011; Garcia et al. 2010; Gobbi et al. 2013; Jolly et al. 2003; Medina et al. 2013; Moreira et al. 2008). Garcia et al. (2010) produced wines from *C. membranifaciens* and *S. cerevisiae* mixed cultures and the sensory analysis indicated that oenologists preferred such wines over the control made with *S. cerevisiae* alone. This preference could be linked back to differences in certain esters and in propanol content. They also observed a decrease in acetic acid production for the *S. cerevisiae* and *C. membranifaciens* yeast pair. Jolly et al. (2003) observed a similar response following the sensory analysis of a wine produced by the fermentation of *S. cerevisiae* and *M. pulcherrima* in three consecutive years. These wines had an increase in quality over wines produced with *S. cerevisiae* only. While being able to contribute to flavour and aroma through the production of certain esters, it was also observed that some of these non-*Saccharomyces* yeasts do not contribute to the production of certain undesired compounds (Moreira et al. 2008). Mixed cultures of *H. uvarum* and *Hanseniaspora guilliermondii* with *S. cerevisiae* led to similar amounts of higher alcohols and sulphur-containing compounds as the *S. cerevisiae* pure cultures. Recently, sequential fermentations of Chardonnay grape juice using *H. vineae* and then *S. cerevisiae* after 6 days also noted an increase in flavour and aroma, when compared to *S. cerevisiae* pure cultures and spontaneous fermentations (Medina et al. 2013). A chemical and sensory analysis of these wines revealed increases in acetate esters, some ethyl esters and decreases in isovaleric acid and some higher alcohols which lead to increased fruity characters in the wine. Specifically, a 17-fold higher concentration of 2-phenylethyl acetate than the sensory threshold was observed for the mixed cultures and a 5- and 10-fold higher concentration in wines produced by a *S. cerevisiae* starter culture and spontaneous fermentation. This compound contributes to ‘rose’, ‘honey’, ‘fruity’ and ‘flowery’ notes in wine (Swiegers et al. 2005). Consequently, this wine was described as being more full bodied, more complex in the palate

and more intense in terms of fruity characters in the nose before MLF was completed. Sequential fermentations seem to be the best option for such mixed culture fermentations, since it allows the non-*Saccharomyces* yeast inoculated to contribute significantly to wine flavour and aroma before it is outcompeted by *S. cerevisiae*. The combination of *P. fermentans* and inoculation of *S. cerevisiae* after 2 days also resulted in wines with a more complex flavour and aroma profile (Clemente-Jimenez et al. 2005; Domizio et al. 2011). While many non-*Saccharomyces* yeasts have a low tolerance to sulphur dioxide, Clemente-Jimenez et al. (2005) selected this yeast species because of its high tolerance to this compound, which was similar to that of *S. cerevisiae* in YPD medium. Sequential fermentations of this yeast pair resulted in increases in the concentration of acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl caprylate, 2,3-butanediol and glycerol (Clemente-Jimenez et al. 2005), while Domizio et al. (2011) reported increases in the total polysaccharide concentration of these wines. The latter compounds have been shown to contribute to wine taste, body and aroma persistence (Domizio et al. 2011). *L. thermotolerans* can also contribute positively to wine complexity through the increased production of glycerol and 2-phenyl ethanol (Comitini et al. 2011), which has been linked to desirable ‘floral’ and ‘rose’ aromas (Swiegers et al. 2005). Gobbi et al. (2013) confirmed the above-mentioned results and following a sensory analysis of these wines, they detected ‘spicy’ notes (which could be linked back to an increase in ester formation). Furthermore, these authors confirmed previous studies which demonstrated that mixed cultures with *L. thermotolerans* have the potential to reduce the pH of a wine as a result of a high production of L-lactic acid (Kapsopoulou et al. 2005, 2007; Mora et al. 1990). Gobbi et al. (2013) suggested that this characteristic may be used as a biological acidifying agent for wines with undesirably high pH levels instead of a chemical solution, which is not allowed in wines from certain regions. This non-*Saccharomyces* yeast also produces low levels of acetic acid and in mixed cultures with *S. cerevisiae*, acetic acid is also lower than in pure *S. cerevisiae* cultures (Ciani et al. 2006; Mora et al. 1990). This is usually attributed to the fact that *L. thermotolerans* could consume the acetic acid produced by *S. cerevisiae*.

2.2.3. Reduced acetic acid levels

The latter characteristic has also been observed for sequential and mixed fermentations of *T. delbrueckii* and *S. cerevisiae* (Bely et al. 2008; Ciani et al. 2006). Furthermore, Bely et al. (2008) concluded that the best option for using multistarter winemaking practices was to inoculate *T. delbrueckii* with *S. cerevisiae* at a ratio of 20:1, since they observed a significant drop in volatile acidity and acetaldehyde production compared to *S. cerevisiae* pure cultures and mixed culture fermentations. However, the behaviour of these yeasts in such wine fermentations are strain

specific (Bely et al. 2008). Rantsiou et al. (2012) also noticed a reduction in acetic acid levels for sequential and mixed culture fermentations of *Starmerella bacillaris* (formerly known as *Candida zemplinina*) and *S. cerevisiae.*

2.2.4. Reduced ethanol levels

Recently, there has been a higher demand for wines with reduced ethanol levels. Mixed cultures of *S. cerevisiae* and non-*Saccharomyces* yeasts might be a natural way of achieving this. Garcia et al. (2010) noticed a significant reduction in ethanol production in co-fermentations of *C. membranifaciens* and *S. cerevisiae* compared to the *S. cerevisiae* control (from 15.6 down to 12.6 %) and speculated that this might be due to competition between these two species. Gobbi et al. (2013) noted a decrease in ethanol concentration (0.7-0.9 %) for sequential fermentations of *L. thermotolerans* and *S. cerevisiae* at lower temperatures. More recently, Morales et al. (2015) saw an optimized decrease of 2.2 % ethanol content in mixed cultures of *S. cerevisiae* and *C. pulcherrima* after aeration of the culture for the first 48 h of fermentations, keeping it under anaerobic conditions for the rest of the fermentation duration.

2.2.5. Increased varietal thiol levels

Another positive contribution to wine that has been linked to some non-*Saccharomyces* yeasts is the increased production of varietal thiols in *Pichia kluyveri* (Anfang et al. 2009). Co-cultures of this yeast with *S. cerevisiae* at a ratio of 9:1 resulted in a higher concentration of 3MHA (3-mercaptohexyl acetate) in Sauvignon blanc wines when compared to the *S. cerevisiae* control. This compound is known to contribute to fruity notes, such as ‘passion fruit’ and ‘grapefruit’ in white and rosé wines (Roland et al. 2011).

2.3. Yeast interactions in wine

The winemaking environment is characterised by a complex microbial ecosystem, consisting of many species and strains of yeasts, bacteria and filamentous fungi. These organisms have the potential to interact with each other within this ecosystem and the effect of such interactions on the final wine composition cannot be ignored. Specifically, yeast-yeast interactions are of great interest because of their dominant role in conducting alcoholic fermentation (Fleet 2003). In general, it has been accepted that the early death of non-*Saccharomyces* yeasts (after 2-3 days) in wine fermentation is as a result of rising ethanol concentrations. However, recent studies suggest otherwise, since some non-*Saccharomyces* yeast species have been found to possess a relatively high tolerance to ethanol (Pina et al. 2004). While not much research has focused on

the role that yeast interactions and other contributing factors may play during wine fermentation, some studies have improved our understanding of the mechanisms behind such interactions (Albergaria et al. 2010; Bely et al. 2008; Ciani et al. 2006; Nissen et al. 2003, 2004; Pérez-Nevado et al. 2006; Renault et al. 2013; Strehaiano et al. 2010). The results of these studies have contributed to a better understanding of the early death of non-*Saccharomyces* yeasts and how such microorganisms interact with *S. cerevisiae* in mixed culture fermentations. Nevertheless, more research is needed regarding the specific mechanisms through which these yeast interact with each other, the specific genes that are involved and the effect this may have on the final wine composition and sensorial profiles. There are two ways in which these yeasts may interact with each other: 1. in a direct way through physical, cell-cell interactions, or 2. in an indirect way through the secretion of certain molecules or specifically evolved systems (like killer toxins and quorum sensing).

2.3.1. Direct interactions

While it seems obvious that indigenous and inoculated yeasts (especially in multistarter fermentations) would interact physically, few studies have focused on revealing such interactions. Eleven years ago, Nissen et al. (2003) hypothesised such an interaction, but few studies have elaborated on this. However, with the search for finding non-*Saccharomyce*s and *S. cerevisiae* multistarter yeast pairs that might introduce positive characteristics into wines, more recent studies have revealed new information regarding a possible physical interaction.

Bely et al. (2008) tested the response to high sugar fermentations of *S. cerevisiae* and *T. delbrueckii* mixed cultures and observed a reduced volatile acidity in these fermentations. They speculated that this might be due to an interaction between the two yeasts whereby the growth of *S. cerevisiae* was somewhat suppressed by high cell concentrations of *T. delbrueckii*, but that more research would be needed to confirm this hypothesis. Ciani et al. (2006) also noticed a reduced maximum cell count for *S. cerevisiae* in mixed cultures compared to its pure cultures and Comitini et al. (2011) observed that this influence on *S. cerevisiae* was highly dependent on the inoculum ratios and the yeast species involved. In a 1:1 ratio, the growth of non-*Saccharomyces* yeasts did not appear to have any effect on that of *S. cerevisiae*, but its growth was delayed or reduced at ratios of 100:1 and 1000:1 (non-*Saccharomyces*/*S. cerevisiae*). These results were similar to what Ciani et al. (2006) and Mendoza et al. (2007) observed. Furthermore, these authors also observed that both the non-*Saccharomyces* yeasts and *S. cerevisiae*'s maximum biomass production was lower in mixed cultures compared to their individual pure cultures, which might indicate a physical (or metabolic) interaction between the two. Comitini et al. (2011) also observed that *M. pulcherrima* had no effect on the growth of *S.*

cerevisiae, indicating that this interaction is specific to certain yeast species. However, these studies did not specifically aim at studying interactions and indeed, few have done so.

In 2003, Nissen et al. conducted a study specifically aimed at investigating interactions between *S. cerevisiae* and *T. delbrueckii* and/or *L. thermotolerans* mixed cultures. As was expected, both non-*Saccharomyces* yeasts died off earlier in the fermentations than *S. cerevisiae*. The cause of this phenomenon was investigated through some supplementary experiments:

1. **Nutrient limitation** was ruled out since growth arrest followed even after oxygen availability was increased and fresh medium was added.
2. The presence of **growth inhibitory compounds** (such as ethanol, killer toxins and medium chain fatty acids) was also ruled out by adding supernatants from mixed cultures at the time of growth arrest to the respective non-*Saccharomyces* pure cultures in late exponential phase. After doing this, no growth arrest was observed.
3. The impact of a **quorum sensing** effect was considered, but later ruled out based on the experiment listed above. The mixed culture supernatant contained no compound in solution that impacted negatively on the growth of the non-*Saccharomyces* yeast.
4. The **presence of *S. cerevisiae* cells** at a high concentration was confirmed to cause cellular death in *T. delbrueckii* and *L. thermotolerans*. This was achieved by the addition of a high concentration (5 X 10^7 cells/mL) of viable *S. cerevisiae* cells (metabolically and enzymatically active cells) to pure cultures of *T. delbrueckii* and *L. thermotolerans* in late and early exponential phase which then led to the immediate growth arrest of these two non-*Saccharomyces* yeasts. To prove that this theory was correct, the same experiment was performed with the addition of a high concentration of dead *S. cerevisiae* cells (metabolically and enzymatically inactive cells) and *S. cerevisiae* cell debris (metabolically inactive and enzymatically active cells) and in both cases growth of the non-*Saccharomyces* yeasts carried on for 24 h after the additions.
5. With the use of a dialysis tube fermentation method, it was confirmed that the early deaths of the non-*Saccharomyces* yeasts were also mediated by **cell-cell contact** with *S. cerevisiae* cells. The latter was inoculated into a dialysis tube (containing 10mL medium) and submerged into 70 mL medium which was inoculated with the respective non-*Saccharomyces* yeast. The yeast populations were physically separated, but the dialysis tube was permeable to nutrients and metabolites. During these fermentations, the non-*Saccharomyces* populations reached stationary phase cell concentrations close to that of their pure cultures (and therefore higher than the mixed cultures where they

were in physical contact with *S. cerevisiae*). After including other *S. cerevisiae* strains to these experiments (and observing the same trends), it was concluded that the ability of *S. cerevisiae* to induce death in *T. delbrueckii* and *L. thermotolerans* is a cell-cell mechanism dependant on high concentrations of viable *S. cerevisiae* cells which is a common feature in this species.

The above-mentioned method proved helpful and gave insight into the underlying mechanism through which non-*Saccharomyces* yeasts and *S. cerevisiae* may interact in mixed culture wine fermentations. However, there was a disequilibrium between the two compartments (since the volume of both was 10 and 70 mL respectively) and therefore population growth could only be monitored in the external compartment. The yeast population and medium composition of the internal compartment (containing the *S. cerevisiae* population) could only be assessed after fermentation was complete. Therefore, the effect of the metabolism of non-*Saccharomyces* on *S. cerevisiae* was excluded as the latter population could not be monitored throughout fermentation. Other studies, following the work of Nissen et al. (2003) have further elaborated on this topic. While it remains unclear what causes this cell-cell mediated death, Nissen et al. (2004) showed in a different study that the early death of *T. delbrueckii* is also regulated by the availability of oxygen and its glucose uptake ability. A cell-cell interaction was also suggested by Arneborg et al. (2005) when the close proximity of *S. cerevisiae* cells caused a delay in growth of non-*Saccharomyces* yeast. However, it is only recently that this cell-cell mediated death in non-*Saccharomyces* yeasts could be studied and confirmed by utilising a method that would rule out the above-mentioned limitation in the work of Nissen et al. (2003) (Renault et al. 2013).

With the specific aim of studying the effect of physical separation of *S. cerevisiae* and *T. delbrueckii* mixed cultures under wine making conditions, the latter authors designed a double compartment bioreactor which separated the two yeast populations, while still allowing the flow of medium between the two compartments. Therefore, *S. cerevisiae* and *T. delbrueckii* cells were physically separated, but were still able to share the fermentation medium and exchange metabolites. The medium was kept homogenised through mixing between the compartments with magnetic stirrer bars and a peristaltic pump (therefore eliminating a disequilibrium between the two compartments), fermentation kinetics was monitored through weight loss and growth kinetics was monitored independently in both compartments on agar plates. In all fermentations, *S. cerevisiae* dominated, while *T. delbrueckii* struggled more (compared to the study done by Nissen et al. (2003)), because of harsher conditions more similar to wine making conditions. Nevertheless, these authors observed that when separated physically from *S. cerevisiae*, the viability of *T. delbrueckii* could be maintained until the end of fermentation (at 90 g/L CO_2

produced), while it dropped earlier in the fermentation (35 g/L CO_2 produced) than where it was not separated. These results correspond to what Nissen et al. (2003) found. While uncertain as regarding the mechanism behind such a cell-cell induced death, these authors proposed that it was either due to direct physical contact through receptor/ligand interactions or that such a cell-cell interaction may lead to a metabolic response in *S. cerevisiae*, leading to the expulsion of a soluble molecule lethal to non-*Saccharomyces* yeasts at high concentrations.

2.3.2. Indirect interactions

In wine making, the most common indirect interactions between microorganisms are competition and amensalism (Strehaiano et al. 2010). The former is an indirect interaction defined as the competition for a common substrate (such as sugar) and where the organism with the promoted growth has a higher growth rate. The latter is also an indirect interaction whereby a molecule secreted by one organism has a negative influence on another organism's growth. The organism that secretes said molecule does not benefit directly, nor does it harm itself. A possible commensalism interaction has been reported by Mills et al. (2002) whereby the selective consumption of fructose by a *Candida* isolate aided the fermentative capacity of *S. cerevisiae* through an increased glucose/fructose ratio.

One example of amensalism is the secretion of killer toxins (extracellular glycoproteins) by one (killer) yeast in order to stimulate cellular death (through damage to the cell membrane) in another (sensitive) yeast species present in the wine (Strehaiano et al. 2010). Several wine yeast genera exhibiting killer activity have been identified: *Saccharomyces*, *Hanseniaspora*, *Pichia*, *Candida*, *Lachancea*, *Zygosaccharomyces*, *Metschnikowia* and *Cryptococcus* (Albergaria et al. 2009; Ciani and Fatichenti 2001). The killer toxins that have been identified for *S. cerevisiae* are however only toxic for other sensitive strains of the same species (Ciani and Fatichenti 2001). Nevertheless, Albergaria et al. (2010) were able to show that *S. cerevisiae* produced one or more proteinaceous molecules that proved deadly to *H. guilliermondii*. Apart from the killer toxin as signal molecule, other studies have proposed different compounds acting in a quorum sensing-like manner to stimulate specific responses in yeast (Hayashi et al. 1998; Hornby et al. 2001; Ohkuni et al. 1998; Palková et al. 1997; Richard et al. 1996) and this phenomenon has also been proposed to act in an amensalism interaction to induce cell death in yeast (Nissen et al. 2004; Renault et al. 2013).

Quorum sensing has been well described in bacteria as a response with alterations in gene expression to a threshold value of chemical signal molecules, termed autoinducers, which are produced by bacteria and accumulate in the environment (Waters and Bassler 2005). While the latter phenomenon has not been well described in yeasts, some early studies have proposed

how it might occur between yeast species (Hayashi et al. 1998; Hornby et al. 2001; Ohkuni et al. 1998; Palková et al. 1997; Richard et al. 1996). Palková et al. (1997) proposed that unprotonated volatile ammonia acts as signal molecule between different yeast species on agar plates to notify the population of incoming nutrient starvation. Richard et al. (1996) suggested that acetaldehyde acts as signal molecule to synchronise the glycolytic oscillation of individual *S. cerevisiae* cells at high cell densities. It has also been proposed that bicarbonate may stimulate meiosis and sporulation in *S. cerevisiae* (Hayashi et al. 1998; Ohkuni et al. 1998) and that farnesol may prevent mycelial development in *Candida albicans* (Hornby et al. 2001). More recent studies have studied this phenomenon in mixed culture fermentations of different wine yeast. While Nissen et al. (2004) speculated that the early death of *T. delbrueckii* and *L. thermotolerans* in mixed cultures with *S. cerevisiae* might be due to a specific signal molecule, they were unable to confirm this and attributed it to a different mechanism. Recently, Renault et al. (2013) saw similar results by making use of a double compartment bioreactor where *S. cerevisiae* and *T. delbrueckii* cells were physically separated. Apart from confirming a cell-cell interaction that stimulated cell death in *T. delbrueckii*, these authors also saw that even though *T. delbrueckii* growth had seized towards the end of alcoholic fermentation, its remaining metabolic activity had an indirect effect on the growth and viability of *S. cerevisiae*. Here, it was observed that *S. cerevisiae* growth was delayed (confirmed by a lower V_{max}).This is the only study of its kind to confirm such a metabolic interaction and therefore it would be of great value to further elaborate on these results and test this kind of interaction on other non-*Saccharomyces*/*S. cerevisiae* yeast pairs.

