Evaluating the effect of oxygen addition on yeast physiology, population dynamics and wine chemical signature in controlled mixed starter fermentations

by

Kirti Shekhawat

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Supervisor: Dr ME Setati
Co-supervisor: Prof FF Bauer

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Declaration

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Summary

The use of commercial starter cultures of non-*Saccharomyces* yeast, usually together with *Saccharomyces cerevisiae*, has become a trend in the global wine industry in the past decade. Depending on the specific species of non-*Saccharomyces* yeast, the procedure may aim at enhancing aroma and flavour complexity of the wine, reduce acetic acid levels, and/or lower the ethanol yield. However, the contribution of non-*Saccharomyces* yeast strains depends on several factors, and in particular on the strains ability to establish significant biomass and to persist for a sufficient period of time in the fermentation ecosystem. For an effective use of these yeasts, it is therefore important to understand the environmental factors that modulate the population dynamics of such environments. In this study, we evaluated the effect of oxygen addition on yeast physiology, population dynamics and wine chemical signature in controlled mixed starter fermentations. The population dynamic in co-fermentations of *S. cerevisiae* and three non-*Saccharomyces* yeast species namely, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima*, revealed that oxygen availability strongly influences the population dynamics and chemical profile of wine. However, results showed clear species-dependent differences. Further, experiments were confirmed in Chardonnay Grape juice, inoculated with *L. thermotolerans* and *S. cerevisiae* with different oxygen regimes. The results showed a trend similar to those obtained in synthetic grape juice, with a positive effect of oxygen on the relative performance of *L. thermotolerans*. The results in this study also indicates that continuous stirring supports the growth of *L. thermotolerans*.

We further analysed the transcriptomic signature of *L. thermotolerans* and *S. cerevisiae* in single and mixed species fermentations in aerobic and anaerobic conditions. The data suggest the nature of the metabolic interactions between the yeast species, and suggests that specific stress factors are more prominent in mixed fermentations. Both yeasts showed higher transcript levels of genes whose expression is likely linked to the competition for certain metabolites (copper, sulfur and thiamine), and for genes involved in cell wall integrity. Moreover, the transcriptomic data also aligned with exo-metabolomic data of mixed fermentation by showing higher transcripts for genes involved in the formation of aroma compounds found in increased concentration in the final wine. Furthermore, the comparative transcriptomics analysis of the response of the yeasts to oxygen provides some insights into differences of the physiology of *L. thermotolerans* and *S. cerevisiae*. A limited proteomic data set aligned well with the transcriptomic data and in particular confirmed a higher abundance of proteins involved in central carbon metabolism and stress conditions in mixed fermentation.

Overall, the results highlight the role of oxygen in regulating the succession of yeasts during wine fermentations and its impact on yeasts physiology. The transcriptomics data clearly showed metabolic interaction between both yeasts in such ecosystem and provide novel insights into the adaptive responses of *L. thermotolerans* and *S. cerevisiae* to oxygen availability and to the presence of the other species.

This dissertation is dedicated to my Papa Ji

Biographical sketch

Kirti Shekhawat was born in Khetri Nagar, Rajasthan, India on 26 March 1988. She completed her high school in Biology, Chemistry and Physics at Rajasthan Senior Secondary High School, Khetri Nagar. She pursued a Bachelor of Science degree in Biotechnology from Kurukshetra University, Kurukshetra. She obtained her Master's degree in Agricultural Microbiology in 2010 from Haryana Agricultural University. After her Master's she gained one year experience as a senior researcher in Central Soil Salinity Research Institute, Karnal. In 2012, she was accepted as PhD student under the supervision of Dr Evodia Setati and Prof Florian Bauer at the Institute for Wine Biotechnology, Stellenbosch University.

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Preface

This dissertation is presented as a compilation of seven chapters and two appendixes

Chapter 1	**General Introduction and project aims**
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Chapter 1

Introduction and project aims

1.1 Introduction

Natural alcoholic wine fermentation involves a continuous succession of yeast species. In this process, many non-*Saccharomyces* yeast species that vary between grape musts dominate the early stages of fermentation, while *Saccharomyces* species, predominantly *S. cerevisiae* generally dominate the later stage of fermentation. Yeast succession is governed by several factors such as ethanol concentration, toxic secondary metabolites, temperature, pH, physical contact of yeast cells and rapid development of anaerobiosis (Fleet 2003; Wang et al., 2016). For the past decades, most wine fermentations globally are inoculated with specific strains of *S. cerevisiae*, ensuring early dominance by this species and providing a level of predictability to the process. However, several non-*Saccharomyces* yeast species have more recently been commercialized for co-inoculations with *S. cerevisiae*. Such mixed fermentations have become a growing trend in the wine industry because they are considered to offer various opportunities, including improving wine sensorial properties, reducing ethanol yields or diminishing the levels of volatile acidity, depending on the co-inoculated species. The positive contribution of non-*Saccharomyces* yeasts such as *Lachancea thermotolerans*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* has been scrutinized in the analytical profiles of wines (Albergaria and Arneborg 2016; Ciani et al., 2016; Masneuf-Pomarede et al., 2016). Although these yeasts can contribute positively to wine quality, their contribution may be restricted by factors that inhibit their growth or metabolic activity such as high ethanol concentration, interactions with other species and low oxygen levels (Ciani et al., 2016). However, some winemaking practices may allow wine makers to influence the population dynamics between co-inoculated species, and therefore modulate the contribution of individual species to the final wine character. One such practice is oxygen management, which is applied in different ways at several steps particularly in red wine making. Typically, oxygenation is employed to extract color and phenolics, as well as to stimulate yeast growth and biomass formation in the early stages of fermentation, but also throughout the fermentation process to avoid sluggish fermentations. Oxygen addition, therefore, may be a promising tool to modulate co-fermentations, since the many of the non-*Saccharomyces* wine yeast species display higher oxygen demands than *S. cerevisiae*. Indeed, a few studies have shown that oxygenation can improve the growth and persistence of wine yeasts such as *Torulaspora delbrueckii* and *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*). Oxygen can be provided through practices such as pumping over, topping up and racking (Hansen et al., Moenne et al., 2014). Recently, Luyt (2015) demonstrated that even at small dosages e.g. 30 min oxygen pulses once or twice a day can extend the viability of *L. thermotolerans* during wine fermentation.

The incorporation of oxygen in wine fermentation and its impact on wine chemical composition and quality, as well as on yeast physiology is reasonably understood (Ciani et al., 2016a; Morales et al., 2015; Moenne et al., 2014; Verbelen et al., 2009). However, regarding yeast physiology, previous studies have primarily focused on *S. cerevisiae*, and have generated valuable insights on how this yeast regulates gene expression and adjusts its metabolism as a function of oxygen availability. *S.*

cerevisiae meets the energy demand either using fermentation, respiration or both (Aceituno et al., 2012). Besides the central carbon metabolism, oxygen availability also influences synthesis of ergosterol and unsaturated fatty acids, proline uptake, heme synthesis (Rosenfeld et al., 2003; Aceituno et al., 2012). However, the impact of oxygen provision on non-*Saccharomyces* yeasts has been studied only in a few genera such as *Pichia*, *Kluyveromyces lactis* and requires more understanding on other non-*Saccharomyces* yeasts.

Due to positive contribution of non-*Saccharomyces* yeasts to wine aroma, some of non-*Saccharomyces* such as strains of *M. pulcherrima*, *T. delbrueckii* and *L. thermotolerans* have been commercialized. Although these studies provide importance of non-*Saccharomyces* yeasts in mixed fermentation, a number of important characteristics remain unclear. In this regard, in the fermentation ecosystem, these non-*Saccharomyces* yeasts interact with *S. cerevisiae* in various ways but the mechanisms underlying these interactions still remain blurred and requires further investigation. Such studies are challenging because of the complexity of mixed culture fermentations and of ecological interactions. Recently, the development of novel high-throughput DNA sequencing techniques has provided a new method for quantifying transcriptomes. This method, called RNA-Seq (RNA sequencing), has clear advantages over existing approaches such as microarray. One particularly powerful advantage of RNA-Seq is that it can capture transcriptome dynamics across different conditions and determines RNA expression levels more accurately than microarrays (Wang et al., 2009). This technique offers researchers a great opportunity to investigate microbial interactions in complex mixed culture fermentation on a molecular level. Such molecular techniques can provide the knowledge of mechanisms involved in yeasts adaptation to oxygen availability and mixed fermentation under winemaking conditions. Such knowledge of the mechanisms involved in response to oxygen and yeast-yeast interaction at the molecular level is essential in order to control the mixed culture fermentations better. In current dissertation, we sought to understand the effect of oxygenation on growth of three non-*Saccharomyces* yeasts in mixed fermentations with *S. cerevisiae*. We further unravel the interaction between *Lachancea thermotolerans* and *S. cerevisiae* at the molecular level using RNA-seq. We used *L. thermotolerans* as non-*Saccharomyces* yeast in mixed fermentation with *S. cerevisiae* as that yeast has already been commercialised and the genome of this yeast has been sequenced and has been partially annotated. Furthermore, previous studies have shown that *L. thermotolerans* and *S. cerevisiae* show metabolic interactions, but that direct physical contact also impacted on the growth of the two species and played an important role in the ecologic interaction of the two species. (Luyt, 2015; Nissen et al., 2003). Such interactions have not been characterised at the molecular level.

1.2 Project aims

The overall aims of this study are therefor to characterize the impact of oxygen on yeast growth and volatile compounds production in single and mixed species wine fermentation, while also providing insights into yeast-yeast interaction at the molecular level (*Saccharomyces cerevisiae* and *Lachancea thermotolerans*) in mixed and single culture fermentation. To achieve this, four objectives were set as follows:

1.1 To assess the impact of oxygenation on the growth of three non-*Saccharomyces* yeast species and volatile compounds profile in mixed wine fermentation with *Saccharomyces cerevisiae.*

1.2 To evaluate the effect of oxygen pulses on yeast growth and aroma profile of inoculated Chardonnay grape must.

1.3 To investigate the transcriptional response of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed and single species fermentation and to assess the impact of oxygen on these molecular responses.

1.4 To analyze the proteome of mixed and single fermentation of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* under aerobic and anaerobic conditions.

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Chapter 2

Literature review

Wine fermentation and oxygenation: Influence on yeast physiology and population dynamics

Wine fermentation and oxygenation: Influence on yeast physiology and population dynamics

2.1 Introduction

Oxygen is an important environmental parameter in winemaking as it can have both negative and positive effects on wine quality. In standard winemaking procedures, grape juice is naturally exposed to oxygen during crushing and pressing. However, due to the risk of oxidation, especially in white winemaking, it is common practice to use dry ice or nitrogen gas blanketing to protect the juice during crushing, pressing and juice transfer. On the contrary, oxygen addition in red winemaking is standard practice. For instance, oxygen is regularly introduced to fermenting musts through pump-overs, punch-downs, and délestage (Moenne et al., 2015; du Toit et al., 2006; Sacchi et al., 2005). This is mainly done to enhance extraction of phenolic compounds and to promote yeast viability.

Oxygen is a key factor in sugar metabolism of yeast as it is an electron acceptor in the generation of energy via mitochondrial respiration. Moreover, oxygen is also required in several biosynthetic pathways, such as those for heme, sterols, unsaturated fatty acids, pyrimidines, and deoxyribonucleotides. Under anaerobic conditions, yeast can import sterols and unsaturated fatty acids from the growth medium and employ alternative pathways to synthesize other molecules required for growth and viability (Ingledew et al., 1987; Rosenfeld et al., 2003). However, studies investigating the effects of oxygen on the metabolism of facultative anaerobes, including the Crabtree positive yeast *Saccharomyces cerevisiae* (the main fermentation driver) and some Crabtree negative non-*Saccharomyces* yeasts such as *Wickerhamomyces anomalus* (formerly *Pichia anomala*), *Komagataella pastoris* (formerly *Pichia pastoris*), and *Scheffersomyces stipitis* (formerly *Pichia stipitis*), show that the adaptive responses of yeasts to oxygen availability can be quite diverse (Bauman et al., 2011; Walker 2011; Cho and Jeffries 1999; Orellana et al., 2014). These responses are evident in both the primary and secondary metabolism of yeasts. In the case of primary metabolism, the availability of oxygen mainly influences the central carbon metabolism and depending on the Crabtree nature of species, yeast may redirect metabolism towards fermentation, respiration and respiro-fermentation to meet the energy demand. The differential gene expression as a function of oxygen is well studied for *S. cerevisiae*; the genes that encode enzymes involved in heme synthesis, TCA cycle and electron transport are known to be up-regulated in the presence of oxygen. Under anaerobic conditions, genes that participate in the fermentation process and in glycerol production are up-regulated to satisfy energy demand and to maintain the intracellular redox balance (Aceituno et al., 2012; Rosenfeld et al., 2003).

The secondary metabolism and the production of yeast-derived wine aroma compounds are also influenced by oxygen availability. For example, the production of higher alcohols (2-phenylethanol, isobutanol, isoamyl alcohol) and some acetate esters (ethyl acetate, ethyl lactate) is well known to be influenced by the presence of oxygen (Valero et al., 2002). Likewise the expression pattern of some of the genes encoding metabolic enzymes for these compounds is also well known to be influenced by the availability of oxygen. In the presence of oxygen, higher expression was reported for genes that encode alcohol dehydrogenases (*AHD1*), pyruvate decarboxylases (*PDC1*) and amino acid permeases (*BAP2*). Similarly, down-regulation was reported for *ATF1* gene that encodes the acetyl transferase enzyme involved in the formation of esters (Walker et al., 2011; Fujiwara et al., 1998; Verbelen et al., 2009).

Yeasts are major contributors to wine chemical composition as they drive the alcoholic fermentation, a complex biochemical process in which grape constituents are converted to CO_2, ethanol and a broad diversity of by-products derived from the yeast secondary metabolism. The wine yeast consortium comprises several yeast species expressing different metabolic capabilities and different oxygen requirements. Depending on their abundance and persistence, the individual species contribute to the aroma and flavour complexity of wine to varying intensities. Several studies have shown that when inoculated at a high dosage in grape juice, non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima* result in the increased levels of glycerol, esters, and higher alcohols, while reduced levels of volatile acidity and ethanol are observed. Furthermore, oxygen addition supports the growth and promote the persistence of most non-*Saccharomyces* yeasts (Ciani et al., 2016). Although there is no information on the gene expression patterns during wine fermentation with these yeasts, it is clear from their behaviour and chemical contributions in mixed culture fermentations with *S. cerevisiae*, that their transcriptional profiles and metabolomes are quite distinct. This review aims to highlight the importance of oxygen in winemaking, yeast physiology and population dynamics, with emphasis on data obtained on *S. cerevisiae*.

2.2 Oxygen addition in standard winemaking practices

The grape juice is naturally exposed to oxygen, starting at crushing and pressing. In addition, oxygen may be added intentionally throughout the fermentation, especially in red wine making to enhance fermentation efficiency and stabilize wine color. Typically, at the beginning of the vinification process, the juice is exposed to oxygen in order to optimize yeast biomass production (du Toit et al., 2006; Rosenfeld et al., 2003). Methods employed by winemakers for the addition of oxygen during fermentation include punch-downs, pump-overs and/or délestage and racking (two-step “rack-and-return” process) (Sacchi et al., 2005). There are several reasons for using these methods i.e. to provide oxygen to yeast cells to start the fermentation process (also if the fermentation is stuck), to

submerge the skins so that carbon dioxide is pushed to the surface of the juice and released, and to facilitate extraction of color and flavour. Among these methods, pump-over is the most commonly used method in winemaking. The amounts of oxygen entering the system by different methods, depends on factors such as the temperature and composition of must, the concentration of solids, and the mixing provided by the bubbles of CO_2 produced by the yeast cells (Moenne et al., 2014; Singleton 1987). For instance, pumping over adds about 2 mg L^{-1} of oxygen; while other methods like, a transfer from tank to tank achieves up to 6 mg L^{-1}, filtration 4-7 mg L^{-1}, racking 3-5 mg L^{-1} of oxygen, respectively (Boulton et al., 1996). This addition of oxygen at various stages of fermentation affects the metabolic activities of the yeats, population dynamics, fermentation kinetics as well as the chemical composition of the final wine.

2.3 Effect of oxygen on yeast physiology

Yeasts are classified into three main groups based on their metabolic behaviour and their dependence on oxygen: (i) obligate aerobes (ii) facultative anaerobes and (iii) obligate anaerobes (Rosenfeld et al., 2003). The facultative anaerobes display both respiratory and fermentative metabolism. Yeasts are well-known to redirect their metabolism according to oxygen availability in order to generate energy. Under aerobic conditions, yeasts generate energy through respiration and produce CO_2 and biomass. In contrast, under anaerobic growth, the energy supply is supported by fermentative process, resulting in the production of CO_2, ethanol and biomass (Fig 2.1). Facultative anaerobes comprise two groups, Crabtree positive and Crabtree negative, based on their ability to perform aerobic fermentation. As illustrated in Fig 2.1, under aerobic conditions with high sugar level, Crabtree positive yeasts can generate energy via oxidative phosphorylation and fermentative process, while Crabtree negative yeasts follow only oxidative phosphorylation (Aceituno et al., 2012; Hagman and Piskur 2015).

Oxygen is not only a key factor in the regulation of sugar metabolism in yeasts, but it is also required in several biosynthetic pathways, such as those for heme, sterols, unsaturated fatty acids, pyrimidines and deoxyribonucleotides (Fig. 2.1). Consequently, the expression of a significant number of yeasts genes, is regulated by oxygen levels.

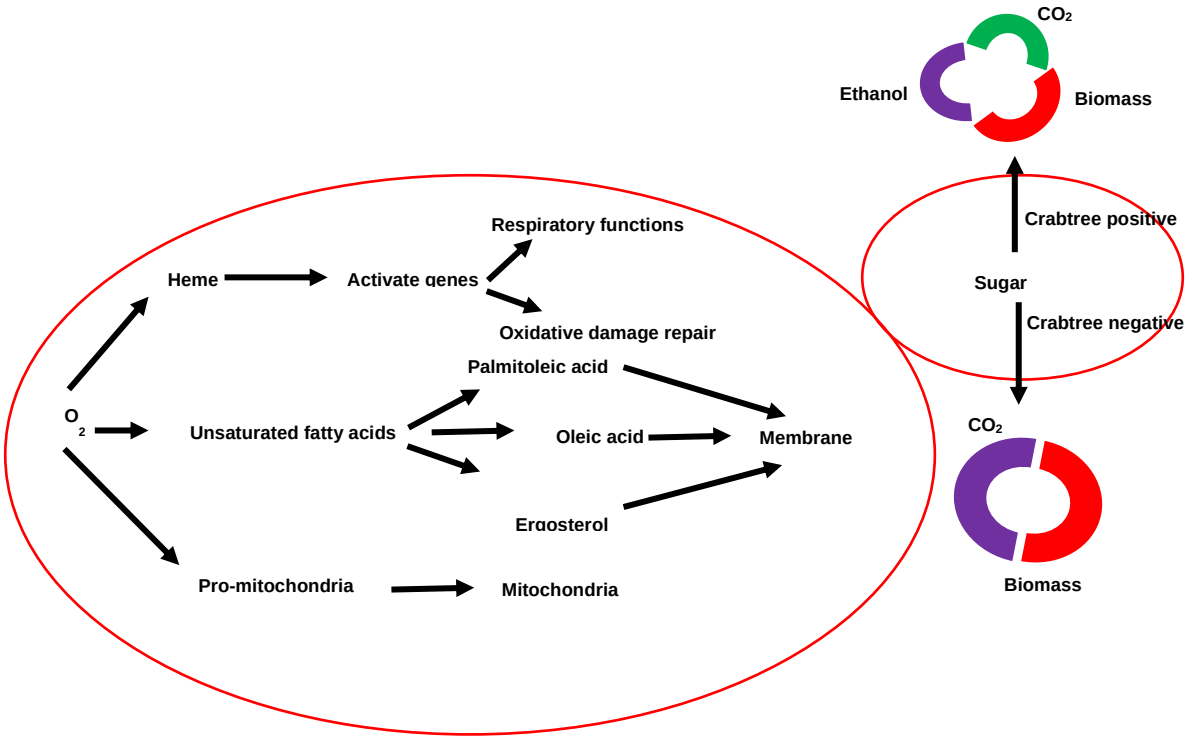


Figure 2.1: A general overview of impact of oxygen provision of yeast physiology

2.3.1 Oxygen sensing and gene regulation in response to oxygen

The mechanism of oxygen sensing and influence on gene expression has been mainly described in *S. cerevisiae*. This yeast adapts to oxygen availability by changing the expression of many genes, called ‘‘aerobic and hypoxic genes’’, which encode enzymes involved in oxygen-related functions e.g. respiration, heme, lipid and cell wall biosynthesis. For the regulation of these genes, *S. cerevisiae* senses oxygen availability through cellular heme. As demonstrated in Fig 2.2, the synthesis of cellular heme takes place in the presence of oxygen, which is further detected by a transcriptional activator called *HAP1*; this transcriptional activator activates *ROX1* transcriptional repressor which represses anaerobic genes (Fig. 2.2). In anaerobiosis, about one-third of gene expression is known to be controlled by the Rox1p transcriptional repressor. This is achieved when the synthesis of oxygen-dependent heme decreases, which leads to decrease in Hap1p mediated activation of *ROX1* and decrease in expression of Rox1p and increase in the expression of genes responsible for hypoxic conditions. In *S. cerevisiae,* the expression of typical anaerobic signature genes (*DAN1-3 TIR1-4, PAU2,3,4,5,8,9,14,18*) is known to be regulated by *ROX1* transcriptional repressor (Cohen et al., 2001; Kwast et al., 1996; Rintala et al., 2009; Snoek et al., 2007).

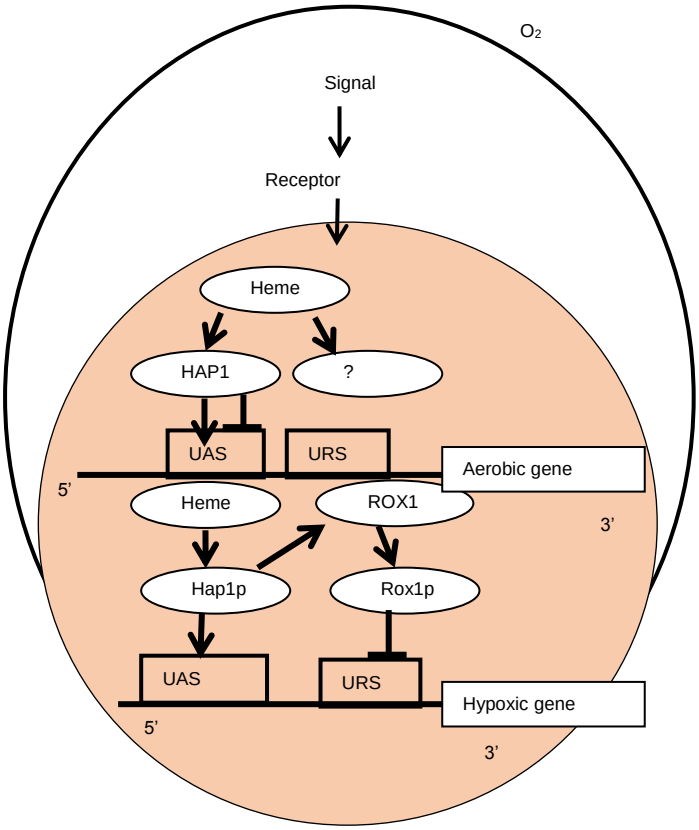
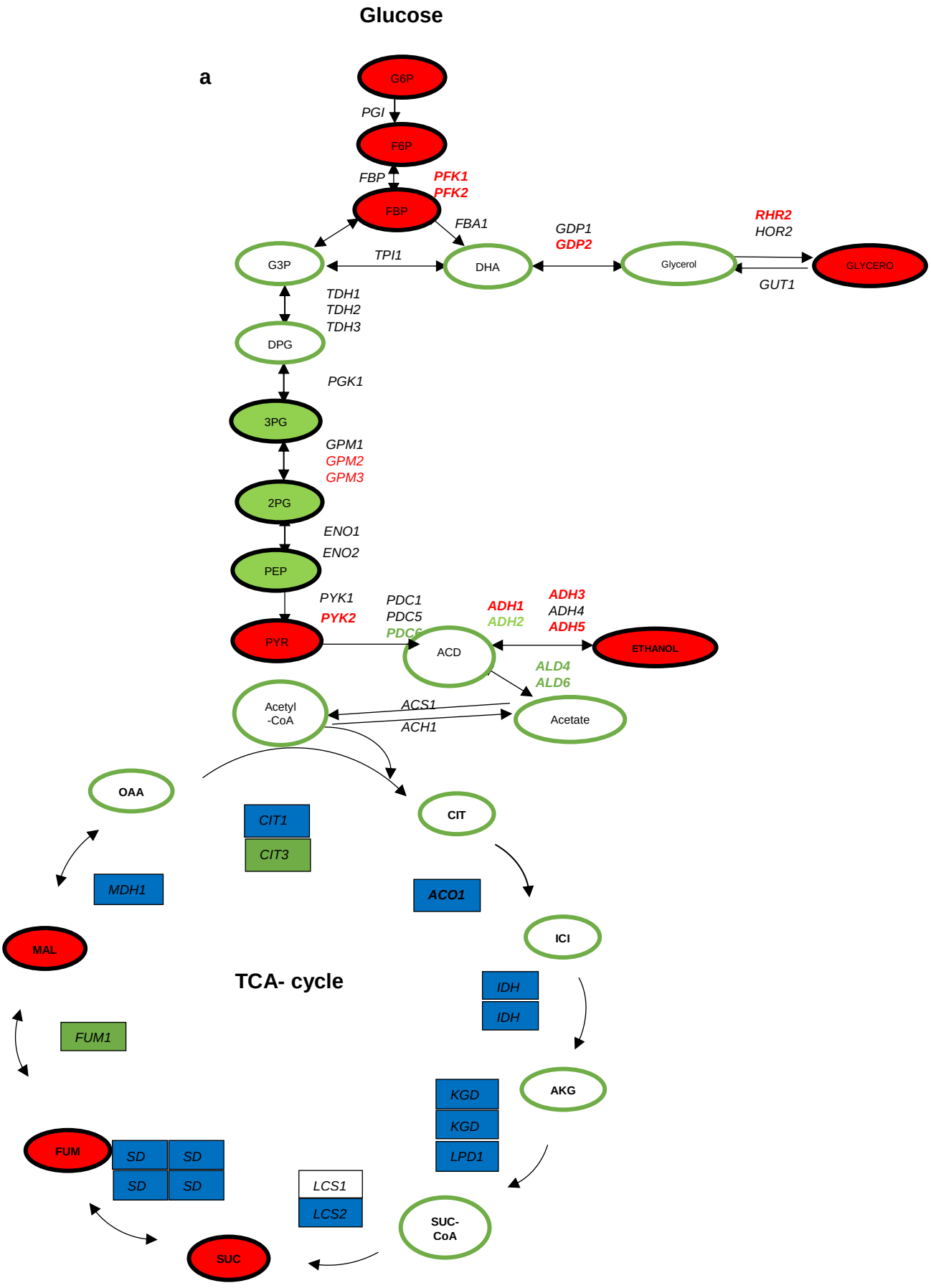


Figure 2.2: Oxygen sensing and regulation of genes in response to oxygen availability in *S. cerevisiae*

2.3.2 Effect of oxygen on central carbon metabolism

The central carbon metabolism is the core metabolism in the yeast and provides precursors for the biosynthesis of amino acids, fatty acids, reducing agents in the form of NAD(P)H, $FADH_2$ and energy. Oxygen availability largely affects central carbon metabolism. For example, under aerobic condition, the pyruvate produced from glycolysis is decarboxylated to enter the TCA cycle and generates NADH and $FADH_2$ which enters the respiratory chain. The respiratory chain synthesizes ATP by using electrons from NADH and $FADH_2$ with the help of an ATP synthase enzyme situated in the inner mitochondrial membrane. In the case of anaerobic conditions, yeast generates energy via fermentation process and produces CO_2 and ethanol from pyruvate (Aceituno et al., 2012). However, depending on nutrients and oxygen, yeast can manipulate its metabolism towards respiro-fermentation (Jouhten et al., 2008; Tai et al., 2005). This switch to different metabolism is accomplished with the help of a change in gene expression in response to oxygen availability. For the central carbon metabolism, in the absence of oxygen, higher expression has been reported for genes that encode enzymes of the TCA cycle and glycolytic pathway (genes highlighted in red colour Fig. 2.3a). Also, depending on the amount of oxygen, some genes that encode for enzymes of the TCA cycle also up-regulates in respiro-fermentation (genes highlighted in blue colour Fig. 2.3a). In contrast, under anaerobic conditions, genes responsible for encoding enzymes of fermentation process mainly up-regulates (*ADH1*, *ADH3*, *ADH5*, *GDP1*, *RHR1*) as demonstrated in Fig. 2.3a.