2.4. Inhibiting factors

While it is hard to ignore the effect that yeast interactions have on the persistence of non-*Saccharomyces* yeasts in mixed culture wine fermentations, studies have highlighted the role that other factors may play on the survival of non-*Saccharomyces* yeasts (Bisson 1999; Cartwright et al. 1986; Ciani and Comitini 2006; Gobbi et al. 2013; Hansen et al. 2001; Nissen et al. 2003; Pina et al. 2004; Sá-Correia et al. 1989; Viegas et al. 1989; Xufre et al. 2006) The most important of these include the components of the grape juice, fermentation methods (such as the addition of SO_2) and conditions (temperature and oxygen content) and the ethanol concentration (Jolly et al. 2006). Through these studies it has become apparent that our understanding of the ability of non-*Saccharomyces* yeasts to compete with *S. cerevisiae* needs improvement and that these factors should be considered before making use of these yeasts for wine making purposes.

2.4.1. Ethanol and temperature

It is generally accepted that the rising ethanol concentrations in wine are the main cause for the observed death of non-*Saccharomyces* yeasts early in fermentation (Fleet 2008). After their death, *S. cerevisiae* usually takes over and completes the fermentation. Ethanol's toxicity stems from its ability to either impair the cell's ability to maintain pH homeostasis within the cytoplasm (Bisson 1999; Cartwright et al. 1986) or its role in the disruption of protein function within the plasma membrane and consequent cell leakage (Bisson 1999; Sá-Correia et al. 1989). In the past, it was observed that yeasts belonging to the genera *Hanseniaspora*, *Candida*, *Pichia*, *Lachancea* and *Metschnikowia* could not survive ethanol concentrations of 5-7 % (Heard and Fleet 1988, Gao and Fleet 1988), but as mentioned above, recent reports have revealed some wine isolates with ethanol tolerance levels close to that of *S. cerevisiae* (Pina et al. 2004; Xufre et al. 2006), which has highlighted the need to re-evaluate our understanding of what influences the survival of non-*Saccharomyces* yeasts in wine fermentation. Pina et al. (2004) observed strains of *H. guilliermondii* and *S. bombicola* with ethanol tolerance levels close to that of *S. cerevisiae* and these yeasts were able to persist under an ethanol stress of 25 % (v/v). In laboratory scale wine fermentations, Xufre et al. (2006) observed that even though *S. cerevisiae* had dominated the fermentation, some non-*Saccharomyces* yeasts (*L. thermotolerans*, *Lachancea marxianus* and *S. bombicola*) still had relatively high cell densities up to 98 h into the fermentation when ethanol was at ± 70 g/L. Other studies have observed that temperature can alter some non-*Saccharomyces* yeasts' tolerance to ethanol (Ciani and Comitini 2006; Gao and Fleet 1988; Gobbi et al. 2013). Gao and Fleet (1988) observed that at 10°C and 15°C, *S. bombicola* could tolerate a maximum ethanol level of 12.5 % (v/v) and that this tolerance decreased at 30°C. They also noted that *Kloeckera apiculata* could survive ethanol levels of 10-12 % (v/v) at 10°C and that this tolerance decreased at 15°C and 30°C. Ciani and Comitini (2006) reported similar results for *S. bombicola.* When immobilized cells of this yeast were used in sequential fermentations with *S. cerevisiae* at 16°C, it had the best fermentation ability (compared to sequential fermentations at 20°C) and was able to survive until the end of fermentation at concentrations similar to *S. cerevisiae* and a final ethanol concentration of 8.9 % (v/v). Gobbi et al. (2013) recently confirmed the same trend for *L. thermotolerans* at 20°C which reached a final ethanol concentration of 14 % (v/v). In the latter study, it was observed that this yeast had an increased persistence throughout fermentation and even had an inhibitory effect on *S. cerevisiae*, which was not observed for mixed culture fermentations at 30°C. This increase in tolerance to ethanol at lower temperatures seems to be a major influence on the survival rate

of non-*Saccharomyces* yeasts and could be exploited in the wine industry for wines produced at lower temperatures (Fleet 2008).

2.4.2. Other growth inhibitory compounds

It is believed that the production of certain compounds (such as acetic acid and medium chain fatty acids) by *S. cerevisiae* may also lead to the early death of non-*Saccharomyces* yeasts (Bisson 1999; Fleet 2003; Ludovico et al. 2001). Indeed, Viegas et al. (1989) found that decanoic acid and octanoic acid were toxic during alcoholic fermentation of two laboratory media by *S. cerevisiae* and artichoke juice by *L. marxianus.* In both types of fermentations, a decrease in maximum specific growth rate and biomass yield was observed at 30°C. This decrease could be correlated back to the amount of each acid added. Furthermore, they noticed that decanoic and octanoic acids were more toxic than ethanol and that their toxicity increased with a drop in pH. More recently, Pérez-Nevado et al. (2006) also noticed that ethanol could not be the only contributing factor to the early death of non-*Saccharomyces* yeast. Indeed, by inoculating the supernatants of 3- and 6-day-old mixed culture fermentations with *H. guilliermondii*, they observed immediate growth arrest of this yeast. The death rate was also higher in 6 day old supernatants. By performing these experiments, they had ruled out the possibility of a cell-cell induced death by *S. cerevisiae* and concluded that it was due to one or more toxic compounds produced by this yeast. The nature of these compounds is, however, yet to be unravelled.

The addition of SO_2 in wine is commonly used to avoid the development of spoilage yeasts and bacteria on the wine. However, some studies have shown that this effect is concentration dependent and that some yeasts can withstand high concentrations of this compound (Constantí et al. 1998; Jolly et al. 2006; Rementeria et al. 2003) After SO_2 addition to their spontaneously fermented wines over two vintages, Rementeria et al. (2003) noticed that *Saccharomyces bayanus* became more frequent compared to previous years and *Candida glucosophila* dominated both vintages. In general, they also concluded that while the addition of SO_2 affected the yeast population, it had no effect on species diversity. It has been seen that low sulphur addition (20 mg/L) suppresses non-*Saccharomyces* yeasts, but higher levels (40-50 mg/L) still allow for growth of certain yeasts, such as *H. uvarum*, *S. bombicola, Candida guilliermondii* and *Zygosaccharomyces* spp. (Constantí et al. 1998; Jolly et al. 2006).

2.4.3. Dissolved Oxygen

Recent studies have evaluated the effect of dissolved oxygen on the survival and performance of non-*Saccharomyces* yeasts (Brandam et al. 2013; Ciani and Comitini 2006; Hansen et al. 2001; Nissen et al. 2004). While Ciani and Comitini (2006) could only observe major differences

in the survival of *S. bombicola* in mixed cultures when temperature was altered (and not oxygen), Hansen et al. (2001) reported a different result in their studies. These authors compared mixed culture fermentations of *S. cerevisiae* with *T. delbrueckii* and *L. thermotolerans* in two fermentation systems. System 1 was closed off with a silicone stopper and system 2 with sterile paper and gauze only (allowing for some oxygen to penetrate). It was observed that both non-*Saccharomyces* yeast died off later in the fermentation and once death had commenced, the rate at which it occurred was slower, thereby indicating that an increased oxygen availability augmented the survival time and decreased the death rate of *T. delbrueckii* and *L. thermotolerans* in mixed culture with *S. cerevisiae.* Nissen et al. (2004) made use of similar fermentation systems and the same yeast pairs for mixed culture fermentations. These authors observed the same trend and also documented that *T. delbrueckii* and *L. thermotolerans* have higher oxygen requirements than *S. cerevisiae.* If this holds truth for all non-*Saccharomyces* yeasts, it would explain their early death in fermentation as oxygen is depleted rapidly by SO_2 addition and *S. cerevisiae* within the first few days of fermentation. Furthermore, these authors also concluded that the presence of oxygen enhanced the ability of *T. delbrueckii* and *L. thermotolerans* to compete for nutrients with *S. cerevisiae*. This might be explained by differences in relative glucose uptake abilities between these species, which also affects their ability to compete for nutrients. However, more research is needed to confirm this hypothesis. Recently, Brandam et al. (2013) tested the effect of constant aeration on pure *T. delbrueckii* cultures of a synthetic grape must. In doing so, they observed a significant increase in biomass, while still obtaining a good ethanol yield (0.50 g/g). There have not been many studies that tested the effect of oxygen on these mixed culture fermentations and it would be valuable to do so. If the above-mentioned results were true for other non-*Saccharomyces* yeasts, the application of small oxygen doses in wine making (in order to facilitate the survival of non-*Saccharomyces* yeast, without the formation of undesirable compounds) could be a helpful tool. Recently, Morales et al. (2015) saw no significant increase in volatile acidity after sparging mixed cultures of *C. pulcherrima* and *S. cerevisiae* with air for the first 48 h of fermentation, since a final acetic acid concentration of 0.35 g/L was achieved. As mentioned before, this method led to a reduction in ethanol of 2.2 % (v/v).

2.5. Conclusion

The ability of non-*Saccharomyces* yeasts to contribute positively to mixed culture wine fermentations has been proven in several studies. Some studies have evaluated the types of yeast interactions that may take place within these fermentations and although a physical, cell-cell interaction has been confirmed for one yeast pair (*S. cerevisiae* and *T. delbrueckii*), it has

not been validated for others. A similar interaction has been hypothesised for *S. cerevisiae* and *L. thermotolerans*. Furthermore, the effect of metabolic interactions and the identity of the compounds that specifically induce them, remain largely unknown. To date, no studies have focused on the effect that such interactions have on gene expression level i.e. which genes are expressed or suppressed as a result of such interactions. Future studies should therefore focus on both physical and metabolic interactions (more specifically which compounds lead to the latter) and the genes that are involved. It would also be interesting to confirm these interactions for *S. cerevisiae* and *L. thermotolerans*, especially since the latter yeast is a good candidate for mixed culture fermentations and is already being used for this purpose in the wine industry.

The effect of other inhibitory factors (part of the wine making process) on mixed culture fermentations cannot be ignored. While ethanol has been believed to be the main cause of early death of non-*Saccharomyces* yeasts, we now know that this phenomenon is much more complex and is not only related to interactions with *S. cerevisiae*, but other factors, such as dissolved oxygen. The latter has especially been proven to affect the ability of non-*Saccharomyces* yeasts to last throughout fermentation and to compete with *S. cerevisiae*. However, few studies have focused on this. Therefore, it would also be of interest for future studies to focus on the decline in oxygen concentration as wine fermentation progresses and how this affects the growth of all yeasts involved, whether small oxygen dosages could facilitate non-*Saccharomyces* growth and ability to compete with *S. cerevisiae* and if such a solution would be viable for application in the wine industry.

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Chapter 3

Interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed culture fermentations of synthetic grape must using a double compartment bioreactor

Chapter 3 –Interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed culture fermentations of synthetic grape must using a double compartment bioreactor

3.1. Introduction

The wine making process involves complex reactions, primarily linked to the conversion of grape juice sugars and of other nutrients into ethanol, CO_2 and secondary metabolites, many of which contribute to wine flavour and aroma (Moreno-Arribas and Polo 2005). Traditional winemaking practices make use of appropriate *Saccharomyces cerevisiae* starter cultures in order to control the process better and of the addition of SO_2 to eliminate potential spoilage yeast that might be present in the grape juice (Fleet 2008). In the last few decades, there has been more research into the potential use of certain non-*Saccharomyces* yeasts in conjunction with *S. cerevisiae* to ferment wine in the hopes of creating new wines with more complex sensorial profiles (Ciani & Comitini 2011; Fleet 2008; Gobbi et al. 2013; Jolly et al. 2003). Some have been found to possess qualities worth exploiting (Bely et al. 2008; Ciani and Comitini 2006; Gobbi et al. 2013). *L. thermotolerans* occurs naturally in the grape must and has such positive characteristics that could be exploited for winemaking purposes (Comitini et al. 2011; Gobbi et al. 2013; Kapsopoulou et al. 2005, 2007; Mora et al. 1990). In particular, it has been found to produce high levels of L-lactic acid (Mora et al. 1990) and depending on how the wine is produced, this can lead to an overall reduction in the pH of the wine (Kapsopoulou et al. 2005, 2007). Depending on the fermentation parameters, it has also been seen to produce high levels of glycerol and 2-phenyl-ethanol (Comitini et al. 2011), lower levels of ethanol and increased production of certain esters which are linked to desirable spicy notes in wine (Gobbi et al. 2013). However, before implementation into commercial wine making, it is important to evaluate how *S.cerevisiae* and non-*Saccharomyces* yeasts interact with each other during alcoholic fermentation and how it can impact on wine flavour. Consequently, some research has focused on such aspects (Bely et al. 2008; Nissen et al. 2003, 2004; Renault et al. 2013; Strehaiano et al. 2010). An innovative tool in which to study these interactions has been developed recently: fermentation bioreactors consisting of two chambers which are separated by a membrane (Albasi et al. 2001; Renault et al. 2013; Salgado-Manjarrez et al. 2000). In these bioreactors, the yeast cells of the mixed culture can be separated, while still allowing the yeast to share the fermentation medium. This allows separating the effects of physical and metabolic interaction within a mixed culture fermentation. Nissen et al. (2004) presented data suggesting that there is a physical cell-cell interaction between *S. cerevisiae* and the non-*Saccharomyces* yeasts

T. delbrueckii and *L. thermotolerans* when co-fermented, and recently Renault et al. (2013) confirmed these data for the pair *S. cerevisiae*-*T. delbrueckii.* Furthermore, it has been well documented that the early death of non-*Saccharomyces* yeasts in mixed culture fermentations of wine is due to their sensitivity to high concentrations of ethanol (Fleet 2008). However, some studies have identified non-*Saccharomyces* species exhibiting ethanol tolerances close to those of *S. cerevisiae* (Pina et al. 2004; Xufre et al. 2006). Many other factors may influence the survival of these non-*Saccharomyces* yeasts in the early stages of fermentation (Fleet 2008). In particular, the effect of dissolved oxygen has been reported (Hansen et al. 2001).

Taking into account the positive contributions that *L. thermotolerans* may bring to mixed culture wine fermentations with *S. cerevisiae* and the fact that the modes of interaction have not been confirmed for *L. thermotolerans*-*S. cerevisiae*, this study focused on shedding light onto possible modes of interactions between the two. Using a Double Compartment Bioreactor (DCB), mixed culture fermentations were performed in an attempt to evaluate whether a physical cell-cell interaction would take place between these two species in mixed culture fermentations. The effect of dissolved oxygen on these fermentations was also investigated.

3.2. Materials and methods

3.2.1. Microorganisms and media

L. thermotolerans strain IWBT Y1240 from the yeast culture collection of the Institute for Wine Biotechnology at Stellenbosch University and *S. cerevisiae* strain Lalvin EC1118 (Lallemand Inc.) were used in this study. The strains were grown on YPD agar (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar) and maintained at 4°C. The medium used to calculate viable cell counts during fermentations was Wallerstein (WL) Nutrient agar and was also used to differentiate between colonies of *L. thermotolerans* and *S. cerevisiae* (the former appears green while the latter appears white on the agar). This medium was used for both mixed and pure culture fermentations.

3.2.2. Bioreactor fermentations

A series of fermentations were performed with the use of a DCB (Salgado-Manjarrez et al. 2000; Albasi et al. 2001) (Figure 3.2.1). All were carried out in duplicate. Conditions were identical in all fermentations, the only exception being the removal of the hollow-fibre membrane for pure cultures and mixed fermentations where the yeasts were in physical contact. A synthetic grape must medium was used for the fermentations (Table 3.2.1).

3.2.3. Inoculation strategies

Each compartment contained 1.5 L of medium (giving a total of 3 L) and was co-inoculated to obtain a total cell count of 2 x 10^6 cells/mL. Pre-cultures were prepared by inoculating 100 mL YPD broth with a yeast colony and allowing it to grow overnight (±16 hours) at 30°C on agitation at 100 rpm on a shaker. The cultures were first washed with a 0.9 % NaCl solution before inoculation took place. For pure cultures, 1 x 10^6 cells/mL of the same strain (either *L. thermotolerans* Y1240 or *S. cerevisiae* EC1118) were inoculated into each vessel (giving a total of 2 X 10^6 cells/mL) and for mixed culture fermentations, 1 x 10^6 cells/mL of each strain were inoculated into each vessel (*L. thermotolerans* Y1240 into Vessel A and *S. cerevisiae* EC1118 into vessel B).

3.2.4. Fermentation conditions and oxygenation strategies

The fermentations were conducted at 30°C and at 250 rpm stirring (magnetic stirrer bars). The medium was transferred between vessels with the use of nitrogen gas applied at a pressure of 0.3 bars. Twice a day (8 am and 4 pm), immediately before sampling, air was sparged through the medium for 30 min at a flow rate of 0.56 vvm (volume of air per volume of medium per minute). This oxygenation strategy was considered as the control. Further fermentations were conducted to test the effect of oxygen on the fermentation dynamics by sparging air for 30 min once a day at 8 am. Samples of 20 mL were taken twice a day.

3.2.5. Sample analysis

Samples were used to monitor optical density at 600 nm (pathway of 1 mm) with the use of a Jenway 7135 spectrophotometer, viable cell counts on WL agar, and total and viable cell counts with the use of a Thoma haemocytometer counting chamber and methylene blue staining using a method by Alfenore et al. (2004). A 0.1% Methylene blue solution (1 g of Methylene Blue powder (Merck, Fontenay-sous-Bois, France) dissolved in 1 L of a 2% Sodium citrate solution) was used to stain the appropriate dilutions of yeast suspensions in a 1:1 ratio (dilution factor of 2). The concentration of yeast suspension was adjusted so that 40-60 cells were present per microscope field. Any count outside of the range of 150-300 was regarded as inaccurate. Since the methylene blue solution is temperature and light sensitive, it was stored at 4°C in an amber bottle. A CHS light microscope from Olympus was used to determine cell counts. The rest of the sample was spun down at 7,500 rpm at 4°C, 2 mL at a time. The supernatant was kept at -4°C for analytical determinations. Dry weight was determined on the last day of fermentation by spinning down 2 mL samples at 7,500 rpm at 4°C, washing the pellet with 1 mL of distilled water, repeating the centrifugation step and drying the pellet at 100°C for 48 hours. As a quick (yet

somewhat inaccurate) way to monitor sugar concentrations, a DNS (3,5-Dinitrosalicylic acid) method was used approximately 5-7 days into the fermentations. This was a quick test to determine whether fermentations were complete or not. The DNS solution (30 g/L Potassium & Sodium tartrate, 16 g/L NaOH, 10 g/L DNS) was used to draw a standard curve. Nine dilutions of a 50 g/L solution of fructose were prepared to have final concentrations of 0, 0.5, 1, 5, 10, 20, 30, 40 and 50 g/L respectively. 50 µL of each dilution were placed into 2 mL microcentrifuge tubes. To this, 950 µL of DNS solution was added and placed into a water bath at 80°C for 3 min. Each tube was then placed on ice for 5 min and absorbance was measured at 580 nm (0.5 cm pathway). Absorbance values were plotted against different fructose concentrations to obtain the standard curve's equation. The same procedure was followed as above with 50 µL of fermentation samples. These absorbance values were then used to calculate sugar concentration with the use of the standard curve. Fermentations were considered complete when total sugar concentrations were less than 5 g/L. Unless stated otherwise, all compounds used were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and Fisher Scientific (Strasbourg, France).