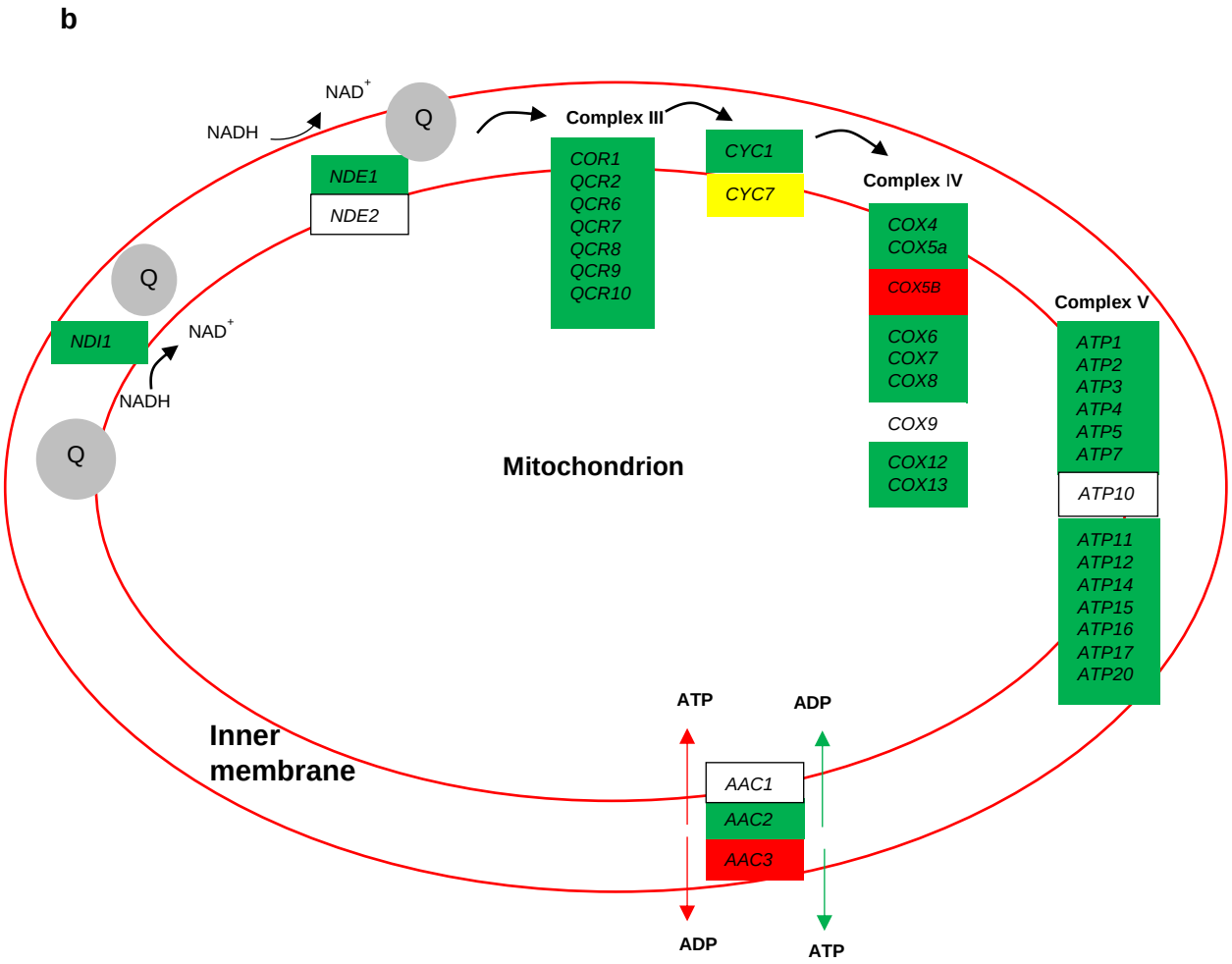


Figure 2.3: Schematic representation of differentially gene expression of central carbon metabolism under aerobic and anaerobic condition. The red color shows the highest expression under anaerobic conditions, green colour shows higher transcripts in respiration while blue color shows high expression in respiro-fermentation (Fig. 2.3a). In mitochondrion, green color shows higher transcripts, red shows down-regulation, in aerobic condition, yellow shows high expression in both, while white stays unchanged in aerobic as well as anaerobic conditions (Fig. 2.3b).

Similarly, expression of genes that encode enzymes of oxidative phosphorylation also changes in response to oxygen availability as presented in Fig. 2.3b. Apart from a few genes such as *COX5b*, *AAC3* (down-regulates), *CYC7* (induced in both), *NDE2, ATP10, COX9* and *AAC1* (does not change) the rest of the genes have been shown to be up-regulated under aerobic condition and down-regulated under anaerobic condition (Kwast et al., 2002; Rintala et al., 2009; Snoek et al., 2007).

2.3.3 Role of oxygen in unsaturated fatty acid metabolism

The biosynthesis of unsaturated fatty acids (UFA) plays an essential role in the lipid metabolism of yeast. The synthesis of unsaturated acids is an oxygen dependent mechanism; however, in the absence of oxygen yeast manages to grow by importing these sterols and unsaturated fatty acids from the medium by remodelling the cell wall (Rosenfeld et al., 2003). Under anaerobic conditions, *S. cerevisiae* forms a functional complex of fatty acid transport proteins (FATP) and a cognate long-chain acyl-CoA synthetase (ACSL) at the plasma membrane which helps in the transport and

activation of exogenous fatty acids (Concetta et al., 2005). In winemaking conditions, studies have shown that if oxygen is available in low concentration then it gets used for sterol synthesis. Rosenfeld et al., (2003) mentioned that when the respiratory chain is inhibited, approximately 40% of oxygen accounts for ergosterol biosynthesis. From the transcriptomics analysis, many studies done in *S. cerevisiae* under different conditions have shown higher expression of genes that encode enzymes which play a significant role in ergosterol biosynthesis in response to oxygen. In the presence of high oxygen concentration expression is reported for *ERG2, ERG3, ERG5, ERG6, ERG9, ERG10, ERG11, ERG13, ERG20* and *OLE1* genes (Abramova et al., 200; Klis et al., 2002; Kwast et al., 2002; Snoek et al., 2007). However, the response of UFAs pathway is also species dependent, for instance, in comparison to *S. cerevisiae*, the gene expression analysis of *K. pastoris* (*Pichia pastoris*) showed higher transcript levels of *ERG1, ERG3, ERG11* and *ERG25* genes in hypoxia, while *ERG27, ERG6* and *ERG4* were down-regulated. In contrast, studies on *S. cerevisiae* reported down-regulation of these genes under anaerobic conditions (Baumann et al., 2011).

2.3.4 Oxygen and amino acid utilization

Amino acids are the key nitrogen source in yeast. Yeast can synthesize and assimilate most of the amino acids needed to build cellular proteins. Few studies have shown the effect of oxygen on amino acid utilization. For instance, among amino acids, proline is one of the main nitrogen source in grape juice (20% of total nitrogen), which is utilized by the yeast only in the presence of oxygen via *PUT1* gene encoding protein. Put1p is a membrane bound FAD-dependent proline oxidase enzyme, and this enzyme stops working in anaerobic conditions, since membrane bound FAD is not available. The higher fermentation activity due to oxygen addition (in sluggish fermentation) is also attributed to proline uptake (Orellana et al., 2014; Rosenfeld et al., 2003). Similarly, the higher transcript level of *BAP2* (gene involved in uptake of branched chain amino acids) has been observed in the presence of oxygen in *S. cerevisiae* (Fujiwara et al., 1998; Verbelen et al., 2009).

2.3.5 Cell wall remodelling under anaerobic conditions

The cell wall of yeast is a stiff structure which determines the cell morphology and serves as a protective barrier by providing a mechanical shield and enabling selective uptake of macro molecules. Under anaerobiosis, yeast remodels the cell wall in order to adapt to such conditions. This remodelling action is mainly due to change in expression of genes encoding proteins involved in lipid synthesis, protein secretion and vesicle trafficking (Cohen et al., 2001). A large number of genes that are up-regulated under anaerobic condition are cell wall associated genes. For example, higher expression of nearly the entire seripauperin encoding (*PAU*) gene family has been shown under anoxia. The higher expression of *PAU* gene family (*PAU1*, *PAU2*, *PAU5*, *PAU6*, *PAU7*) is known to help the yeast to thrive under anaerobic conditions, to maintain the cell wall integrity (Abramova et al., 2001, Luo et al., 2009). Recently studies have also shown higher expression of *PAU5* gene in response to the killer activity of another yeast (Rivero et al., 2015). Furthermore, the

DAN/TIR (DAN1, DAN2, DAN3, DAN4, TIR1, TIR2, TIR3 and TIR4) genes are known to encode nine cell wall mannoproteins in *S. cerevisiae* which are highly expressed in anaerobically grown cells while the major cell wall proteins encoding genes *CWP1* and *CWP2* are up-regulated in aerobic conditions. The exact role of these genes is unknown; however, it is expected that change in the expression of these genes could perhaps influence the cell wall porosity and membrane fluidity under anaerobiosis. The regulation of all these genes involved in cell wall remodelling is assisted by *ROX1* transcriptional repressor (Cohen et al., 2001).

2.3.6 Oxygen and fermentation metabolites

Oxygen availability also influences the synthesis of metabolic products of fermentation such as fusel alcohols, medium chain fatty acids and esters. Valero et al. (2002) compared the concentration of higher alcohols and esters in oxygenated and non-oxygenated grape must. This study showed that pre-oxygenation of grape juice before the fermentation results in higher concentration of higher alcohols. Incorporation of oxygen during the fermentation process also leads to high concentration of higher alcohols such as, Isoamyl-alcohol and 2-phenylethanol; while a decrease in concentration of some esters such as ethyl acetate, isoamyl acetate and medium chain fatty acids (Verbelen et al., 2009). The addition of oxygen has been shown to influence expression of genes accountable for encoding enzymes involved in sysnthesis of these secondary metabolites. The synthesis of higher alcohols takes place using branched chain amino acids (leucine, isoleucine and valine) via the Ehrlich pathway. Studies have shown higher expression of genes that encode for branched chain amino acid permeases such as *BAP2,* and pyruvate decarboxylases *PDC5* in the presence of oxygen (Verbelen et al., 2009). Esters are important secondary metabolites in yeast which contribute to aroma profile of wine (Swiegers et al., 2005). The synthesis of acetate esters is catalysed by the enzyme called alcohol acetyltransferases and encoded by *ATF1* and *ATF2*. These are membrane-bound enzymes responsible for the synthesis of esters using higher alcohols and acetyl-CoA as substrates (Sumby et al., 2010). The expression of these genes under enological conditions in response to oxygen has been reported in *S. cerevisiae*. It has been proposed that oxygen addition leads to increase in the unsaturated fatty acid content and this can result in the inhibition of enzymatic activity of acetyl transferases and down-regulation of *ATF1* gene (Fujiiwara et al., 1997), which explains to some extent the reduction in the concentration of esters because of oxygen addition. Despite this knowledge, the actual contribution of oxygen in the production of secondary metabolites at genome level is still unclear.

2.3.7 A combined effect of oxygen and nutrients on yeast transcriptome

It is important to highlight that the differential gene expression in yeast under different oxygen conditions also depends on the limiting nutrient source in the media (Tai et al., 2005). A study by Piper et al. (2002) compared the aerobic and anaerobic transcriptome of *S. cerevisiae* under glucose limitation; results showed a total of 877 differentially expressed transcripts, these genes were mainly

responsible for encoding enzymes involved in respiration, oxygen toxicity and fatty acid oxidation. Tai et al. (2005) analysed the expression under micronutrient limitation and found that only 155 of these genes responded consistently to anaerobiosis under four different macronutrient limitations. These genes include those responsible for transport, cell wall organisation, metabolism and energy functions and 55 of them were of unknown function. Similar work was also performed by Lai and co-workers (2005) using galactose and glucose as a carbon source; where they found different transcriptional responses as a function of carbon source in two different conditions of oxygen. Transcriptome analysis on galactose as carbon source resulted in down-regulation of genes responsible for DNA replication and repair, cell cycle, rRNA processing. Rintala et al. (2011) did time dependent transcriptomic analysis of *S. cerevisiae* to sudden oxygen depletion in carbon limited conditions. They observed a transient upregulation of genes related to fatty acid oxidation, peroxisomal biogenesis, oxidative phosphorylation, TCA cycle, response to oxidative stress, and pentose phosphate pathway only in the initial oxygen-limited cultures.

Some studies performed under nitrogen limited conditions have shown up-regulation of genes involved in nitrogen metabolism such as transport of ammonia and amino acids and nitrogen metabolism. These reported genes include*: DAN1, DAN2, DAN3, DAN4, DAN5* (cell wall mannoprotein encoding genes)*, PUT1, PUT2, PUT3, PUT4* (involved in proline utilization) and*, MEP2* (responsible for ammonia uptake). Similarly, in sulfur limited conditions, up-regulation of genes involved in sulfur uptake and assimilation is reported, such as *SUL1, SUL2* (High affinity sulfate permease)*, SAM1, SAM2, SAM3, SAM4* (S-adenosylmethionine synthetase, involved in sulfur assimilation pathway)*, MET3, MET4, MET9* (methionine synthase also involved in sulfur assimilation pathway) (Boer et al., 2003) Thus, the major common impact of oxygen on yeast physiology occurs in central carbon metabolism, sterols and unsaturated fatty acids, cell wall integrity, however, the differences can be seen on the availability of nutrients.

2.4 Wine microbial ecosystem

The wine has a complex microbial ecology including yeasts, filamentous fungi and bacteria. Some species are only found on grape berry surface, while others can survive and grow in wines, constituting the wine microbial consortium. The composition of wine is determined by the interplay between several factors including microbial dynamics, environmental factors, viticulture practices as well as the grape varietal (Ciani and Comitini, 2015, Setati et al., 2012). In particular, wine aroma, which comprises hundreds of different compounds and is an important contributor to wine quality, is derived from the interactive growth and biochemical activities of a mixture of yeast species and strains. Most of these compounds arise from the alcoholic fermentation process, which in natural and mixed-starter fermentations, is characterized by a successional development of species and strains. In the past decade, use of mixed-starter fermentation has become a common practice in the

global wine industry and gained significant interest due to the yeast-yeast interaction which plays a fundamental role in wine aroma profile.

2.4.1 Yeast-Yeast interaction

Yeasts are the main driver of wine fermentation and determine the final composition of the wine. Yeasts originate on grape berry surface from the vineyard and participate until the end of the wine fermentation. Although *S. cerevisiae* is the main alcoholic agent, other non-*Saccharomyces* yeasts also play a significant role in determining the final composition of the wine. Interactions between the different species occur at various stages including grape berry surface to throughout the fermentation process. These interactions are known to have a significant impact on the final composition of the wine. In past decade, yeast-yeast interactions, including neutralism, commensalism, mutualism/ synergism, amensalism or antagonism have gained significant attention because of their main role in conducting the wine fermentation (Fleet, 2003).

In wine ecosystem, the ecological interactions start at the surface of grape berry and contribute to the species diversity during the wine fermentation. Usually, very few yeasts (10-10^3 cfu g^{-1}) are detected on the surface of unripe grape berries, but the population of yeast species increases gradually as the grapes mature to harvest due to sugars leach from the inner tissues of the grape to the surface. The surface of unripe grapes berry mainly consists of non-fermentative yeasts such as *Rhodotorula, Cryptococcus* and yeast-like fungus *Aureobasidium pullulans*. These yeast species are also isolated from ripe grapes, however, at this stage, oxidative or less fermentative yeasts species such as *Hanseniaspora*, *Metschnikowia*, *Candida* are mostly predominant (Barata et al., 2012; Setati et al., 2012). Indeed, this is surprising that why certain yeast species dominate on the surface wine grapes, and others are not. Perhaps the main reason behind the dominance of these yeasts could be due to possible yeast-yeast interaction on the surface grape berry, *M. pulcherrima*, commonly found on grapes, has been shown as an inhibitory yeast to a range of other yeasts, including *S. cerevisiae* (Nguyen and Panon, 1998). Some other reason also includes high tolerance of these yeasts towards several factors such as natural stresses of temperature, sunlight, irradiation; tolerance to chemical inhibitors from the application of agrichemicals (Fleet et al., 2002; Andrews and Buck, 2002). Therefore, the overall composition of yeasts on grape berry impacts the yeast ecology of wine production. However, the interaction on the surface of grape beery remains largely unknown.

During alcoholic fermentation, different yeast species and/or strains interact with each other directly or indirectly through the production of toxic compounds, via cell-cell contact or because of competition for nutrients (Ciani et al., 2015; Wang et al., 2015; Perrone et al., 2013). Of the indirect interaction, *S. cerevisiae* has known to produce toxic metabolites including ethanol to exert selective pressure towards non-*Saccharomyces* yeasts, medium-chain fatty acids on its own and together

with ethanol are also known to decrease the growth rate of non-*Saccharomyces* yeasts due to their toxicity (Fleet, 2003). Proteinaceous compounds such as killer toxins secreted by *S. cerevisiae* are found to be death-inducing factors for non-*Saccharomyces*, e.g. enzymes with glucanase activity (Magliani et al., 1997), and antimicrobial peptides derived from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein of *S. cerevisiae* (Branco et al., 2014). It has been suggested that during the inoculated fermentation with *S. cerevisiae* strain, *S. cerevisiae* does not only interact with non-*Saccharomyces* but also with indigenous *S. cerevisiae* strains present in grape juice, by modifying fermentation products. For instance, metabolic interaction has been shown between two *S. cerevisiae* strains, where acetaldehyde produced by one yeast was metabolized by the other strain of *S. cerevisiae* (Cheraiti et al., 2005).

Of the direct interaction, cell-cell contact appears to be involved in the interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts. Nissen et al. (2003) postulated that an early decline in growth of *T. delbrueckii* or *L. thermotolerans* occurs due to physical interaction with *S. cerevisiae*. In mixed culture fermentation of *T. delbrueckii* and *S. cerevisiae*, *S. cerevisiae* has shown to produce some unknown metabolites to resist the growth of *T. delbrueckii* and the data showed a phenomenon of amensalism exerted by *S. cerevisiae* towards *T. delbrueckii* (Taillandier et al., 2014; Renault et al., 2013). Cell-cell mediate dominant behavior is also studied in *S. cerevisiae*, surprisingly the dominant strain of *S. cerevisiae* remains dominant only when it senses the presence of another strain of *S. cerevisiae* in co-fermentation (Perrone et al., 2013). In a study by Luyt (2015) showed that the metabolic interaction led to a reduction in biomass of *L. thermotolerans* in mixed culture fermentation with S. cerevisiae. However, the study also confirms that the loss in viability was greater for *L. thermotolerans* when this yeast was in physical contact with *S. cerevisiae*. There was no significant loss in viability of *S. cerevisiae* was observed in all mixed cultures, therefore, this suggests that *S. cerevisiae* highly influences the survival of *L. thermotolerans* throughout fermentation. This study further investigated the role of oxygen pulses on the growth of these two yeasts in single and mixed culture fermentation and results indicated that an increase was observed in viable cell count of *L. thermotolerans* when oxygen pulses were added. However, this increase was less in mixed culture fermentations in comparison to single culture fermentations. The study concluded that the combined effect of oxygen and physical contact with *S. cerevisiae* could have led to declining of *L. thermotolerans* in the mixed culture fermentation. Furthermore, the degree of interaction between different yeasts is also influenced by several abiotic factors (oxygen, pH, temperature, ethanol etc.), biotic factors and the management of mixed fermentations, such as cell concentration, inoculation modalities (pure or mixed fermentation).

2.4.2 Influence of interactions on aromatic profile of wine

The different interactions which exist between the different yeasts have shown to have a synergistic, passive and negative effect on an aromatic compound produced at the end of the fermentation. The metabolic interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts such as *T. delbrueckii*, *L. thermotolerans*, *Hanseniaspora uvarum* in mixed fermentation, have shown an increase in the quantity of desirable compounds, such as higher alcohols and esters (Zohre and Erten, 2002; Viana et al., 2009). In these studies, the production of these compounds was not compared with the biomass produced and simply identified the change in aroma profile due to yeast-yeast interaction. However, the normalization of generated biomass with produced aroma compounds also indicated strong yeast-yeast interaction and its impact on the metabolic profile of the wine. The synergistic effect on aroma profile was found in mixed fermentation when *M. pulcherrima* was in co-culture with *S. cerevisiae*. Although *M. pulcherrima* did not pursue till the end of the fermentation, the presence of this yeast significantly changes the aroma profile with an increase in fatty acids, ethyl esters, acetates, and terpenol profile. While a negative interaction was observed between *C. zemplinina* and *S. cerevisiae*, the mixed fermentation of these two yeasts led to a decrease in terpene and lactone content. These interactions are independent of biomass production. In contrast, the biomass dependent interaction showed a passive effect on aroma profile due to mixed fermentation with *T. delbrueckii* and *S. cerevisiae*. The aroma profile in mono-culture and in co-culture of *T. delbrueckii*/ *S. cerevisiae* resulted very similarly, reflecting a neutral interaction (Howell et al., 2006; Sadoudi et al., 2012). These results indicate the occurrence of metabolic interaction between different yeast species and strain which determines the final flavor of the wine produced by the co-culture reaction. However, to obtain a complete picture of yeast interaction in multispecies fermentations a multifactorial approach using “omics” methodologies would be more helpful.

2.5 Influence of oxygen on yeast dynamics

Alcoholic fermentation of grape juice is typically characterized by the successional development of yeast species. The yeast succession is influenced by many factors such as the composition of initial yeast species in juice, the chemical composition of juice, pesticide residues, sulfur dioxide levels, the concentration of dissolved oxygen, ethanol, temperature and interaction between yeasts (Fleet, 2003; Fleet and Heard 1993). More recently, studies have highlighted the role of dissolved oxygen in yeast population dynamics. Generally, at the beginning of wine fermentation, the amount of dissolved oxygen present in grape must vary between 0 and 8 mg L^{-1} (du Toit et al., 2006). The gradual increase in yeast metabolic activity depletes the dissolved oxygen quickly and creates anaerobic conditions. Under these conditions, *S. cerevisiae* can grow in media supplemented with anaerobic factors (Ergosterol and Tween). In contrast, non-*Saccharomyces* spp. such as *Torulaspora delbrueckii*, *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*), *Metschnikowia pulcherrima*, *Hanseniaspora* spp. *Candida* spp. and *Pichia* spp. struggle to survive

in anaerobic conditions due to higher biosynthetic oxygen requirements than *S. cerevisiae* (Brandam et al., 2013; Hanl et al., 2005; Hansen et al., 2001; Luyt, 2015; Quiros et al., 2014; Renault et al., 2015; Visser et al., 1990). Several studies have shown that oxygen is a key factor which influences the growth of non-*Saccharomyces* yeasts. Low availability of oxygen decreases the survival rate of non-*Saccharomyces* spp. such as *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* (Contreras et al. 2015; Hansen et al. 2001; Morales et al., 2015). Hansen et al. (2001) showed an early decline in growth of *L. thermotolerans* and *T. delbrueckii* in sytem with less oxygen, while higher persistence was observed when both yeast species provided with oxygen. Likewise, Quirós et al. (2014) showed an enhanced growth rate of *M. pulcherrima* and *L. thermotolerans* when fermentations were supplemented with oxygen regimes. Similarly, the decreased oxygen feed rate perturbed the energy metabolism of *T. delbrueckii* more than *S. cerevisiae*, and suggested oxygen as the main reason for the poorer growth of *T. delbrueckii* under anaerobiosis (Hanl et al., 2005; Mauricio et al., 1998). Nevertheless, more research needs to be performed regarding the specific mechanisms and genes that are involved in the impact of oxygen on the growth of these yeasts and the mechanisms through which these yeasts interact with each other and the final composition of the wine.

2.6 Employing mixed-starter fermentations under oxygenation to lower ethanol in wine

Typically, in winemaking processes grape juice is fermented by selected strains of *S. cerevisiae* for better microbiological control of the alcoholic fermentation (AF), which gives the wine a reliable, consistent and predictable style and quality. However, some non-*Saccharomyces* species, such as *Hanseniaspora uvarum* (anamorph *Kloeckera*), *L. thermotolerans*, *T. delbrueckii*, *M. pulcherrima*, and *Starmerella bacillaris* (*Candida zemplinina)*, are predominant during the initial stages of wine fermentation and (Fleet, 2003; Gobbi et al., 2013; Wang et al., 2016) may persist during other fermentative stages, and contribute to a desirable flavour and aroma of the final product. The use of non-*Saccharomyces* yeasts has been emphasized more for their beneficial aspect in wine such as an increase in glycerol content, higher alcohols, esters, improved aroma profile and a decrease in ethanol (Andorrà et al., 2012; Comitini et al., 2011; Masneuf-Pomarede et al., 2016). Central carbon metabolism is one of the essential metabolism in all yeast species; however, the mechanism for the regulation of central carbon metabolism significantly differs between different yeasts (Flores et al., 2000). As mentioned previously, yeasts are classified into two distinct categories based on Crabtree effect: Crabtree positive and Crabtree negative yeasts (Crabtree, 1928). The Crabtree-positive yeasts, such as *S. cerevisiae*, still ferment under aerobic conditions when sugar is present in higher concentration, while the extent of fermentation in Crabtree negative yeasts (*M. pulcherrima*, *Scheffersomyces stipitis* or *Candida utilis*) is limited and the carbon flows more towards the biomass generation via respiration (Quirós et al., 2014). Therefore, the combination of non-*Saccharomyces* yeasts and oxygen could help in reducing ethanol levels in wine. Recently the use of non-

Saccharomyces yeasts with *S. cerevisiae* has been considered to reduce ethanol levels in wine. The use of some non-*Saccharomyces* yeasts such as *M. pulcherrima*, *Schizosaccharomyces malidevorans* and *Candida stellata* in sequential inoculations with *S. cerevisiae* was shown to produced less ethanol than *S. cerevisiae* alone (Contreras et al., 2014). The inoculation of Shiraz with *M. pulcherrima,* in sequential fermentation with *S. cerevisiae,* to complete alcoholic fermentation was shown to reduce ethanol concentration by up to 1.6% (v/v) (Contreras et al., 2014). Also, *C. zemplinina* and *S. uvarum* with *S. cerevisiae* showed a reduction of 0.34% and 0.90% vol of ethanol comparing to *S. cerevisiae* control fermentation (Bely et al., 2013). This available literature suggests the feasibility of using the non-*Saccharomyces* yeasts at the industrial level for reducing alcohol levels in wine. However, a better understanding of the metabolism of these alternative yeast species, as well as of the interactions between different yeast starters during the fermentation requires further investigation.

2.7 Additional benefits of using mixed-starter fermentations

The use of the non-*Saccharomyces* in mixed fermentation is not only beneficial for ethanol reduction but it is becoming a growing practice due to its influence on overall wine aroma profile and flavour (Table 2.1). The wines derived from mixed culture fermentations are known to have distinct profiles than single culture fermentation of *S. cerevisiae*; these distinct profiles are mainly due to change in major volatiles. These changes in aroma profile are associated with the type of non-*Saccharomyces* species and strain used in mixed fermentation. For example, yeasts of the genus *Hanseniaspora* are considered to be great producers of esters in mixed fermentation with *S. cerevisiae*; however, it again depends upon species used in mixed fermentation. Wines with *H. uvarum* showed increased concentration of isoamyl acetate, whereas *H. guilliermondii, H. osmophila* and *H. vineae* resulted in increased concentration of 2-phenylethyl acetate (Medina et al., 2003; Moreira et al., 2005; 2008; Viana et al., 2009). Similarly, the positive oenological contribution of *T. delbrueckii* has also been described in many reports. The impact of sequential *T. delbrueckii/ S. cerevisiae* mixed cultures in high sugar fermentation was evaluated to determine whether it could improve the quality of wines and reduce the acetic acid content (Bely et al., 2008). *T. delbrueckii/ S. cerevisiae* cultures at a ration of 20:1, produced 53% and 60% reductions in the volatile acidity and acetaldehyde, respectively, while sequential cultures showed lower effects on the reduction of these metabolites. Loira et al. (2014) demonstrated the benefit of using *T. delbrueckii* in fermentation with *S. cerevisiae* where these fermentations produced larger quantities of diacetyl, ethyl lactate and 2-phenylethyl acetate than single culture *S. cerevisiae* fermentation. Contreras et al. (2014) analyzed fermented Chardonnay grape must and reported increase in total concentration of esters and higher alcohols in mixed sequential fermentation of *M. pulcherrima* and *S. cerevisiae* (the significant increases was seen for ethyl acetate, 2- and 3 methyl butyl acetate among higher alcohols the increase was observed for 2-methyl propanol and 2- and 3-methyl butanol). A chemical and sensory analysis by

Swiegers et al. (2005) reported advantage of these compounds in wine as they add different kind of flavours to wine; higher alcohols are known to add rose, honey, flowery aroma in wine while presence of volatile fatty acids gives sweet, cheesy and a fatty smell in nose; in this study wines with high concentration of esters were the most preferred, as presence of esters add fruity smell and improve the complexity of the wine.

The increase in complexity to final wines has been credited to the enzymatic activity of some non-*Saccharomyces* yeasts; the glycosidase activity of non-*Saccharomyces* has been known to enhance the varietal composition of wine. The non-*Saccharomyces* yeasts are reported to hydrolyse the glycosidically-bound form of monoterpens which can be converted to free odours forms e.g. linalool, nerol, geraniol, a-terpineol and citronella (Ferreira et al., 2001; Mateo and Di Stefano 1997; MikloÂsy and PoÈloÈs, 1995; Rosi et al., 1994).

Furthermore, the use of certain non-*Saccharomyces* yeasts in mixed fermentation also results in wine with less acetic acid. Acetic acid constitutes 90% of the total volatile acids present in wine and plays an important for wine quality (Lambrechts and Pretorius 2000). Radler (1993) has shown that the amount of acetic acid produced by *S. cerevisiae* strains varies between 100 mg L^{-1} and 2 g L^{-1}. However, articles on some non-*Saccharomyces* yeasts have shown that in comparison to *S. cerevisiae*, mixed fermentation with non-*Saccharomyces* results in less acetic acid concentration. The mixed fermentation of *S. cerevisiae* with C. *zemplinina*, *L. thermotolerans* and *T. delbrueckii* showed significant reduction in comparison to *S. cerevisiae* mono-culture fermentation (Bely et al., 2008; Ciani et al., 2016; Sadoudi et al., 2012), certain strains of *M. pulcherrima* in combination with *S. cerevisiae* have also showed a reduction in acetic acid (Comitini et al., 2011; Sadoudi et al., 2012). The use of non-*Saccharomyces* yeasts is also recommended for increasing glycerol content in wine, which positively contributes to the wine quality (Nieuwoudt et al., 2002). For instance, Ciani & Ferraro (1996, 1998) proposed the use of immobilized *Candida* cells to enhance the glycerol content in wine. In addition, the combination of *S. cerevisiae* with *M. pulcherrima* and *Starmerella bacillaris* (*C. zemplinina)* also showed greater glycerol levels in comparison to *S. cerevisiae* pure cultures (Comitini et al., 2011; Rantsiou et al., 2012). Based on several studies which have shown the positive impact of non-*Saccharomyces* yeasts in winemaking, some of these yeast species are currently being commercialized. However, there are no studies available which enlighten the reason behind the change in aroma complexicity of wine resulting from mixed culture fermentations at the molecular level, a knowledge that could be used to guide the development of mixed culture starter fermentations.

Table 2.1. The positive contribution of non-*Saccharomyces* mixed culture fermentation with *S. cerevisiae* in the final wine.

Species	Positive contribution in wine	Process	References
***Starmerella bacillaris* (*C. zemplinina*)**	Reduced acetic acid and higher glycerol	Mixed	Comitini et al., 2011, Rantsiou et al., 2012;
T. delbrueckii	Low acetic acid and higher concentration of glycerol, increased fruitiness and complexity due to higher esters concentration	Sequential and mixed	Loira et al., 2014, Renault et al., 2015
L. thermotolerans	Reduced volatile acidity, high concentration of glycerol increase in higher alcohol concentrations, increased in spiciness	Sequential and mixed	Mora et al., (1990); Ciani et al., (2006); Kapsopoulou et al., (2007)
M. pulcherrima	Increase in esters and higher alcohols. Reduced ethanol concentration	Mixed and sequential (oxygenation)	Comitini et al., 2011; Contreras et al., 2014; Morales P., 2015
H. uvarum	Increase in esters and reduced heavy sulphur compounds	Mixed	Moreira et al., 2008
H. vineae	Increased flavour diversity in chardonnay must due to higher esters production	Sequential	Medina et al., 2013
C. zemplinina* *S. uvarum* *Schizosaccharomyces malidevorans* *Candida stellata	Reduced ethanol	Sequential and mixed	Quirós et al., 2014; Bely et al., 2013 Contreras et al., 2014
Candida membranifaciens* *H. uvarum* *H. guilliermondii* *Zygosaccharomyces fermentati	Increased complexity and wine flavours due to ethyl esters and higher alcohols	Sequential and mixed	Moreira et al., 2005, 2008; Garcia et al., 2010)
Schizosaccharomyces pombe* *Issatchenkia orientalis	Reduced malic acid	Mixed	Peinaud et al., 1962, Rankine et al., 1966; Munvon et al., 1977; Kim et al., 2008

2.8 Conclusion

Mixed fermentation of non-*Saccharomyces* and *S. cerevisiae* has become a growing trend in wine industry since the procedure intends to enhance unique aroma and flavour profiles of wine. However, mixed-culture wine fermentations tend to become rapidly dominated by *S. cerevisiae*, reducing the contribution of non-*Saccharomyces* yeast. In this regard, oxygen addition appear a promising tool, since the growth of non-*Saccharomyces* yeasts is highly oxygen dependent. The addition of oxygen is a common practice at some points in winemaking to support the generation of initial biomass and to avoid sluggish or stuck fermentation. Incorporation of oxygen in wine fermentation has a reasonably well understood impact on wine quality and yeast physiology. Previous studies on the impact of oxygen on yeast physiology have primarily focused on *S. cerevisiae*, however, the impact

of oxygen on non-*Saccharomyces* yeasts still needs to be investigated under enological conditions. Despite the knowledge of mixed culture fermentation, little is known about how these yeasts interacts with each other metabolically which might have an impact on wine-relevant issues such as fermentation kinetics, nitrogen use, and aroma production. To date, there are no studies on gene expression of non-*Saccharomyces* in mixed fermentation with *S. cerevisiae* under different physiological conditions. Therefore, it is necessary to gain a clear understanding of gene behaviour of non-*Saccharomyces* yeasts under different physiological conditions. This understanding will help in future to control the mixed culture fermentation to improve the complexity of wine aroma.

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Chapter 3

Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*

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Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*

3.1 Abstract

The sequential or co-inoculation of grape must with non-*Saccharomyces* yeast species together with *Saccharomyces cerevisiae* wine yeast strains has recently become a common practice in winemaking. The procedure intends to enhance unique aroma and flavor profiles of wine. The extent of the impact of non-*Saccharomyces* strains depends on their ability to produce biomass and to remain metabolically active for a sufficiently long period. However, mixed-culture wine fermentations tend to become rapidly dominated by *S. cerevisiae*, reducing or eliminating the non-*Saccharomyces* yeast contribution. For an efficient application of these yeasts, it is therefore essential to understand the environmental factors that modulate the population dynamics of such ecosystems. Several environmental parameters have been shown to influence population dynamics, but their specific effect remains largely uncharacterized. In this study, the population dynamic in co-fermentations of *S. cerevisiae* and three non-*Saccharomyces* yeast species, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima*, is investigated as a function of oxygen availability. In all cases, oxygen availability strongly influenced the yeast growth, but clear species-dependent differences were observed. Our data show that *L. thermotolerans* required the least oxygen, followed by *T. delbrueckii* and *M. pulcherrima*. Distinct species-specific chemical volatile profiles correlated in all cases with increased persistence of non-*Saccharomyces* yeasts, with in particular increases in some higher alcohols and medium chain fatty acids. The results highlight the role of oxygen in regulating the succession of yeasts during wine fermentations and suggest that more stringent aeration strategies would be necessary to support the persistence of non-*Saccharomyces* yeasts in real must fermentations.

Key words: non-*Saccharomyces* yeast, dissolved oxygen, yeast growth, mixed-culture fermentation, wine fermentation

3.2 Introduction

The majority of commercial wine fermentations are performed by inoculating *Saccharomyces cerevisiae* active dry yeast starter cultures. The advantages of inoculation include more predictable fermentation properties and aromatic profiles (Ciani et al., 2006, 2010; Comitini et al., 2011; Gobbi et al., 2013; Sadoudi et al., 2012; Soden et al., 2000). However, anecdotal evidence suggests that the extensive use of single strains, inoculated at high cell density and therefore dominating the natural microbiota from the start may reduce the sensorial complexity of the finished wine in comparison with spontaneously fermented wines where multiple yeast species may contribute significantly to the final aromatic features. Consequently, the last decade has seen a re-evaluation

of the role of non-*Saccharomyces* yeast species in wine fermentation with the aim of identifying alternative starter cultures to be used in mixed fermentation regimes (Ciani et al., 2010). The desirable attributes of such yeast species may include increasing the fruitiness and complexity of wine, reducing ethanol and acetic acid content, or alleviating sluggish/stuck fermentation of high sugar musts (Ciani et al., 2006; Comitini et al., 2011; Gobbi et al., 2013; Sadoudi et al., 2012). The contribution of these yeasts to the final organoleptic characteristics of wine will primarily depend on their ability to be metabolically active and to maintain a high cellular concentration during a significant part of the fermentation process (Ciani et al., 2006; Zuzuarregui et al., 2006). However, data regarding the impact of fermentation conditions on the relative performance of these species when competing with *S. cerevisiae* are limited. It is well established that in wine fermentation, whether spontaneous or inoculated, strains of *S. cerevisiae* tend to dominate the later stages of fermentation. This pattern also persists in multi-starter fermentations, even when non-*Saccharomyces* yeast species are inoculated at higher concentrations prior to *S. cerevisiae* to ensure a significant contribution (Andorra et al., 2010).

The relative decline of non-*Saccharomyces* yeast species during wine fermentation has been attributed to various factors including low ethanol tolerance, absence or low levels of oxygen, cell-to-cell contact inhibition, presence of proteinaceous antifungal compounds and killer toxins (Hansen et al., 2001; Hanl et al., 2005; Nissen et al., 2003; Panon, 1997; Pérez-Nevado et al., 2006; Visser et al., 1990). Recently, studies demonstrated that oxygen limitation in particular exerts a strong selective pressure during wine fermentation, and that the growth and persistence of non-*Saccharomyces* yeast species such as *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima* is strongly dependent on oxygen availability (Hansen et al., 2001; Hanl et al., 2005; Morales et al. 2015; Pérez-Nevado et al., 2006). Some studies have evaluated the impact of dissolved oxygen and have demonstrated the positive influence of oxygen addition on the cell physiology of *S. cerevisiae* and *T. delbrueckii* during fermentation (Aceituno et al., 2012; Brandam et al., 2013; Rintala et al., 2009; Varela et al., 2012). In addition to affecting yeast growth, oxygen also affects the production of major wine volatile compounds especially the ratio of esters to higher alcohols (Valero et al. 2002), and oxygenation of mixed starter fermentations employing *M. pulcherrima* and *S. cerevisiae* reduced the final ethanol levels in wine (Morales et al., 2015). However, the research regarding the impact of oxygen on yeast growth is still in its infancy and our understanding of the influence of oxygen on the overall yeast growth and contribution of non-*Saccharomyces* yeast species to the organoleptic properties of wine remains limited. In particular, data regarding the response of mixed fermentation to different levels of oxygenation is limited. Therefore, the present study aimed to evaluate the effect of three different levels of dissolved oxygen on the growth and fermentation kinetics of *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima* during co-fermentation with *S. cerevisiae*. We also investigated the influence of these conditions on the volatile chemical profiles derived from these fermentations. Our study clearly suggests the potential of oxygen manipulation strategies to steer

yeast population growth and ensure a desirable contribution to wine sensorial signatures by different non-*Saccharomyces* yeast species.

3.3 Materials and methods

3.3.1 Yeast Strains and media

S. cerevisiae (Cross evolution-285), and *T. delbrueckii* (Biodiva) are commercial strains from Lallemand SAS (Blagnac, France) while *M. pulcherrima* (IWBT-Y1337) and *L. thermotolerans* (IWBT-Y1240) were obtained from the culture collection of the Institute for Wine Biotechnology (Stellenbosch University).The selection criteria for these particular non-*Saccharomyces* yeasts was on the basis of their positive contribution reported in pervious literature and their commercialization (Ciani et al., 2006, 2010; Comitini et al., 2011; Gobbi et al., 2013; Sadoudi et al., 2012; Soden et al., 2000). Cryogenically maintained (-80°C) strains were reactivated by streaking out on YPD agar plates containing 10 g yeast extract, 20 g peptone and 20 g glucose per liter. Cultures were stored at 4°C for short term use.

3.3.2 Fermentations and Sampling

Fermentations were performed in synthetic grape juice (pH 3.5) containing (per liter) 100 g glucose, 100 g fructose, 1 g yeast extract (Oxoid, Thermo Fisher Scientific, Hampshire, United Kingdom), 2 g $(NH_4)_2SO_4$, 0.3 g citric acid, 5 g L-malic acid, 5 g L-tartaric acid, 0.4 g $MgSO_4$, 5 g KH_2PO_4, 0.2 g NaCl, 0.05 g $MnSO_4$ and anaerobic factors (ergosterol 10 mg L^{-1}, Tween 80 0.5 mL L^{-1}) (Henschke and Jiranek, 1993; Ough et al., 1989). Fermentations were conducted in 1.3 L BioFlo 110 bench top bioreactors (New Brunswick, NJ, USA) using 900 mL of final working volume, a temperature of 25°C and an agitation speed of 200 rpm. Fermentations were carried out anaerobically and with three different levels of oxygenation corresponding to 1% (0.08 mg L^{-1}), 5% (0.41 mg L^{-1}) and 21% (1.71 mg L^{-1}) of dissolved oxygen (DO). The anaerobic conditions were created by initially sparging N_2 to bring down the DO level to 0%, and then to minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation set-up was equipped with Norprene tubing. For aerobic fermentation, the DO probe was calibrated by adding oxygen to the medium as compressed air, using a peristaltic pump and with air flow rate of 1 vvm (volume per volume per minute). The three different DO levels were maintained through supplementary addition of gas mixture (CO_2, N_2, O_2 and compressed air at 1vvm) from which O_2 was introduced into the fermentation whenever required, using an automated gas flow controller. To minimize the gas variability in each vessel, same gas mixture module was used for all the vessels and experiments were performed in duplicate at the same time with. The dissolved-oxygen concentration in the cultures was monitored with an oxygen electrode. Samples were collected at 24 h intervals to monitor growth and fermentation progress. In all experimental conditions both non-*Saccharomyces* and *S. cerevisiae* were inoculated simultaneously with cell number 10^7: 10^6 (non-*Saccharomyces*: *S. cerevisiae*). All fermentations were conducted in duplicate.

3.3.3 Analysis of yeast population growth and dry biomass

Serial dilutions of the cell suspensions were performed with 0.9% (w/v) NaCl. One hundred microliter samples were spread on YPD agar and incubated at 30°C for 2-3 days. For yeast enumeration in mixed culture fermentations, the individual species were distinguished based on colony morphology (the pictures illustrating colony morphologies are provided in Supplementary Fig. S3.1). Colony counts were performed on plates with 30-300 colonies. The dry weight biomass was determined by separating the cells from the liquid by centrifugation at 5000 × *g* (4 mL of volume in triplicate) in tubes. The empty tubes were pre-weighed and then kept at 90°C. After reaching a constant weight, the dry biomass was obtained by subtracting the weight of empty tubes.

3.3.4 Analytical methods

Cell free supernatants were obtained by centrifuging cell suspensions at 5000 × *g* for 5 min. Glucose, fructose, glycerol, acetic acid and acetaldehyde were measured using specific enzymatic kits, Enytec^{TM} *Fluid* D-glucose, fructose, acetic acid (Thermo Fisher Scientific Oy, Helsinki, Finland), Boehringer Mannheim / R-Biopharm-acetaldehyde (R-Biopharm AG, Darmstadt, Germany) and analyzed using Arena 20XT photometric analyzer (Thermo Electron Oy, Helsinki, Finland) (Schnierda et al., 2014). Ethanol was analyzed by high performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Agilent RID and UV detectors were used in tandem for peak detection and quantification. Final analysis was done using the HPChemstation software (Rossouw et al., 2012). Liquid-liquid extraction method was used for volatile compound analysis using GC-FID, where five mL sample of synthetic must was added with internal standard 4-methyl-2-pentanol (final concentration 5 mg L^{-1}). To perform liquid-liquid extraction, 1 mL diethyl ether was added to each sample and sonicated for 5 min. The wine/ether mixture was then centrifuged at 4000 × *g* for 5 min, and the ether layer (supernatant) removed and dried on Na_2SO_4 to remove excess water. For gas chromatography (GC) a DB-FFAP capillary column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 0.32 mm i.d. × 0.5 µm film thickness and a Hewlett Packard 6890 Plus GC instrument (Little Falls, USA) equipped with a split/splitless injector and a flame ionisation detector (FID) was used. The initial oven temperature was 33°C, held for 17 min, after which the temperature was increased by 12°C min^{-1} to 240°C, and held for 5 min. Three µL of the diethyl-ether extract was injected at 200°C in split mode. The split ratio was 15:1 and the split flow rate 49.5 mL min^{-1}. The column flow rate was 3.3 mL min^{-1} using hydrogen as carrier gas. The detector temperature was 250°C (Louw et al., 2010).

3.3.5 Statistical analysis

All chemical analyses were performed in duplicate technical repeats on two independent fermentations and all the values were expressed as means ± S.D. Differences between measurements within different treatments were determined using analysis of variance (a least-

significant-difference[LSD]test) with the statistical software Statistica version 13.0 (StatSoft Inc., Tulsa, Oklahoma, USA) and differences were considered significant when *p* values were less than 0.05. For multivariate data analysis, principle component analysis (PCA) was constructed using SIMCA-P software version 14.0 (Umetrics, Umea, Sweden).

3.4 Results

3.4.1 Impact of aeration on yeast growth

The fermentation kinetics and growth of *S. cerevisiae*, *L. thermotolerans, T. delbrueckii* and *M. pulcherrima* in single (at anaerobic and 21% DO) or in mixed cultures (at anaerobic, 1%, 5% and 21% DO) were evaluated. In single species, anaerobic fermentations, *S. cerevisiae* completed the fermentation (sugar levels < 2 g L^{-1}) in 96 h with final cell counts at 7.6 × 10^9 CFU mL^{-1}. The non-*Saccharomyces* yeast, *L. thermotolerans* and *T. delbrueckii* completed fermentation in 120 h, with cell counts of 1.5 × 10^7 and 7.0 × 10^7 CFU mL^{-1}, respectively, on completion while the fermentations with *M. pulcherrima* became stuck with 55 g L^{-1} residual sugar and final cell counts of 2.19 × 10^6 CFU mL^{-1} (Fig. 3.1) when the fermentation was stopped. Under aerobic condition at 21% DO level, all single species culture completed the fermentation faster than in anaerobic conditions and reached higher cell counts. *S. cerevisiae* completed fermentation within 48 h with a viable cell count of 4.14 × 10^{11} CFU mL^{-1} at end of the fermentation, followed by *L. thermotolerans* and *T. delbrueckii* within 72 h, and end-point CFUs of 3.8 × 10^{10} and 6.7 × 10^{10} mL^{-1}, while *M. pulcherrima* achieved dryness after 144 h and final viable cell count of 3.75 × 10^9 CFU mL^{-1} (Fig. 3.1). The growth rate of *S. cerevisiae* was 0.37 in single anaerobic fermentation which reduced to 0.21, 0.23 and 0.28 in mixed anaerobic fermentation *with M. pulcherrima*, *L. thermotolerans* and *T. delbrueckii*, respectively (Table 3.1.-3.2). Of the non-*Saccharomyces* yeasts, like *S. cerevisiae*, all three non-*Saccharomyces* yeasts also showed a decrease in their specific growth rate in mixed anaerobic fermentations. Overall, oxygen input resulted in increased growth rates of all species especially in single culture fermentations (Table 3.1). In mixed fermentation with 21% DO level, the specific growth rate of *S. cerevisiae* and *T. delbrueckii* decreased in comparison to their single fermentation with 21% of DO, while *L. thermotolerans* and *M. pulcherrima* exhibited almost similar growth rates as their single fermentations. Comparing to anaerobic mixed fermentation, in mixed culture-fermentation with *T. delbrueckii* and *L. thermotolerans*, oxygen inputs improved the growth rate of the non-*Saccharomyces* yeasts while that of *S. cerevisiae* was reduced in comparison to their anaerobic mixed fermentations. For instance, at 5% and 21% DO, the two yeasts show approximately 3-fold faster growth rate than at 1% DO or anaerobic conditions, while the growth rate of *S. cerevisiae* slowed down (Table 3.2). In contrast, in mixed culture fermentation with *M. pulcherrima*, similar growth rates (≈ 0.3) were observed for the two yeasts under different oxygen inputs. However, in comparison with anaerobic conditions, the growth rate of *M. pulcherrima* increased almost 3-fold while that of *S. cerevisiae* is only 1.5-fold higher under aerobic conditions.

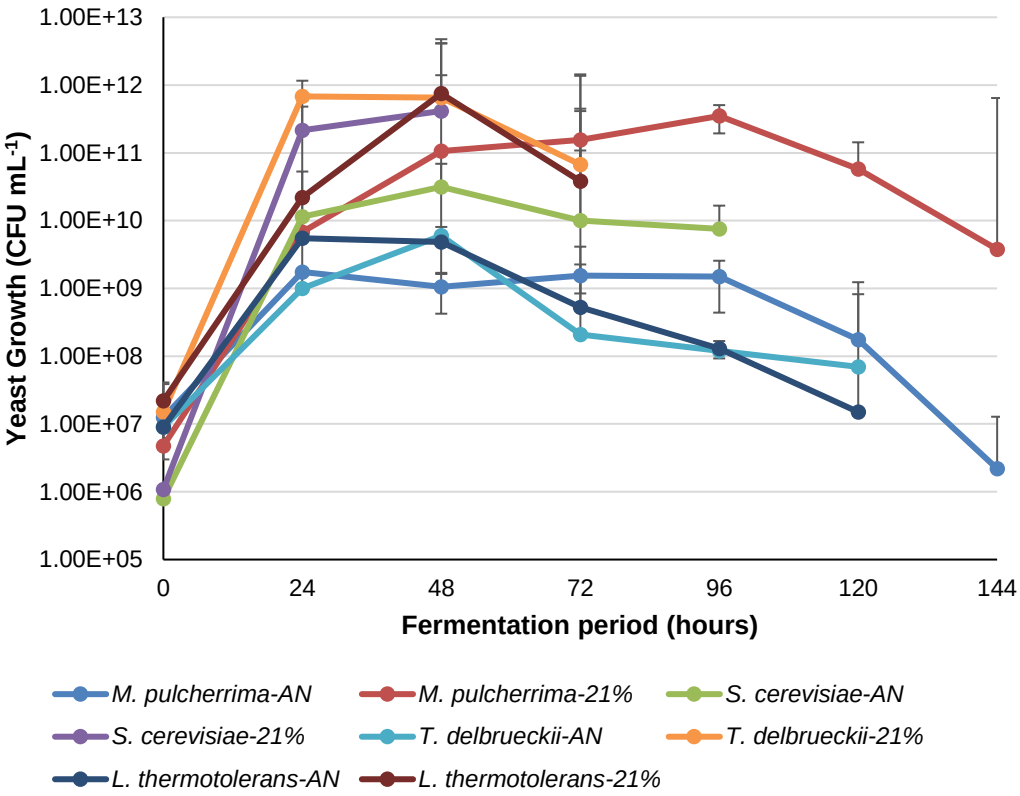

Figure 3.1 Growth of three non-*Saccharomyces* and *S. cerevisiae* single-culture fermentations in anaerobic and 21% DO level

Table 3.1 Specific growth rates of three non-*Saccharomyces* and *S. cerevisiae* in singe-culture fermentation under anaerobic and aerobic (21% DO) conditions

Single culture fermentations	Specific growth rate (h^{-1})
M. pulcherrima-AN	0.20
M. pulcherrima-21%	0.30
S. cerevisiae-AN	0.37
S. cerevisiae-21%	0.43
T. delbrueckii-AN	0.19
T. delbrueckii-21%	0.42
L. thermotolerans-AN	0.26
L. thermotolerans-21%	0.28

Table 3.2 Specific growth rates of non-*Saccharomyces* yeasts and *S. cerevisiae* in mixed culture fermentation under anaerobic (AN) and aerobic (1%, 5% and 21% DO) conditions

Mixed fermentations	Fermentation-AN Specific growth rate (h^{-1})	Fermentation- 1% Specific growth rate (h^{-1})	Fermentaion-5% Specific growth rate (h^{-1})	Fermentaion-21% Specific growth rate (h^{-1})
S. cerevisiae	0.21	0.32	0.32	0.31
M. pulcherrima	0.09	0.36	0.35	0.29
S. cerevisiae	0. 28	0.30	0.25	0.09
T. delbrueckii	0.11	0.18	0.28	0.29
S. cerevisiae	0.23	0.31	0.17	0.15
L. thermotolerans	0.1	0.25	0.25	0.30

The yeast growth in mixed fermentations showed species-specific differences in response to different oxygen conditions. Under anaerobic conditions, all mixed fermentations were completed in 120 h, and throughout the fermentation *S. cerevisiae* established itself rapidly as the dominant yeast, maintaining viable cell counts of 10^9 CFU mL^{-1}. However, significant differences were observed regarding the ability of the non-*Saccharomyces* species to grow and persist in these conditions: *L. thermotolerans* (Fig. 3.2a) and *T. delbrueckii* (Fig. 3.3a) persisted until the end of fermentation and attained 1.7×10^7 and 2.3×10^6 CFU mL^{-1}, respectively at the end point of fermentation (120 h). In contrast, *M. pulcherrima* grew in the first 24 h reaching 1.25×10^8 CFU mL^{-1} but could no longer be detected after 48 h of fermentation (Fig. 3.4a).

Increasing levels of DO favored growth and persistence of the non-*Saccharomyces* yeast species to varying degrees. As expected, in comparison to anaerobic mixed fermentations, all aerobic mixed fermentations generated higher total CFU counts, mainly due to increased CFU counts of the non-*Saccharomyces* yeasts. Among the three-species assessed here, *L. thermotolerans* achieved the highest CFU counts, exceeding the cellular concentrations of *S. cerevisiae* at all three levels of oxygenation, reaching maximum viable count of 9×10^9, 9.8×10^9 and 2.8×10^{10} CFU mL^{-1} at 1%, 5% and 21% DO, respectively (Fig. 3.2-b, c, d). This numerical dominance of *L. thermotolerans* over *S. cerevisiae* was maintained until the end of fermentation. *T. delbrueckii*, on the other hand, was outcompeted by *S. cerevisiae* at 1% DO (Fig. 3.3b), but achieved higher cell counts than *S. cerevisiae* at 5% and 21% DO and reached a maximum cell density of 1.06×10^{10}, 1.89×10^{10} CFU mL^{-1} respectively (Fig. 3.3-c, d). Similarly, *M. pulcherrima* showed rapid growth in the first 24 h at 1%, 5% and 21% DO levels and generated maximum viable cell count of 9.8×10^9, 6.5×10^{10} and 9.8×10^{10}, respectively (Fig. 3.4-b, c, d). These levels were 10-fold higher than *S. cerevisiae* and were maintained at all the DO levels for 72 h. However, at 1% DO the population of *M. pulcherrima* declined steadily after 72 h reaching 2×10^5 CFU mL^{-1} at the end of fermentation, while at 5% a decline was only observed after 96 h (Fig. 3.4-b, c). In contrast, at 21% DO *M. pulcherrima* displayed

higher cell counts than *S. cerevisiae*, reaching 9.8 × 10^{10} CFU mL^{-1} in the middle of fermentation and maintaining this numeric dominance until the end of fermentation (Fig. 3.4d).

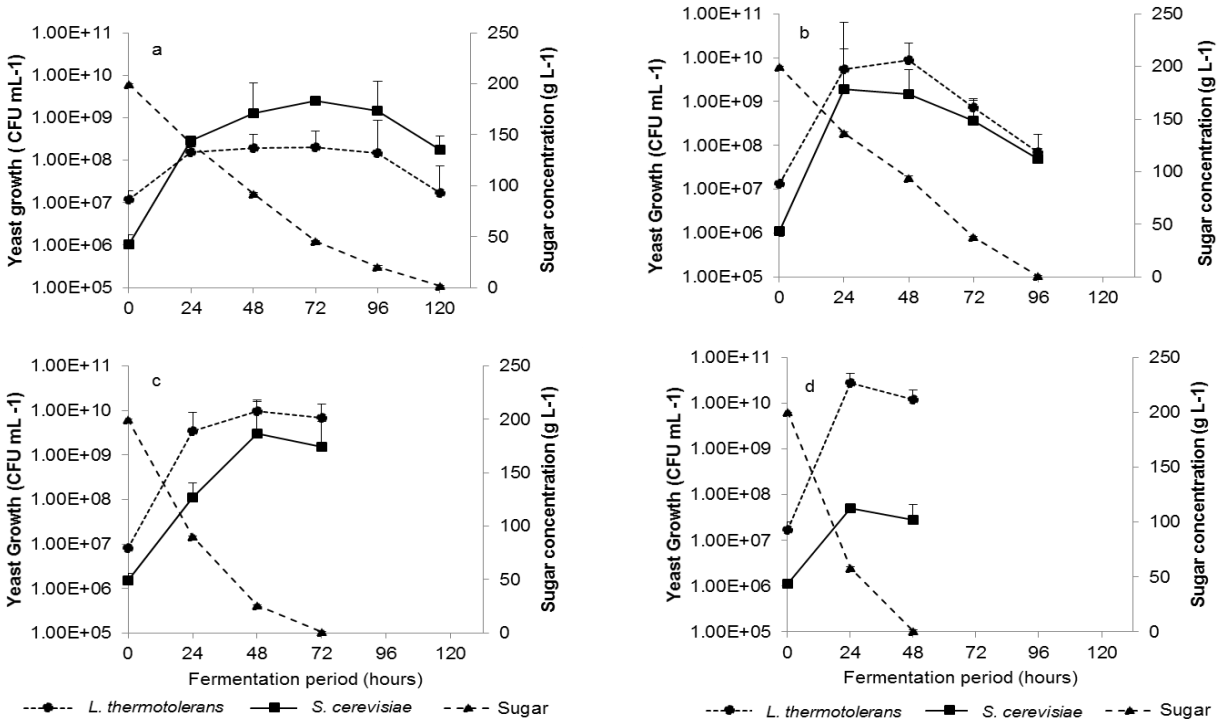

Figure 3.2. Growth of *L. thermotolerans* (round) and *S. cerevisiae* (square) in mixed fermentation under anaerobic (a), 1% (b), 5% (c), and 21% (d) level of dissolved oxygen conditions. Secondary y-axis indicates utilization of sugar (triangle) in grams per liter.