Table 3.2.1: Synthetic grape must medium. The composition was amended from Henschke and Jiranek (1993) and Bely et al. (1990) adjusted pH of 3.5 with 10M KOH

Carbon Sources	**[g/L]**
Glucose	*115
Fructose	*115
Acids	**[g/L]**
KH Tartrate	2.5
L-Malic acid	3
Citric Acid	0.2
Salts	**[g/L]**
K_2HPO_4	1.14
$MgSO_4.7H_2O$	1.23
$CaCl_2.2H_2O$	0.44
***Nitrogen Sources**	**[g/L]**
Tyrosine	1.4
Tryptophane	13.7
Isoleucine	2.5
Aspartic Acid	3.4
Glutamic Acid	9.2
Arginine	28.6
Leucine	3.7
Threonine	5.8
Glycine	1.4
Glutamine	38.60
Alanine	11.10
Valine	3.40
Methionine	2.40
Phenylalanine	2.90
	6.00

Serine	2.50
Histidine	1.30
Lysine	1.00
Cystein	46.80
Proline	
Trace Elements	**[µg/L]**
$MnCl_2.4H_2O$	200
$ZnCl_2$	135
$FeCl_2$	30
$CuCl_2$	15
H_3BO_3	5
$Co(NO_3)_2.6H_2O$	30
$NaMoO_4.2H_2O$	25
KIO_3	10
Vitamins	**[mg/L]**
Myo-Inositol	100
Pyridoxine.HCl	2
Nicotinic Acid	2
Calcium Pentothenate	1
Thiamin.HCl	0.5
PABA.K	0.2
Riboflavin	0.2
Biotin	0.125
Folic Acid	0.2
Lipids/Oxygen	**Amount per litre**
Ergosterol	10 mg
Tween 80	0.5 ml
Air Saturated or	0-9 ppm
O_2 free	

*Amended Values

3.2.6. Bioreactor

A schematic representation of the DCB is shown in Figure 3.2.1. This reactor system was designed and patented by the Laboratoire de Génie Chimique (LGC), which is a research group part of the National Polytechnic Institute of Toulouse (INPT) situated in Toulouse, France. It is a system that can be used to study the indirect interactions between different species of microorganisms and has been tested and described in full (Albasi et al. 2001; Salgado-Manjarrez et al. 2000). The system consists of two vessels which are interconnected by a hollow-fibre membrane. The membrane is submerged in the media of one of the two vessels. Throughout fermentation, compressed, filter-sterilized air or nitrogen gas is applied into the headspace of one of the two compartments, which in turn transports liquid from said compartment into the other. This transport of liquid occurs until a specific level is reached. The liquid level is picked up by conductivity probes also submerged into the media and this sends a signal to a system of valves which alternates the pressure in both vessels.

The membrane, manufactured by Polymem (Castanet-Tolosan, France), is made up of Polysulfone, U-shaped fibres which are held together at the top with an epoxy resin. The filtering section of the membrane is submerged into the media. The fibres have a pore size of 0.1 µm with an internal and external diameter of 0.25 mm and 0.45 mm each. The water permeability has been estimated to be 3.5 x $10^{-9}m^3.m^{-2}.s^{-1}.Pa^{-1}$. The total filtering surface was fixed to be 0.1 m^2. This was decided since the authors concluded that in order for such a study to be successful, a mixing time of less than 10 min from one vessel to the other was needed. Following a theoretical study, it was calculated that in order to achieve this, a dilution rate higher than 8 h^{-1} was needed. This can be achieved with a surface area greater than 0.05 m^2.

3.2.7. Analytical determinations

The ethanol, glycerol, glucose and fructose concentrations were determined with the use of a High Performance Liquid Chromatography (HPLC) method (Fernandez Lopez et al. 2014). An HPLC-equipped Phenomenex ROA Organic column was used. The liquid phase was 10 mM of sulphuric acid solution which circulated at 0.170 mL/min at 30°C. The volume of the injection loop was 25 µL. The peaks of ethanol, glycerol, acetic acid, glucose and fructose were detected by an infra-red detector.

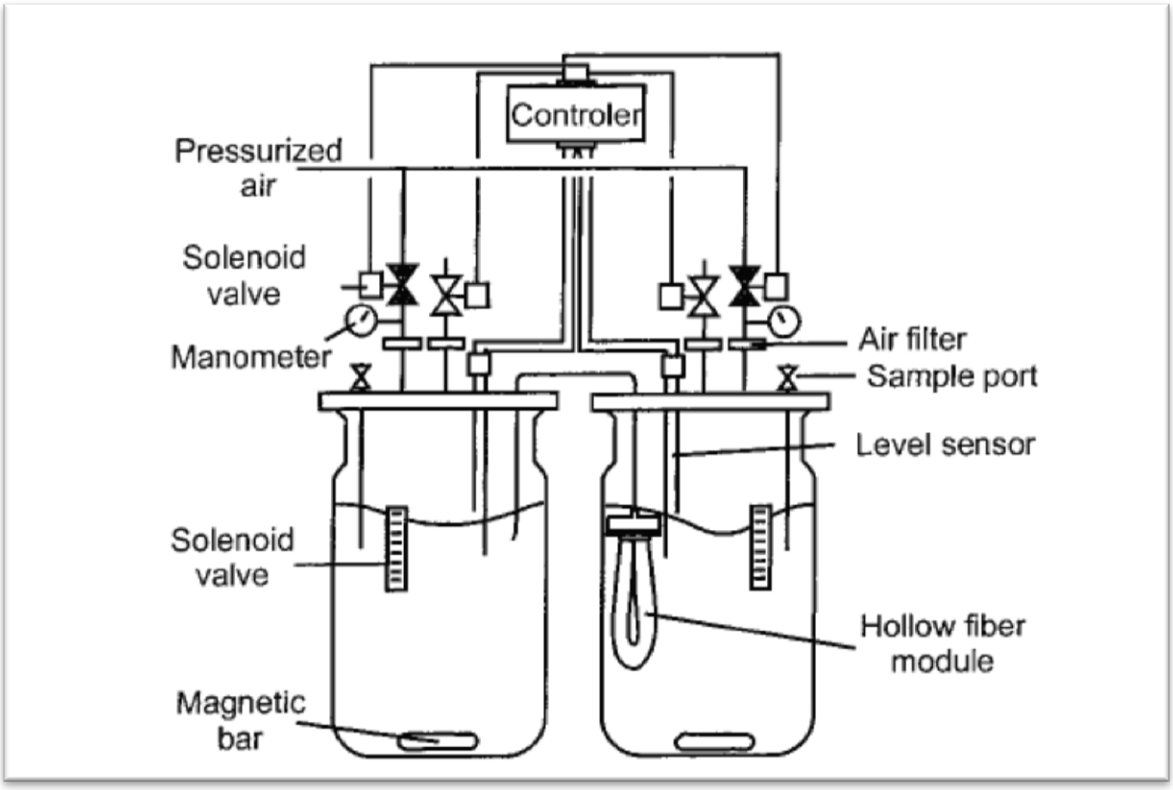

Figure 3.2.1: Schematic representation of the Double Compartment Bioreactor that was used to carry out fermentations (Albasi et al. 2001; Salgado-Manjarrez et al. 2000).

3.3. Results

3.3.1. DCB: interaction studies

To observe possible interactions between *S. cerevisiae* and *L. thermotolerans*, a DCB (Figure 3.2.1, section 3.2) was used to perform fermentations. Pure and mixed culture fermentations were conducted. For the latter, the two yeast cultures were separated by a central filtration membrane which allowed for yeast cells to be separated, while still being able to share the medium. When the filtration membrane was present in mixed cultures, the yeast were in indirect contact and when absent, the yeast were in direct contact. Fermentation parameters were kept the same in each type of fermentation (refer to section 3.2). Nitrogen gas was used to facilitate mixing of the fermentation medium between the two compartments. Following preliminary fermentation trials, it was observed that the yeast struggled to grow in the presence of this gas (which was applied at a pressure of 0.3 bars). For this reason, air was sparged through the medium twice a day which allowed the yeast to ferment to dryness. It was observed that after each oxygenation event, the yeast consumed the oxygen rapidly and within 30 min the levels of dissolved oxygen (DO) were at or near to 0%. It should be noted that the application of such oxygen pulses during fermentation might more imitate the effects of commonly used wine making practices such as pump-overs.

Pure and mixed culture (indirect contact) fermentations of *L. thermotolerans* and *S. cerevisiae* were followed by measuring the OD to determine total biomass, and methylene blue stained cells counted on a microscope, to determine viability. The data show that all cultures follow the same broad trends in terms of the accumulation of biomass as measured by OD (Figure 3.3.1). While the actual values differed, all fermentations reached stationary phase around 60 h into the fermentation. After this point, a gradual decline in optical density was observed until the end of fermentation (after ± 200 h). Pure cultures of these two yeasts both reached a maximum OD of ± 25, while mixed cultures reached a significantly lower maximum OD of ± 15 (graphs A & B, Figure 3.3.1). From these graphs, the decline in cell viability can also be observed. This decline was gradual up until the 90 h mark, whereafter it accelerated for both *L. thermotolerans* and *S. cerevisiae* but to a lesser extent for the latter. When the yeasts were in indirect contact, *L. thermotolerans* accumulated less biomass when compared to *S. cerevisiae* (graph C, Figure 3.3.1).

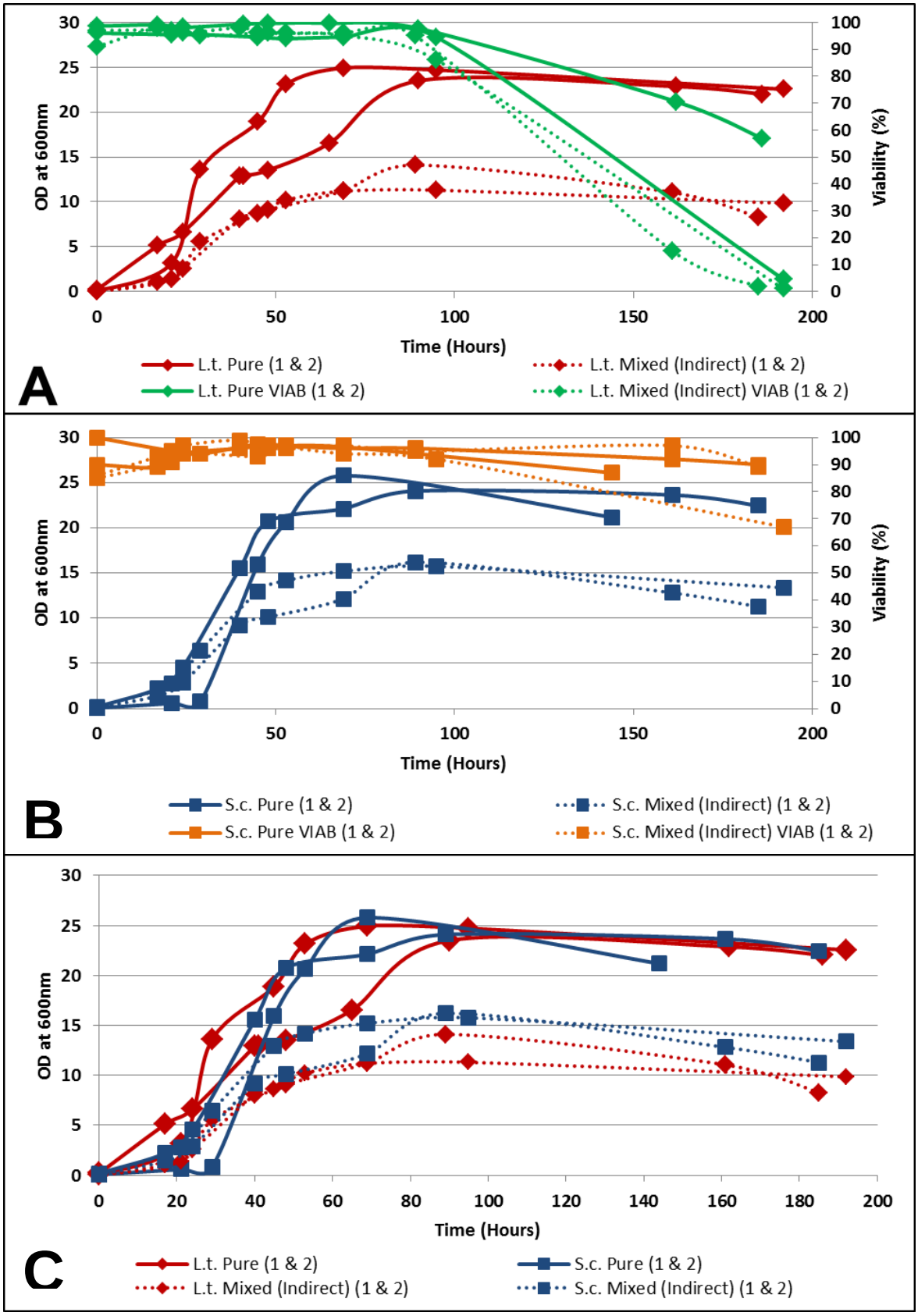

Figure 3.3.1: Fermentation growth represented in OD_{600nm} plotted against viable cells as a percentage of total cells present at a specific time point [A and B] of *L. thermotolerans* [A] and *S. cerevisiae* [B] in pure cultures (indicated in solid lines) and mixed cultures where the cells were in indirect physical contact

(indicated in dashed lines). Two biological repeats are presented for each type of fermentation. Graph C represents the combination of graphs A and B for OD_{600nm}.

The viable cell concentrations for pure and mixed culture (direct and indirect contact) fermentations all followed the same trend on WL plates (Figure 3.3.2). While the actual values differed, after inoculation a sharp increase of cells occurred and within 60 h (onset of stationary phase) the cell counts leveled out at more or less 10^8 cells/mL.

After ± 90 h, a sharp decrease in viable cell concentrations was observed for all *L. thermotolerans* cell populations (graph A, Figure 3.3.2).This decline occurred at a faster rate in the mixed culture where *L. thermotolerans* was in direct contact with *S. cerevisiae*, at a slower rate in the mixed culture where the two yeasts were in indirect contact and the slowest rate of this decline in cellular concentration was observed in the pure culture fermentation of *L. thermotolerans*.

This trend was not observed for *S. cerevisiae* (graph B, Figure 3.3.2). Here, after ± 90 h, a decline in cell concentrations was only observed in the mixed culture fermentation where the two yeasts were in indirect contact. No decline in cells can be observed for the mixed culture where they were in direct contact and a slight increase after 90 hours is observed for pure culture fermentations of *S. cerevisiae.*

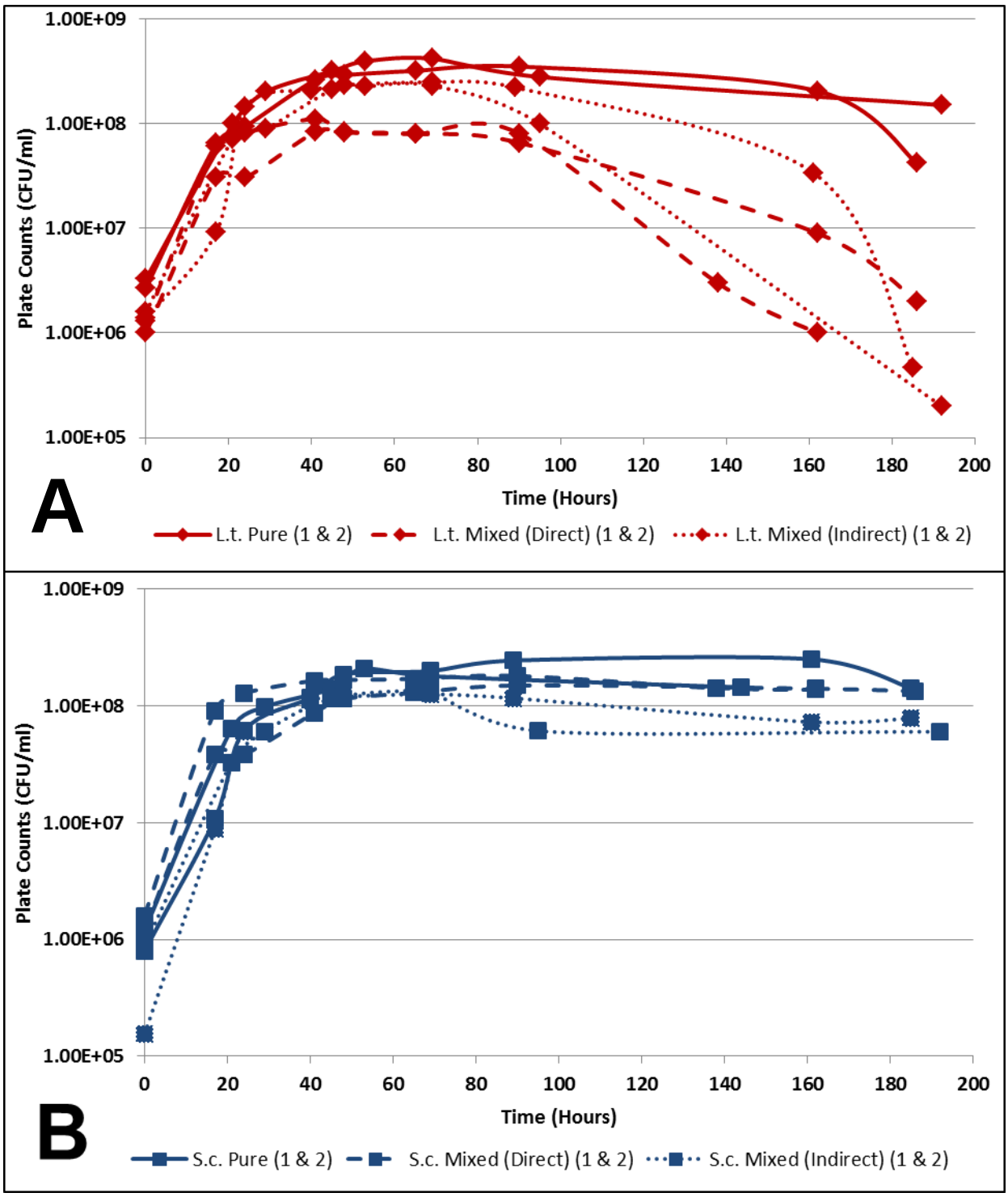

Figure 3.3.2: Fermentation behaviour represented as Plate counts (CFU/ml) (logarithmic scale) of *L. thermotolerans* [A] and *S. cerevisiae* [B] in pure cultures (indicated in solid lines) and mixed cultures where the cells were in direct and indirect contact (indicated in dashed lines) between two biological repeats.

Abbreviations for graphs: **L.t.**: *L. thermotolerans*; **S.c.**: *S. cerevisiae*; **VIAB**: Viability; **Mixed (Direct)**: Mixed culture of *L. thermotolerans* and *S. cerevisiae* where the yeast cells were in direct physical contact; **Mixed (Indirect)**: Mixed culture fermentation of *L. thermotolerans* and *S. cerevisiae* where the yeast cells were not in direct physical contact.

3.3.2. DCB: effect of oxygen on bioreactor fermentations

The effect of oxygen on the behaviour of these yeasts was evaluated in pure and mixed (direct contact) cultures by assessing the impact of a single oxygen pulse, as compared to two oxygen pulses applied in all the previous fermentations. However, it must be noted that because of time constraints, only a single fermentation could be done for some fermentations (*L. thermotolerans* and *S. cerevisiae* pure cultures). The treatment could also only be applied to pure and mixed direct contact cultures.

Following the onset of fermentation, OD increased gradually and levelled out after ± 60 h in all pure culture fermentations where oxygen was given twice a day at an OD of ± 25 (graph A and B, Figure 3.3.3). In pure culture fermentations where oxygen was given once a day, stationary phase was only reached after ± 90 h at an OD of ± 15 (graph A and B, Figure 3.3.3). In mixed culture fermentations where oxygen was given twice and once a day, stationary phase was reached after ± 90 h (graph C, Figure 3.3.3). Here a maximum OD of ± 22 and 19 was reached for fermentations where oxygen was given twice and once a day, respectively.

To compare the growth of *L. thermotolerans* and *S. cerevisiae* in pure and mixed cultures and to assess the effect of a lowered oxygen availability, dry weight at the end of fermentation was also measured. Here large differences were observed.

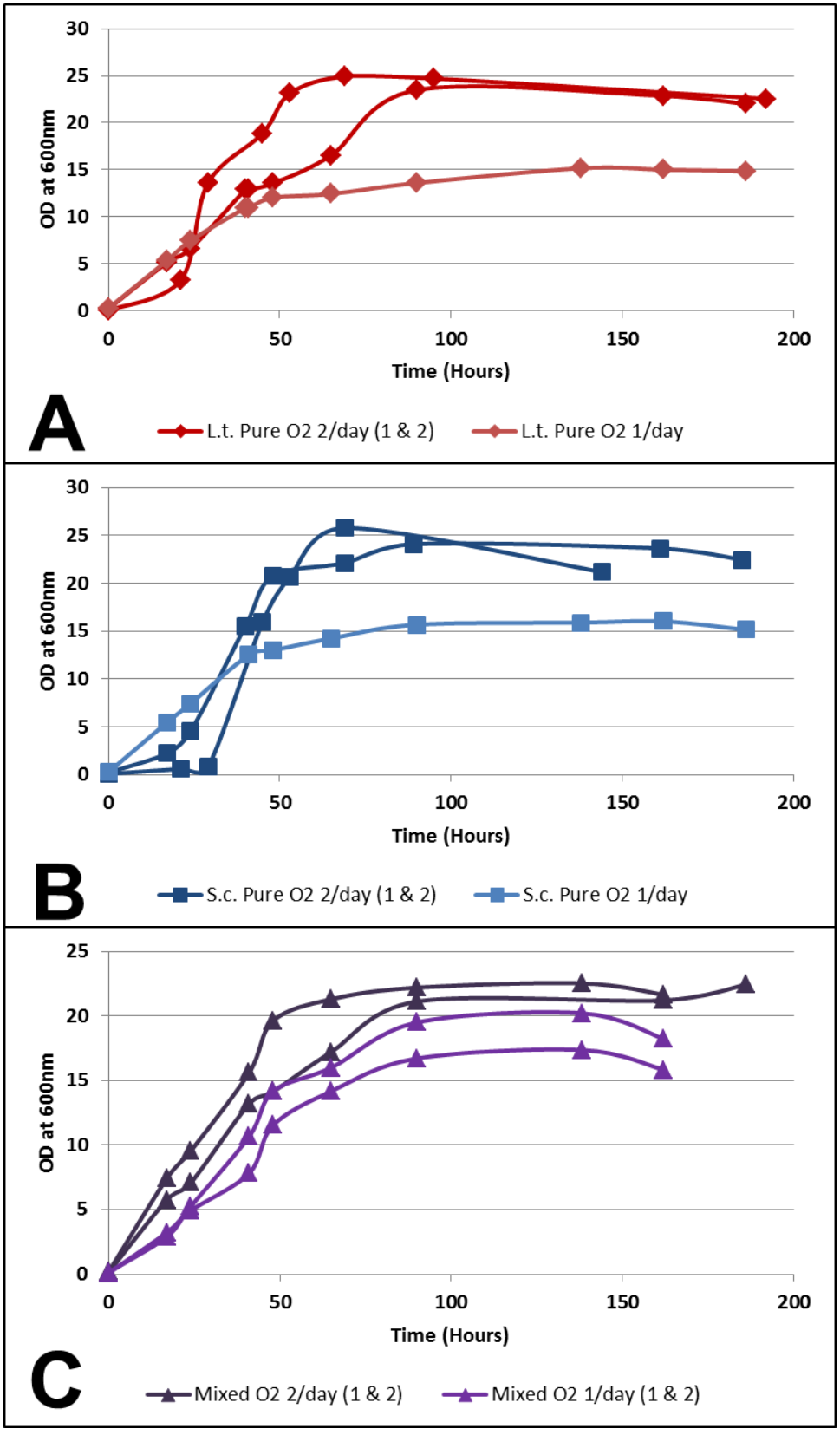

Figure 3.3.3: Fermentation growth represented in OD_{600nm} of *L. thermotolerans* [A] and *S. cerevisiae* [B] in pure culture and mixed culture fermentations [C] where the cells were in direct contact and different oxygen treatments were applied. For all fermentations where oxygen was supplied twice a day, two

biological repeats are represented, for pure culture fermentations where oxygen was supplied once a day, one repeat is represented and for mixed culture fermentations (direct contact) where oxygen was supplied once a day, two repeats are represented.

When given oxygen twice and once a day, pure cultures for *L. thermotolerans* reached a final dry weight of 8.5 g/L and 3.5 g/L respectively (Figure 3.3.4). A similar trend was also observed for *S. cerevisiae* (Figure 3.3.4). Here, final dry weight when given oxygen twice and once a day reached 7.5 g/L and 4.5 g/L, respectively. In mixed cultures of *L. thermotolerans* and *S. cerevisiae* where the yeast were in direct contact, a final dry weight of 6.4 g/L (oxygenation twice a day) and 4.6 g/L (oxygenation once a day) were reached. In mixed cultures of *L. thermotolerans* and *S. cerevisiae* where the yeasts were in indirect contact, *L. thermotolerans* reached a final dry weight of 3.4 g/L (oxygenation twice a day) and *S. cerevisiae* reached a final dry weight of 6.6 g/L (oxygenation twice a day). These values are lower than the dry weight obtained in pure culture fermentations. This decrease in dry weight was more pronounced for *L. thermotolerans* compared to *S. cerevisiae.*

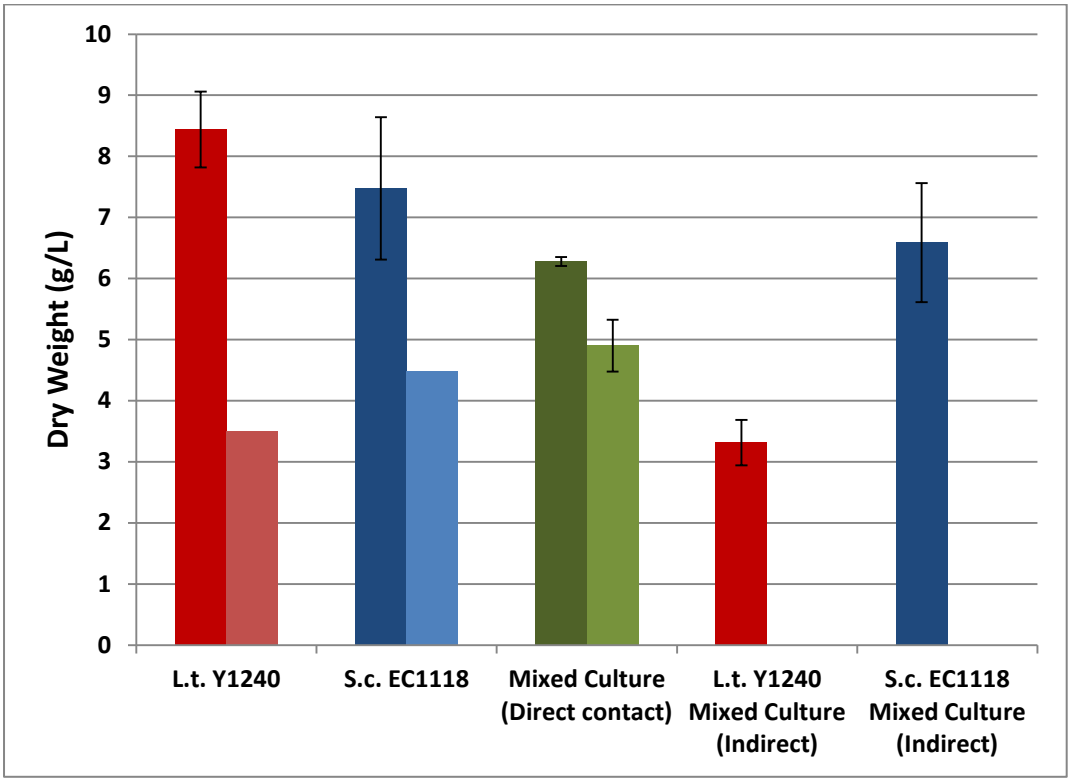

Figure 3.3.4: Dry weight measured on the last day of fermentation for *L. thermotolerans* (red bars) and *S. cerevisiae* (blue bars) in pure and mixed cultures where cells were not in direct physical contact and mixed cultures where the yeast cells were in direct contact (green bars). Darker and lighter shades of red, blue and green indicate oxygenation of twice and once a day respectively. Error bars are present for fermentations which could be repeated.

The fermentation where oxygen was supplied once a day could not be repeated for the mixed culture where the yeast were in indirect contact because of instrumental faults.

For all fermentations, ethanol levels were measured. All fermentations (except the pure culture fermentation of *L. thermotolerans* where oxygen was given once a day) fermented to or near to dryness (total sugar concentration of 5 g/L) (Table 3.3.1). When given oxygen twice a day (control fermentations), *L. thermotolerans* pure cultures produced 64.3 g/L of ethanol by the end of fermentation (Figure 3.3.5). This translates into an ethanol yield of 0.28 (g/g) and is somewhat lower compared to all other fermentations (*S. cerevisiae* pure culture, mixed culture where the yeast were in direct and indirect contact) which produced ethanol concentrations of 68.9 g/L, 69.9 g/L and 69.9 g/L respectively. These all translate into ethanol yields of 0.30 (g/g). When given less oxygen (once a day), no difference for *L. thermotolerans* pure culture fermentations could be observed, because the fermentation where less oxygen was provided did not ferment to dryness. This fermentation also had an ethanol yield of 0.28 (g/g), even though less oxygen was provided. This stands in contrast to *S. cerevisiae* pure culture and mixed culture fermentations where the cells were in direct contact. Here, after given less oxygen (once a day), ethanol levels increased to 98.5 g/L and 72.2 g/L respectively. These translate into ethanol yields of 0.42 (g/g) and 0.31 (g/g). Ethanol levels and yields are relatively lower than one would expect and there is variation between repeats.

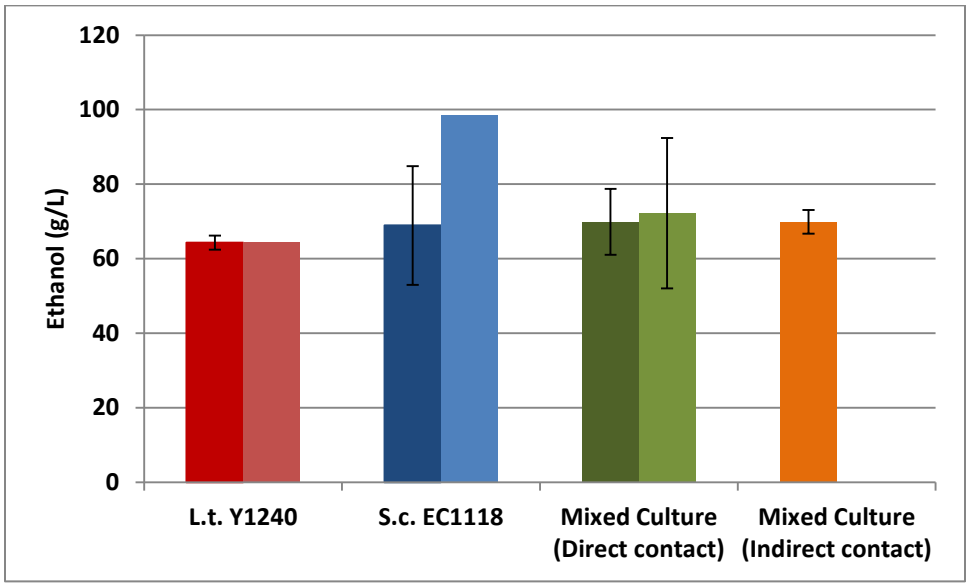

Figure 3.3.5: Ethanol measured on the last day of fermentation for *L. thermotolerans* (red bars) and *S. cerevisiae* (blue bars) in pure cultures, mixed cultures where the cells were in direct contact (green bars) and mixed cultures where the cells were in indirect contact (orange). Darker and lighter shades of red, blue, green and orange indicate oxygenation of twice and once a day respectively. Error bars are present for fermentations which could be repeated.

Control fermentations of both *L. thermotolerans* and *S. cerevisiae* where oxygen was given twice a day lasted 144 h (Table 3.3.1). Compared to these, mixed culture fermentations where the cells were in direct contact did not last as long (138 h) and mixed culture fermentations where

the cells were in indirect contact lasted longer (185 h). From this table, it can also be observed that pure cultures of *L. thermotolerans* and *S. cerevisiae* produced 6.6 g/L and 5.9 g/L of glycerol, respectively. In control fermentations of mixed cultures where the cells were in direct contact significantly higher levels were produced (11.8 g/L). In contrast to this, much lower levels of glycerol were produced when the cells were in indirect contact (4.6 g/L).

When given less oxygen, fermentations tended to last longer, with one exception being the mixed culture fermentations where cells were in direct contact. Here oxygenation twice and once a day resulted in the fermentation lasting 138 h. For pure cultures of *L. thermotolerans* and *S. cerevisiae*, when given less oxygen, more glycerol was produced. This increase was higher for *S. cerevisiae* (from 5.9 g/L to 11.9 g/L). A decrease in glycerol levels was observed for mixed culture fermentations where the cells were in direct contact and less oxygen was provided (from 11.8 g/L to 9.2 g/L). However, it must also be noted that for the fermentations where oxygen was given once a day, large variation between repeats occurred.

Table 3.3.1: Final concentrations for Total sugars (Glucose and Fructose), Ethanol and Glycerol on the last day of fermentation indicated in g/L. Where fermentations could not be repeated, no standard deviation is specified (-).

Fermentation	Oxygenation	Time point (h)	Total Sugars (g/L)	Std dev	Ethanol (g/L)	Std dev	Glycerol (g/L)	Std dev
L.t. Pure	2/day	144	**7.6**	1.0	**64.3**	1.9	**6.6**	1.0
	1/day	162	**41.7**	-	**64.4**	-	**7.0**	-
S.c. Pure	2/day	144	**5.0**	3.8	**68.9**	15.9	**5.9**	0.6
	1/day	186	**5.3**	-	**98.5**	-	**11.9**	-
Mixed (Direct)	2/day	138	**6.2**	0.6	**69.9**	8.8	**11.8**	0.8
	1/day	138	**4.9**	4.0	**72.2**	20.2	**9.2**	4.1
Mixed (Indirect)	2/day	185	**2.7**	0.6	**69.9**	3.2	**4.6**	0.5

Abbreviations for graphs: **L.t.**: *L. thermotolerans*; **S.c.**: *S. cerevisiae*; **VIAB**: Viability; **Mixed**: Mixed Culture of *L. thermotolerans* and *S. cerevisiae* where the yeast cells were in direct physical contact; **O2 2/day**: Fermentation oxygenated twice a day; **O2 1/day**: Fermentation oxygenated once a day.

3.4. Discussion

3.4.1. Interaction studies

Although it has been shown that *S. cerevisiae* dominates and completes wine fermentation, non-*Saccharomyces* yeasts occur naturally in the grape must (Clemente-Jimenez et al. 2004; Fleet 2003; Pretorius 2000) and that they contribute significantly to the final composition of the wine (Ciani et al. 2010; Clemente-Jiminez et al. 2004; Fleet 2008). As mentioned above, we

evaluated the yeast pair *L. thermotolerans*-*S. cerevisiae* in mixed culture fermentations and used a DCB approach to investigate possible yeast interactions.

Figure 3.3.1 illustrates how these yeasts reacted to the presence of each other in terms of fermentation growth. When *S. cerevisiae* and *L. thermotolerans* were grown in pure cultures, a high OD of ± 25 was reached and the fermentation was completed after ± 200 hours. For *S. cerevisiae*, a loss in viability was observed, but never dropped below 80%, while the loss in viability was more drastic for *L. thermotolerans*, dropping to ± 60% and ± 10% in the two repeats. This could be attributed to the fact that some non-*Saccharomyces* yeasts have been found to be more sensitive to growth-inhibitory compounds like ethanol and medium-chain fatty acids (Fleet 2008; Nissen et al. 2003; Viegas et al. 1989). By incorporating a filtration membrane in mixed cultures, the cells were physically separated and consequently, the yeasts were in indirect contact. While the fermentation duration did not seem to be affected by this, the accumulation of biomass was. In these fermentations, both *S. cerevisiae* and *L. thermotolerans* saw a reduction in OD to ± 16 and ± 14, respectively. The data suggests that the yeasts interacted on a metabolic level (since the effect of a physical interaction was excluded) and that this interaction affected the yeasts' ability to accumulate biomass, but not their ability to remain viable throughout fermentation. This is supported by Figure 3.3.4, which compared dry weight at the end of fermentations. *L. thermotolerans* pure cultures accumulated the most biomass between all fermentations, but when co-fermented with *S. cerevisiae* (indirect contact), this dry weight was reduced significantly (from ± 8.5 to 3.3 g/L). The same reduction in dry weight was observed for *S. cerevisiae* (from ±7.5 to 6.6 g/L), but was less compared to *L. thermotolerans*.