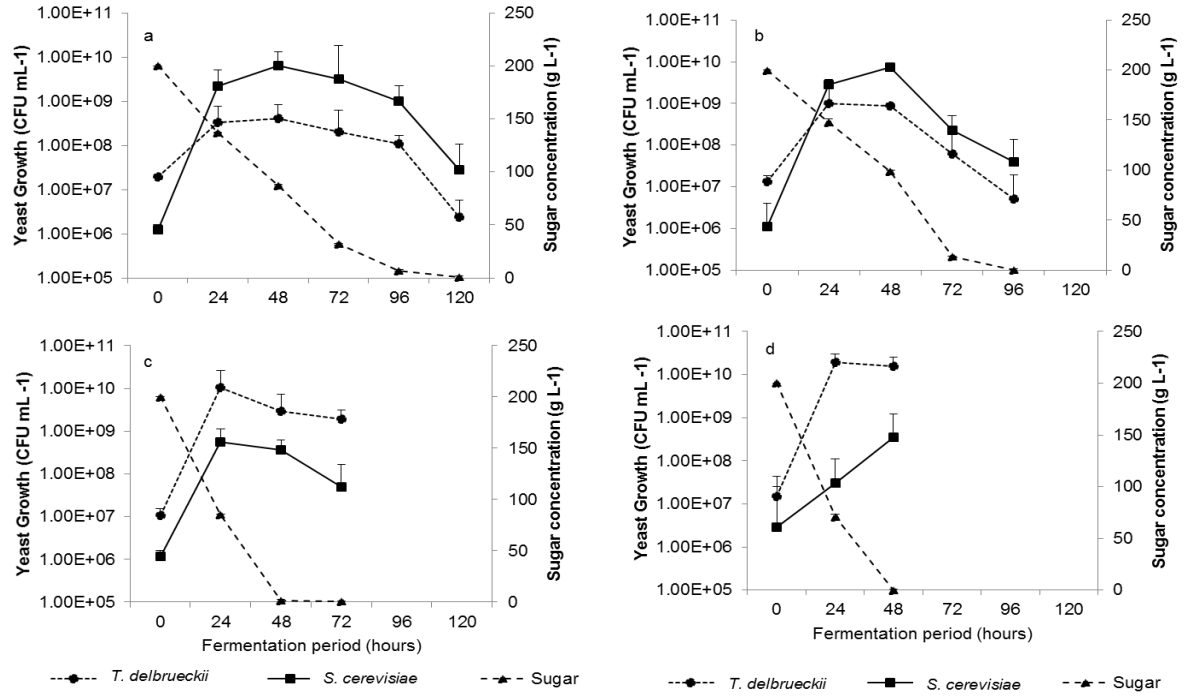

Figure 3.3 Growth of *T. delbrueckii* (round) and *S. cerevisiae* (square) in mixed fermentation under anaerobic (a), 1% (b), 5% (c), and 21% (d) level of dissolved oxygen conditions. Secondary y-axis indicates utilization of sugar (triangle) in grams per liter.

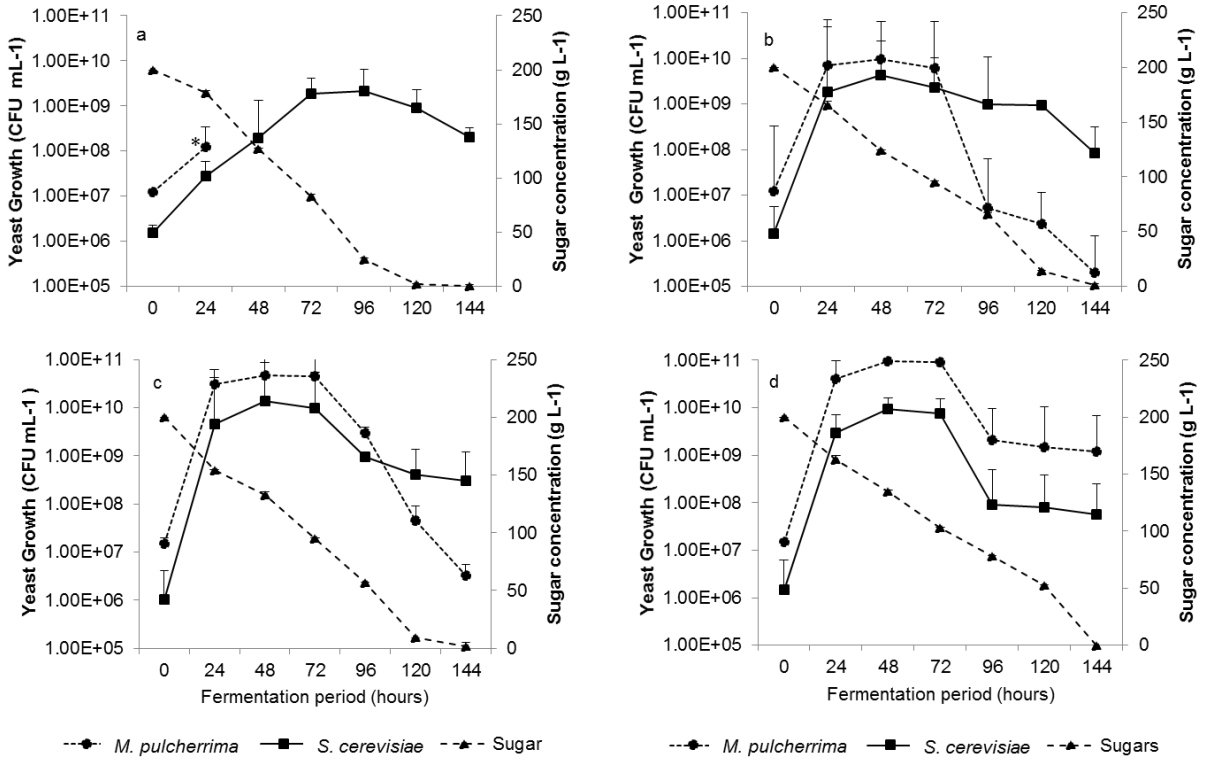

Figure 3.4 Growth of *M. pulcherrima* (round) and *S. cerevisiae* (square) in mixed fermentation under anaerobic (a), 1% (b), 5% (c), and 21% (d) level of dissolved oxygen conditions. Secondary y-axis indicates utilization of sugar (triangle) in grams per liter. In anaerobic condition, * indicates that *M. pulcherrima* could not be detected

The effect of aeration on biomass generation was also evaluated by measuring the dry biomass of samples from fermentations under anaerobic conditions and at 5% DO. Overall, the supply of oxygen at 5% DO resulted in approximately a two-fold increase in biomass production compared to fermentation under anaerobic conditions (Fig. 3.5). The anaerobic fermentations with *S. cerevisiae*, *S. cerevisiael L. thermotolerans*, *S. cerevisiael T. delbrueckii*, and *S. cerevisiael M. pulcherrima* generated 6.1, 5.1, 6.1, 6.0 g L^{-1}, respectively, while at 5% level of DO the biomass was 11.0, 10.73 and 11.73 g L^{-1}, respectively (Fig. 3.5).

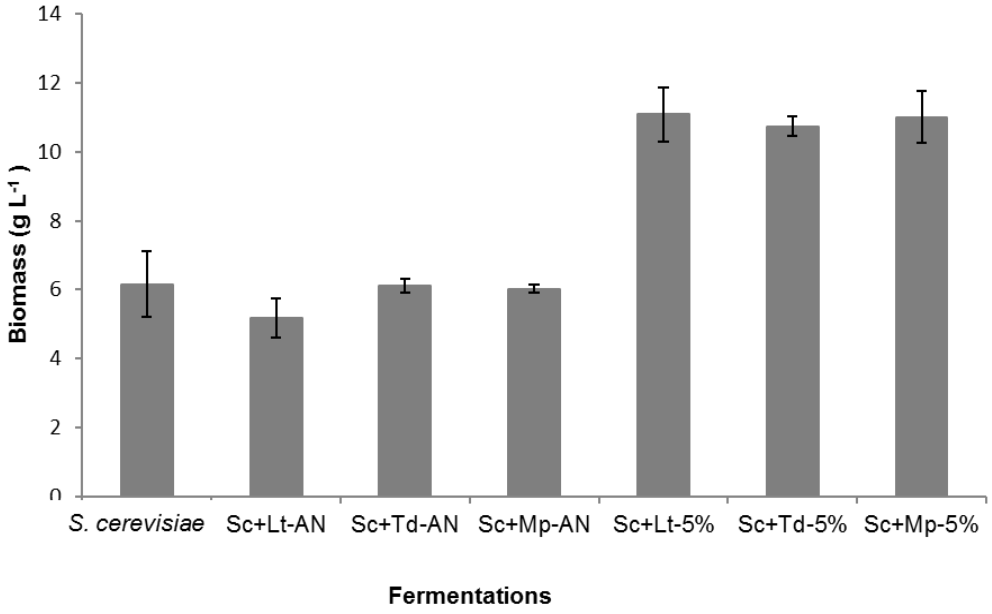

Figure 3.5 Dry mass produced by control *S. cerevisiae* and mixed fermentation of three non-*Saccharomyces* and *S. cerevisiae* in anaerobic and 5% OD level aerobic fermentation. Values are in grams per liter.

3.4.2 Production of metabolites under anaerobic and aerobic fermentation conditions

Regarding the primary products of fermentative metabolism, in comparison to anaerobic fermentations, oxygenation at all three DO levels resulted in ethanol and glycerol reduction (Table 3.3). In the *S. cerevisiae* single culture fermentation, the ethanol yield decreased from 0.50 (under anaerobic conditions) to 0.36 (at 21% DO). Similarly, the anaerobic mixed culture fermentation of *S. cerevisiae*/ *L. thermotolerans* resulted in an ethanol yield of 0.49, which was reduced to 0.44, 0.40 and 0.29 at 1%, 5% and 21 % DO levels, respectively (Table 3.3). In the case of *S. cerevisiae*/ *T. delbrueckii* co-fermentations, the ethanol yield decreased from 0.49 under anaerobic conditions to 0.46 at 1 % and 5% DO, and 0.23 at 21% DO. The *S. cerevisiae*/ *M. pulcherrima* mixed fermentations displayed a similar trend, resulting in a reduction in ethanol yield from 0.50 under anaerobic conditions to 0.44, 0.39 and 0.23 at 1%, 5% and 21% DO, respectively (Table 3.3). A general decrease in glycerol levels was evident in mixed culture fermentations with a 6-fold reduction in *S. cerevisiae*/ *T. delbrueckii* fermentations under 21% DO compared to anaerobic conditions, while in *S. cerevisiae*/ *L. thermotolerans* and *S. cerevisiae*/ *M. pulcherrima* fermentations a 1.6-fold reduction in glycerol concentrations was observed (Table 3.3). In comparison to the *S. cerevisiae* fermentation, all anaerobic mixed fermentation had lower acetic acid. The mixed fermentation with *M. pulcherrima* produced the lowest acetic acid concentration followed by the *L. thermotolerans*, *T. delbrueckii* mixed fermentation (Table 3.3). The concentration of acetic acid and acetaldehyde gradually increased from anaerobic to 1%, 5% and 21% DO level, and more than two-folds increase was observed at 21% DO for all fermentations (Table 3.3).

Table 3.3 Ethanol, acetic acid, acetaldehyde and glycerol concentrations in non-*Saccharomyces* anaerobic individual control *S. cerevisiae* and their mixed fermentations in anaerobic and three aerobic fermentations for mixed culture and 21% for control *S. cerevisiae*.

Fermentations	Ethanol yield (g ethanol/ g sugar)	Ethanol (g L^{-1})	Acetic acid (g L^{-1})	Acetaldehyde (mg L^{-1})	Glycerol (g L^{-1})
S. cerevisiae-AN	0.50	100.23±0.06	1.06±0.041	49±4.42	4.36±1.83
S. cerevisiae-21%	0.36	72.00±0.03	1.70±0.012	85±7.07	4.86±0.92
L. thermotolerans-AN	0.49	98.00±0.08	0.63±0.32	30±2.43	7.3±0.37
Sc+Lt-AN	0.49	98.79±0.10	0.94±0.06	41±7.07	7.05±0.39
Sc+Lt-1%	0.44	89.35±0.04	0.91±0.051	84±8.48	6.80±0.728
Sc+Lt-5%	0.40	81.87±0.06	2.03±0.031	285±7.70	4.12±0.59
Sc+Lt-21%	0.29	59.08±0.04	3.84±0.04	369±5.65	4.57±1.04
T. delbrueckii-AN	0.47	94.16±0.09	0.89±0.11	42±1.42	6.79±0.93
Sc+Td-AN	0.49	99.64±0.08	0.79±0.17	54±2.82	6.84±0.45
Sc+Td-1%	0.46	92.88±0.13	0.71±0.014	70±2.82	6.46±1.41
Sc+Td-5%	0.40	80.12±0.09	1.03±0.05	399±7.10	1.74±1.02
Sc+Td-21%	0.23	46.91±0.13	2.06±0.10	551±8.84	1.09±1.62
M. pulcherrima-AN	0.38	56.19±0.20	0.24±0.61	28±1.09	7.1±1.45
Sc+Mp-AN	0.50	100.22±0.03	0.69±0.19	37±7.07	7.93±2.01
Sc+Mp-1%	0.44	88.04±0.08	1.44±0.072	39±16.90	5.53±1.73
Sc+Mp-5%	0.39	78.75±0.06	2.06±0.02	117±12.70	4.70±1.40
Sc+Mp-21%	0.23	46.96±0.04	2.05±0.78	471±6.91	4.41±0.63

All the compounds are average of two biological duplicates ± SD

3.4.3 Non-*Saccharomyces* and oxygenation derived changes in volatile compounds profile

Volatile compounds produced during the fermentations were measured at the end of the process. Significant differences were observed for the different yeast combinations and for different oxygen levels. The non-*Saccharomyces* single species fermentations generally exhibited a high production of higher alcohols (mainly 2-phenylethanol, isoamyl alcohol and isobutanol) in anaerobic fermentations (Table 3.4-3.6). In addition, *L. thermotolerans* produced significantly high levels of 3-ethoxy-1-propanol, isobutyric acid (Table 3.4), and *T. delbrueckii* contributed higher levels of propionic acid (Table 3.5), while, *M. pulcherrima* contributed high levels of ethyl acetate, diethyl succinate and ethyl lactate in both mono- and mixed-culture fermentations (Table 3.6). The *S.*

cerevisiae single culture fermentation generally showed higher levels of MCFAs (medium chain fatty acids) than the non-*Saccharomyces* species single fermentations.

The metabolic profile of the *S. cerevisiae* single species anaerobic fermentations differed significantly from its mixed anaerobic fermentations. Anaerobic mixed fermentations with *S. cerevisiae*/ *L. thermotolerans* and *S. cerevisiae*/ *T. delbrueckii* showed higher concentration of the higher alcohols, MCFAs and esters (2-phenylethyl acetate, diethyl-succinate, 2-isoamyl-acetate, ethyl-hexanoate, ethyl-caprylate, and ethyl-phenylacetate). For the *S. cerevisiae*/ *M. pulcherrima* fermentation the concentration of MCFAs reduced while that of isoamyl alcohol, 2-phenylethanol, isobutanol, and esters (2-phenyethyl lacetate, diethyl-succinate, ethyl acetate and ethyl lactate, 2-isoamyl-acetate, ethyl-hexanoate, ethyl-caprylate, ethyl-phenylacetate) increased (Table 3.4-3.6).

Oxygenation of both single and mixed culture fermentations resulted in a general increase in higher alcohols, particularly in isoamyl-alcohol, 2-phenylethanol, isobutanol, and a decrease was observed in MCFAs and 2-phenylethyl acetate. Moreover, the incorporation of oxygen enhanced production of 3-ethoxy-1-propanol and isobutyric acid in *S. cerevisiae*/ *L. thermotolerans* fermentation (Table 3.4), while increase in butyric acid and propionic acids in the mixed fermentation with *T. delbrueckii* (Table 3.5). In addition, the oxygenation in *S. cerevisiae*/ *M. pulcherrima* mixed fermentation enhanced the production of diethyl-succinate, ethyl acetate, ethyl lactate, 3-ethoxy-1-propanol, propanol, while the levels of isoamyl acetate, ethyl hexanoate, ethyl caprate, ethyl-phenyl acetate were reduced (Table 3.5). Further analysis of the yields of these compounds on the basis of biomass shows that the increase in some higher alcohols, propionic acid and butyric acid, as well as the decrease in MCFAs and esters is also due to increase in cell biomass (Supplementary Table S3.1-S3.4).

Table 3.4 Major volatile compounds detected with significant differences at end of the fermentation in *S. cerevisiae*/ *L. thermotolerans* single culture and their mixed cultures.

Major volatiles	*S. cerevisiae* (AN*)	*L. thermotolerans* (AN*)	Sc+Lt (AN*)	Sc+Lt- 1%	Sc+Lt- 5%	Sc+Lt- 21%
2-Phenylethanol	6.30±1.24^{d}	39.84±9.31^{c}	33±3.02^{c}	92±3.48^{b}	104±4.14^{b}	105±4.65^{a}
Isoamyl alcohol	50.8±8.75^{d}	86.49±0.10^{d}	120.5±2.81^{c}	203±4.42^{b}	215±1.96^{b}	322±1.12^{a}
Isobutanol	12.5±2.85^{e}	20.79±2.80^{d}	33.57±0.24^{c}	125±2.82^{bc}	139±3.02^{b}	197±2.21^{a}
Propanol	31.18±5.48^{a}	17.59±1.41^{b}	0.0±0.00^{a}	0.0±0.01^{a}	25.53±4.37^{ab}	0.0±0.00^{a}
Butanol	0.0±0.00^{c}	3.34±0.25^{a}	0.98±0.03^{bc}	0.65±0.08^{bc}	1.05±0.07^{bc}	1.40±0.69^{b}
Pentanol	1.56±0.00^{a}	0.96±0.01^{b}	0.0±0.00^{d}	0.90±0.01^{c}	0.96±0.02^{b}	0.0±0.00^{d}
Hexanol	0.89±0.03^{b}	0.59±0.02^{b}	0.0±0.00^{c}	0.0±0.00^{c}	10.55±0.090^{a}	0.0±0.00^{c}
3-Ethoxy-1-propanol	1.15±0.40^{d}	28.51±1.91^{a}	4.28±0.23^{bc}	6.56±0.11^{cd}	8.27±0.45^{c}	9.87±0.12^{c}
3-Methyl-1-pentanol	Nd	Nd	Nd	Nd	Nd	Nd
Propionic acid	1.69±0.12^{c}	4.55±0.20^{b}	2.03±0.09^{c}	7.28±0.67^{a}	9.46±1.27^{a}	1.84±0.16^{c}
Isobutyric acid	1.57±0.55^{b}	3.19±0.30^{b}	1.44±0.05^{b}	8.84±0.61^{a}	9.70±0.27^{a}	10.87±1.06^{a}
Butyric acid	1.30±0.04^{b}	0.98±0.11^{c}	0.98±0.03^{c}	1.80±0.04^{a}	1.77±0.10^{a}	0.92±0.01^{c}
Isovaleric acid	1.07±0.00^{a}	0.79±0.27^{b}	0.0±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}	0.67±0.05^{b}
Valeric acid	0.66±0.06	1.42±0.08^{a}	0.70±0.04^{b}	0.80±0.00^{b}	0.66±0.07^{b}	0.85±0.01^{b}
Hexanoic acid	1.96±0.20^{b}	0.83±0.02^{c}	2.16±0.01^{a}	1.03±0.01^{d}	0.71±0.02^{d}	0.28±0.09^{e}
Octanoic acid	2.64±0.03^{b}	1.49±0.12^{c}	3.29±0.74^{a}	1.02±.020^{ab}	1.06±0.05^{ab}	0.93±0.63^{d}
Decanoic acid	3.76±0.35^{b}	2.19±0.05^{c}	8.34±0.34^{a}	1.27±0.04^{d}	1.24±0.06^{d}	0.90±0.96^{d}
2-Phenylacetate	1.07±0.03^{bc}	1.4±0.81^{b}	2.25±0.07^{a}	0.96±0.11^{c}	0.0±0.00^{c}	0.79±0.80^{c}
2-Isoamyl acetate	0.80±0.01^{a}	0.49±0.03^{abc}	0.78±0.00^{a}	0.15±0.22^{c}	0.30±0.00^{bc}	0.55±0.06^{ab}
Hexyl acetate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl-hexanoate	1.05±0.23^{a}	0.15±0.01^{abc}	0.69±0.02^{bc}	0.25±0.36^{ab}	0.0±0.00^{c}	0.54±0.01^{abc}
Ethyl-caprylate	0.34±0.03^{a}	0.51±0.07^{a}	0.26±0.01^{a}	0.29±0.28^{a}	0.0±0.00^{a}	0.14±0.03^{a}
Ethyl acetate	24.15±2.04^{b}	23.61±1.35^{b}	39.85±1.44^{a}	36.64±1.82^{a}	34.12±3.14^{ab}	30.94±4.89^{ab}
Ethyl butyrate	0.75±0.27^{a}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}
Ethyl lactate	0.0±0.00^{b}	0.51±0.07^{a}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}
Ehtyl-3-hydroxybutanoate	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	1.80±0.05^{a}	0.0±0.00^{b}	0.0±0.00^{b}
Ethyl-caprate	0.51±0.04^{a}	0.15±0.05^{b}	1.14±0.03^{a}	0.80±0.02^{ab}	0.0±0.00^{c}	0.19±0.10^{c}
Ethyl phenylacetate	0.0±0.00^{d}	1.55±0.03^{b}	1.15±0.00^{c}	1.22±0.00^{c}	1.75±0.02^{a}	1.21±0.00^{c}
Diethyl succinate	0.0±0.00^{d}	1.05±0.08^{a}	1.06±0.02^{a}	3.02±0.61^{a}	1.87±0.12^{b}	1.05±0.03^{c}

Mean values bearing differing superscript letters showed significant differences and mean values bearing the same letter were statistically similar
All the compounds are presented in mg L^{-1} and are average of two biological duplicates ± SD
AN* indicating anaerobic conditions
Nd- Not detected

Table 3.5 Major volatile compounds detected with significant differences at end of the fermentation in *S. cerevisiae*/ *T. delbrueckii* single culture and their mixed cultures.

Major Volatiles	*S. cerevisiae* (AN*)	*T. delbrueckii* (AN*)	Sc+Td (AN*)	Sc+Td -1%	Sc+Td-5%	Sc+Td-21%
2-Phenylethanol	6.30±1.24^{c}	18.29±7.21^{c}	36±0.40^{cb}	54±0.02^{b}	213±0.54^{a}	222±18.00^{a}
Isoamyl alcohol	50.8±8.75^{c}	87.82±0.58^{c}	119±0.85^{b}	132±2.50^{a}	198±2.26^{a}	208±16.40^{a}
Isobutanol	12.5±2.85^{d}	16.17±2.09^{d}	26.2±2.75^{d}	87±0.26^{c}	128±2.70^{b}	169±1.34^{a}
Propanol	31.18±5.48^{ab}	20.65±4.51^{b}	0.0±0.00^{c}	0.0±0.00^{c}	37.87±2.17^{a}	0.0±0.00^{c}
Butanol	0.0±0.00^{b}	0.69±0.09^{b}	0.71±0.00^{b}	0.52±.07^{b}	3.56±0.56^{a}	0.73±0.03^{b}
Pentanol	1.56±0.00^{a}	0.48±0.13^{c}	0.0±0.00^{d}	0.88±0.02^{b}	1.11±0.01^{b}	0.0±0.00^{d}
Hexanol	0.89±0.00^{b}	0.04±0.02^{c}	0.0±0.00^{c}	0.0±0.00^{c}	10.32±0.01^{a}	0.0±0.00^{c}
3-ethoxy-1-propanol	1.15±0.40^{c}	16.89±4.44^{ab}	8.68±0.44^{bc}	6.41±0.08^{bc}	25.07±3.43^{a}	8.04±0.76^{bc}
3-Methyl-1-pentanol	0.0±0.00^{c}	0.48±0.00^{b}	0.0±0.00^{c}	0.0±0.00^{c}	1.02±0.00^{a}	0.0±0.00^{c}
Propionic acid	1.69±0.12^{b}	2.96±0.55^{b}	2.10±0.06^{b}	4.2±1.34^{b}	7.6±1.15^{a}	7.4±0.50^{a}
Isobutyric acid	1.57±0.55^{b}	1.58±0.33^{b}	2.08±0.09^{b}	7.8±0.54^{a}	5.8±1.20^{a}	2.08±0.08^{b}
Butyric acid	1.30±0.04^{a}	1.38±0.32^{a}	1.24±0.02^{a}	1.91±0.04^{a}	2.08±0.57^{a}	2.12±0.13^{a}
Isovaleric acid	1.07±0.00^{a}	0.91±0.08^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.33±0.47^{bc}	0.0±0.00^{b}
Valeric acid	0.66±0.06^{a}	0.46±0.05^{a}	0.56±0.00^{a}	0.68±0.01^{a}	0.82±0.47^{a}	0.57±0.00^{a}
Hexanoic acid	1.96±0.20^{a}	0.68±0.03^{b}	2.28±0.04^{a}	0.92±0.07^{b}	1.05±0.02^{b}	0.38±0.54^{b}
Octanoic acid	2.64±0.03^{b}	0.88±0.22^{c}	3.18±0.01^{a}	0.96±0.02^{b}	0.97±0.08^{b}	0.45±0.01^{c}
Decanoic acid	3.76±0.35^{b}	2.02±0.02^{c}	6.33±0.02^{a}	1.19±0.03^{a}	1.22±0.14^{d}	1.00±0.02^{d}
2-Phenylacetate	1.07±0.03^{b}	0.89±0.03^{b}	2.47±0.11^{a}	0.78±0.26^{b}	1.13±0.10^{b}	1.32±0.32^{b}
2-Isoamyl acetate	0.80±0.01^{a}	0.43±0.07^{c}	0.59±0.03^{bc}	0.0±0.00^{a}	0.0±0.00^{a}	0.61±0.06^{b}
Hexyl acetate	0.0±0.00^{b}	0.66±0.03^{a}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}
Ethyl-hexanoate	1.05±0.23^{a}	0.49±0.00^{c}	0.27±0.03^{c}	0.0±0.00^{a}	0.09±0.01^{a}	0.63±0.06^{b}
Ethyl-caprylate	0.34±0.03^{b}	2.07±0.63^{a}	0.63±0.01^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.20±0.02^{b}
Ethyl acetate	24.15±2.04^{bc}	20.71±1.73^{c}	26.43±3.36^{bc}	22.43±0.22^{bc}	28.18±1.35^{ab}	33.79±0.36^{a}
Ethyl butyrate	0.75±0.27^{a}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}	0.0±0.00^{b}
Ethyl lactate	0.0±0.00^{a}	0.71±0.17^{a}	0.0±0.00^{a}	0.0±0.00^{a}	0.29±0.42^{a}	0.0±0.00^{a}
Ethyl-3-hydroxybutanoate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl-caprate	0.51±0.04^{ab}	0.29±0.01^{ab}	0.96±0.09^{a}	0.0±0.00^{b}	0.14±0.04^{ab}	0.53±0.51^{ab}
Ethyl phenylacetate	0.0±0.00^{b}	1.37±0.03^{a}	1.15±0.00^{ab}	1.17±0.00^{ab}	1.40±0.08^{ab}	0.57±0.81^{ab}
Diethyl succinate	0.0±0.00^{c}	1.05±0.00^{b}	1.29±0.02^{b}	2.70±0.08^{a}	1.52±0.26^{b}	1.40±0.17^{b}

Mean values bearing differing superscript letters showed significant differences and mean values bearing the same letter were statistically similar
All the compounds are presented in mg L^{-1} and are average of two biological duplicates ± SD
AN* indicating anaerobic conditions
Nd- Not detected

Table 3.6 Major volatile compounds detected with significant differences at end of the fermentation in *S. cerevisiae*/ *M. pulcherrima* single culture and their mixed cultures.