The plate counts for mixed culture (direct contact) fermentations revealed a different interaction. Indeed, in pure cultures, viable cells accumulated rapidly within the first 24 h for *L. thermotolerans* and reached a maximum count of ± 4 x 10^8 CFU/mL. This concentration was higher than for *S. cerevisiae* pure cultures, which reached a maximum of ± 2 x 10^8 CFU/mL. The presence of *S. cerevisiae* had a major effect on the growth and viability of *L. thermotolerans* in fermentations where the two species were both in direct and indirect contact. This effect can be visualized in Figure 3.3.2 (graph A). In both cases, a lower maximum viable count was reached for *L. thermotolerans*. When *L. thermotolerans* was in direct contact with *S. cerevisiae*, the lowest viable cell counts were observed. Therefore, the former yeast's growth was affected more when it was in direct contact with *S. cerevisiae* compared to being in indirect contact. The accumulation of viable cells for *S. cerevisiae* in all fermentations was moreover unaffected by the presence of *L. thermotolerans* (graph B, Figure 3.3.2). In pure and mixed cultures it was able to reach a maximum of more than 10^8 CFU/mL. After 90 h, a slight decrease in viability was observed for the indirect contact mixed culture, but the yeast was still able to maintain a high

viability until the end of fermentation (which can also be visualized from Figure 3.3.1). The loss in viability (and subsequent cell lysis) of the *L. thermotolerans* population, could have provided *S. cerevisiae* with an extra source of nutrients, which facilitated its ability to dominate the fermentation and maintain a high viability until the end. In both mixed culture fermentations, there was a sharp decline in viable cells for *L. thermotolerans* after ± 90 h. This suggests that the growth and survival of *S. cerevisiae* was only affected by *L. thermotolerans* on a metabolic level as a result of competition for nutrients (Strehaiano et al. 2010). This phenomenon has been reported in mixed cultures of *S. cerevisiae* and *T. delbrueckii* (Renault et al. 2013). In the latter article, it was hypothesized that *T. delbrueckii* affected the growth of *S. cerevisiae* in an indirect way (through the production of a certain molecule) in a quorum sensing-like mechanism. In contrast, *L. thermotolerans* was affected negatively by molecules being secreted by *S. cerevisae* as well as the physical presence of this yeast, which had major impacts on *L. thermotolerans*' ability to generate biomass and viable cells throughout the fermentations. Therefore, a physical, cell-cell interaction between *S. cerevisiae* and *L. thermotolerans* also exists. This interaction triggered another type of interaction, amensalism. In wine making conditions, this refers to a molecule being produced by one organism with the specific aim of harming another organism, without benefiting from it or harming itself in the process (Strehaiano et al. 2010). A cell-cell interaction has been hypothesized and reported before in co-fermentations of *S. cerevisiae* with *T. delbrueckii* and *L. thermotolerans* (Nissen et al. 2003). These authors concluded that this cell-cell mechanism induced cell death in *T. delbrueckii* and *L. thermotolerans* and that it was dependent on a high concentration of viable *S. cerevisiae* cells. Although this mechanism has not been confirmed elsewhere for *L. thermotolerans*-*S. cerevisiae* mixed culture fermentations, it has been done so for *T. delbrueckii* and *S.cerevisiae* (Renault et al. 2013). By using a similar DCB than ours, these authors found that *S. cerevisiae* induced cell death in *T. delbrueckii* either through cell receptors or ligand-like molecules located on the cell surface or through the production of a soluble molecule lethal at high concentrations.

The direct and indirect presence of both yeasts did not have any significant impact on the production of ethanol (Figure 3.3.5). Although a different experimental layout was used, ethanol levels for the mixed cultures corresponds to what Hansen et al. (2001) found (67 g/L) and for *L. thermotolerans* to what Kapsopoulou et al. (2005) found (± 60 g/L). For *S. cerevisiae*, ethanol production was lower than expected and could have been because of evaporation or experimental error. If the DCB is fitted with a gas condenser, this could be avoided in future as it will allow any ethanol that has evaporated to condense back into the media. Furthermore, the loss in ethanol could have been caused by the sparging of the system with air, but this should be tested further by performing more fermentations. Table 3.3.1. suggests that the direct and

indirect presence of both yeasts might have impacted on glycerol production. When the yeasts were in direct physical contact, the highest glycerol content was detected (11.8 g/L). This is much higher than the concentration normally found in wines (1.0 – 9.0 g/L) and the amount needed to detect an increase in sweetness in wines (5.2 g/L) (Noble and Bursick 1984). When the yeasts were in indirect contact, the lowest level was produced (4.6 g/L), which was lower than the detection limit needed to pick up an increase in sweetness in the wine (Noble and Bursick 1984). When compared to glycerol levels produced in the pure cultures (6.6 g/L for *L. thermotolerans* and 5.9 g/L for *S. cerevisiae*), there was a significant increase in glycerol production in the mixed cultures where the yeast were in direct contact and slight decrease in the mixed cultures where they were in indirect contact. Taking this into account, it appears as though a cell-cell interaction could have stimulated glycerol production in one of the two yeast or both. Although *Starmerella bombicola* (formerly known as *Candida stellata*) has been used in co-fermentations with *S. cerevisiae* to enhance the glycerol content of wines (Ciani and Ferraro 1998; Soden et al. 2000), it has not been done with *L. thermotolerans*. Mainly because most studies performing mixed culture fermentations with these two yeasts did not pick up any significant changes in the glycerol content when co-fermented (Gobbi et al. 2013). This could be attributed to differences in experimental layout. Therefore, it is difficult to make similar conclusions from this data.

3.4.2. The effect of oxygen on DCB fermentations

In the DCB system, N_2 was used to exchange the medium between the two compartments. As mentioned in section 3.3, it was observed that the yeasts struggled to grow in the presence of N_2. One explanation for this could be the presence of H_2CO_3 which formed as a result of trapped CO_2 in the system. The presence of this acid could have been detrimental to the yeasts' growth. Therefore, it was decided to provide the fermentations with air twice a day. The yeasts were able to grow well under these conditions and all fermentations were able to ferment to or close to dryness (Table 3.3.1). After each oxygen pulse, it was observed that oxygen was consumed rapidly and within a few minutes the Dissolved Oxygen (DO) percentage had gone down to 0%. This trend was also observed by Brandam et al. (2013). Only when the yeasts had reached stationary phase DO could be saturated to 100%. Therefore, these fermentations were always under anaerobic conditions, except during and directly after every oxygen pulse. It was decided to do supplementary experiments, using different oxygenation strategies, to evaluate the effect on yeast growth. For these fermentations, all but one (*L. thermotolerans* pure culture provided with oxygen once a day) fermented to or close to dryness.

In our experiments, when oxygen was given twice a day, pure cultures of *L. thermotolerans* and *S. cerevisiae* reached stationary phase after ± 60 h with a max OD of ± 25 (graphs A and B, Figure 3.3.3). When given less oxygen, there was a significant decrease in biomass formation in pure cultures of both species, decreasing from ± 25 to 15. It must however be noted that these fermentations could not be replicated. This decrease in biomass was also observed in mixed culture fermentations where the yeast were in direct contact. When given less oxygen, OD dropped from ± 22 to 19 (graph C, Figure 3.3.3). What is interesting to note is that the mixed culture fermentations were less affected by this change in oxygenation strategies. This can also be observed in Figure 3.3.4. when comparing the accumulated dry weight at the end of each fermentation. *L. thermotolerans* pure cultures had the highest dry weight when given oxygen twice a day and this decreased by more than half when less oxygen was supplied (± 8.5 to 3.5 g/L). This decrease in biomass was less for *S. cerevisiae* pure cultures (± 7.5 to 4.5 g/L), while an even smaller difference in biomass was observed for mixed cultures (± 6.4 to 4.6 g/L). Brandam et al. (2013) also observed this decrease in biomass when less oxygen was provided for *T. delbrueckii* pure cultures. Therefore, it appears as though *L. thermotolerans* was more affected by the change in oxygen availability and indeed, it has been reported that this yeast has higher oxygen requirements than *S. cerevisiae* (Nissen et al. 2004).The different oxygen treatments also had an impact on the fermentation duration (Table 3.3.1). For pure cultures of *L. thermotolerans* and *S. cerevisiae*, fermentations tended to last longer, increasing from 144-162 h and 144-186 h, respectively. This indicated that less oxygen could have impacted the rate at which sugar was consumed. Brandam et al. (2013) reported the same trend. Nissen et al. (2004) observed a similar response to a lower oxygen availability: a decreased maximum specific growth rate (μ_{max}) for *T. delbrueckii*, *L. thermotolerans* and *S. cerevisiae* pure cultures. Furthermore, these authors found that oxygen can increase *L. thermotolerans*' ability to compete for nutrients when co-fermented with *S. cerevisiae.* If this is the case, it might further explain why *L. thermotolerans* was able to last so long throughout the fermentations in our study and indeed, Hansen et al. (2001) reported this as well.

The effect of a lowered aeration strategy on ethanol and glycerol production is depicted in Table 3.3.1. It is difficult to draw clear conclusions from this data, since some experiments could not be repeated. However, it seems as though the differences in oxygenation had little effect on the production of ethanol, which was also reported by Brandam et al. (2013) and Hansen et al. (2001) and although the former authors observed a decrease in glycerol content, when more oxygen was supplied, our study showed the opposite. When more oxygen was supplied, the production of glycerol increased in the pure cultures, but decreased in the mixed cultures (Table 3.3.1). Ciani & Comitini (2006) reported increases in glycerol in mixed cultures of

S. bombicola and *S. cerevisiae* when a higher temperature was used and more oxygen was provided. These differences can be attributed to differences in experimental layout and in our study, some experiments could not be repeated. To compare these results, the experiments testing the effect of oxygen needs to repeated in the DCB. Furthermore, the differences in results for these studies highlight the fact how each specie and strain reacts differently to the smallest of differences in experimental layouts.

3.5. Conclusions

While some experiments could not be repeated and vast differences between the experimental layouts were observed compared to other studies, some interesting and useful information has been gathered in this work.

The results confirmed that *S. cerevisiae* and *L. thermotolerans* interact in two ways in co-fermentations of synthetic grape must. Firstly, there was a cell-cell interaction which triggered an amensalism response from *S. cerevisiae* resulting in a loss in viability of *L. thermotolerans*. The specific response and consequent molecule produced by *S. cerevisiae* which lead to the death of *L. thermotolerans* remains unknown. To a lesser extent, there was also a metabolic interaction between the two. The latter had a smaller effect on both yeasts and only impacted biomass production. The effect of these interactions on ethanol and glycerol production was less obvious. Specifically, it is difficult to draw conclusions on the observed increase in glycerol production in direct contact mixed cultures, because it has not been reported before in such DCB fermentations or for *L. thermotolerans* and *S. cerevisiae* co-fermentations.

Furthermore, when provided with less oxygen, the yeasts were still able to ferment to dryness, although it took them longer to do so. The most significant effect that it had was on biomass production. A reduced oxygen availability led to a decrease in total biomass; this result correlates with previous studies (Brandam et al. 2013). Ethanol production largely remained unaffected when less oxygen was given, while glycerol increased in mixed cultures (direct contact) and decreased in pure cultures of *S. cerevisiae* and *L. thermotolerans*. This data is however not supported by previous findings and because some of the fermentations could not be repeated, should not be interpreted as fact. It has become clear that it is not just the rising ethanol concentrations in mixed culture fermentations that influence the early death of non-*Saccharomyces* yeast, but that dissolved oxygen concentrations also play a part.

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Chapter 4

Interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans* and the impact of oxygen

Chapter 4 –Interactions between *Saccharomyces cerevisiae* and *Lachancea thermotolerans* and the impact of oxygen

4.1. Introduction

As mentioned in Chapter 3, recent studies have focused on finding alternative methods of wine making in the attempt to create wines with more complex sensorial profiles (Ciani and Comitini 2011; Fleet 2008; Gobbi et al. 2013; Jolly et al. 2003). In this context, a selection of a few non-*Saccharomyces* yeasts have been identified as suitable for use of mixed culture fermentations with *S. cerevisiae* (Bely et al. 2008; Ciani and Ferraro 1996; Clemente-Jimenez et al. 2005; Comitini et al. 2011; Gobbi et al. 2013; Kapsopoulou et al. 2005, 2007; Mora et al. 1990). However, more research is needed regarding the specific interactions taking place between yeast species in mixed culture wine fermentations. Some studies have already focused on this issue (Bely et al. 2008; Nissen et al. 2003, 2004; Renault et al. 2013). Nissen et al. (2004) hypothesised that a physical cell-cell interaction occurs between *S. cerevisiae* and *T. delbrueckii* and/or *L. thermotolerans* when co-inoculated and Renault et al. (2013) confirmed this for *T. delbrueckii*. The work conducted in Chapter 3 was aimed at studying such interactions for *S. cerevisiae* and *L. thermotolerans* and after its completion, it was decided to conduct further mixed culture fermentations in South Africa with the use of a Single Compartment Bioreactor (SCB) in the hopes of aligning these data sets. Although some trends were the same, we were unable to align the data and therefore decided to present them in separate chapters. In wine fermentations, it is often also observed that these non-*Saccharomyces* yeasts do not survive more than 3 days and recently, some research has focused on the cause of their early decline (Hansen et al. 2001). Indeed, these authors highlighted the important role that dissolved oxygen plays on the survival of non-*Saccharomyces* yeasts and that their decline might not just be because of interactions with *S. cerevisiae*. Preliminary investigations on the effect of oxygen on these mixed cultures fermentations revealed that dissolved oxygen might indeed impact on their behaviour (chapter 3). It was therefore decided to further investigate this aspect in the SCB fermentations.

The aim of this study was thus to perform mixed culture fermentations using *S. cerevisiae* and *L. thermotolerans*, in an attempt to study the interactions that may take place between these 2 species and to further elaborate on the results obtained in Chapter 3. Furthermore, following the observed impact of oxygen on these fermentations, this study

was also aimed at testing the impact of dissolved oxygen on these mixed culture fermentations.

4.2. Materials and methods

4.2.1. Microorganisms and media

For the next set of fermentations, the same microorganisms were used as referred to in Chapter 3, section 3.2.1. The strains were maintained at 4°C on YPD agar (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 20 g/L agar). In order to differentiate between *S. cerevisiae* and *L. thermotolerans* and to enumerate viable cells, the yeasts were grown on Wallerstein (WL) Nutrient agar at 30°C. This method was used for both mixed and pure culture fermentations.

4.2.2. Bioreactor fermentations

For the purpose of the next set of fermentations, a different system from that described in Chapter 3 was used. This system, the BioFlo® 110 reactor manufactured by New Brunswick Scientific (Enfield, CT), is a SCB (Figure 4.2.1). All fermentations were carried out in duplicate. In each fermentation, conditions were kept the same. A synthetic grape must medium was used (refer to chapter 3, Table 3.2.1).

4.2.3. Inoculation strategies

For each fermentation, the bioreactor vessel contained 1 L of medium. The preparations of the starter cultures and inoculum size were carried out as in chapter 3, section 3.2.3. YPD broth from Merck (Modderfontein, South Africa) was used.

4.2.4. Fermentation conditions & oxygenation strategies

The fermentations were conducted at 30°C and at 250 rpm stirring. In contrast to DCB fermentations, the medium was not transferred between two vessels. In order to keep fermentation conditions as constant as possible between the two types of bioreactor experiments, the head space of the SCB was saturated with nitrogen gas at a flow rate of 0.5 vvm (volume of air per volume of medium per minute). Twice a day (at 8 am and 4 pm), immediately before sampling, air was sparged through the medium for 30 min at a flow rate of 0.5 vvm. This oxygenation strategy was considered as the control. Further fermentations were conducted to test the effect of oxygen on the fermentation dynamics by sparging air for 30 min once a day at 8 am and once at the beginning of fermentation at a flow rate of 0.5 vvm. Samples of 20 mL were taken twice a day.

4.2.5. Sample analysis

Samples were used to monitor optical density at 600 nm (pathway of 1 mm) using a UV – 1601 Spectrophotometer (Shimadzu, Kyoto, Japan), viable cell counts on WL agar, and total and viable cell counts with the use of a Neubauer Spencer® Bright-Line™ haemocytometer, light microscope from Zeiss (Iena, Germany) and methylene blue staining using a method by Alfenore et al. (2004). Refer to chapter 3, section 3.2.5 for methylene blue staining and cell counting method. The rest of the sample was spun down at 7,500 rpm at 4°C. The supernatant was stored at -4°C. Dry weight was determined using the same method as described in Chapter 3, section 3.2.5.

4.2.6. Bioreactor

A schematic representation of the SCB is shown in Figure 4.2.1. This reactor system has been designed so that specific experimental needs can be met. Agitation is achieved with Rushton-style impellers. Temperature is controlled with the use of a temperature probe submerged into the medium within a metal casing and a system of heating and cooling with an external heating blanket and cooling coil immersed into the vessel. Dissolved oxygen (DO) and pH can be measured and controlled with the use of probes which are also submerged into the medium and connected to a Primary Control Unit (PCU). All data captured by these probes can be stored electronically. A gas mix controller is present and air flow into the system can be measured and controlled.

4.2.7. Analytical determinations

Ethanol concentrations were determined with the use of a High Performance Liquid Chromatography (HPLC) method (Eyéghé-Bickong et al. 2012). The Agilent 1100 system was obtained from Agilent Technologies©, Palo Alto, CA. Frozen, centrifuged samples were thawed and analysed on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. To detect and quantify peaks, an Agilent Refractive Index Detector (RID) and Diode Array Detector (DAD) were used simultaneously. The data were analysed with the use of the HPChemstation software package.

Glycerol, acetic acid, glucose and fructose concentrations were determined with the use of enzymatic assays (Arena 20XT Photometric Analyzer obtained from Thermo Electron Oy, Finland).

Volatile compounds were determined with the use of gas chromatography–flame ionization detector (GC-FID) as described by Styger et al. (2011). 5 mL frozen, centrifuged samples were thawed and spiked with 100 µl of internal standard (0.5 mg/mL 4-methyl-2-pentanol in 12% (v/v) ethanol) after which volatiles were extracted by adding 1 mL of diethyl

ether and vortexing with three short pulses to ensure all liquids were mixed well. This mixture of fermentation sample and ether was then placed in an ultrasonic bath for 5 min and then centrifuged at 4,000 rpm for 3 min. The ether layer was removed and dried on anhydrous $NaSO_4$. These extractions were injected into the GC-FID in duplicate. The analysis was performed according to Styger et al. (2011).

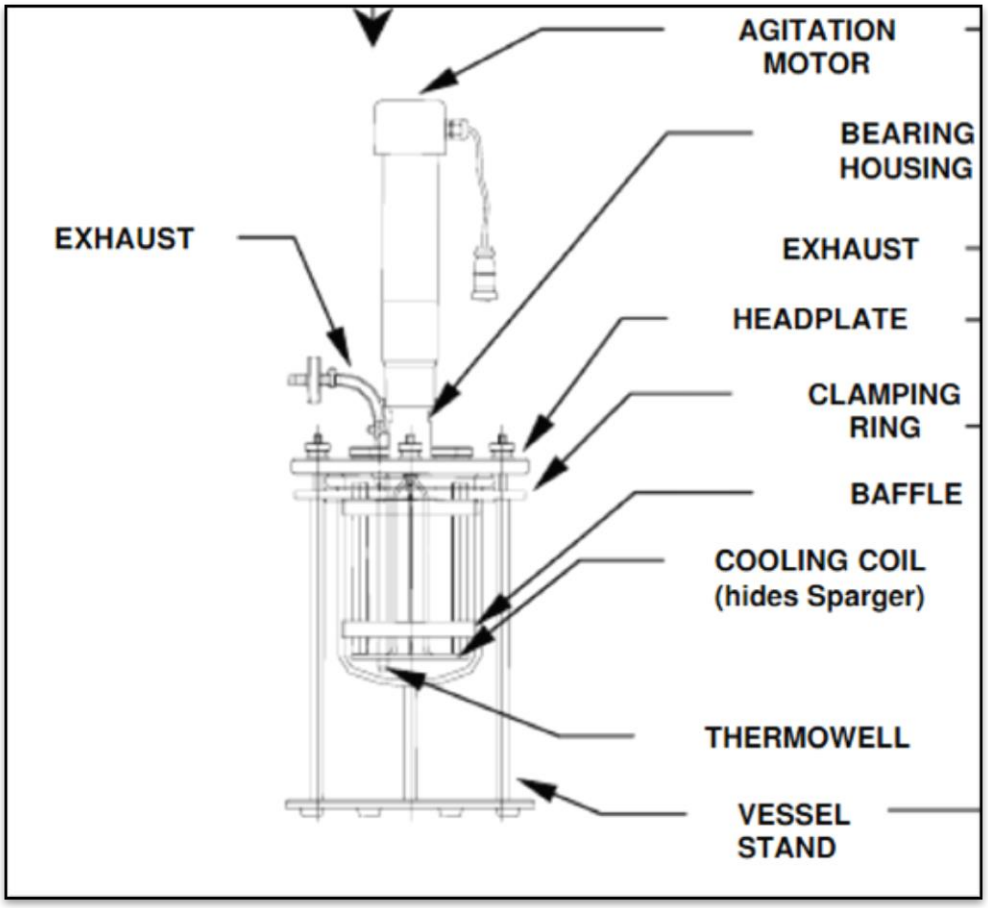

Figure 4.2.1: Schematic representation of the SCB used to perform fermentations (New Brunswick Guide to Operations, Manual nr. M1273-005).

4.3. Results

4.3.1. SCB: interaction studies

As described in section 4.2, a SCB was used to conduct a series of fermentations using a synthetic grape must medium. *S. cerevisiae* and *L. thermotolerans* pure culture and mixed culture fermentations were conducted in duplicate. The setup of the two reactor systems (i.e. in Chapter 3 and in this chapter) differ vastly, nevertheless fermentation conditions were kept the same as far as possible. Therefore, the headspace of the SCB was saturated with N_2 and oxygen was supplied twice a day.

From the onset of fermentation, cellular growth entered the exponential phase immediately (i.e. no lag phase was observed) and reached stationary phase between 50 and 60 h after inoculation (Figure 4.3.1). This trend could be observed for pure cultures of *L. thermotolerans* and *S. cerevisiae* as well as the mixed culture fermentations of these two species. The pure culture fermentations of *S. cerevisiae* lasted 69 h and mixed cultures of *S. cerevisiae* and *L. thermotolerans* lasted 93 h. The former achieved the highest cellular density and the latter the second highest, reaching maximum OD values of 21 and 14, respectively. The pure culture fermentation of *L. thermotolerans* lasted longer (144 h) and achieved the lowest cellular density at an OD of 11.

After ± 50 h, a steady drop in cell viability was observed for the *S. cerevisiae* pure culture fermentations and the mixed culture fermentations of *S. cerevisiae* and *L. thermotolerans* (Figure 4.3.1). This drop in viability occurred steadily over the next 40 h, going from a total of ± 100 % to ± 80 % living cells. For *L. thermotolerans* pure culture fermentations, a drop in cell viability was also observed, but it only occurred after the 70 h mark. Thereafter, it dropped steadily, then steeply until a viability of 33% was reached by the end of fermentation.

The concentration of viable cells present in each fermentation was also monitored on WL plates. After inoculation, the viable cell count in all fermentations increased rapidly and reached 1 x 10^7 CFU/mL within 24 h (Figure 4.3.2).

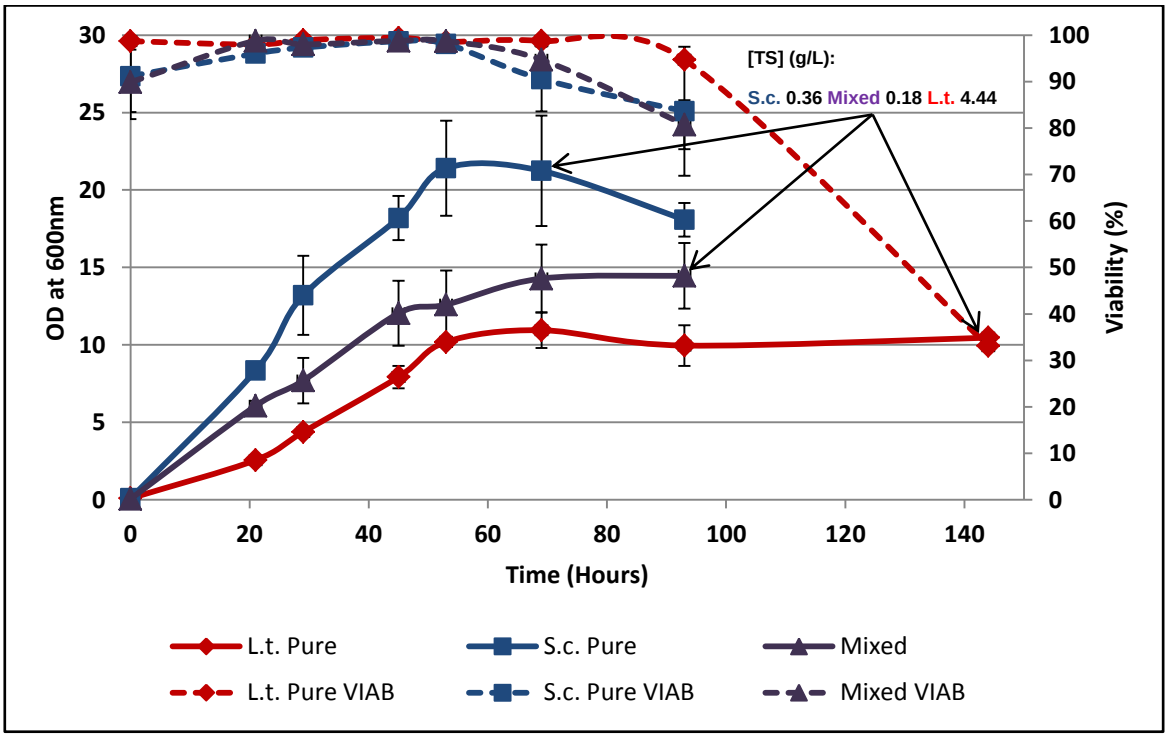

Figure 4.3.1: Fermentation growth represented in OD_{600nm} (indicated in solid lines) and plotted against the concentration of viable cells as a percentage of total cells present at a specific time point

(indicated in dashed lines) of *L. thermotolerans* (red) and *S. cerevisiae* (blue) in pure cultures and in mixed cultures (purple) where the cells were in physical contact.

Pure cultures of *S. cerevisiae* produced the highest viable cell counts of all fementations at ± 1.1 x 10^8 CFU/mL. In the mixed culture, the amount of viable *S. cerevisiae* cells was lower and never surpassed the 1 x 10^8 CFU/mL mark. For *L. thermotolerans* pure cultures, the increase in viable cells was also lower compared to the pure cultures of *S. cerevisiae*. When this fermentation reached stationary phase, the concentration of viable cells was ± 8 x 10^7 CFU/mL. After ± 90 h, a drop in viability occurred, which was not observed for *S. cerevisiae*. When *L. thermotolerans* was co-inoculated with *S. cerevisiae*, the amount of viable cells generated was significantly lower. Here, the growth rate of *L. thermotolerans* was much lower compared to all other fermentations. In mixed culture, *L. thermotolerans* could only reach a max viable cell concentration of ± 1 x 10^7 CFU/mL and after 50 h a significant drop in viability was observed, while *S. cerevisiae* was able to maintain a viable cell concentration of almost 1 x 10^8 CFU/mL up until the end of fermentation.

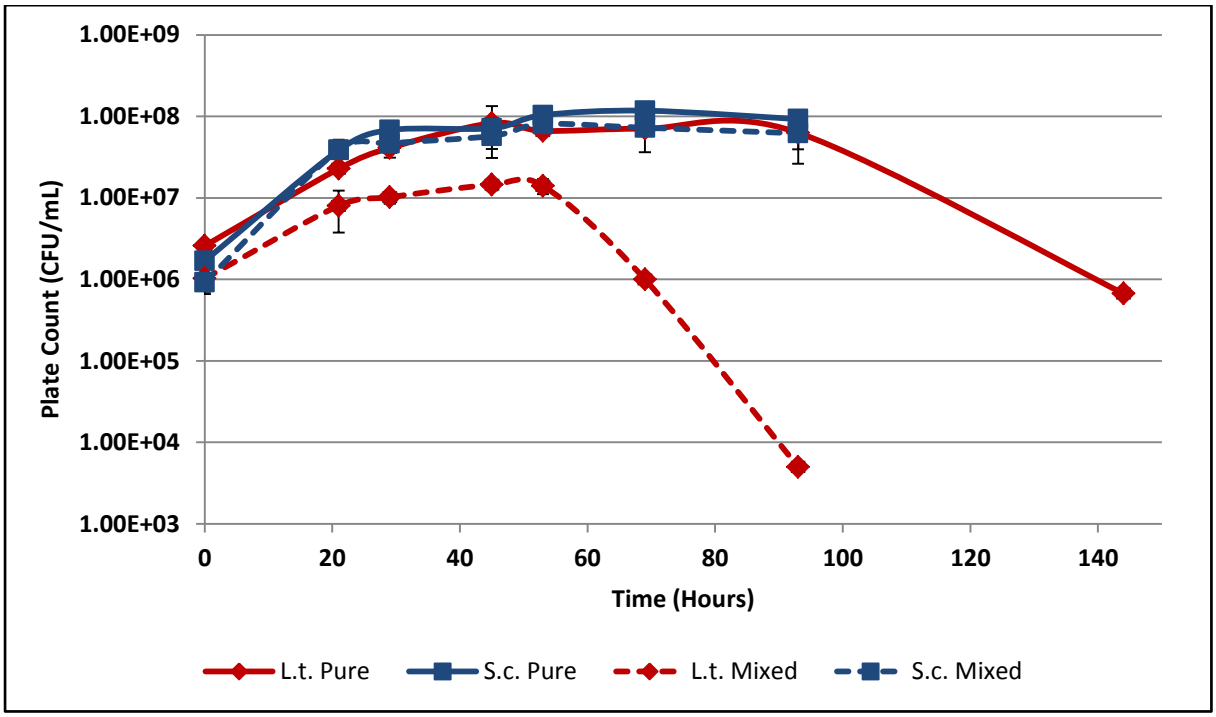

Figure 4.3.2: Population dynamics represented as Plate counts (CFU/mL) (logarithmic scale) of *L. thermotolerans* (red) and *S. cerevisiae* (blue) in pure cultures (indicated in solid lines) and mixed cultures where the cells were in physical contact (indicated in dashed lines).

Abbreviations for graphs: **L.t.**: *L. thermotolerans*; **S.c.**: *S. cerevisiae*; **VIAB**: Viability; **Mixed**: Mixed culture of *L. thermotolerans* and *S. cerevisiae* where the yeast cells were in physical contact; **[TS]**: Total sugar concentration.

4.3.2. SCB: effect of oxygen on bioreactor fermentations

Fermentations using the SCB were conducted as above with the exception of two additional oxygen treatments (oxygenation once a day and once at the beginning of fermentation) being added. This was done to evaluate what the effect of less oxygen would be on the behaviour of the yeasts in pure cultures and mixed cultures.

Different oxygen treatments (feeding air twice and once a day and once at the beginning of fermentation) had a major impact on the fermentation behaviour of *L. thermotolerans* and *S. cerevisiae* in pure culture fermentations as well as co-fermentations of these two yeasts (Figure 4.3.3). When *L. thermotolerans* pure cultures were given oxygen twice a day, the fermentations were completed in 144 h (Table 4.3.1) and reached an OD of ± 11 (graph A, Figure 4.3.3). Giving less oxygen (once a day and once at the beginning of fermentation) resulted in a reduction in cell density and an increase in fermentation duration. The fermentation where oxygen was given once a day and once at the beginning of fermentation reached a maximum OD of ± 8 and 7.5 and lasted 168 and 192 h, respectively (Table 4.3.1). While there was not much difference in maximum OD values for the latter fermentations, the effect of less oxygen was greater on the time it took to complete the fermentation. For each oxygen treatment, the viability was high up until ± 90 h, after which a significant drop in viable cell counts was observed (graph A, Figure 4.3.3). This decrease in viable cell counts for *L. thermotolerans* was somewhat slower for the fermentations where oxygen was given once a day and once at the beginning of fermentation. By the last day of fermentation, viability had dropped from 100% to between 40 and 30%.

A similar trend was observed for the pure culture fermentations of *S. cerevisiae* (graph B, Figure 4.3.3). Here, when given oxygen twice a day, a maximum OD of ± 22 was reached and the fermentation lasted 69 h (Table 4.3.1). When given oxygen once a day and once at the beginning of fermentation, these values dropped to a max OD of ± 16 and 10 and the fermentations lasted 93 and 144 h, respectively (Table 4.3.1). When oxygen was given twice a day, a steady decrease in viability occurred from ± 50 h until the percentage of viable cells left over by the end of fermentation was at ± 80% (graph B, Figure 4.3.3). A slight drop in viability was also recorded when given oxygen once a day which occurred after ± 60 h until it dropped to ± 90% by the end of fermentation. The trend was similar when given oxygen once at the beginning of fermentation up until ± 90 h after which there was a sharp drop in viability to ± 50% by the end of fermentation.

When different oxygen treatments were administered, mixed culture fermentations of these two yeasts resulted in a similar trend in terms of differences in OD values and loss in viability (graph C, Figure 4.3.3). Oxygenation twice a day resulted in a maximum OD of ± 14 and fermentation duration of 93 h (Table 4.3.1).

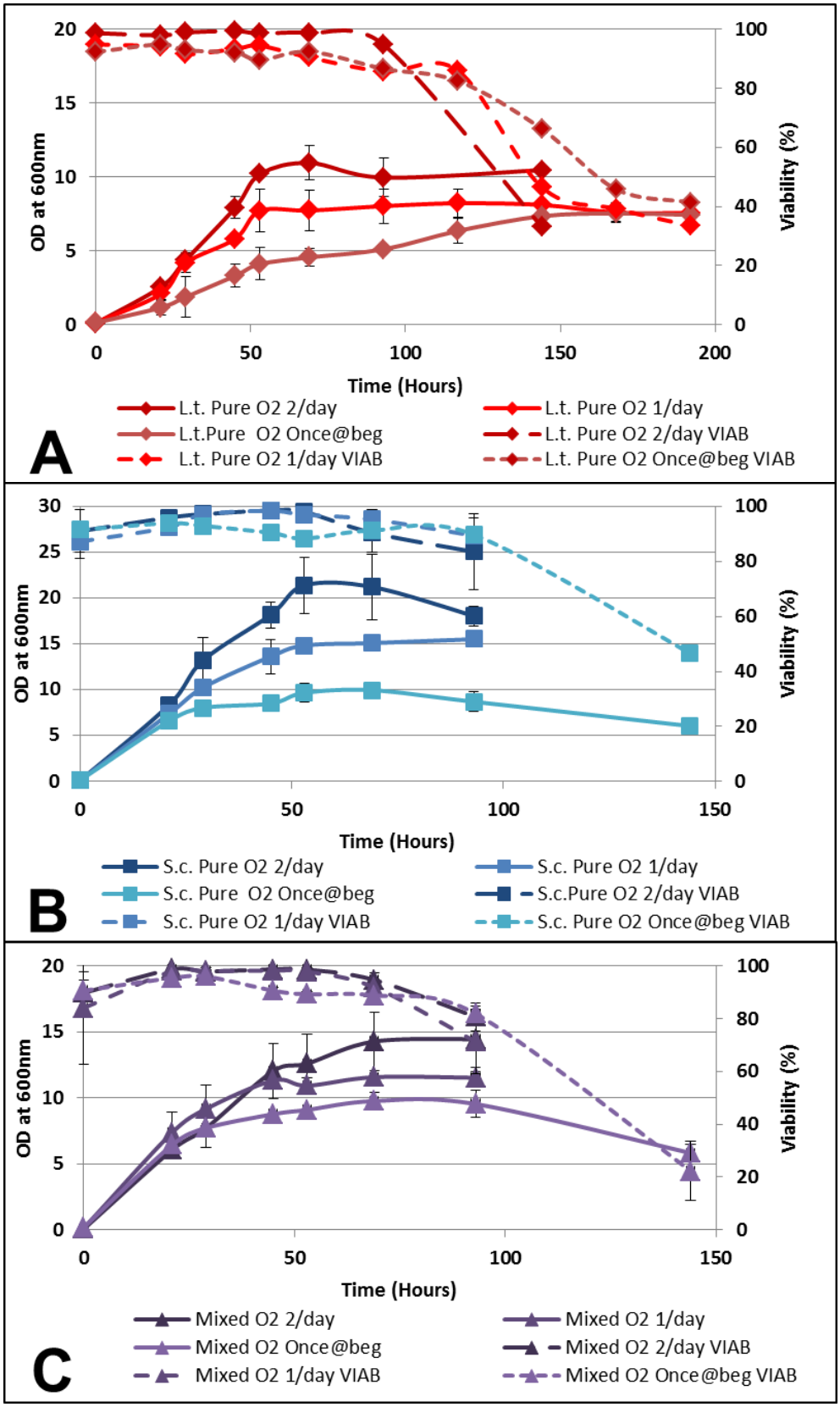

Figure 4.3.3: Fermentation growth represented in OD_{600nm} (indicated in solid lines) and plotted against Viable cells as a percentage of total cells present at a specific time point (indicated in dashed

lines) for *L. thermotolerans* [A] and *S. cerevisiae* [B] in pure cultures and mixed cultures where the cells were in physical contact [C]. Different Oxygen treatments are indicated in different shades of red [A], blue [B] and purple [C].

The viability dropped gradually to ± 80% at the end of fermentation. When given less oxygen, the same reduction in OD could be seen as in pure cultures. These differences in OD values were however less.

Oxygenation once a day saw a maximum OD of just lower than 12 and the viability dropped gradually to ± 70% by the end of fermentation. No difference in fermentation duration could be seen. When only supplied with oxygen at the beginning of fermentation, a maximum OD of ± 10 was reached and here the viability dropped more drastically by the end of fermentation to ± 20%. This fermentation lasted longer (144 h) (Table 4.3.1).

For these fermentations, viable cell concentrations were also monitiored on WL plates (see section 4.2 for method). After the onset of fermentation, the viable cell counts for both *L. thermotolerans* (graph A, Figure 4.3.4) and *S. cerevisiae* (graph B, Figure 4.3.4) increased rapidly within the first 24 h. This occurred in all fermentations. For *L. thermotolerans*, the highest count was achieved in the pure culture where oxygen was given twice a day at just under 10^8 cells/mL (graph A, Figure 4.3.4). Here, when given less oxygen (once a day and once at the beginning of fermentation), the maximum viable cells that were produced in pure cultures were lower. Viability dropped after ± 90 h in all three types of oxygen treatments for the pure culture fermentations. When co-cultivated with *S. cerevisiae* and given oxygen twice a day (control), *L. thermotolerans* produced less viable cells than the pure cultures of *L.* thermotolerans (graph A, Figure 4.3.4). This effect was more pronounced in the fermentations where less oxygen was given (once a day and once at the beginning of fermentation). Here, the viability dropped earlier compared to pure cultures and after 144 h and 90 h no more colonies could be counted on WL plates for the fermentations where oxygen was given once a day and once at the beginning of fermentation, respectively.