Major Volatiles	*S. cerevisiae* (AN*)	*M. pulcherrima* (AN*)	Sc+Mp (AN*)	Sc+Mp -1%	Sc+Mp -5%	Sc+Mp -21%
2-Phenylethanol	6.30±1.24^{b}	24.37±1.89^{b}	27±2.25^{b}	133±0.42^{a}	119±14.00^{a}	141±7.70^{a}
Isoamyl alcohol	50.8±8.75^{d}	137.43±0.56^{c}	79±7.57^{a}	152±3.80^{bc}	196±8.56^{b}	276±29.00^{a}
Isobutanol	12.5±2.85^{e}	227.44±5.16^{a}	124±1.45^{d}	122±2.56^{d}	145±4.26^{c}	167±8.60^{b}
Propanol	31.1±5.48^{c}	22.3±0.00^{c}	51±0.33^{c}	63±6.30^{bc}	77±1.52^{b}	107±2.25^{a}
Butanol	0.00±0.00^{a}	0.51±0.00^{b}	0.83±0.07^{c}	2.07±0.13^{d}	0.67±0.03^{bc}	0.69±0.40^{bc}
Pentanol	1.56±0.00^{b}	0.37±0.00^{a}	1.62±0.03^{b}	0.98±0.00^{c}	0.94±0.01^{c}	0.97±0.00^{c}
Hexanol	0.89±0.00^{a}	0.41±0.00^{a}	0.84±0.06^{a}	10.84±0.09^{b}	0.71±0.24^{a}	0.22±0.31^{a}
3-Ethoxy-1-propanol	1.15±0.40^{ab}	00.0±0.00^{a}	2.28±0.11^{abc}	4.5±0.00^{a}	4.6±1.80^{a}	4.67±0.00^{a}
3-Methoxy-1-pentanol	0.00±0.00^{c}	0.00±0.00^{c}	1.56±0.01^{a}	0.0±0.00^{c}	0.97±0.00^{b}	0.97±0.00^{b}
Propionic acid	1.69±0.12^{ab}	0.78±0.02^{a}	1.25±0.58^{ab}	1.49±0.81^{ab}	1.42±0.11^{ab}	2.62±0.34^{b}
Isobutyric acid	1.57±0.55^{ab}	0.72±0.01^{a}	1.35±0.19^{ab}	1.47±0.04^{ab}	0.95±0.01^{a}	2.30±0.02^{a}
Butyric acid	1.30±0.04^{c}	0.89±0.01^{c}	1.50±0.12^{c}	3.39±0.33^{a}	1.06±0.13^{b}	2.23±0.17^{a}
Isovaleric acid	1.0±0.09^{a}	0.00±0.04^{d}	1.05±0.05^{a}	0.73±0.02^{b}	0.63±0.00^{c}	0.75±0.01^{d}
Valeric acid	0.62±0.06^{a}	0.41±0.03^{c}	0.64±0.04^{a}	0.54±0.03^{ab}	0.40±0.00^{c}	0.42±0.00^{bc}
Hexanoic acid	1.96±0.20^{a}	0.61±0.06^{b}	1.55±0.22^{a}	1.52±0.20^{a}	1.62±0.31^{a}	1.58±0.28^{a}
Octanoic acid	2.64±0.03^{a}	0.73±0.00^{b}	1.67±0.05^{ab}	1.72±0.34^{ab}	1.16±0.98^{ab}	1.63±0.46^{ab}
Decanoic acid	3.76±0.35^{a}	1.96±0.01^{b}	2.27±0.03^{b}	2.27±0.32^{ab}	2.34±0.58^{b}	2.28±0.40^{b}
2-Phenylethy acetate	1.07±0.03^{a}	0.95±0.00^{c}	0.93±0.05^{a}	0.47±0.00^{a}	1.07±0.09^{c}	0.47±0.25^{a}
2-Isoamyl acetate	0.80±0.01^{a}	0.03±0.00^{a}	0.77±0.04^{b}	0.0±0.00^{c}	0.39±0.12^{b}	0.2±0.04^{ab}
Hexyl acetate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl-hexanoate	1.05±0.23^{a}	0.00±0.00^{e}	0.80±0.01^{b}	0.25±0.03^{b}	0.15±0.01^{c}	0.09±0.00^{d}
Ethyl-caprylate	0.34±0.03^{a}	0.00±0.01^{c}	0.12±0.02^{b}	0.08±0.00^{b}	0.0±0.00^{c}	0.0±0.00^{c}
Ethyl acetate	24±2.04^{f}	157.16±7.21^{d}	84±0.90^{e}	310±7.00^{b}	265±6.34^{c}	366±1.67^{a}
Ethyl butyrate	0.64±0.02^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.10±0.14^{c}
Ethyl lactate	0.0±0.00d	0.64±0.30^{d}	11±2.51^{b}	12.4±0.88^{c}	15±1.40^{cd}	18±1.14^{a}
Ethy-3-hydroxybutanoate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl-caprate	0.51±0.04^{a}	0.00±0.02^{c}	0.16±0.01^{b}	0.0±0.00^{c}	0.05±0.00^{c}	0.04±0.00^{c}
Ethyl phenylacetate	0.0±0.00^{c}	0.00±0.03^{c}	1.56±0.03^{a}	1.20±0.01^{b}	0.47±0.09^{b}	1.07±0.25^{b}
Diethyl succinate	0.0±0.00^{e}	0.60±0.00^{cb}	1.26±0.03^{bc}	2.3±0.14^{cd}	2.22±0.73^{cd}	2.7±0.05^{a}

Mean values bearing differing superscript letters showed significant differences and mean values bearing the same letter were statistically similar
All the compounds are presented in mg L^{-1} and are average of two biological duplicates ± SD
AN* indicating anaerobic conditions
Nd- Not detected

Principal component analysis (PCA) biplot showed that the first two principal components explain 41% of the variability shown in the fermentations studied (Fig. 3.6-a, b). PC1 differentiates the fermentations according to the yeast dominance profiles, resulting in the *S. cerevisiae* and *M. pulcherrima* dominated fermentations forming distinct groups separate from the *L. thermotolerans* and *T. delbrueckii* dominated fermentations. The fermentations are further separated along PC2 which explains 18% of the variance, and separates according to levels of aeration.

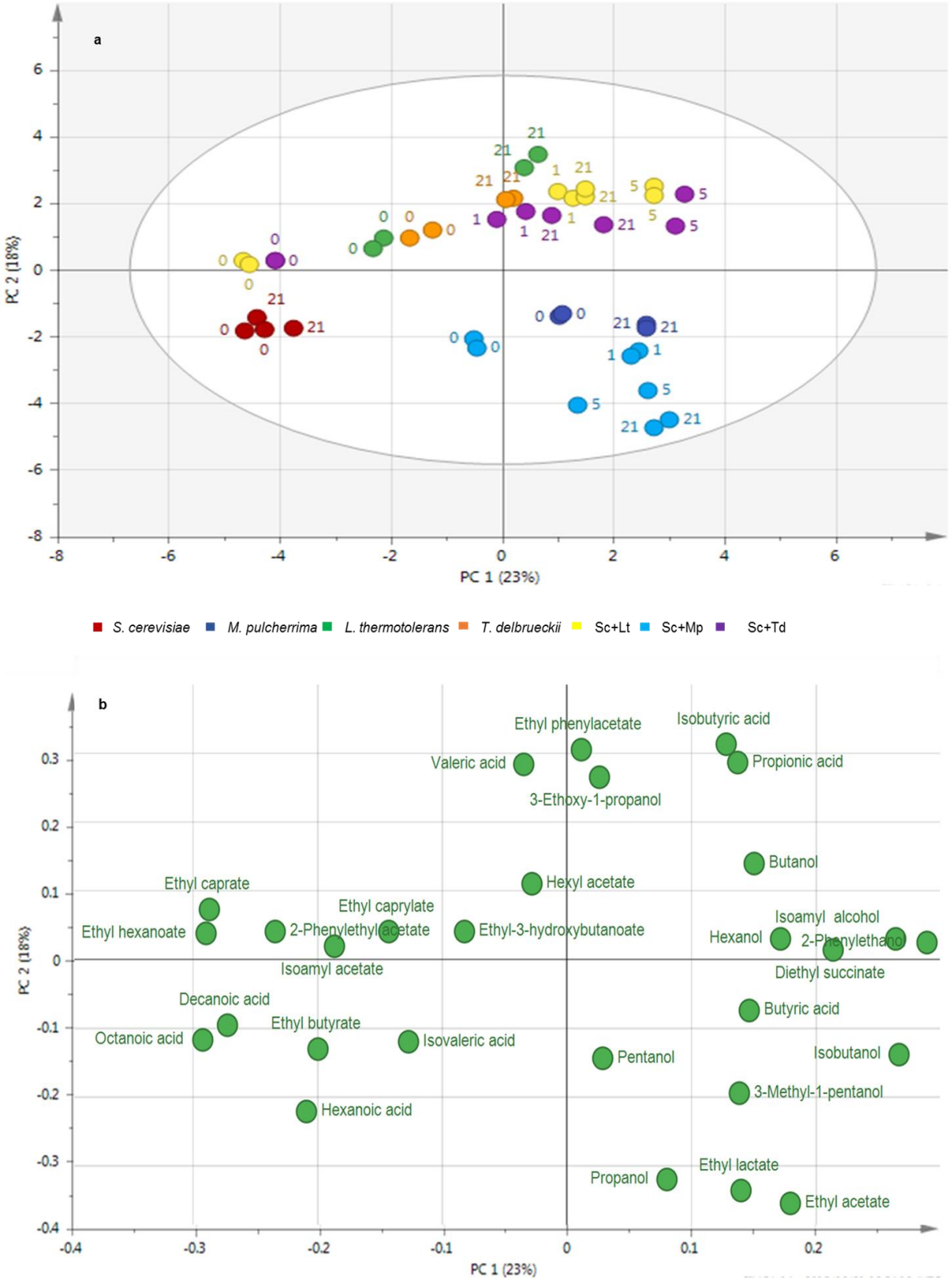

Figure 3.6 PCA score plot (a) and loading plot (b) of the first principle components showing major volatiles produced by different single species and mixed fermentations with and without oxygen. The numbers (0, 1%, 5% and 21%) indicates the DO levels in the fermentations.

The metabolic profile of the anaerobic *S. cerevisiae*/ *L. thermotolerans*, *S. cerevisiae*/ *T. delbrueckii* is close to the *S. cerevisiae* single culture fermentation, while their aerated mixed cultures exhibit a distinct chemical profile from the anaerobic mixed fermentations, however, similar to the aerated *L. thermotolerans*, *T. delbrueckii* single fermentations. The fermentation profiles of *L. thermotolerans*, *T. delbrueckii* mixed fermentation with *S. cerevisiae* could not show a clearly separation on the basis of oxygenation levels. In contrast, the *S. cerevisiae*/ *M. pulcherrima* mixed fermentations showed a clear separation between the 1%, 5% and 21% DO treatments. The separation of the *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* single anaerobic fermentations and their mixed cultures along PC1 was mostly driven by the production of medium chain fatty acids. While, the separation of the aerated *L. thermotolerans, T. delbrueckii*, *S. cerevisiae/ L. thermotolerans* and *S. cerevisiae/ T. delbrueckii* from the anaerobic cultures was strongly associated with the accumulation of higher alcohols. In contrast, the separation of the aerated *S. cerevisiae/ M. pulcherrima* fermentation was mainly driven by acetate esters and higher alcohols. For more clear understanding PC3 and PC 4 were also performed, however again no clear separation was seen from there.

3.5 Discussion

The current study evaluated the effect of three different levels of oxygen on yeast growth and volatile compound production by applying a co-fermentation strategy with *S. cerevisiae* with either *T. delbrueckii*, *L. thermotolerans* or *M. pulcherrima*.

3.5.1 Effect of oxygen on persistence of non-*Saccharomyces* yeasts

Our data show that oxygenation had a positive effect on yeast population growth especially on the growth and persistence of non-*Saccharomyces* yeasts. However, all three non-*Saccharomyces* yeasts responded very differently to oxygen availability, perhaps due to their different oxygen requirement. Of the three, *M. pulcherrima* displayed the strongest dependence on oxygen, and its ability to contribute significantly to the outcome of the fermentation strongly depended on the amount of oxygen supplied. Indeed, in anaerobic conditions, this yeast could only be detected in the first 24 to 48 h, and its contribution to the final aroma compound levels was insignificant. However, at 1%, 5% and 21% DO this yeast displayed protracted persistence with viable cell count reaching up to 10^{10} CFU mL^{-1}. In contrast, *L. thermotolerans* and *T. delbrueckii* could grow and persist in anaerobic conditions albeit at relatively low cell numbers of 10^{7} and 10^{6} CFU mL^{-1}, respectively. The growth of both yeasts was significantly enhanced under oxygenation resulting in cell numbers reaching upto 10^{9} and 10^{10} CFU mL^{-1} in *T. delbrueckii* and *L. thermotolerans*, respectively, at 1% DO and 10^{10} CFU mL^{-1} in both yeasts at 5% and 21% DO. The difference in response to oxygen in the three yeasts could be due to different oxygen demands of these yeasts and different respiratory quotient (RQ). Indeed, previous studies have shown that *M. pulcherrima* displays a fully respiratory glucose metabolism, with respiratory quotient (RQ) values of 1.04-1.26 (Contreras et al., 2014; Morales et al., 2015; Quirós et al., 2014).

The three non-*Saccharomyces* yeasts not only responded differently to oxygenation but also influenced the growth of *S. cerevisiae* in different ways. In the conditions used here (taking into consideration a 1:10 *S. cerevisiae*, non-*Saccharomyces* inoculation ratio), the non-*Saccharomyces* yeasts were able to numerically dominate the fermentations for extended periods of time. For instance, the growth rate of *S. cerevisiae* at 5% DO was slower in the presence of *L. thermotolerans* such that the maximum cell concentration of 10^9 CFU mL^{-1} was only achieved after 48 h while in the presence of *T. delbrueckii* a similar effect only becomes apparent at 21% DO. Both *L. thermotolerans* and *T. delbrueckii* displayed a competitive growth advantage over *S. cerevisiae* at 21% DO as *S. cerevisiae* only managed to grow to 10^7 and 10^8 CFU mL^{-1} in co-fermentation with *L. thermotolerans* and *T. delbrueckii*, respectively. In contrast, *S. cerevisiae* reached similar maximum growth levels of 10^9 CFU mL^{-1} under anaerobic and aerobic conditions (at all DO levels) in the presence of *M. pulcherrima*. Although both *L. thermotolerans* and *T. delbrueckii* are Crabtree-positive and facultative anaerobes like *S. cerevisiae*, it is clear that under oxygenated conditions they display a greater intrinsic growth rate than *S. cerevisiae*. The higher cell counts of non-*Saccharomyces* yeasts in the presence of oxygen is likely a consequence of the greater proportion of carbon flow through respiratory metabolism in these strains (Brandam et al., 2013; Morales et al., 2015; Visser et al., 1990). Indeed, our data show that at 21% DO *S. cerevisiae* in monoculture generated 72 g L^{-1} ethanol while in the presence of *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima* only 59.08, 46.91 and 46.96 g L^{-1} ethanol was produced suggesting that in the mixed cultures most of the sugar is respired. This finding is congruent with previous studies which showed that under oxygenated conditions, *S. cerevisiae* only respires 25% of the sugar while most some non-*Saccharomyces* yeasts such as *T. delbrueckii* can respire 40 – 100% of the sugar without concomitant production of ethanol. Overall our data show that the ethanol yield decreases with increase in aeration and that most of the carbon flux is channeled towards biomass and acetic acid production. However, it is also possible that minor levels of ethanol could escape although for the current experimental setup this was minimized by fitment of a condenser (maintained at -4°C) and through low gas flow rate, standardized for all fermentations.

A further look at the primary metabolites shows that the three non-*Saccharomyces* yeasts have very distinct metabolic responses to oxygenation. For instance, the *S. cerevisiae*/ *M. pulcherrima* fermentations generated excessive amounts of acetic acid (> 1200 mg L^{-1}) at all DO levels, followed by the *S. cerevisiae*/ *L. thermotolerans* fermentation which at 5% and 21% DO also produced undesirable levels. In contrast, the *S. cerevisiae*/ *T. delbrueckii* fermentations only produced high acetic acid levels at 21% DO, while at 1% and 5% DO the levels were lower, and in fact lower than even the *S. cerevisiae* monoculture under anaerobic conditions. In addition, our data show that *T. delbrueckii* which is often described as a low acetic acid producer under standard winemaking conditions maintains this trait even under continuous oxygen supply. Regular punch-downs and pump-overs which are standard practices in red wine fermentations can incorporate varying amounts

of DO upto 5.6 mg L^{-1} into grape must depending on the stage of fermentation (Moenne et al., 2014). Hence, the 1% DO (0.08mg L^{-1}) which is favorable for the all three non-*Saccharomyces* yeasts can be used during winemaking to sustain their growth and reduce the ethanol levels in wine without negative influence on quality, except for *M. pulcherrima* for which lower levels might be preferable to keep the acetic acid level lower.

3.5.2 Overall effect of mixed fermentation and aeration on yeast specific growth rate

The growth rate of all four-yeast species showed species specific differences in single and mixed fermentations under anaerobic and aerobic conditions. The comparison of anaerobic versus aerobic single fermentation showed that overall, the addition of oxygen increased the growth rate of all four yeasts. Increased growth rate for all yeasts in single fermentation agree with previous literature reports that addition of oxygen led to increase in yeast cell growth due to synthesis of cell wall component which enhances yeast cellular tolerance to ethanol and provides healthier growth (Aceituno et al., 2012; Morales et al., 2015; Rosenfeld et al., 2003). Comparing to anaerobic mixed fermentation, the growth rate of three non-*Saccharomyces* yeasts increased with the addition of oxygen, while the growth rate of *S. cerevisiae* decreased (at 5% and 21% DO level), except *S. cerevisiae*/ *M. pulcherrima* fermentation. Besides the increase in specific growth rate of non-*Saccharomyces* yeasts, the addition of oxygen reduced the stationary growth phase in aerobic mixed fermentation, again with the exception of mixed fermentation with *M. pulcherrima*. Although, *S. cerevisiae* displays faster growth rate in single aerobic fermentation, in mixed aerobic fermentation with high DO levels, *L. thermotolerans* and *T. delbrueckii* grow faster than *S. cerevisiae* and maintained higher viable cell counts, suggesting that non-*Saccharomyces* yeasts generate more biomass than *S. cerevisiae* in presence of oxygen (Ciani et al., 2016; Morales et al., 2015). However, in the presence of *M. pulcherrima*, *S. cerevisiae* displayed similar growth rate as *M. pulcherrima* and exhibited longer exponential and stationary phase indicates more competitive environment for both yeasts. Our results agree with previous studies that the specific growth rate and different growth phases of yeasts depend on yeasts and environmental factors such as oxygen (Quirós et al., 2013; Werner-Washburne et al., 1993).

In anaerobic mixed fermentation, the decrease in growth rate for all four-yeast species shows that presence of yeast species together makes the anaerobic mixed fermentation a more hostile environment in comparison to single anaerobic fermentation. This unfriendly environment in anaerobic mixed fermentation could be due to competitive milieu for the yeast species because of nutrients etc. Among the four non-*Saccharomyces* yeast species presence of *M. pulcherrima* influenced most the growth of *S. cerevisiae*, similarly *S. cerevisiae* affected the growth of *M. pulcherrima* most in anaerobic mixed fermentation. In S. cerevisiae/ *M. pulcherrima* mixed anaerobic fermentation, *M. pulcherrima* stayed only for 48 hours but the growth rate of *S. cerevisiae* still decreased with a longer stationary phase. Such behavior is intriguing because, in co-culture, *M. pulcherrima* did not persist after 48 h of alcoholic fermentation. This suggests a metabolic interaction

between *S. cerevisiae* and *M. pulcherrima*. The antagonistic effect of *M. pulcherrima* against other yeasts has been shown via production of pulcherrimin pigment which causes iron sequestration and medium and arrests growth of other yeasts (Oro et al., 2014). Therefore, the decrease in growth rates of non-*Saccharomyces* yeasts could be due to anaerobic condition while the longer stationary phase in *M. pulcherrima* fermentation suggests a metabolic interaction between both yeasts.

3.5.3 Impact of oxygen on formation of volatile compounds

In mixed culture fermentations, the chemical compositions of the synthetic wines at the end of fermentation clearly showed the contribution of each non-*Saccharomyces* yeasts. *L. thermotolerans* and *T. delbrueckii* showed similar behavior and resulted in higher production of 2-phenylethanol, isoamyl-alcohol, isobutanol, hexanoic, decanoic, octanoic acids, while *M. pulcherrima* also affected ethyl lactate, ethyl acetate. The higher production of these compounds by *M. pulcherrima* have been attributed to high cell density ratios between non-*Saccharomyces* and *S. cerevisiae* yeasts in co-inoculation (Contreras et al., 2014; Sadoudi et al., 2012). In addition, it is important to note that *M. pulcherrima* generates high levels of ethyl acetate (> 300 mg L^{-1}) in all aerobic fermentations, which could suggest that this yeasts mainly uses ethyl acetate production as a detoxification mechanism to remove ethanol and acetate from cells. Ethyl acetate at levels above 100 mg L^{-1} contributes a solvent, balsamic aroma and is not desirable at high levels in wine. The incorporation of oxygen in both mixed and single fermentations showed significant increase in higher alcohols (particularly 2-phenylethanol, isoamyl-alcohol and isobutanol), revealing a positive correlation between the production of higher alcohols, the growth of non-*Saccharomyces* yeasts and oxygen levels. The yield data of volatile compounds normalized with biomass at 5% DO show an increase in higher alcohols (isoamyl-alcohol, 2-phenylethanol and isobutanol) as well as propionic acid and butyric acid (*S. cerevisiae*/ *L. theromotolerans* and *S. cerevisiae*/ *T. delbrueckii*), while a decrease in MCFA and esters was observed. This increase in the yield of these compounds can be in part due to increased biomass (Supplementary Table S3.1-S3.4) under aerobic conditions, but also due to increased uptake of branched chain amino acids such as leucine, isoleucine and valine. Indeed, the expression of *BAP2*, which encodes branched chain amino acid permeases, is upregulated under aerobic conditions (Verbelen et al., 2009). Evidently, the total sum of the three higher alcohols (isoamyl-alcohol, 2-phenylethanol and isobutanol) accumulated at 5% and 21% levels ranged between 300 and 500 mg L^{-1} depending on the DO levels. At such high levels these alcohols are known to impart harsh, spirituous, nail polish-like aroma, which are not desirable in wine (Panon, 1997; Sun et al., 2014). The impact of oxygenation in all aerobic fermentations resulted in a decrease in MCFAs, reflecting the incorporation of fatty acids into long chain fatty acids biosynthesis through the acetyl-CoA carboxylase and fatty acid synthetase activity (Lambrechts and Pretorius, 2000; Sumper, 1974). Overall, our data clearly show that the impact of non-*Saccharomyces* yeast on wine fermentation and aroma can be managed through controlled oxygen supply, and that the level of oxygen will largely determine the degree of impact of the non-*Saccharomyces* yeast including on the aromatic

contribution of these strains. The impact is significant already at relatively low levels of oxygen supply, and such DO level can be managed within a winery through various strategies such as micro-oxygenation or regular pump-overs in red winemaking.

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3.7 Compliance with ethical standards

3.7.1 Funding

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3.7.2 Conflict of interest

The authors declare that they have no conflict of interest.

3.7.3 Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

3.8 References

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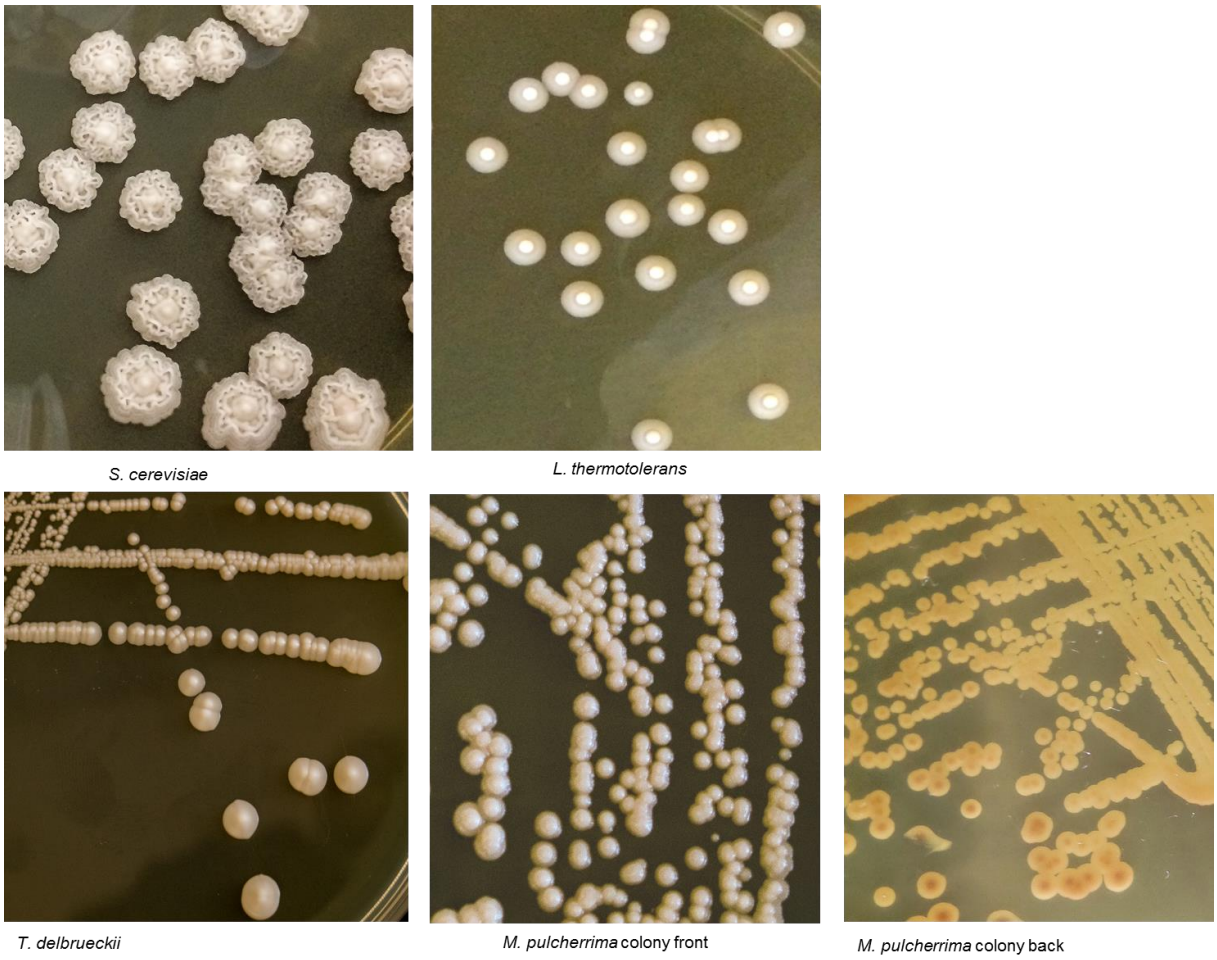

Figure S 3.1 Colony characteristics of *S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima* (front and back side) on YPD plates

Table Supplementary 3.1. Major volatile compound's yield in *S. cerevisiae*/ *L. thermotolerans* mixed fermentations under anaerobic (AN) and 5% DO aerobic condition

Major volatiles	Sc+Lt (AN)	Sc+Lt (5%)
	µg/µg of Sugar	µg/µg of Sugar
2-Phenylethanol	0.165	0.52
Isoamyl alcohol	0.60	1.075
Isobutanol	0.167	0.695
Propanol	0.0	0.127
Butanol	0.0049	0.0052
Pentanol	0.0	0.0048
Hexanol	0.0	0.0527
3-Ethoxy-1-propanol	0.0214	0.0413
3-Methyl-1-Pentanol	0.0	0.0
Propionic acid	0.010	0.047
Isobutyric acid	0.0072	0.048
Butyric acid	0.0049	0.0088
Isovaleric acid	0.0	0.0
Valeric acid	0.0035	0.0033
Hexanoic acid	0.0108	0.0035
Octanoic acid	0.0164	0.0053
Decanoic acid	0.0417	0.0062
2-Phenylacetate	0.0112	0.0
2-Isoamyl acetate	0.0039	0.0015
Hexyl acetate	0.0	0.0
Ethyl-hexanoate	0.0034	0.0
Ethyl-caprylate	0.0013	0.0
Ethyl acetate	0.199	0.1706
Ethyl butyrate	0.0	0.0
Ethyl lactate	0.0	0.0
Ethyl-3-hydroxybutanoate	0.0	0.0
Ethyl-caprate	0.0057	0.0
Ethyl phenylacetate	0.0057	0.0087
Diethyl succinate	0.0053	0.0093

Table Supplementary 3.2. Major volatile compound's yield in *S. cerevisiae*/ *T. delbrueckii* mixed fermentations under anaerobic (AN) and 5% DO aerobic condition

Major volatiles	Sc+Td (AN)	Sc+Td (5%)
	µg/µg of Sugar	µg/µg of Sugar
2-Phenylethanol	0.180	1.065
Isoamyl alcohol	0.595	0.99
Isobutanol	0.131	0.64
Propanol	0.0	0.189
Butanol	0.0035	0.017
Pentanol	0.0	0.0056
Hexanol	0.0	0.0516
3-Ethoxy-1-propanol	0.0434	0.1253
3-Methyl-1-pentanol	0.0	0.0051
Propionic acid	0.0105	0.038
Isobutyric acid	0.0104	0.029
Butyric acid	0.0062	0.010
Isovaleric acid	0.0	0.0016
Valeric acid	0.0028	0.0042
Hexanoic acid	0.0114	0.0052
Octanoic acid	0.0159	0.0048
Decanoic acid	0.0316	0.0061
2-Phenylacetate	0.0123	0.0056
2-Isoamyl acetate	0.0029	0.0
Hexyl acetate	0.0	0.0
Ethyl-hexanoate	0.0013	0.0004
Ethyl-caprylate	0.0031	0.0
Ethyl acetate	0.132	0.1409
Ethyl butyrate	0.0	0.0
Ethyl lactate	0.0	0.0014
Ehthyl-3-hydroxybutanoate	0.0	0.0
Ethyl-caprate	0.0048	0.0007
Ethyl phenylacetate	0.0057	0.0070
Diethyl succinate	0.0064	0.0076

Table Supplementary 3.3. Major volatile compound's yield in *S. cerevisiae*/ *M. pulcherrima* mixed fermentations under anaerobic (AN) and 5% DO aerobic condition

Major volatiles	Sc+Mp (AN) µg/µg of Sugar	Sc+Mp (5%) µg/µg of Sugar
2-Phenylethanol	0.135	0.595
Isoamyl alcohol	0.395	0.98
Isobutanol	0.62	0.725
Propanol	0.255	0.385
Butanol	0.0041	0.0033
Pentanol	0.0081	0.0047
Hexanol	0.0042	0.0035
3-Ethoxy-1-propanol	0.0114	0.023
3-Methyl-1-pentanol	0.0078	0.0048
Propionic acid	0.0062	0.0071
Isobutyric acid	0.0067	0.0047
Butyric acid	0.0075	0.0053
Isovaleric acid	0.0052	0.0031
Valeric acid	0.0032	0.0020
Hexanoic acid	0.0077	0.0081
Octanoic acid	0.0084	0.0058
Decanoic acid	0.0113	0.0117
2-Phenylacetate	0.0046	0.0053
2-Isoamyl acetate	0.0038	0.0019
Hexyl acetate	0.00	0.0
Ethyl-hexanoate	0.004	0.0007
Ethyl-caprylate	0.0006	0.0
Ethyl acetate	0.42	1.325
Ethyl butyrate	0.00	0.0
Ethyl lactate	0.055	0.075
Ethyl-3-hydroxybutanoate	0.0	0.0
Ethyl-caprate	0.0008	0.0002
Ethyl phenylacetate	0.0078	0.0023
Diethyl succinate	0.0063	0.0111

Table Supplementary 3.4. Major volatile compound's yield, normalized with biomass obtained in anaerobic and 5% DO level condition in *S. cerevisiae* mixed fermentation with three non-*Saccharomyces* yeasts

Major volatiles	Sc+Lt-AN µg/µg of biomass	Sc+Lt-5% µg/µg of biomass	Sc+Td AN µg/µg of biomass	Sc+Td 5% µg/µg of biomass	Sc+Mp AN µg/µg of biomass	Sc+Mp5% µg/µg of biomass
2-Phenylethanol	6.400	9.429	5.913	19.906	4.592	10.86
Isoamyl alcohol	23.319	19.43	19.464	18.504	13.176	17.83
Isobutanol	6.494	8.274	4.288	11.961	20.657	13.24
Propanol	0.000	2.304	0.000	3.530	8.626	7.029
Butanol	0.190	0.095	0.117	0.332	0.139	0.061
Pentanol	0.000	0.087	0.000	0.103	0.270	0.085
Hexanol	0.000	0.953	0.000	0.961	0.140	0.065
3-Ethoxy-1-propanol	0.829	0.747	1.420	2.336	0.471	0.423
3-Methyl-1-pentanol	0.000	0.000	0.000	0.095	0.260	0.088
Propionic acid	0.394	0.854	0.344	0.708	0.208	0.129
Isobutyric acid	0.278	0.875	0.341	0.542	0.224	0.087
Butyric acid	0.190	0.160	0.202	0.194	0.249	0.096
Isovaleric acid	0.000	0.000	0.000	0.031	0.175	0.057
Valeric acid	0.135	0.059	0.092	0.076	0.106	0.036
Hexanoic acid	0.419	0.064	0.373	0.098	0.257	0.147
Octanoic acid	0.636	0.090	0.520	0.091	0.278	0.106
Decanoic acid	1.613	0.112	1.035	0.114	0.377	0.213
2-Phenylacetate	0.435	0.063	0.404	0.105	0.159	0.043
2-Isoamyl acetate	0.151	0.027	0.097	0.000	0.128	0.036
Hexyl acetate	0.000	0.000	0.000	0.000	0.000	0.000
Ethyl-hexanoate	0.133	0.000	0.103	0.000	0.134	0.014
Ethyl-caprylate	0.051	0.000	0.044	0.009	0.021	0.000
Ethyl acetate	7.710	3.080	4.323	2.626	13.990	24.175
Ethyl butyrate	0.000	0.000	0.000	0.000	0.000	0.000
Ethyl lactate	0.000	0.000	0.000	0.027	1.904	1.449
Ethyl-3-hydroxybutanoate	0.000	0.000	0.000	0.000	0.000	0.000
Ethyl-caprate	0.222	0.000	0.157	0.013	0.027	0.004
Ethyl phenylacetate	0.223	0.158	0.188	0.130	0.260	0.000
Diethyl succinate	0.206	0.168	0.211	0.142	0.210	0.202

Chapter 4

Research results II

Employing oxygen pulses to modulate *Lachancea thermotolerans*-*Saccharomyces cerevisiae* Chardonnay fermentations

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Employing oxygen pulses to modulate *Lachancea thermotolerans-Saccharomyces cerevisiae* Chardonnay fermentations

4.1 Abstract

Oxygen is sometimes deliberately introduced in winemaking at various stages to enhance yeast biomass formation and prevent a stuck fermentation. However, there is limited information on how such interventions affect the dynamics of yeast populations. The previous study in synthetic grape juice showed that oxygen supply enhances the persistence *Lachancea thermotolerans*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*. The three species however differ in their response to oxygen, impacting on other wine-relevant characteristics such as aroma production and ethanol yields. The present study focused on evaluating the influence of short oxygen pulses on population dynamics and the aroma profile of Chardonnay wine inoculated with *L. thermotolerans* and *Saccharomyces cerevisiae*. The results confirmed a positive effect of oxygen on the relative performance of *L. thermotolerans*. The results also indicate that continuous stirring supports the growth of *L. thermotolerans* independently of the specific oxygen treatment. The mixed culture fermentation with *L. thermotolerans* with *S. cerevisiae* developed a distinct aroma profile when compared to monoculture *S. cerevisiae*. Specifically, a high concentration of esters, medium chain fatty acids and higher alcohols was detected in the mixed culture fermentation. The data also showed that the longer persistence of *L. thermotolerans* due to addition of oxygen pulses influenced the formation of major volatile compounds such as ethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl caprylate, ethyl caprate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, propanol, isobutanol, butanol, isoamyl alcohol, hexanol, isobutyric acid, butyric acid, iso-valeric acid, hexanoic acid, octanoic acid, and decanoic acid. This influence was mainly an increase in higher alcohols while decrease in medium chain fatty acids.

Keywords: non-*Saccharomyces* yeast, winemaking, oxygenation regimes, mixed cultures, yeast population

4.2 Introduction

Wine fermentation is typically characterized by low pH, a rapid development of anaerobiosis, an increase in ethanol and in some cases an increase in temperature. Under these conditions, *S. cerevisiae* displays a better fitness than non-*Saccharomyces* yeast species and tends to rapidly dominate the wine microflora (Albergaria and Arneborg 2016; Williams et al., 2015). Several physiological or metabolic features contribute to the dominance of *S. cereviaise*. *S. cerevisiae* indeed shows better fermentative capacity in anaerobic conditions and higher ethanol tolerance than all other species that are present in the wine ecosystem (Brandam et al., 2013; Ciani et al., 2016; Hanl et al., 2005; Hansen et al., 2001; Jolly 2006). Besides such broad physiological adaptations, *S.*

cerevisiae also relies on some more targeted mechanisms such as the production of toxic metabolites including anti-microbial peptides that target specific competing species. For instance, antimicrobial peptides which are derived from reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase has been reported as the main contributing factor in *S. cerevisiae* competitions against *Hanseniaspora* spp. (Branco et al., 2014; Ciani et al., 2016).

Previous study in our laboratory showed that a continuous supply of oxygen at 1 and 5% dissolved oxygen allowed *L. thermotolerans* and *T. delbrueckii* to dominate mixed fermentations with *S. cerevisiae*, thus confirming that *S. cerevisiae* niche construction and ecological dominance against these two yeasts was due to anaerobiosis (Brandam et al., 2013; Hanl et al., 2005; Hansen et al., 2001). Recently, non-*Saccharomyces* yeasts have become increasingly popular as co-inoculants in mixed-starter fermentations. Indeed, in the past 10 years several species including *L. thermotolerans*, *T. delbrueckii*, *M. pulcherrima* and *P. kluyveri* have been commercialised and are available as monoculture active dry yeasts or as blends (Jolly et al., 2014). Our current study employed *L. theromotolerans* as it was found to require the least amount of oxygen to dominate *S. cerevisiae* compared to *T. delbrueckii* and *M. pulcherrima*. This yeast is known to enhance the concentration of higher alcohols (particularly 2-phenylethanol), L-lactic acid, glycerol, and esters in wine. Moreover, using *L. thermotolerans* in sequential fermentation with *S. cerevisiae* at low temperatures was reported to bring down the levels of ethanol in wine (Gobbi et al., 2013).

Oxygen is typically introduced in winemaking especially in the production of red wine through punch downs, pump over and transfers. Such methods can add up to 6 mg L^{-1} of oxygen (du Toit et al., 2006; Moenne et al., 2014). These oxygen additions are common practice in most wineries as they promote yeast biomass synthesis and contribute to sound wine fermentation and enhance the aroma profile of wine. However, there is little to no information on how they influence the growth and development on non-*Saccharomyces* yeast inoculants.

In the current study, we employed *L. thermotolerans* to evaluate the effect of low oxygen input on its persistence and contribution to the aroma of Chardonnay as higher doses oxygen can be detrimental to wine (Moenne et al., 2014). Therefore, the aim of the current study was to evaluate the impact of commercially realistic oxygen input on the growth and persistence of *L. thermotolerans* in mixed culture fermentation with *S. cerevisiae*, and to evaluate the impact of the changed yeast population dynamics on the organoleptic properties of Chardonnay.

4.3 Materials and methods

4.3.1 Microorganisms and media

A strain of *L. thermotolerans* (IWBT Y-1240) was obtained from the yeast culture collection of Institute for Wine Biotechnology (Stellenbosch University), while *S. cerevisiae* (Cross evolution 285) was obtained from Lallemand SAS (Blagnac, France). The cryogenically (-80°) maintained yeast strains were streaked out on YPD agar plates containing (per litre) 20 g glucose, 20 g peptone, 10 g yeast extract, 20 g bacteriological agar. For further use, cultures were maintained at 4°C for a short period. The chemical analysis of Chardonnay grape juice was obtained from Fourier transform infrared (FT-IR) spectroscopy using the Grape Scan 2000 instrument (FOSS Electric, Denmark). The analysis revealed a total sugar concentration of 215 g L^{-1}, and pH 3.7.

4.3.2 Yeast enumeration and Isolation

For initial yeast identification of Chardonnay grape juice, serial dilutions were prepared in 0.9% (w/v) NaCl solution and spread on WL agar plates (Wallerstein laboratory nutrient, Sigma-Aldrich). Yeast enumeration and isolation were performed from plates that contained yeast colonies between 30 and 300. Yeast colonies with different features (color, texture, size, shape, margin) were further isolated. At least three representative colonies per colony morphology were streaked out from each plate. The obtained isolates were further stored in glycerol 20% (v/v) at -80°C (Bagheri et al., 2015). For further yeast enumeration of mixed and single culture inoculated fermentations, samples were taken every second day; both species were distinguished based on colony morphology on YPD plates as described in chapter-3. Colony counts were performed on plates with 30-300 colonies.

4.3.3 Yeast Identification

For yeast identification, the genomic DNA was extracted from 1 mL of the sample using the rapid yeast DNA extraction method (Hoffman, 2003). The ITS1-5.8S rRNA-ITS2 region amplification was performed by PCR using the primer set ITS1 (5′-TCCGTAGGTGAACCTCGCG-3′) and ITS4 (5′-TCCTCCGCTTTATTGATATGC-3′) (Esteve- Zarzoso et al., 1999). PCR amplification was done in a final volume of 25 µL containing 0.4 mM dNTP mix, 0.25 µM of each primer, 1 U of Ex-Taq polymerase (TaKara), 1× buffer, 1 mM $MgCl_2$ and 100 ng template DNA. Further, the PCR products were purified using the ZymocleanTM Gel DNA recovery kit (Zymo Research Corporation, Irvine, CA, USA) following the manufacture's instruction. Restriction fragment length polymorphisms (RFLP) digestion was performed on of the ITS- 5.8S rRNA PCR product using *Hae*III, *Hin*fI, and *Cfo*I in separate reactions as described by Esteve-Zarzoso et al. (1999). For further identification, the yeast isolates were grouped according to distinct restriction patterns, and previously sequenced species were digested with the same enzymes and used as references to identify the current isolates (Bagheri et al., 2015).

4.3.4 Fermentations

Fermentations were performed in Chardonnay grape juice. Two fermentation procedures were implemented. In the first set-up, 4 L of clarified juice was inoculated in triplicate and fermentations were performed in 5 L bottles sealed with fermentation caps. Oxygen was added to the bottles with the help of Norprene tubing using an oxygen cylinder. The oxygen concentration was monitored by using oxygen sensor spots (Pst-3; PreSens, Regensburg, Germany) fitted inside each bottle. The fermentation kinetics were monitored by weighing the bottles every second day until the weight was stable (Fig S 1).

In the second set-up, controlled fermentations were carried out in 1.3 L BioFlo 110 bench top bioreactors (New Brunswick, NJ, USA) with controlled oxygenation regimes. Fermentations in the bioreactors were carried out in 900 mL clarified juice and were done in duplicate under three different conditions: anaerobically, oxygen pulses once a day and three times a day. Oxygen was added to the cultures using a peristaltic pump. The air flow rate was 1 vvm (volume per volume per minute). To minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation set-up was equipped with Norprene tubing. The dissolved-oxygen concentration in the cultures was monitored with an oxygen electrode. Fermentations were carried out at 20°C, with continuous stirring at 200 rpm.

4.3.5 Inoculation strategies

The yeast strains were first inoculated in 5 mL of YPD broth overnight followed by a transfer of 1 mL to 100 mL of YPD broth which was allowed to grow overnight (±16 h) at 30°C with agitation at 100 rpm. To obtain a higher cell concentration; the 100-mL pre-culture was re-cultured into 1 L YPD broth and incubated until mid-exponential growth phase. Cells were harvested by centrifugation at 5000 x *g* for 5 min, and re-suspended into 0.9% (w/v) NaCl solution. For single culture fermentations, *S. cerevisiae* CE 285 and *L. thermotolerans* Y1240 were inoculated into separate vessels at 10^6 and 10^7 cells mL^{-1}, respectively, while for mixed culture fermentations, *S. cerevisiae* CE 285 and *L. thermotolerans* were co-inoculated simultaneously in the same vessel with a cell density of 10^6 and 10^7 cells mL^{-1}, respectively.

4.3.6 Sample analysis

Samples were collected after every 24 h from the bioreactors and 48 h from the fermentation bottles. To enumerate the yeast population, serial dilutions were performed in 0.9% (w/v) NaCl and 100 µL was plated on YPD agar. The plates were incubated at 30°C for 3-4 days. Glucose, fructose, glycerol and acetic acid were measured using specific enzymatic kits, Enytec™ *Fluid* D-glucose, fructose, acetic acid (Thermo Fisher Scientific Oy, Finland), Boehringer Mannheim / R-Biopharm-acetaldehyde (R-Biopharm AG, Darmstadt) and analyzed using Arena 20XT photometric analyser (Thermo Electron Oy, Helsinki, Finland) (Schnierda et al., 2014). Ethanol was analyzed by high-

performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Agilent RID and UV detectors were used in tandem for peak detection and quantification. The final analysis was done using the HPChemstation software (Rossouw et al., 2012). The liquid-liquid extraction method was used for volatile compound analysis using GC-FID, where 5 mL sample of synthetic grape juice was added with internal standard 4-methyl-2-pentanol (final concentration 5 mg L^{-1}). To perform liquid-liquid extraction 1 mL of diethyl ether was added to each sample and sonicated for 5 min. The wine/ether mixture was then centrifuged at 4000 x *g* for 5 min, and then ether layer (supernatant) was removed and dried on Na_2SO_4 to remove the excess of water. For gas chromatography (GC) a DB-FFAP capillary column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length x 0.32 mm i.d. x 0.5 µm film thickness and a Hewlett Packard 6890 Plus GC instrument (Little Falls, USA) equipped with a split/splitless injector and a flame ionisation detector (FID) was used. The initial oven temperature was 33°C, held for 17 min, after which the temperature was increased by 12°C min^{-1} to 240°C, and +held for 5 min. Three µL of the dieth+yl-ether extract was injected at 200°C in split mode. The split ratio was 15:1 and the split flow rate 49.5 mL min^{-1}. The column flow rate was 3.3 mL min^{-1} using hydrogen as carrier gas. The detector temperature was 250°C (Louw et al., 2010).

4.3.7 Statistical analysis

The chemical analysis of all compounds was performed in duplicate technical repeats on three independent biological repeats of fermenttaions in bottels , and all the values are stated as means ± S.D. The significant differences between measurements within different treatments were determined using analysis of variance (a least-significant-difference[LSD]test) with the statistical software Statistica version 13.0 (Stat Soft Inc., USA) and differences were considered significant when p values were ≤ 0.05. To analyse the significant differences in major volatiles due to aeration and mixing, the two-way ANOVA was performed using XLSTAT 2017 software (Addinsoft, NY, USA). For multivariate data analysis, principle component analysis (PCA) was created using SIMCA-P software version 14.0 (Umetrics, Umea, Sweden).

4.4 Results

4.4.1 Grape juice analysis: Initial yeast identification

The analysis of the initial yeast diversity of the Chardonnay grape juice revealed the presence of 9 different yeast species (Fig. 4.1). *Hanseniaspora uvarum* was the by far most abundant species (74%, 5.8×10^4), followed by *Candida apicola* (7%, 1.2×10^4 cfu mL^{-1}), *Candida oleophila* (5%, 4×10^3 cfu mL^{-1}), *Starmerella bacillaris* (5%, 3.67×10^3 cfu mL^{-1}), *Candida intermedia* (4%, 3.3×10^3 cfu mL^{-1}), and *Candida californica* (2%, 1.66×10^3 cfu mL^{-1}), while *Metschnikowia pulcherrima, Zygoascus meyerae, Bandoniozyma visegradensis* each accounted for approximately 1% (0.67×10^3 cfu mL^{-1}) of the population.

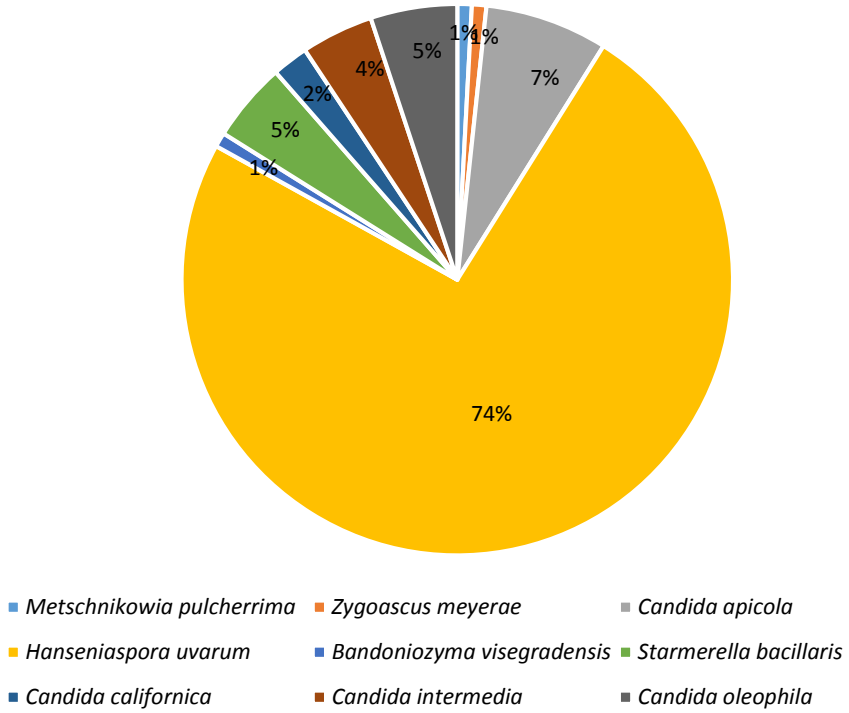

Figure 4.1: The identified initial yeast percentage in Chardonnay grape juice

4.4.2 Yeast dynamics

In the current study, small-scale wine fermentation was performed in two different systems *viz.* 5 L fermentation bottles without agitation and 1.5 L bioreactor units with continuous stirring at 200 rpm. Oxygen pulses were performed either once a day or three times a day. In bottles, the monoculture fermentations of *L. thermotolerans* and *S. cerevisiae* with both types of oxygen pulses reached dryness (< 5 g L^{-1} sugar) in 10 days while fermentation without oxygen addition took 12 days to achieve dryness (Fig. 4.2a). In bottles, *L. thermotolerans* could only be detected for the first 4 days under anaerobic conditions, before the indigenous yeast became dominant. In contrast, in fermentations pulsed three times a day with oxygen, *L. thermotolerans* persisted for 6 days before the indigenous population surpassed it (Fig. 4.2a). In bioreactors, which had constant agitation, *L. thermotolerans* showed persistence until the end of the fermentation even under anaerobic conditions where the fermentation took 7 days to finish (Fig. 4.2b). Similarly, under anaerobic conditions, *S. cerevisiae* reach to dryness in 7 days (Fig. 4.2b).

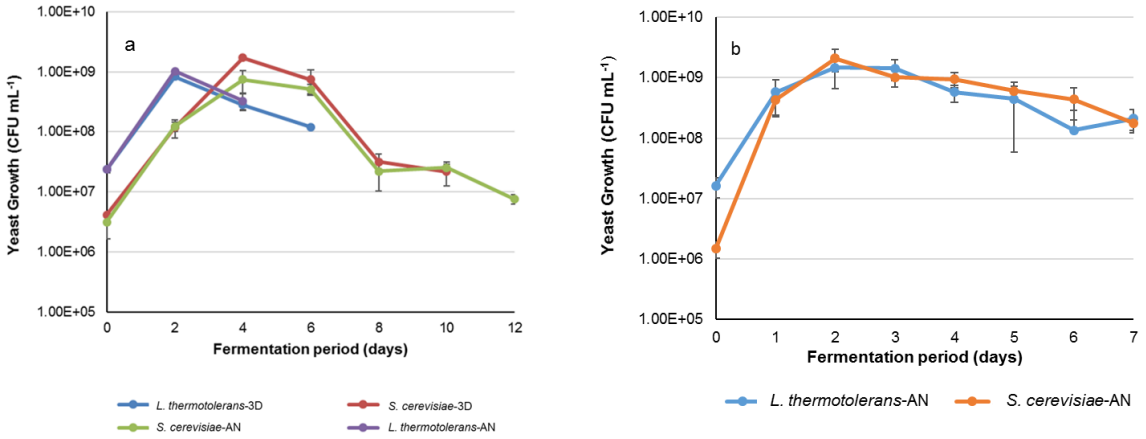

Figure 4.2: Population kinetics of *L. thermotolerans* and *S. cerevisiae* single culture fermentation in bottles (a) under anaerobic (AN) and three times oxygenation per day (3D) condition and in bioreactors (b) under anaerobic conditions.

In bottles, mixed fermentation with oxygen pulses reached dryness in 10 days, while under anaerobic conditions, the fermentation finished in 12 days. In bioreactors, mixed fermentations were performed for 5-days, and the residual sugar concentrations of anaerobic fermentation, once a day and three times a day was 62.6, 44.80 and 28.46 g L^{-1}, respectively. The two systems resulted in different yeast growth. Under anaerobic conditions, in bottles, *L. thermotolerans* maintained viability at the initial inoculum level for four days and then declined below detection (Fig. 4.3), while in bioreactors, it persisted until the fermentations were stopped (fermentations were stopped after 5 days) (Fig. 4.4). In bottles, when oxygen was pulsed once a day, *L. thermotolerans* showed a slight increase in the first two days of fermentation and could be detected until 6 days of fermentation. Incorporation of oxygen pulses 3 times a day, resulted in an increase in growth of *L. thermotolerans* from the initial inoculum level of 10^7 cfu mL^{-1} to 10^9 cfu mL^{-1} within the first two days. *S. cerevisiae* displayed a steady increase in growth from 10^6 cfu mL^{-1} to a maximum of 6.3×10^8 cfu mL^{-1} and 5.2×10^8 cfu mL^{-1} in four days under anaerobic conditions and with oxygen pulsed once a day, respectively (Fig. 4.3). In contrast, when oxygen was pulsed three times a day, *S. cerevisiae* increased to a maximum of 7.0×10^8 cfu ml^{-1} within two days and remained stable for four days before starting to decline.

In bioreactors, under anaerobic conditions, *L. thermotolerans* achieved maximum levels of 1.8×10^9 cfu mL^{-1} within 2 days followed by a slight declined (Fig. 4.4), while *S. cerevisiae* maintained viability at 1.5×10^9 cfu mL^{-1} until the fermentations were stopped (Fig. 4.4). Oxygen provision once and three times a day increased the *L. thermotolerans* cell concentrations to a maximum of 3.3×10^9 cfu mL^{-1} and 1.40×10^{10} cfu mL^{-1}, respectively (Fig. 4.4). Similarly, *S. cerevisiae* displayed a 100-fold increase from the initial 10^6 cfu mL^{-1} inoculated to a maximum of 1.1×10^9 cfu mL^{-1} and 3.2×10^9 cfu mL^{-1} under 1 day and 3-day oxygen pulses, respectively (Fig. 4.4). Overall, in comparison to anaerobic fermentations the persistence of *L. thermotolerans* was increased in fermentation with oxygen pulses in bottles as well as bioreactors.

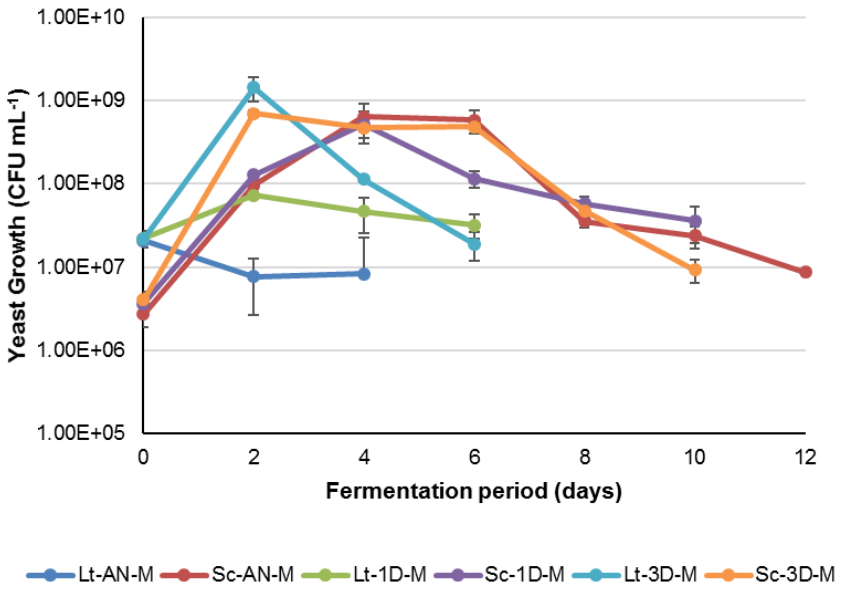

Figure 4.3: Population kinetics of *L. thermotolerans* and *S. cerevisiae* mixed culture fermentation in bottles, under anaerobic (Lt-AN-M, Sc-AN-M) oxygenation once per day (Lt-1D-M, Sc-1D-M) and three times per day (Lt-3D-M, Sc-3D-M).

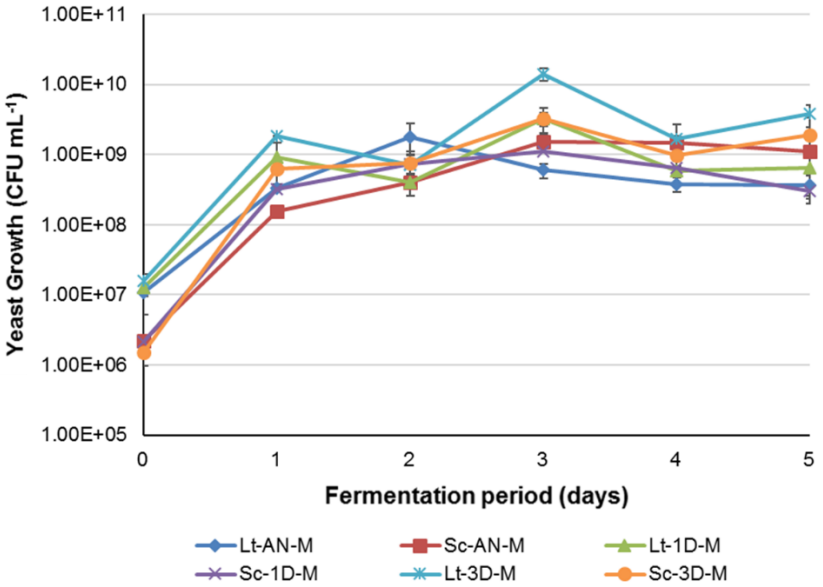

Figure 4.4: Population kinetics of *L. thermotolerans* and *S. cerevisiae* mixed culture fermentation in bioreactors, under anaerobic (Lt-AN-M, Sc-AN-M) oxygenation once per day (Lt-1D-M, Sc-1D-M) and three times per day (Lt-3D-M, Sc-3D-M).