S. cerevisiae was less affected by the presence of *L. thermotolerans* and alteration in oxygen feeding (graph B, Figure 4.3.4). Here, there was not much difference in the maximum viable cell counts that could be produced in pure cultures between different oxygen treatments. When given oxygen twice and once a day in pure cultures, *S. cerevisiae* was able to produce a viable cell count of over 10^8 cells/mL and when given oxygen once at the beginning of fermentation, this value fell just under 10^8 cells/mL. After ± 90 h, the viability dropped in all pure culture fermentations, but never below 10^7 cells/mL. When *S. cerevisiae* was co-cultivated in the presence of *L. thermotolerans*, there did not seem to be much difference in the amount of viable cells that *S. cerevisiae* was able to produce (graph B, figure 4.3.4). For all mixed culture fermentations, the values lied almost in line with the pure

culture fermentations, except for one (the treatment of oxygen once at the beginning of fermentation). Here, the yeast was not able to reach a maximum viable cell count of over 10^8 cells/mL and by the end of fermentation, *S. cerevisiae* viable cell count was under 10^7 cells/mL.

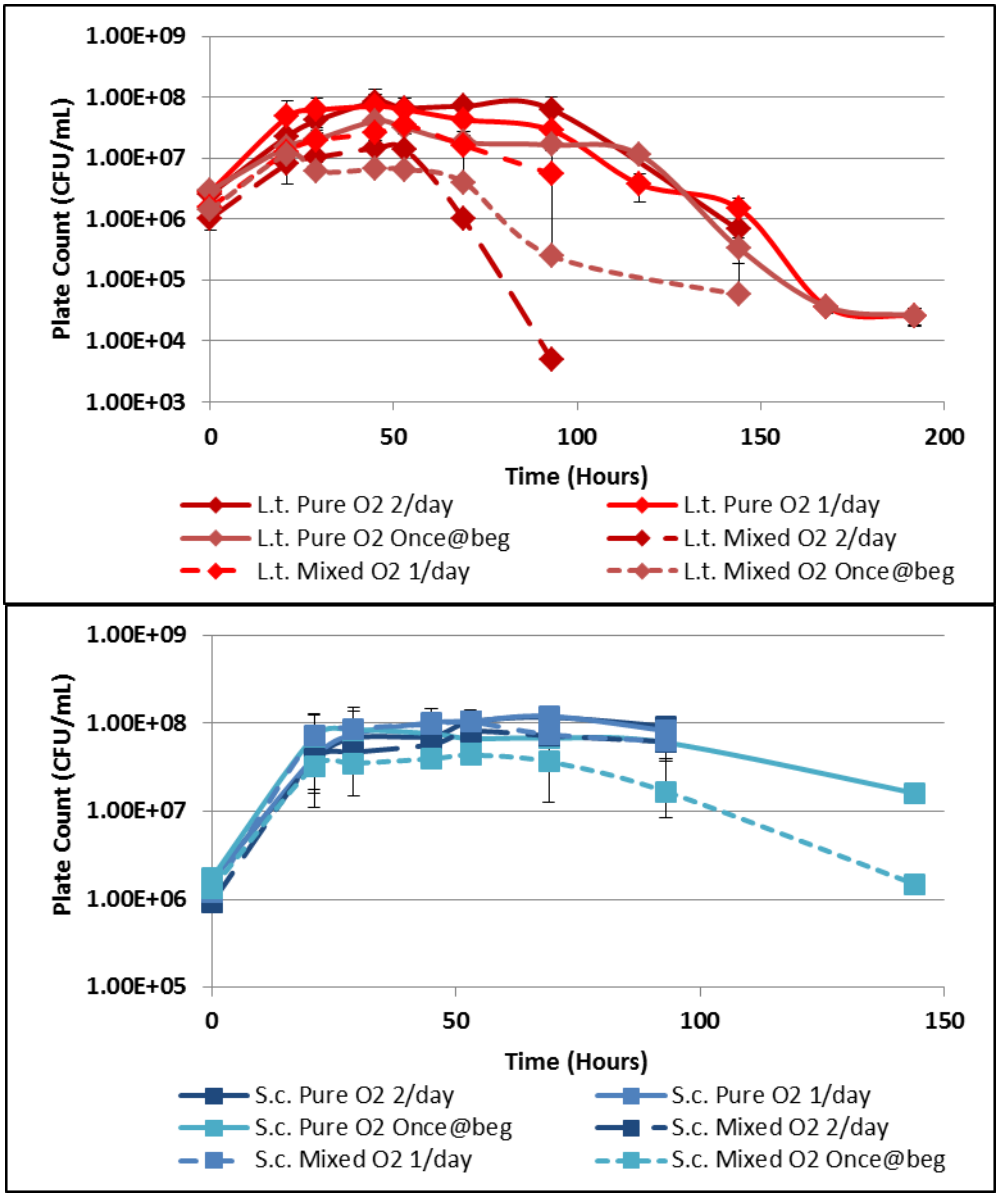

Figure 4.3.4: Population dynamics represented as Plate counts (CFU/mL) (logarithmic scale) of *L. thermotolerans* [A] and *S. cerevisiae* [B] in pure cultures (indicated in solid lines) and mixed cultures where the cells were in physical contact (indicated in dashed lines). Different oxygen treatments are indicated in different shades of red [A] and blue [B].

To observe the accumulation of biomass in each set of fermentations, dry weight was measured on the last day of fermentation. When given oxygen twice a day, the *S. cerevisiae* pure culture fermentations produced the highest dry weight at the end of fermentation while *L. thermotolerans* in the same conditions produced the lowest (Figure 4.3.5). When the two yeasts were co-cultivated and given oxygen twice a day, this maximum dry weight reached

an intermediate value between the two previously mentioned fermentations. When given less oxygen, dry weight decreased in the *S. cerevisiae* pure cultures. For *L. thermotolerans* pure cultures and mixed culture fermentations a decrease in dry weight was also observed, however this decrease was less.

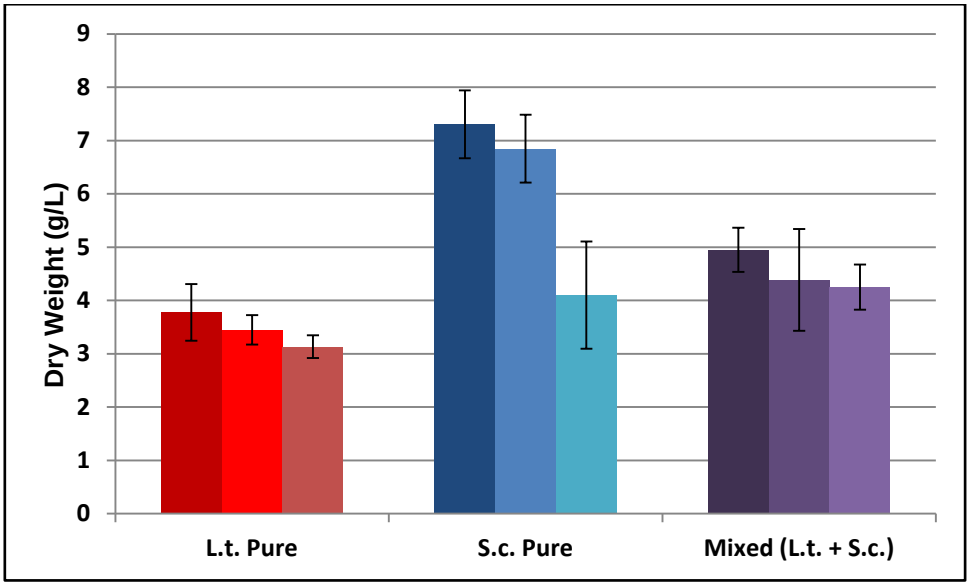

Figure 4.3.5: Dry weight (g/L) measured on the last day of fermentation for *L. thermotolerans* (red bars) and *S. cerevisiae* (blue bars) in pure and mixed cultures where the cells were in physical contact (purple bars). Darker, lighter and lightest shades of red, blue and purple indicate oxygenation of twice, once a day and once at the beginning of fermentation, respectively.

When given oxygen twice a day, *L. thermotolerans* pure cultures lasted the longest (144 h) (Table 4.3.1). The control fermentation for *S. cerevisiae* was completed at a faster rate (69 h). When the two yeasts were co-cultivated, this rate of fermentation was faster when compared to *L. thermotolerans* pure cultures, but slower when compared to *S. cerevisiae* pure cultures. All fermentations were able to ferment to or close to dryness (Table 4.3.1). While it is difficult to identify clear trends in changes in ethanol, glycerol and acetic acid when different oxygen treatments were used, it is possible to look at trends on a more global scale (Table 4.3.1). For all inoculation scenarios and oxygen treatments, final ethanol levels varied extensively and no clear trend could be observed (Table 4.3.1). Within the control fermentations (oxygen given twice a day), *S. cerevisiae* produced the highest amount of glycerol (10.29 g/L), *L. thermotolerans* the lowest (8.71 g/L) and when co-cultivated, these two yeasts produced an intermediate amount of 9.57 g/L (table 4.3.1). When given less oxygen, pure *L. thermotolerans* produced more glycerol. When *L. thermotolerans* and *S. cerevisiae* were co-fermented, the effect of lowered amounts of oxygen was less visible.

Table 4.3.1: Total Sugars, Ethanol, Glycerol and Acetic Acid present at the end of fermentation of *L. thermotolerans* and *S. cerevisiae* pure cultures and mixed cultures for three different oxygen treatments (oxygenation twice and once a day and once at the beginning of fermentation)

Fermentation	Oxygenation	Time Point (h)	Total Sugars (g/L)	Std dev	Ethanol (g/L)	Std dev	Glycerol (g/L)	Std dev	Acetic Acid (g/L)	Std dev
L.t. Pure	2/day	144	**4.44**	0.00	**81.22**	5.69	**8.71**	0.36	**0.65**	0.05
	1/day	168	**5.81**	0.64	**85.77**	3.10	**10.58**	1.02	**0.69**	0.02
	Once@beg	192	**0.06**	0.03	**72.48**	18.73	**10.20**	1.15	**0.71**	0.02
S.c. Pure	2/day	69	**0.36**	0.12	**87.69**	27.52	**10.29**	0.53	**0.91**	0.01
	1/day	93	**0.48**	0.48	**64.16**	31.24	**11.06**	2.35	**0.97**	0.19
	Once@beg	144	**0.66**	0.84	**69.43**	8.38	**9.19**	1.75	**0.94**	0.00
Mixed	2/day	93	**0.18**	0.22	**84.95**	12.60	**9.57**	0.38	**1.14**	0.10
	1/day	93	**0.20**	0.28	**74.48**	3.22	**9.15**	0.33	**0.87**	0.12
	Once@beg	93	**0.49**	0.60	**75.51**	8.31	**8.73**	0.35	**1.18**	0.29

When looking at the acetic acid values (Table 4.3.1), in a broad view, pure *L. thermotolerans* produced the lowest amounts, pure *S. cerevisiae* produced slightly higher and the highest amount of acetic acid could be observed for the fermentations where the two yeasts were fermenting together (Table 4.3.1).

For each inoculation scenario and all oxygen treatments, the concentrations of higher alcohols, short- and medium-chain fatty acids and esters were measured. Differences in higher alcohols can be observed when one considers the trends between different fermentations (Figure 4.3.7). *S. cerevisiae* produced the highest amounts of 2-phenyl ethanol and 3-ethoxy-1-propanol compared to *L. thermotolerans* and mixed cultures of the two yeasts (which yielded similar amounts). *L. thermotolerans* produced significantly higher amounts of butanol when compared to *S. cerevisiae* and mixed cultures of the two. When comparing isobutanol and isoamyl alcohol, the trends were similar. Here, *S. cerevisiae* produced the lowest amount compared to *L. thermotolerans* and mixed cultures. In the case of propanol, the highest amount was produced in mixed cultures, where the pure cultures of *L. thermotolerans* and *S. cerevisiae* produced similar amounts. Large differences in higher alcohol levels when given less oxygen seem to occur only in pure cultures of *L. thermotolerans* and *S. cerevisiae*. In pure *L. thermotolerans*, less isoamyl alcohol and isobutanol was produced when less oxygen was provided. The same trend occurred in pure *S. cerevisiae* cultures for 3-ethoxy-1-propanol.

With the production of small- and medium-chain fatty acids, *S. cerevisiae* produced the highest amounts in the control fermentations (where oxygen was given twice a day) except for decanoic acid (Figure 4.3.8). Here, *L. thermotolerans* produced significantly higher amounts, regardless of which oxygen treatment was given, when compared to *S. cerevisiae* and the mixed cultures of the two. *S. cerevisiae* produced the highest amount of propionic acid compared to *L. thermotolerans* and mixed cultures. For all other fatty acids, the trends are similar in the sense that *S. cerevisiae* and mixed culture values laid more or less within the same range, while the values for *L. thermotolerans* were much lower. Some minor differences could be observed when the yeasts were provided with less oxygen. The largest of these differences could be observed for *S. cerevisiae* pure cultures where a reduction in iso-valeric and propionic acid occurred when less oxygen was provided. To a lesser extent, a reduction in hexanoic and octanoic acid could be observed in the pure cultures of *L. thermotolerans*. It appears that when given less oxygen, these fatty acids did not increase significantly.

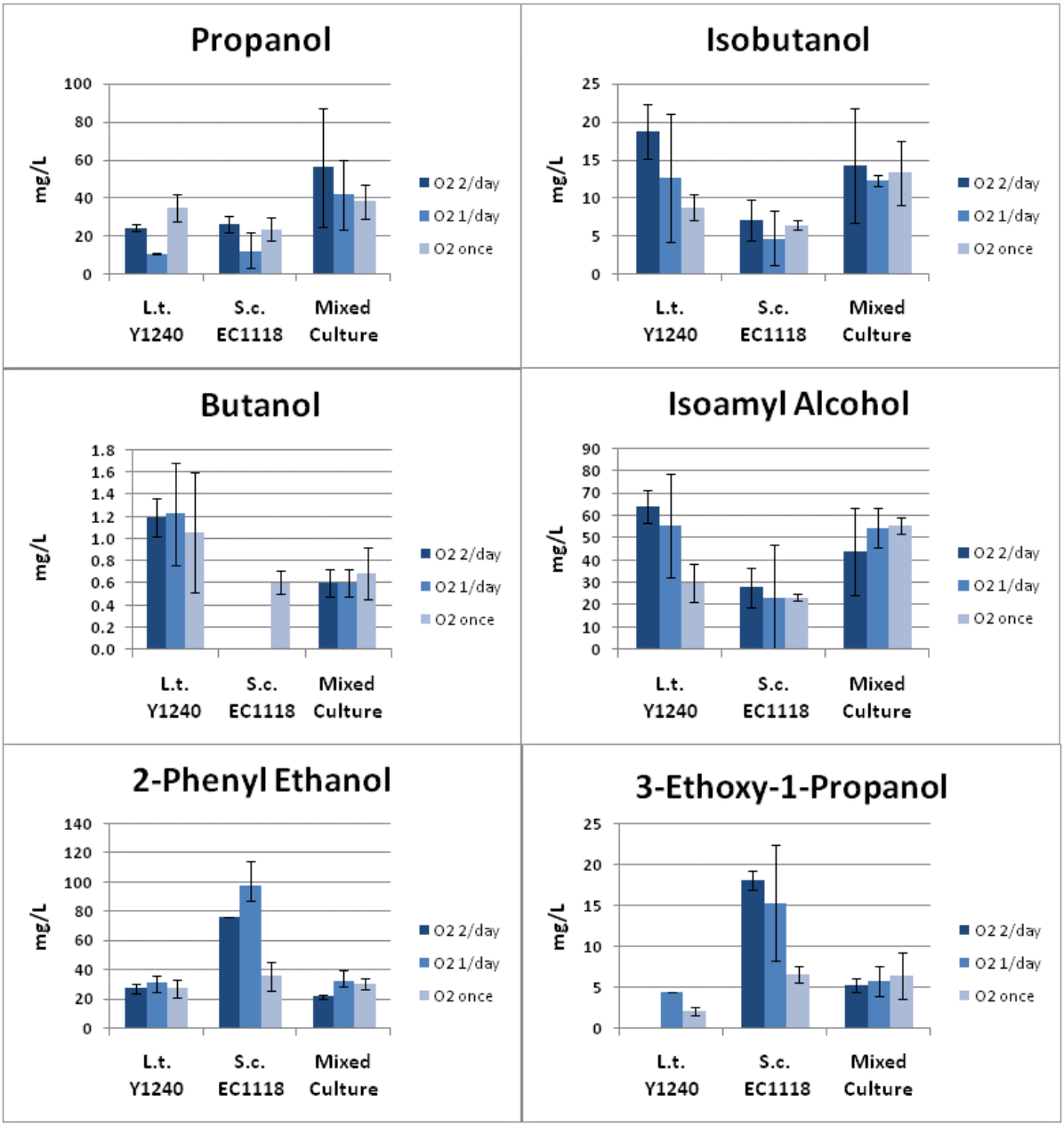

Figure 4.3.7: Concentrations of higher alcohols detected at the end of fermentation for *L. thermotolerans* and *S. cerevisiae* pure and mixed cultures where the cells were in physical contact and different oxygen treatments were used.

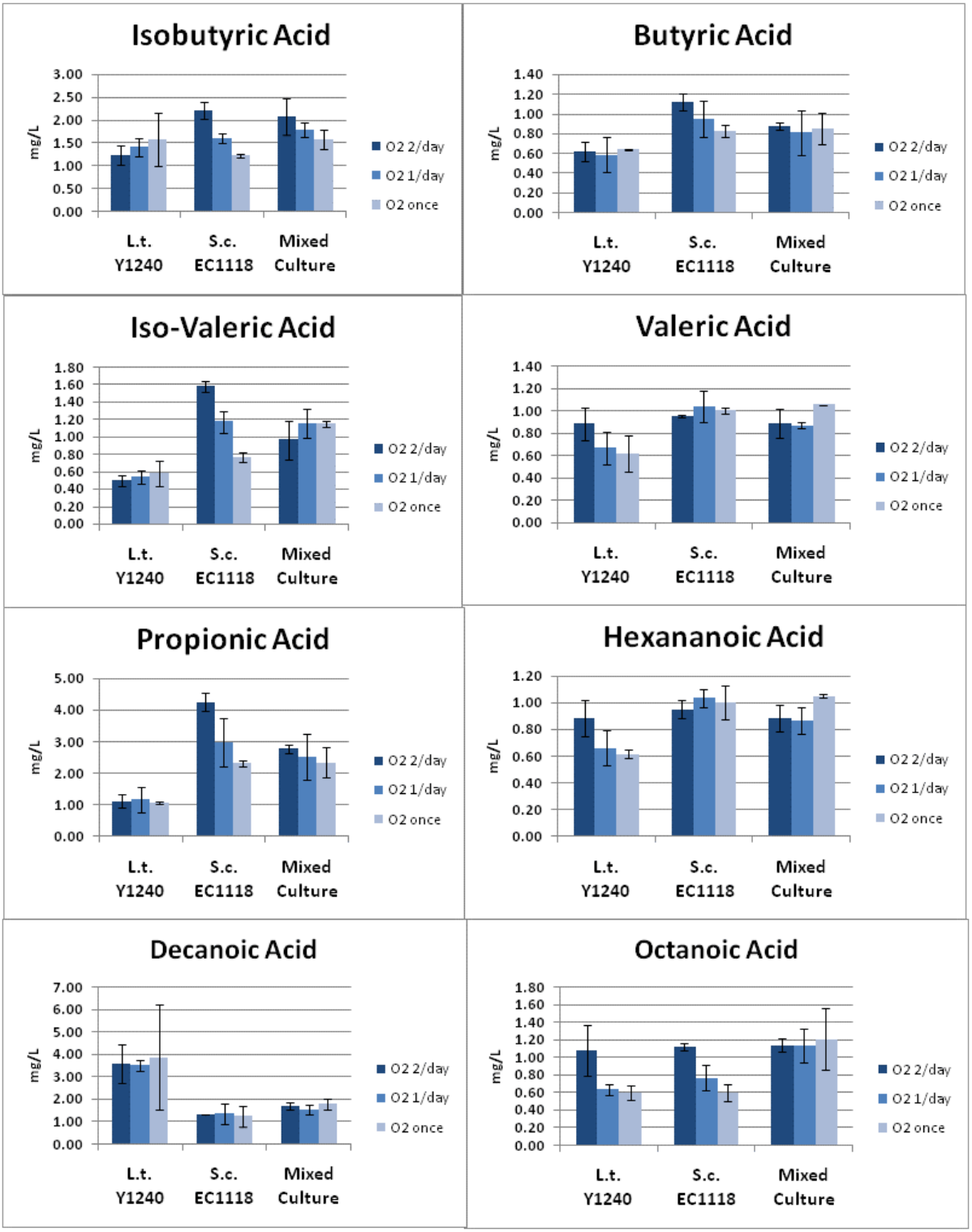

Figure 4.3.8: Concentrations of small- and medium-chain fatty acids detected at the end of fermentation for *L. thermotolerans* and *S. cerevisiae* pure and mixed cultures where the cells were in physical contact and different oxygen treatments were used.