4.4.3 Impact of oxygen pulses on dry biomass and ethanol content

The results of dry biomass and the rest of the chemical analysis will be presented only for fermentations which were performed in bottles, since the mixed fermentation in bioreactors were not performed until the end of the fermentation. The dry biomass was determined only at the end of fermentation. Higher biomass was obtained under aerobic conditions compared to anaerobic conditions (Fig. 4.5). In mixed fermentations, the dry biomass increased from 3.23 g L^{-1} under anaerobic conditions to 3.93, 4.15 g L^{-1}, when oxygen was pulsed once a day and three times a day, respectively (Fig. 4.5). Oxygen pulsing three times a day in mixed fermentations resulted in significant reduction in ethanol levels compared to monoculture inoculations (Table 4.1). There were

no significant differences were observed in the concentration of acetic and glycerol in different fermentations.

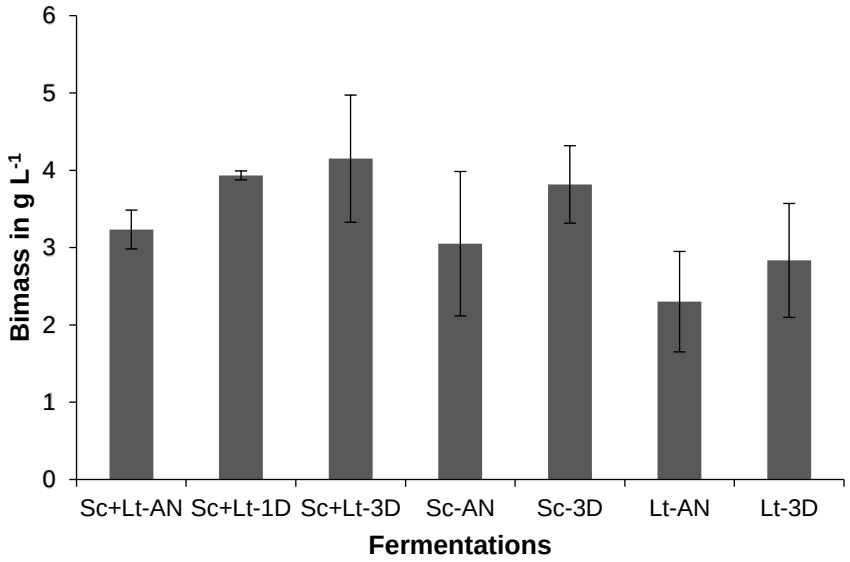

Figure 4.5: Dry mass produced by *S. cerevisiae*/ *L. thermotolerans* mixed and single culture fermentations in bottles with anaerobic condition (AN), oxygenation one time a day (1D) and three times per day (3D).

Table 4.1: Fermentation parameters and products of candidate *L. thermotolerans* and *S. cerevisiae* in mixed and pure culture fermentations in bottles (Values are mean of triplicates).

Fermentation	Ethanol yield (Et g/g sugar)	Ethanol (g L^{-1})	Acetic acid (g L^{-1})	Glycerol (g L^{-1})
Sc+Lt-AN	0.509±0.002	109.8±1.57^{ab}	0.12±0.02^{a}	5.56±0.24^{a}
Sc+Lt-1D	0.503±0.01	108±0.48^{ab}	0.19±0.005^{a}	5.69±0.11^{a}
Sc+Lt-3D	0.49±0.008	107±1.92^{b}	0.22±0.11^{a}	5.75±0.08^{a}
S. cerevisiae-AN	0.51±0.007	110±0.99^{a}	0.19±0.02^{a}	5.54±0.22^{a}
S. cerevisiae-3D	0.49±0.01	109.2±0.79^{ab}	0.14±0.02^{a}	5.59±0.24^{a}
L. thermotolerans-AN	0.50±0.04	109.3±0.45^{ab}	0.20±0.005^{a}	5.80±0.13^{a}
L. thermotolerans-3D	0.49±0.06	108±0.76^{ab}	0.25±0.08^{a}	5.89±0.33^{a}

4.4.4 Impact of oxygen pulses on major volatile compounds of Chardonnay grape juice

The data showed significant increase in the concentration of higher alcohols, esters, and fatty acids in mixed fermentations in comparison to monoculture fermentation of *S. cerevisiae* under anaerobic condition. Two-way ANOVA revealed that the addition of oxygen and co-inoculation of *L. thermotolerans*/ *S. cerevisiae*, influenced the profile of volatile compounds. For instance, the formation of ethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl caprylate, ethyl caprate, ethyl-3-hydroxybutanoate, ethyl phenylacetate, propanol, isobutanol, butanol, isoamyl alcohol, hexanol, isobutyric acid, butyric acid, iso-valeric acid, hexanoic acid, octanoic acid, and decanoic acid was influenced by co-inoculation as well as aeration; while isoamyl acetate, 3-methyl-1-pentanol, valeric acid were mainly influenced by different oxygenation regimes. In contrast, the formation of ethyl

lactate, diethyl succinate, 3-ethoxy-1-propanol, propionic acid was influenced by co-inoculation (Table S 4.1).

Principal component analysis (PCA) showed that the first two principal components explain 68% of the variability between different fermentations (Fig. 4.6). PC1 explained 52% variability and separated the fermentations according to different oxygen regimes. The fermentation profiles of anaerobic fermentations were separated from aerobic fermentations, and the separation was mainly driven by higher alcohols (2-phenylethanol, isobutanol, isoamyl-alcohol, hexanol), ethyl phenyl acetate, valeric acid and isobutyric acid (Fig. 4.6). The fermentations are further separated along PC2 with 16% of the variance. The anaerobic fermentation of *L. thermotolerans* clearly formed a separate group from the rest of the fermentations due to higher concentrations of ethyl lactate, ethyl acetate, and ethyl-3-hydroxybutanoate. The anaerobic fermentation of *S. cerevisiae* and mixed anaerobic fermentations clearly formed separate groups from fermentation with oxygenation either once or three times per day. The metabolic profile of the anaerobic monoculture of *S. cerevisiae* and the anaerobic mixed-fermentation grouped together due to a higher concentration of medium chain fatty acids and esters.

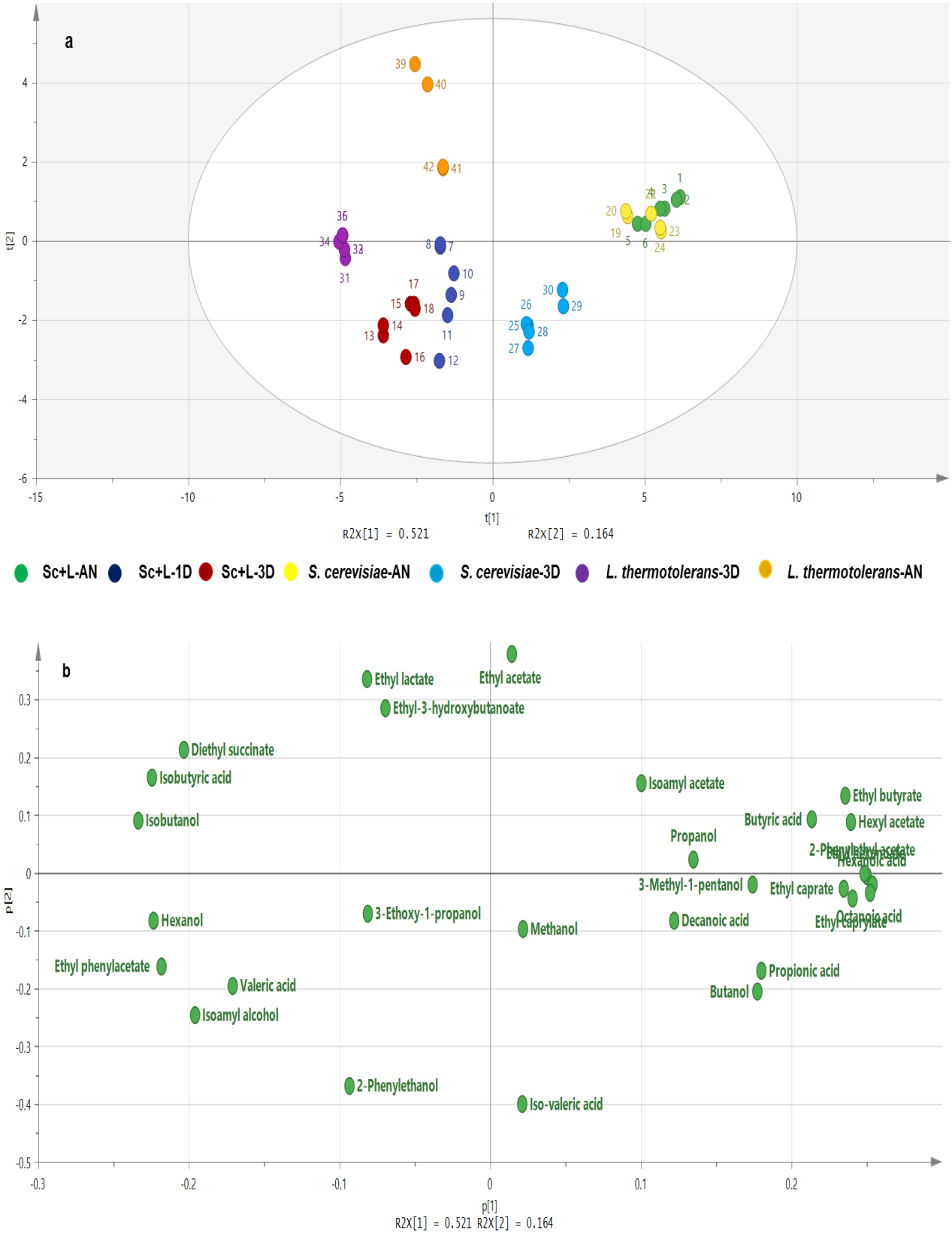

Figure 4.6: PCA score plot (a) and loading plot (b) of the first principle components showing major volatiles produced by *L. thermotolerans* and *S. cerevisiae* single species and mixed fermentations with and without oxygen in bottles.

4.5 Discussion

The incorporation of oxygen at various stages of wine making process has an impact on fermentative rate, wine quality as well as on yeast physiology (Aceituno et al., 2012; Ingledew et al., 1987; Rosenfeld et al., 2003; Valero et al., 2001). Our previous data generated on synthetic grape juice (Chapter-3) demonstrated the influence of oxygen on yeast dynamics and volatile compounds. The addition of oxygen showed the numerical dominance of *L. thermotolerans* and increased the concentration of higher alcohols. However, excessive continuous levels of oxygen were applied which is not a realistic strategy in a commercial cellar. Therefore, the current study applied oxygen levels like those that may be achieved through common winemaking practices such as punch downs and pump-overs. Although in white wine the punch down does not take place, the current study used white grape juice in order to repeat the data of synthetic grape juice which is more close to white grape juice matrix.

4.5.1 Impact of oxygen pulses on persistence of *L. thermotolerans*

In the current study, mixed culture fermentations of *S. cerevisiae* and *L. thermotolerans* carried out under anaerobic condition showed an early decline in the population of *L. thermotolerans* and the rest of fermentation was dominated by *S. cerevisiae*. However, when oxygen was introduced either through pulsing once a day or three times a day, the growth of *L. thermotolerans* was enhanced. In bottles, the single culture inoculated fermentation of *L. thermotolerans*, *L. thermotolerans* declined below detection level after day 4 and day 6 in anaerobic and aerobic conditions, respectively, and the rest of the fermentation was dominated by indigenous yeast. In fermentation with bioreactors, which implemented continuous stirring together with the oxygen input, in *L. thermotolerans* single culture fermentation, *L. thermotolerans* remained viable until the end of fermentations. This marked difference in the growth of *L. thermotolerans* in the two systems could be due to the stirring in bioreactors where the yeast cells have more access to nutrients. Unlike static condition, the continuous stirring in bioreactor distributes the nutrients evenly. Indeed, other studies have also shown enhanced biomass in shaking conditions compared to static conditions (Ali and Khan 2014). In anaerobic mixed fermentation with bioreactors, *S. cerevisiae* dominated throughout the fermentation while *L. thermotolerans* showed a decline after day two. In contrast, with oxygen pulses, *L. thermotolerans* showed higher cell counts than *S. cerevisiae* at day 1, 3 and 5 when oxygen was pulsed once and three times per day, respectively. In bottles, the mixed culture fermentations with oxygen regimes, showed higher cell numbers and an increase in the persistence of *L. thermotolerans* for 6 days compared to 4 days under anaerobic conditions. These results are in accordance with previous studies and clearly show a positive effect of oxygenation and stirring on the growth of *L. thermotolerans* (Hansen et al., 2001; Contreras et al., 2014; Quirós et al., 2014). Nissen et al. (2004) performed population dynamics with *L. thermotolerans* and *S. cerevisiae* and study showed higher oxygen need of *L. thermotolerans* than *S. cerevisiae*. Quirós et al. (2014) showed higher oxygen requirement of *Lachancea* species (*Kluyveromyces lactis*/*marxianus*) with respiratory quotient (RQ)

of 0.8-1.25. This most certainly explains why *L. thermotolerans* seemed to be affected by the changes in oxygen availability. Besides the role of oxygen and stirring on growth of *L. thermotolerans*, considering the previous studies our data of the yeast growth in bottle fermentation also suggests that *L. thermotolerans* died off earlier not only because of less oxygen and stirring, but also possibly because of the presence of *S. cerevisiae* (Luyt 2015; Nissen et al., 2004). Previous studies have presented results where metabolic and cell-cell interaction between *L. thermotolerans*, *T. delbrueckii* with *S. cerevisiae* seems to be responsible for the early decline of these non-*Saccharomyces* yeasts (Nissen et al., 2003; Renault et al., 2013).

In conclusion, our study in both Systems with different oxygen regimes indicate that the growth of *L. thermotolerans* is significantly influenced by multiple factors i.e. oxygen, continuous stirring and presence of *S. cerevisiae*. However, for better understanding more research needs to be done at a molecular level to uncover the underlying facts that which genes and mechanisms responsible for the impact of oxygen on the growth of these yeasts and the mechanism through which these yeasts interact with each other.

4.5.2 Analytical profile of wine

The mixed fermentation of *S. cerevisiae*/ *L. thermotolerans* resulted in less acetic acid production than pure *S. cerevisiae* cultures, confirming our previous results in synthetic grape juice. In mixed culture fermentation, *L. thermotolerans* influenced the final levels of higher alcohols, esters and MCFA in wines, in comparison to *S. cerevisiae* pure culture fermentation. The current dataset of *S. cerevisiae*/ *L. thermotolerans* mixed fermentation is in accordance with our previous study and other literature reports (Howell et al., 2006; Gobbi et al., 2013; Milanovic et al., 2012). The increase in the concentration of secondary metabolites in mixed culture fermentation has been attributed to the metabolic interaction between *S. cerevisiae* and non-*Saccharomyces* yeasts (Barbosa et al., 2015; Luyt 2015). Therefore, the current data set also suggest that perhaps the higher concentration of higher alcohols, esters and medium chain fatty acids in anaerobic mixed culture fermentation could be due to the metabolic interaction between *L. thermotolerans* and *S. cerevisiae*.

Our statistical analysis showed a combined influence of co-inoculation as well as aeration. For instance, the formation of propanol, isobutanol, butanol, isoamyl alcohol, 2-phenyl ethanol, hexanol, isobutyric acid was influenced by co-inoculation as well as aeration. We think that increase in these compounds could be due to increased persistence of *L. thermotolerans* in aerobic fermentation as mixed fermentation even in anaerobic condition led to increasing in the concentration of these higher alcohols.

The formation of these higher alcohols takes places via Ehrlich pathway, which involves uptake of branched-chain amino acids (leucine, isoleucine, and valine) and synthesizes higher alcohols

(isoamyl alcohol, active amyl alcohols and isobutanol respectively). Studies have shown accelerated transcripts of permeases that are responsible for uptake of these amino acids. For instance, higher expression of *BAP2* (branched chain amino acid permease) is reported as result of oxygen addition (Verbelen et al., 2008). Therefore, the higher levels of these alcohols could be due to the higher persistence of *L. thermotolerans* as well more uptake of these amino acids by the yeasts under oxygenation conditions. Similarly, a higher concentration of ethyl phenylacetate, ethyl lactate, diethyl succinate, isobutyric can be linked to longer persistence of *L. thermotolerans* due to oxygenation.
Of the esters, except ethyl phenylacetate, ethyl lactate, diethyl succinate, rest of the esters showed reduction because of oxygen exposure. Volatile esters are enormously important for the flavor profile of the wine. Numerous different enzymes take part in the formation of esters, and the best characterized are the alcohol acetyl transferases I and II, which are encoded by the genes *ATF1* and *ATF2*, respectively (Malcorps and Dufour 1992). The expression of these transferases has been shown to be down-regulated in response to oxygen exposure (Mason and Dufour 2000). Therefore, the reduction in these esters could be due to the result of repression of these genes.

Unsaturated fatty acids (UFAs) are essential for maintaining membrane integrity, function, as well as for adapting to fermentation stresses, such as high sugar and ethanol toxicity. These unsaturated fatty acids are derived from desaturation of small chain and MCFAs in presence of oxygen (Duan et al., 2015). Therefore, in fermentations with oxygenation, the decrease in MCFAs can be explained by the phenomenon of unsaturated fatty acid (UFA), sterols formation. This has been reported to occur in the presence of oxygen, through the action of *ERG1*, which has also been reported previously a reason for partial removal of toxic MCFA (C8-C12) and accelerated the synthesis of long-chain (C16- C18) fatty acids and sterols. These factors can contribute to an enhanced sugar uptake through the cell membrane and accelerated yeast survival (Ingledew 1985; Ribereau-Gayon 1985; Schneider 1998; Varela et al., 2012).

To understand the fermentative behaviour of yeast while fermenting the real grape juice, the use of synthetic grape juice has been considered best approach at laboratory scale. Yet, it is often difficult to extrapolate the behaviour of yeast strains in synthetic grape juice under laboratory conditions to the behaviour of the same yeast strain in real grape juice conditions. It is therefore necessary to validate the behaviour of same yeast strains in both juice compositions. The comparison of the major volatile compounds between the data set obtained from synthetic grape juice (Chapter-3) and Chardonnay grape must (in bottles) for *S. cerevisiae*/ *L. thermotolerans* show a similar trend for oxygen treatments (Fig 2 S). For instance, the total concentration of higher alcohols in Chardonnay grape juice mixed culture fermentation was increased from anaerobic conditions to oxygenation once a day and three times a day: 248, 325 and 336 g L^{-1}, respectively. This trend for higher alcohols was similar to those obtained in Synthetic grape juice under anaerobic, 1%, 5% and 21% levels of oxygen:

191, 426, 466 and 633 g L^{-1}, respectively. In contrast in both matrices the total concentration of esters and MCFA decreased as a result of oxygen addition as showed in Fig S 2.

In conclusion, our data set from real grape juice confirms the result of synthetic grape juice. A reduced oxygen availability greatly affected the growth of *L. thermotolerans* and volatile compounds. However, the presence of *S. cerevisiae* and stirring also influenced the growth of *L. thermotolerans*. Based on the literature, current data set also suggest a possible metabolic interaction between the two yeasts which influences the growth of *L. thermotolerans* and formation of secondary metabolites. Furthermore, the use of oxygen pulses resulted in no major difference in acetic acid concentration. Therefore, use of oxygen pulses in the form of pump-over or punch-down seems more feasible to sustain *L. thermotolerans* for a longer period to attain the higher concentration of volatile compounds, while avoiding the risks of undesirable compounds formation such as acetic acid.

4.6 Funding information

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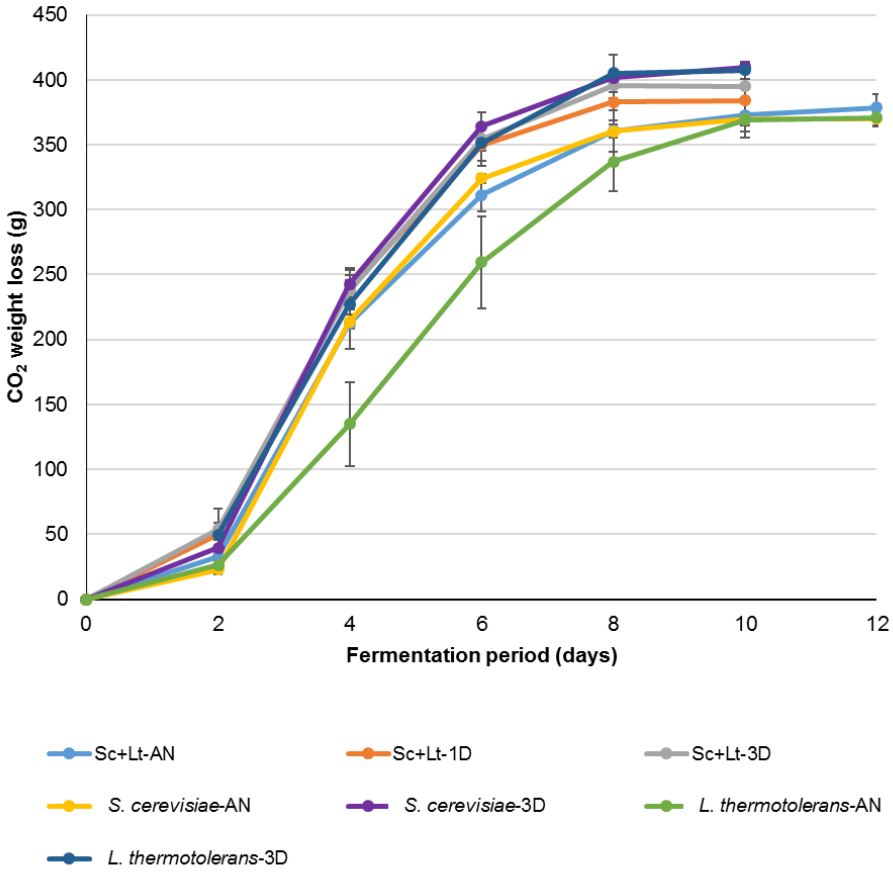

Figure S 1: Fermentation curves of *L. thermotolerans* and *S. cerevisiae* single and mixed culture fermentations in bottles, under anaerobic (AN) oxygenation once per day (1D) and three times per day (3D).

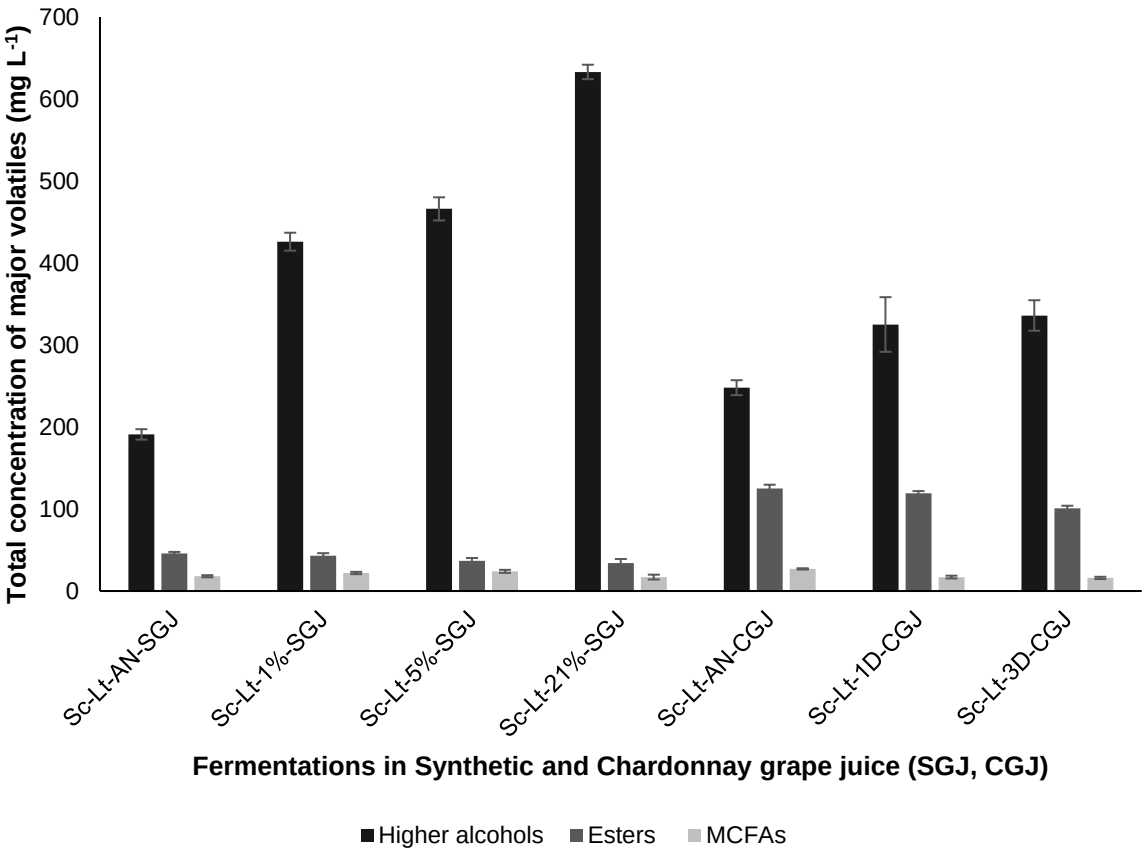

Figure S 2: Total concentration of major volatiles produced by *S. cerevisiael L. thermotolerans* mixed culture fermentations in Chardonnay Grape Juice (CGJ) with anaerobic condition (AN), oxygenation one time a day (1D) three times per day (3D) in bottles and with Synthetic Grape Juice (SGJ) in anaerobic condition (AN), 1%, 5% and 21 % level of oxygenation.

Table S 4.1 Aroma compound's composition of Chardonnay grape must under System I in mixed and pure culture with different fermentation condition.