Abbreviations for graphs: **L.t.**: *L. thermotolerans*; **S.c.**: *S. cerevisiae*; **VIAB**: Viability; **Mixed**: Mixed culture of *L. thermotolerans* and *S. cerevisiae* where the yeast cells were in physical contact; **O2 2/day**: Fermentation oxygenated twice a day; **O2 1/day**: Fermentation oxygenated once a day; **O2 Once@beg:** Fermentation oxygenated once at the beginning of fermentation.

4.4 Discussion

4.4.1. Interaction studies

As mentioned above, there is a need to evaluate the possible interactions between *L. thermotolerans* and *S. cerevisiae* in mixed culture fermentations. A DCB enabled us to study the physical and metabolic interactions directly by separating yeast cultures with a membrane (Chapter 3). In this chapter, the focus was the same, but experiments were conducted with a SCB. These two systems differ vastly, but both are valuable tools for studying microbial interactions.

S. cerevisiae pure cultures accumulated the most biomass (OD of 21) and completed the fermentations the fastest (69 h) compared to all other fermentations. *L. thermotolerans* pure cultures accumulated the lowest biomass (OD of 11) and completed the fermentations the slowest (144 h). In mixed cultures, the values for fermentation duration and accumulated biomass lied in between those of the two types of pure cultures, suggesting that although *S. cerevisiae* still dominated the fermentation, *L. thermotolerans'* presence had a slight negative effect on the growth of *S. cerevisiae*. Dry weight data supports this hypothesis (Figure 4.3.5). Indeed, the same trend for biomass accumulation was observed: dry weights measured for mixed cultures were lower than those of *S. cerevisiae* pure cultures, but more than those of *L. thermotolerans* pure cultures. Figure 4.3.2 illustrates the accumulation of viable colony counts on WL plates and from these graphs, it was also observed that *S. cerevisiae* reached a lower maximum CFU/mL in the mixed cultures compared to its pure cultures. Renault et al. (2013) noticed a similar interaction between *T. delbrueckii* and *S. cerevisiae*: the former yeast's metabolism indirectly slowed down the growth rate of the latter. The plate count data also suggests that *S. cerevisiae* had a direct, negative effect on the growth of *L. thermotolerans*.

L. thermotolerans was more affected by the presence of *S. cerevisiae*. The former yeast reached a max of ± 8 x 10^7 CFU/mL in pure cultures, but this dropped significantly in mixed cultures to ± 1 x 10^7 CFU/mL and by the end of fermentation only ± 3.8 x 10^3 CFU/mL viable colonies could be counted.

All pure and mixed cultures fermented to or close to dryness (Table 4.3.1), but it is difficult to draw clear conclusions from the ethanol data since the standard deviations were high. The SCB is a controlled system, but even so, evaporation could have taken place. Furthermore, the experimental layout for these fermentations has not been tested before and because only two repeats are available, it demonstrates how sensitive the yeasts are to small changes and that at least two more repeats would be of much value. From Table 4.3.1, it can also be seen that *S. cerevisiae* pure cultures produced the highest level of glycerol, mixed cultures the second highest and *L. thermotolerans* pure cultures the lowest. Previous

studies observed an increase in glycerol production in mixed cultures (Comitini et al. 2011; Gobbi et al. 2013), however, this difference could be attributed to differences in experimental layout. Ours provided the yeast with more dissolved oxygen and this has been shown to increase *L. thermotolerans*' ability to compete for nutrients (Nissen et al. 2004), increasing its ability to impact on the metabolism of *S. cerevisiae*. Furthermore, the acetic acid data suggests that this interaction may have led to an increase in production of this compound (since mixed cultures had the highest concentration) and although previous studies did not observe this (Gobbi et al. 2013), it also highlights the role that oxygen could have played in these fermentations.

Volatile compounds were also analyzed. Although the standard deviations for these data were high, one can consider broad trends. In doing so, it was observed that for most compounds, the mixed culture fermentations had an intermediate value compared to both types of pure cultures. Suggesting a decrease/increase in production stimulated by the presence of one of the two yeasts. One exception was propanol (Figure 4.3.7). For this higher alcohol, the amount produced in mixed cultures was more than double the amount produced in pure cultures. When compared to the control fermentation, increases in this compound were observed when *L. thermotolerans* (Gobbi et al. 2013) and *P. fermentans* (Clemente-Jimenez et al. 2005) were fermented sequentially with *S. cerevisiae*.

4.4.2. The effect of oxygen on SCB fermentations

A series of supplementary fermentations testing different oxygenation strategies were performed to investigate what the effect would be on their growth in these bioreactors. In the SCB, we were able to test the effect of pulsing air twice and once a day and once at the beginning of fermentation. Two repeats could be performed.

A reduction in oxygen led to a reduction in biomass formation and an increase in fermentation duration for all fermentations but this effect was somewhat less in the mixed culture fermentations (Figure 4.3.3). In the SCB, a reduction in OD was noticed for the mixed cultures but this reduction was not as pronounced as that in the pure cultures. Furthermore, we observed no difference in fermentation duration for mixed cultures as all fermented to dryness within 93 h. It has been found that *L. thermotolerans* has higher oxygen requirements than *S. cerevisiae* (Nissen et al. 2004). This most certainly explains why this yeast seemed to be more affected by the changes in oxygen availability. Furthermore, it might also explain why mixed cultures were not affected as much. *L. thermotolerans* died off earlier not only because of less oxygen, but also because of the presence of *S. cerevisiae*, which aided the latter's ability to survive longer throughout the fermentations even though less oxygen was available. This reduction in biomass formation was also confirmed by the data for dry weight calculated at the end of fermentation (Figure 4.3.5). Figure 4.3.4.

illustrated the observed colony counts on WL plates for all fermentations and from this graph the same trend could be observed.

For *L. thermotolerans*, it could be seen that there was a slight decrease in viable cell counts when less oxygen was provided and that this decrease was greater in mixed cultures. Here, a loss in viability was observed as early as 60 h into the fermentations and these also obtained the lowest maximum CFU/mL. Although lower colony counts were observed in mixed cultures and when less oxygen was provided for *S. cerevisiae*, once again, this yeast was largely unaffected not only by the presence of *L. thermotolerans*, but also a reduced oxygen availability.

As mentioned above, it was hard to draw conclusions based on the data for ethanol production (Table 4.3.1.) as there were variation between repeats and no clear trends could be observed. Even though all fermentations fermented to or close to dryness, the ethanol levels were somewhat lower than expected. This stresses the fact that the SCB system and our specific experimental layout should be tested more vigorously and repeated to exclude variation. Overall, there was a slight reduction in glycerol for mixed cultures and *S. cerevisiae* pure cultures when less oxygen was provided, while the opposite was observed for *L. thermotolerans* pure cultures. Brandam et al. (2013) saw a significant reduction in glycerol production for *T. delbrueckii* when less oxygen was supplied and this could be attributed to the fact that respiration was favoured to generate NAD^+. However, these authors supplied oxygen throughout the fermentations, while our study made use of oxygen pulses. Our approach (oxygen pulses) never resulted in glycerol levels lower than the amount needed to detect an increase in wine sweetness (Noble and Bursick 1984). Our study and Brandam et al. (2013) saw no significant changes in acetic acid production when less oxygen was provided.

The production of volatile compounds was determined (Figure 4.3.7. and 4.3.8.) and for some, there were small changes in response to less oxygen availability. However, it is difficult to draw clear conclusions since there is some variation in the data. For higher alcohols, overall there was either a small reduction or no change in the specific compound with less oxygen. The same trend was observed for small chain fatty acids (Figure 4.3.8). Here, most of the changes were observed for the *S. cerevisiae* pure cultures. Medium chain fatty acids seemed to be largely unaffected.

4.5. Conclusions

With the use of an SCB, an interaction mechanism between *S. cerevisiae* and *L. thermotolerans* in mixed culture fermentations could be confirmed. In this system, it was

hard to conclude what kind of interaction was taking place. Nevertheless, the data indicated that not only was *L. thermotolerans*' growth greatly affected by that of *S. cerevisiae*'s presence, but that *S. cerevisiae* was also negatively affected by the presence of *L. thermotolerans*. As a result of this interaction, we observed a slight decrease in glycerol and increase in acetic acid in mixed cultures, but further experiments testing the effect of oxygen on the behavior of the yeast are required to confirm this. Furthermore, it was observed that the interaction had a slight impact on the production of volatile compounds, with propanol being increased significantly in the mixed cultures (compared to pure cultures).

A reduced oxygen availability greatly affected the growth of the yeast. A reduction in biomass and increase in fermentation duration was observed for all fermentations, but interestingly, the mixed cultures appeared to be less affected by these changes. This was attributed to the combined effect of *L. thermotolerans*' increased ability to compete for nutrients in the presence of oxygen and the fact that *S. cerevisiae*'s presence induced cell death in *L. thermotolerans*. In doing so, it provided the former yeast with extra nutrients and allowed it to last longer even though less oxygen was available. With less oxygen, a reduction in glycerol and no significant change in acetic acid were observed. Slight reductions in some higher alcohols and small chain fatty acids could also be observed.

These interactions and the effect of dissolved oxygen on the growth of the yeast have a clear impact on the fermentation dynamics, but the effect is less clear on the production of secondary compounds. Further experiments are needed to confirm the results obtained in our study.

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Chapter 5

General Discussion and Conclusions

Chapter 5 – General discussion and conclusions

5.1. General discussion

Grape must is characterized by the presence of many species of bacteria, yeast and filamentous fungi, and by the interactions between these organisms within this complex ecosystem. Many of these organisms contribute to alcoholic fermentation and ultimately, wine flavour and aroma, creating a need to study the ecosystem and to better understand how interactions between organisms impact on oenologically relevant features (Fleet 2008). Within this context, a recent global research focus has been on the application of non-*Saccharomyces* yeasts in wine fermentation, and some interactions between some specific species and the wine yeast *S. cerevisiae* have been described (Albergaria et al. 2010; Bely et al. 2008; Ciani et al. 2006; Pérez-Nevado et al. 2006). Nissen et al. (2003) were the first to hypothesize that a physical cell-cell interaction occurs between *S. cerevisiae* and *T. delbrueckii* and/or *L. thermotolerans*, but were unable to confirm this due to experimental constraints. Recently, Renault et al. (2013) confirmed it for *S. cerevisiae* and *T. delbrueckii* and also observed that even though *T. delbrueckii* was outcompeted and seized growth by the end of fermentation, the growth of *S. cerevisiae* was somewhat delayed by the remaining metabolic activity of *T. delbrueckii*. This study confirmed a physical cell-cell interaction between *S. cerevisiae* and *L. thermotolerans* (Chapter 3). The exclusion of physical interaction revealed the presence of metabolic interaction as well. While the metabolic interaction led to a reduction in biomass in both yeasts, both interactions led to a loss in viability in *L. thermotolerans*. However, this loss in viability was greater in *L. thermotolerans* when it was in physical contact with *S. cerevisiae*. Since no significant loss in viability was observed for S*. cerevisiae* in all mixed cultures, together, this suggests that while *S. cerevisiae* impacts greatly on the survival of *L. thermotolerans*' growth and survival throughout fermentation, the metabolism of *L. thermotolerans* also has an impact on the accumulation of biomass in *S. cerevisiae*. While it was not possible to align the data sets between the DCB and SCB fermentations, some similar trends were observed. The interaction trend described above was also observed in the SCB fermentations (Chapter 4) where *S. cerevisiae* reached a lower maximum CFU/mL on WL plates in mixed cultures compared to its pure cultures, but still maintained a high viability until the end of fermentation. *L. thermotolerans* on the other hand, saw a significant reduction in maximum CFU/mL between pure and mixed cultures. As mentioned before, the data sets from both systems could not be aligned. This was particularly striking for the OD data for *L. thermotolerans* (which was significantly lower in the SCB compared to the DCB). From this it is important to note how sensitive the yeast were to

changes in experimental layout and because the effect of oxygen pulses have not been reported before, it is impossible to conclude what parameter was the key factor in causing these observed differences. Together, our data suggest that while the metabolic activity of both yeasts does impact on the growth of the other yeast, the physical presence of a high concentration of *S. cerevisiae* cells results in low survival and cell lysis of *L. thermotolerans* and consequently, may provide an extra source of nutrients to *S. cerevisiae*. In this study, we also investigated the impact of oxygen, supplied in short pulses, on the yeast's growth. The effect of oxygen has not been studied before and the data show that the yeasts were quite sensitive to these changes. This can be seen in the analytical data for ethanol, acetic acid, glycerol and volatile compounds where variation between repeats were observed. Therefore, it was difficult to establish the actual impact of these interactions on the production of ethanol and other secondary compounds. It was observed that physical interaction might stimulate glycerol and propanol production, but more repeats should be performed to confirm or disprove this. Nevertheless, increases in propanol have been observed when *L. thermotolerans* (Gobbi et al. 2013) and *P. fermentans* (Clemente-Jimenez et al. 2005) were fermented sequentially with *S. cerevisiae*. Furthermore, for most other relevant volatile compounds, the mixed cultures showed intermediate values compared to the two single species cultures, suggesting a balanced contribution to the production of such compounds.

The data clearly confirmed that one of the most important factors impacting on yeast growth was the availability of oxygen. Some early studies already highlighted its effect on the survival of non-*Saccharomyces* yeasts (Hansen et al. 2001; Nissen et al. 2004). Since our data in the DCB confirmed that *L. thermotolerans* was sensitive to changes in oxygen concentration, such changes were studied in more detail in the SCB. Reducing the frequency at which oxygen pulses were administered (twice a day, once a day and once at the beginning of fermentation) impacted greatly on biomass production and fermentation duration in all fermentations. In all pure cultures a significant drop in biomass production and a longer fermentation duration were observed. However, this effect seemed to be reduced in the mixed culture fermentations. Nissen et al. (2004) observed that oxygen increased *L. thermotolerans'* ability to compete for nutrients with *S. cerevisiae* and that the former had a higher oxygen requirement compared to the latter and indeed, this was confirmed in the plate count data where a significant drop in maximum CFU/mL in mixed cultures was observed for *L. thermotolerans* when less oxygen was provided. However, taking this into account and the fact that a cell-cell interaction with *S. cerevisiae* induces *L. thermotolerans*' death, it might further explain why mixed cultures were less affected by the reduced oxygen availability. The combined effect of this could have led to the death of *L. thermotolerans* in the mixed cultures, providing *S. cerevisiae* with an extra source of nutrients

and facilitating its ability to survive longer throughout fermentations, even though less oxygen was provided. Once again, we observed differences between repeats in the analytical data for these experiments, highlighting the sensitivity of the yeasts to these changes in dissolved oxygen and the fact that the impact of oxygen needs to be studied more extensively. Nevertheless, a slight reduction in glycerol was observed for *S. cerevisiae* pure cultures and mixed cultures and a slight increase for *L. thermotolerans* pure cultures. This was also observed in *T. delbrueckii* pure cultures when more oxygen was provided and it was attributed to the fact that the yeasts favoured respiration to generate NAD^+ (Brandam et al. 2013). However, the latter study made use of constant aeration as opposed to oxygen pulses (which never saw glycerol levels lower than the amount needed to detect an increase in sweetness in wine (Noble and Bursick 1984)). The current study and Brandam et al. (2013) also saw no significant changes in acetic acid production when less oxygen was supplied. The volatile data suggests that some small changes occurred in response to less oxygen, but it remains difficult to make clear conclusions since there was some variation between repeats. In general, there was either a small reduction or no change for higher alcohols and small chain fatty acids and medium chain fatty acids seemed largely unaffected by changes in oxygen availability.

5.2. Conclusions

The data show the importance of physical cell-cell interaction between *S. cerevisiae* and *L. thermotolerans* in mixed culture fermentations, leading to increased cell death in the latter. There is also evidence of metabolic interactions between both yeasts, and while the impact of this seems greater on *L. thermotolerans,* it clearly also impacts on the growth of *S. cerevisiae*. These interactions also stimulate the production of some secondary compounds. The dissolved oxygen concentration in mixed culture fermentations impacts greatly on biomass production and fermentation duration in both yeasts and especially on the ability of *L. thermotolerans* to remain viable until the end of fermentation and compete for nutrients with *S. cerevisiae*. In fact, a lowered oxygen availability leads to a greater loss in viability and an earlier death in *L. thermotolerans.* While the impact of this on the production of ethanol and secondary compounds remains elusive, some data suggests that these types of oxygen pulses might be a useful tool in facilitating a non-*Saccharomyces* yeast's ability to survive longer in the fermentation and therefore, significantly contribute to wine flavour and aroma, whilst not increasing the production of undesirable compounds, such as acetic acid.

5.3. Future work

From this work and previous studies, it has become clear that the direct and indirect interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts in multistarter fermentations cannot be ignored (Nissen et al. 2003, 2004; Renault et al. 2013). The DCB system proved to be a useful tool in facilitating the study of such interactions. However, this is only the second study, (aimed at studying wine yeast interactions) that has made use of this specific system which is not distributed widely. There is therefore no standardized system between laboratories/institutions which makes comparisons of data difficult. Future interaction studies should make use of other yeast pairs than those mentioned in this study to specifically investigate indirect and direct interactions between them. It would also be of value for a company to create such a DCB system that can be standardized (like the SCBs of New Brunswick®). These studies should also focus on the effect of these interactions on gene expression, since no previous work has done so. Since we could not align the data between the DCB and SCB systems, this has also stressed the fact that these yeasts are very sensitive to small changes in experimental layout. This is something future work should take into consideration.

Furthermore, these studies should also investigate other contributing factors (such as dissolved oxygen) that are mentioned in the current study. This work should follow up on the work of Hansen et al. (2001) to explore the impact of different dissolved oxygen concentrations on the way non-*Saccharomyces* yeasts interact with *S. cerevisiae* and the consequent effect on their survival ability.

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