Compounds	Sc+Lt-AN	Sc+Lt-1D	Sc+Lt-3D	*S. cerevisiae* AN	*S. cerevisiae* 3D	*L. thermotolerans* AN	*L. thermotolerans* 3D
Ethyl acetate	**98.49±3.07**	**94.44±1.76**	**80.94±2.31**	99.32±3.80	**81.84±10.00**	135.36±3.80	**91.19±1.76**
Ethyl butyrate	**0.87±0.05**	**0.66±0.01**	**0.62±0.01**	0.85±0.04	**0.68±0.03**	0.71±0.04	0.56±0.01
Ethyl hexanoate	**2.09±0.13**	**1.25±0.12**	**1.11±0.09**	1.93±0.09	**1.45±0.13**	1.04±0.09	**0.88±0.12**
Ethyl caprylate	**1.62±0.04**	**0.79±0.22**	**0.73±0.22**	1.50±0.10	**1.02±0.10**	0.51±0.02	**0.35±0.27**
Ethyl caprate	**0.20±0.01**	**0.12±0.01**	**0.10±0.01**	0.20±0.02	**0.18±0.03**	0.12±0.02	**0.06±0.01**
Ethyl-3-hydroxybutanoate	**0.00±0.00**	**0.04±0.00**	**0.00±0.00**	0.00±0.00	**0.00±0.00**	0.19±0.00	**0.00±0.00**
Ethyl phenylacetate	**0.54±0.03**	**0.94±0.05**	**0.98±0.05**	0.54±0.02	**0.72±0.10**	0.73±0.02	**1.05±0.17**
Ethyl lactate	**7.25±0.42**	**7.64±0.44**	**8.03±0.14**	7.44±0.41	7.84±1.09	15.40±0.41	17.48±0.44
Diethyl succinate	**0.40±0.00**	**0.46±0.05**	**0.49±0.05**	0.00±0.00	0.41±0.00	0.81±0.00	0.97±0.05
Hexyl acetate	**1.15±0.05**	**0.55±0.04**	**0.44±0.06**	1.07±0.02	**0.85±0.06**	0.58±0.02	**0.39±0.04**
2-Phenylethyl acetate	2.07±0.10	1.10±0.07	0.90±0.05	1.91±0.26	1.66±0.03	0.94±0.26	0.76±0.07
Isoamyl acetate	11.15±0.45	**11.14±0.12**	**7.49±0.05**	10.22±1.01	**7.53±0.15**	8.81±1.01	**7.40±0.12**
Propanol	**61.75±1.06**	**60.71±1.15**	**46.22±1.19**	65.40±2.47	**54.84±17.08**	50.71±2.47	**50.82±1.15**
Isobutanol	**19.07±0.51**	**26.35±0.35**	**28.62±0.43**	18.60±0.94	**22.62±3.55**	31.20±0.94	**32.35±0.35**
Butanol	**2.08±0.11**	**1.66±0.02**	**1.42±0.01**	2.18±0.13	**1.508±0.29**	1.23±0.13	**1.34±0.02**
Isoamyl alcohol	**138.01±6.51**	**207.24±26.24**	**207.90±12.71**	137.05±7.09	**178.15±10.07**	168.15±7.09	**200.70±26.24**
Pentanol	0.00±0.00	0.17±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
2-Phenylethanol	24.80±0.84	35.61±5.22	36.40±3.64	23.95±3.70	36.17±3.36	24.05±3.70	30.86±5.22
3-Methyl-1-pentanol	0.35±0.00	**0.34±0.00**	**0.00±0.00**	0.36±0.01	**0.36±0.01**	0.41±0.01	**0.00±0.00**
Hexanol	**1.50±0.05**	**2.56±0.22**	**2.70±0.19**	1.53±0.05	**1.61±0.41**	2.10±0.05	**2.74±0.22**
3-Ethoxy-1-propanol	**1.15±0.03**	**1.55±0.14**	**1.81±0.43**	1.23±0.04	1.03±0.02	1.19±0.04	1.61±0.14
Propionic acid	**1.88±0.04**	**1.43±0.22**	**1.46±0.27**	1.88±0.05	1.96±0.44	1.27±0.05	1.09±0.22
Isobutyric acid	**0.96±0.02**	**1.44±0.12**	**1.43±0.07**	0.95±0.04	**1.08±0.08**	1.91±0.04	**1.70±0.12**
Butyric acid	**2.65±0.11**	**2.10±0.22**	**2.00±0.14**	2.64±0.04	**2.15±0.10**	2.11±0.04	**1.82±0.22**
Iso-valeric acid	**1.07±0.02**	**1.15±0.07**	**1.19±0.05**	1.07±0.05	**1.25±0.05**	0.98±0.05	**1.04±0.07**
Valeric acid	0.96±0.01	**1.05±0.03**	**1.06±0.02**	0.97±0.00	**1.02±0.07**	0.98±0.00	**1.08±0.03**
Hexanoic acid	**7.83±0.26**	**4.18±0.25**	**3.73±0.24**	7.59±0.52	**5.79±0.47**	3.40±0.52	**2.82±0.25**
Octanoic acid	**10.32±0.19**	**5.05±0.37**	**4.53±0.35**	9.75±0.58	**7.69±0.77**	3.75±0.58	**3.02±0.37**
Decanoic acid	**1.42±0.08**	**1.41±0.43**	**1.21±0.24**	1.392±0.03	**1.06±0.12**	1.02±0.03	**0.81±0.43**

*Compounds in red code are significantly influenced due to mixing and aeration, compounds in purple code show significant influence of only mixing, while compounds in green color indicate significant influence of aeration

Chapter 5

Research results III

Transcriptional responses of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed fermentations under anaerobic and aerobic conditions

This manuscript will be submitted for publication in
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Transcriptional responses of *Saccharomyces cerevisiae* and *Lachancea thermotolerans* in mixed fermentations under anaerobic and aerobic conditions

5.1 Abstract

The blending of non-*Saccharomyces* yeast with *Saccharomyces cerevisiae* to improve the complexity of wine has become a common practice in wine industries, but the impact of such practices on yeast physiology and on genetic and metabolic regulation has not been investigated in detail. Herein, we describe a transcriptomic and exo-metabolomic analysis of single species and mixed species fermentations. The fermentations were carried out under carefully controlled environmental conditions in a bioreactor to eliminate or reduce any transcriptomic responses that would be due to factors other than the presence of the second species.

The transcriptomic data revealed that the both yeast species showed a clear response to the presence of each other at the molecular level. The genes affected primarily belonged to two groups: genes whose expression can likely be linked to the competition for certain nutrients such as copper, iron, sulfur and thiamine, and genes involved in the maintenance of cell wall integrity. The data also show that the transcriptomic data align well with exo-metabolomic data. The higher concentration of higher alcohols, esters in mixed fermentation is aligned with higher expression of genes that encode for enzymes involved in the formation of these aroma compounds.

The results obtained in the current study suggest that the mixed fermentation created a more competitive and stressful environment for the two species than single strain fermentations. The higher expression of genes that involved in nutrients assimilation and uptake indicates the existence of interaction at the molecular level. The data therefore characterize the ecological and metabolic interactions between *S. cerevisiae* and *L. thermotolerans* and reveal molecular responses of yeast to the presence of competing organisms.

Keywords: Transcriptome, non-*Saccharomyces* yeast, mixed fermentation, metabolic interaction, exo-metabolomics

5.2 Introduction

In the past decade, co-inoculations of two species of yeast, usually a strain of *Saccharomyces cerevisiae* with a strain of a non-*Saccharomyces* yeast, has become a common practice in the global wine industry. These non-*Saccharomyces* yeast are used to enhance the aroma profile and organoleptic characteristics of wine, and may also be useful to reduce ethanol yields Ciani et al., 2006; 2010; 2016; Comitini et al., 2011; Gobbi et al., 2013; Sadoudi et al., 2012; Soden et al., 2000).

As a consequence of such practices, understanding the interaction between *Saccharomyces* and non-*Saccharomyces* yeasts has become a central focus of ecological and of wine-related research. The nature of some of the ecological interactions between two yeast species has been studied. The data show that in the mixed fermentation of *S. cerevisiae* and non-*Saccharomyces* yeasts, *S. cerevisiae* displays antagonistic interaction towards non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii*, *Hanseniaspora guilliermondii*, *Kluyveromyces lactis* Nissen et al., 2003; Pérez-Nevado et al., 2006). The presence of *S. cerevisiae* cells at a high concentration causes cellular death in *T. delbrueckii* and *L. thermotolerans* (Nissen et al., 2003). In our environment study by Luyt (2015) showed a decrease in growth of *L. thermotolerans* due to physical contact with *S. cerevisiae*. These studies suggest the existence of a physical and metabolic interaction between these two-yeast species, but do not provide any insights into the molecular mechanism behind these interactions. However, little is known about the molecular responses and interactions between these yeast species, or about the factors influencing the growth and molecular responses of yeast to the presence of another species in multi-starter fermentations. Such studies are challenging because of the complexity of multispecies systems and of ecological interactions. In particular, very few studies have thus far been published reporting genome-wide data sets for such interactions, and most of these studies have primarily been reporting on the response of *S. cerevisiae* to the presence of another species. For instance, DNA microarray-based transcriptome analyses and mass spectrometry-based proteome analyses have been used to study the interaction between yeast and bacteria as well as between *S. cerevisiae* and non-*Saccharomyces* yeasts under oenological conditions (Barbosa et al., 2015; Bron et al., 2012; de Groot et al., 2007; Gasch et al., 2000; Koskenniemi et al., 2011; Lee et al., 2013; Mostert et al., 2014, Rossouw et al., 2012; Salusjärvi et al., 2003).

In the current study, we evaluated the transcriptomic and exo-metabolomic response of *L. thermotolerans* and *S. cerevisiae* in mixed fermentations when compared to single strain cultures in the same environmental conditions. We selected *L. thermotolerans* as a non-*Saccharomyces* wine yeast as that yeast has already been commercialised for use in mixed starter fermentations. Mixed culture fermentation with *L. thermotolerans* is known for leading to an enhanced concentration of higher alcohols (particularly 2-phenylethanol), L-lactic acid, glycerol and esters, while in some conditions also resulting in lower ethanol wines (Gobbi et al., 2013). The genome of this yeast has been sequenced and the genome sequence has been partially annotated. As demonstrated in chapter-3, 4 and previous studies (Hansen et al., 2001), oxygen enhances the growth and persistence of *L. thermotolerans* in mixed starter fermentations. The current study sought to understand the genetic mechanisms underlying the interactions between these two yeasts under 5% dissolved oxygen and anaerobic conditions.

5.3 Materials and methods

5.3.1 Yeast Strains and Media

S. cerevisiae (Cross evolution-285) was obtained from Lallemand SAS (Blagnac, France), while *L. thermotolerans* (IWBT-Y1240) was obtained from the culture collection of the Institute for Wine Biotechnology (Stellenbosch University). Yeast strains were maintained cryogenically (-80°C) and were reactivated by streaking out on YPD agar plates containing (per litre) 10 g yeast extract, 20 g peptone and 20 g glucose and 20 g bacteriological agar. Cultures were stored at 4°C for short term use.

5.3.2 Batch fermentation

Batch fermentations were performed in synthetic grape juice medium containing (per litre) 100 g glucose, 100 g fructose, 1 g yeast extract (Oxoid), 0.3 g citric acid, 5 g L-malic acid, 5 g L-tartaric acid, 0.4 g $MgSO_4$, 5 g KH_2PO_4, 0.2 g NaCl, 0.05 g $MnSO_4$ and anaerobic factors (ergosterol 10 mg, tween 80 0.5 mL) (22-23) (Henschke and Jiranek 1993; Ough et al., 1989). Fermentations were conducted in 1.3 L BioFlo 110 bench top bioreactors (New Brunswick, NJ, USA) using 900 mL of final working volume, a temperature of 25°C and an agitation speed of 200 rpm. Fermentations were performed under two conditions: anaerobic and aerobic at 5% (0.41 mg L^{-1}) dissolved oxygen (DO). The anaerobic conditions were created by initially sparging N_2 to bring down the DO level to 0%, and then to minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation set-up was equipped with Norprene tubing. The 5% DO level was maintained through the supplementary addition of 4 gasses (CO_2, N_2, O_2 and compressed air) whenever required, using an automated gas flow controller. The DO levels in the cultures were monitored with an oxygen electrode.

5.3.3 Fermentation conditions

In order to maintain similar environmental conditions in mixed and single-culture fermentations, a system similar to continuous fermentation using continuous inflow and outflow of the medium was optimised for single and mixed fermentations. Samples for RNAseq analysis were withdrawn at 48 h when total viable cell count was similar between the mixed and single culture fermentation. The feeding medium was contained glucose and fructose of 50 g L^{-1} each. The working volume was maintained at 0.7 L using a peristaltic effluent pump. All fermentations were conducted in duplicate.

5.3.4 Analysis of yeast growth population

Serial dilutions of the cell suspensions were performed with 0.9% (w/v) NaCl. One hundred microliter samples were spread on YPD agar and incubated at 30°C for 2-3 days. For yeast enumeration in mixed culture fermentations, both species were distinguished based on colony morphology. Colony counts were performed on plates with 30-300 colonies.

5.3.5 Analytical methods

Supernatants were obtained by centrifuging cell suspensions at 5000 × *g* for 5 min. The concentrations of fructose, glucose, acetaldehyde and acetic acid were measured using specific enzymatic kits, Enytec™ *Fluid* D-fructose, glucose, acetic acid (Thermo Fisher Scientific Oy, Finland), Boehringer Mannheim / R-Biopharm-acetaldehyde (R-Biopharm AG, Darmstadt) and analyzed using Arena 20XT photometric analyzer (Thermo Electron Oy, Helsinki, Finland) (Schnierda et al., 2014). Ethanol was analyzed by high performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase as described by Rossouw (Rossouw et al., 2012). The major volatiles analysis was done using liquid-liquid extraction method on GC-FID as described in previous chapters in more detail (Louw et al., 2010).

5.3.6 Sampling, RNA-extraction and RNA-sequencing

Cell samples for RNA-sequencing were obtained from both single and mixed culture fermentations (anaerobic and aerobic, respectively) at 48 h when population and sugar levels were approximately same in all fermentations. Total RNA extractions were performed according to the hot phenol method (Schmitt et al., 1990). Concentration and purity of RNA were determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). The RNA samples with RNA integrity number (RIN) more than 8, and 280:260 ratios more than 2 were further used for the RNA-sequencing purpose. The RNA-sequencing was performed by VIB Nucleomics core, (KU, Leuven (Belgium) using NS2500 next generation sequencing platform.

5.3.7 Data quality assessment

The accuracy of the sequencing was assessed by using the Phred quality score (Q score), which is the most common metric used to assess the accuracy of a sequencing platform. The Phred score< 30 was considered a benchmark for quality of the samples, which was calculated by the ShortRead 1.24.0 package from Bioconductor (http://www.bioconductor.org). Further, the bad sequences and reads less than 35 bp were removed using FASTX and a Phred score of <20 was used to analyse the data for differential gene expression.

5.3.8 RNA-seq data analysis

The RNA-seq data analysis was performed using the reference genomes S288c for *S. cerevisiae* and CBS6340 for *L. thermotolerans*, again in case of *L. thermotolerans* the unknown genes were identified by the homology with *S. cerevisiae* S288c genome. The reads were aligned to the reference genome with Tophat v2.0.13. The obtained *bam* files were further converted in to *gff* files to analyse the data further. The number of reads in the alignments that overlap with gene features were counted with feature Counts 1.4.6 (Liao et al., 2014). The following parameters were chosen: -Q 0 –s 0 –t exon –g gene_id. We removed genes for which all samples had less than 1 count-per-

million. Raw counts were further corrected within samples for GC-content and between samples using full quantile normalization, as implemented in the EDASeq package from Bioconductor (Risso et al., 2011).

5.3.9 Identification and statistical analysis of differentially expressed genes

Gene expression levels were normalized using fragments per kilobase of exon per million mapped reads (FPKM). A negative binomial generalized linear model (GLM) was fitted against the normalized counts using the EdgeR 3.4.0 package of Bioconductor (Robinson et al., 2010). We did not use the normalized counts directly, but worked with offsets. The differential expression was tested with a GLM likelihood ratio test, also implemented in the EdgeR package. The resulting p-values were corrected for multiple testing with Benjamini-Hochberg to control the false discovery rate (FDR) (Benjamini & Hochberg 1995).

5.4 Results

5.4.1. Optimisation of fermentation conditions

Multispecies interaction studies at the molecular, transcriptomic or proteomic level face significant challenges. Indeed, when such studies are carried out in standard batch fermentation conditions, both species continuously modify gene expression to respond to the continuously changing environment, and yeast growth leads to a continuous change in the level of mutual exposure. In such conditions, any specific transcriptional response of one species to the presence of the other species will be hidden within a broader transcriptional response to changes in the environment. To overcome this problem, and to focus the investigation on the transcriptomic signature of the interaction between species, a system similar to a chemostat setup was optimised. The aim of this optimisation was to ensure similar population densities and similar growth medium composition in both single species and mixed species fermentation at the point of sampling. The expression analysis of mixed and single fermentation was performed when the population of *L. thermotolerans* and *S. cerevisiae* had reached equal cell densities as mentioned in table 5.1. The specific growth rate (µMax) was obtained only for single culture fermentation under strictly anaerobic and aerobic conditions (5% oxygen) in synthetic grape must with 100 g L^{-1} sugar (Table 5.1). A similar population number for both yeasts was obtained at a dilution rate of 0.1 h^{-1} and 0.125 h^{-1} for mixed anaerobic and aerobic fermentation, respectively. As Fig 5.1 shows that in these optimised conditions, the species display similar growth rates in both single and mixed fermentations, that the total number of cells in both types of fermentations are almost similar, and that the major environmental factors such as sugar and ethanol concentrations are at similar levels. For *L. thermotolerans* anaerobic fermentation, we obtained similar population at a dilution rate of 0.075 $h^{-1}$and for *S. cerevisiae* at a dilution rate of 0.1 h^{-1}, while for single aerobic fermentation we could obtain the similar biomass at a dilution rate of 0.125 h^{-1} for both yeasts at 48 h of fermentation.

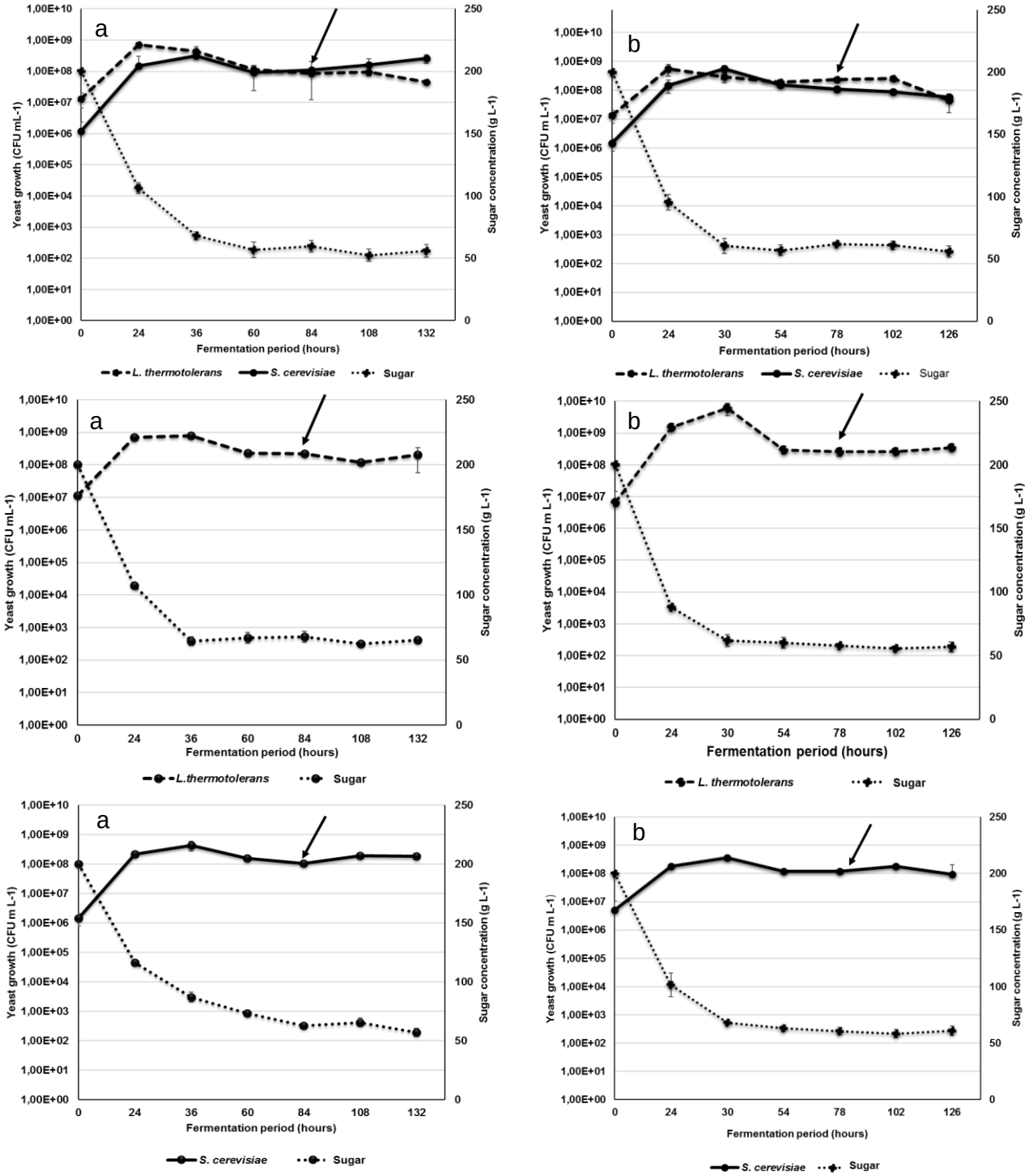

Figure 5.1: Fermentation kinetics and yeast growth of *S. cerevisiae* and *L. thermotolerans* in single and mixed fermentation under anaerobic (a) and aerobic (b) fermentation conditions

*The arrows indicate the sampling points in each graph

5.4.2. Transcriptomic analysis

RNA extractions were performed on samples collected at 48 h (Fig. 5.1). RNA samples with a RIN score of higher than 8 were considered for RNA-sequencing. A total of 41.21 million (150-bp paired-end) reads were generated for the RNA-seq analysis. Similar read counts were obtained from related

fermentation set-ups. For instance, from monoculture fermentations approximately 6.5 Mb of data was obtained; while from mixed-cultures 7.5 Mb were obtained (Table S 5.1). The RNA-sequencing was performed for two biological repeats of each fermentation, and our data show similar expression profiles of two biological repeats (Fig. 5.2). The statistical analysis was done using Benjamini-Hochberg on all highly-expressed genes to control the FDR. The overall gene expression profile of mixed and single fermentations is presented in the heat map (Fig. 5.2).

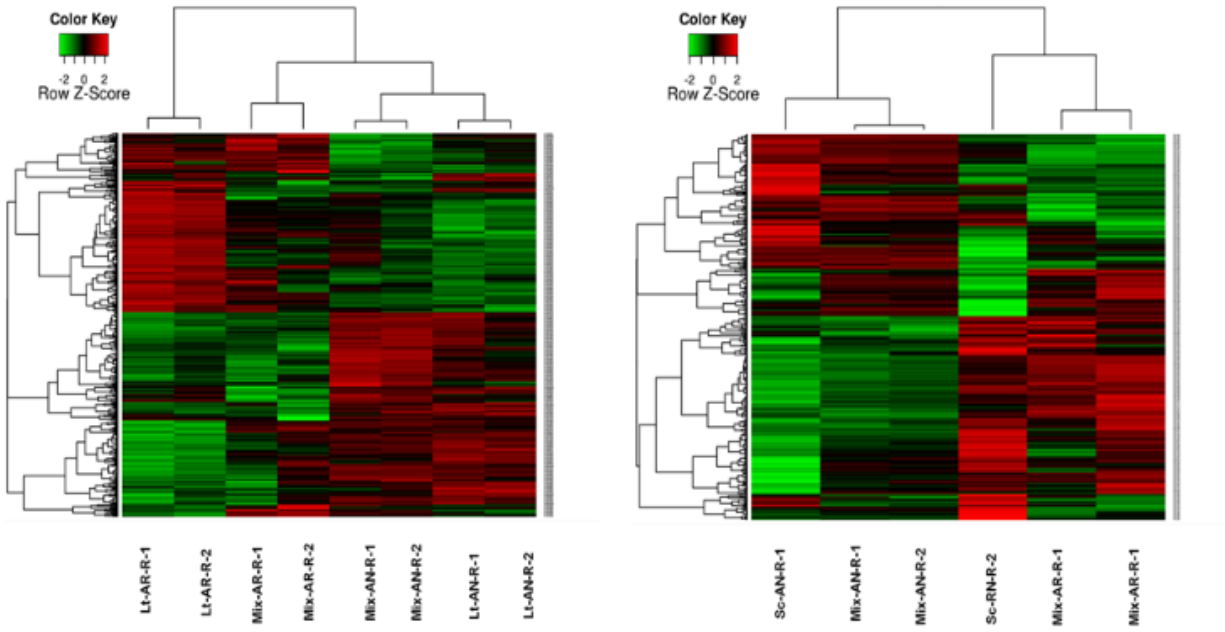

Figure 5.2: The overall representation of differentially expressed genes in the single and mixed fermentation of *S. cerevisiae* and *L. thermotolerans* in anaerobic and aerobic conditions (FDR≤0.05 and log fold change of 2). Red bars denote an increase in expression while green bars indicate a decrease in expression for a given gene.

A gene set analysis was performed using YEASTRACT program (Teixeira et al., 2014) on all up-regulated and down-regulated genes with a minimum log fold change of 1.5 to obtain enriched pathways. In mixed fermentations (anaerobic and aerobic), the up-regulated genes showed enrichment for functional categories such as metal ion, cell wall, sulfur metabolism, oxidation-reduction process and carbohydrate metabolic process in both *S. cerevisiae* and *L. thermotolerans* (Table S 5.2-5.5).

Further statistical analysis using the Benjamini-Hochberg test was performed to control the false discovery rate and only genes with an FDR≤ 0.05 were considered. Based on this analysis 63 genes of *S. cerevisiae* were found to be differentially expressed (31 up-regulated and 32 down-regulated) in response to the presence of *L. thermotolerans* under anaerobic conditions, while under aerobic conditions 337 genes were differentially expressed (120 up-regulated and 217 down-regulated). In contrast, *L. thermotolerans* showed differential expression of 500 genes under anaerobic conditions (320 up-regulated and 180 down-regulated), and 612 under aerobic conditions (227 up-regulated,

385 down-regulated) (Fig. 5.3). For each differentially expressed gene, gene ontology (GO) annotation was obtained with the program YEASTRACT (Teixeira et al., 2014). Further results will be discussed for genes with an FDR≤ 0.05.

Table 5.1 A summary of the dilutions rates, viable cell counts, sugar and glycerol concentrations at the sampling point for transcriptional analysis

Fermentations	Dilution rate h^{-1}	CFU mL^{-1} at 48 h	Sugar concentration at 48 h (g L^{-1})	Glycerol concentration at 48 h (g L^{-1})	µMax at exponential phase (h^{-1})
L. thermotolerans-AN	0.075	2.1E+08	68.0	2.92	0.17
S. cerevisiae-AN	0.10	1.2E+08	62.5	2.40	0.20
Mixed-AN	0.10	9.8E+07	59.6	3.14	
S. cerevisiae-AR	0.125	1.0E+08	60.0	1.09	0.23
L. thermotolerans-AR	0.125	2.5E+08	58.0	1.34	0.24
Mixed-AR	0.125	3.0E+08	62.0	1.12	

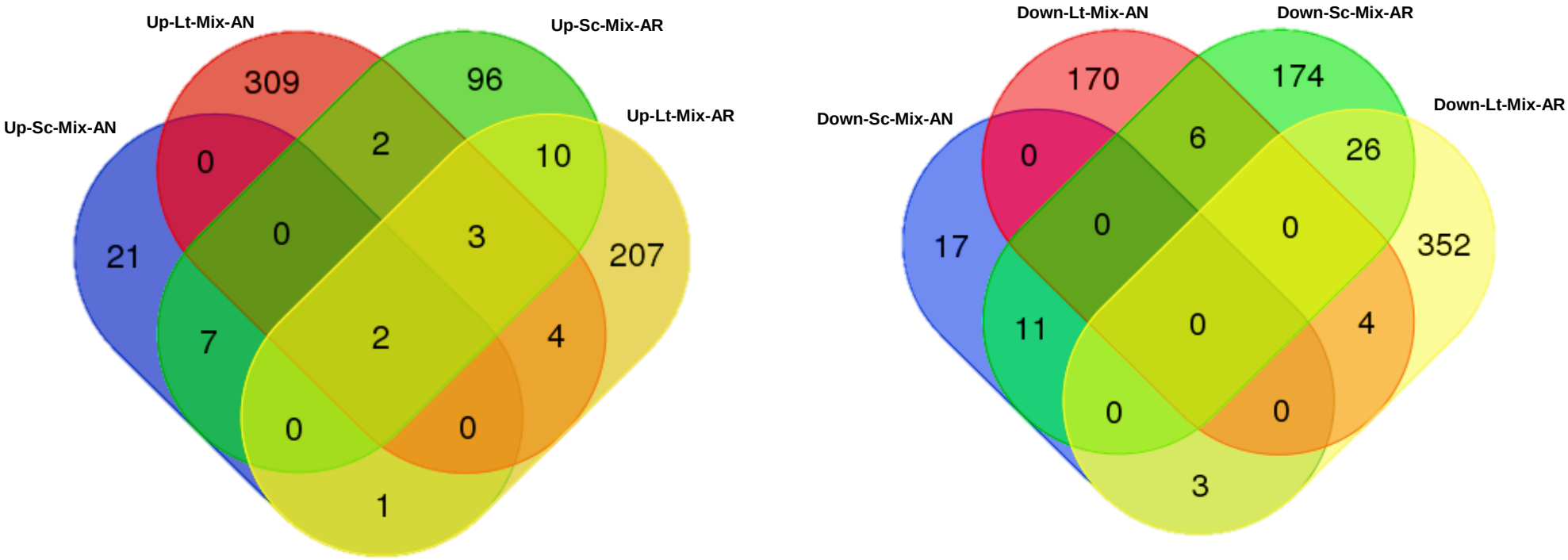

Figure 5.3: Venn diagram representations up-regulated and down-regulated genes of *L. thermotolerans* and *S. cerevisiae* in mixed culture fermentation under aerobic and anaerobic conditions in comparison to their single culture fermentations. The number of genes identified commonly in a compared set of experiments is given in the intersection region of the circles. The number of genes that are unique to *L. thermotolerans* and *S. cerevisiae* is shown outside of the intersection region, along with the total number of genes identified for that individual experiment, shown in parentheses. The table for all common and separate up and down-regulated genes is provided in supplementary materials (Table S 5.6-5.7).

5.4.3 Response of *S. cerevisiae* and *L. thermotolerans* to metal ions

Transcriptomic analysis revealed that both *S. cerevisiae* and *L. thermotolerans* increased transcriptional activity for genes involved in the acquisition of copper and iron. *S. cerevisiae* displays high expression of *CTR1, CTR3, FRE1, FRE7* (Fig. 5.4) and *ENB1* under both aerobic and anaerobic conditions. Furthermore, *FET4, FET3* and *FRE4* are highly expressed under aerobic conditions only (Table S 5.6). In contrast, *L. theromotolerans* shows high expression *of CTR3, FRE1* and *FET4* under anaerobic and aerobic conditions (Table S 5.6). In addition, both yeasts exhibit higher expression of the *FRE5, SIT1* and *ARN1* only under aerobic conditions (Table S 5.6). *CTR1* and *CTR3* encode high affinity copper transporters, while *ENB1, ARN1* and *SIT1* encode the transporters for ferric ions chelated to enterobactin, ferrichromes and ferrioxamine. The genes *FET3* and *FET4* encode a multicopper ferroxidase and a low specificity ionic iron and copper transporter, respectively. The *FRE* genes encode metalloreductases of different specificities (e.g. *FRE1* codes for a low specificity ferrireductase induced in low iron and low copper, *FRE4* codes for a siderophore iron reductase).

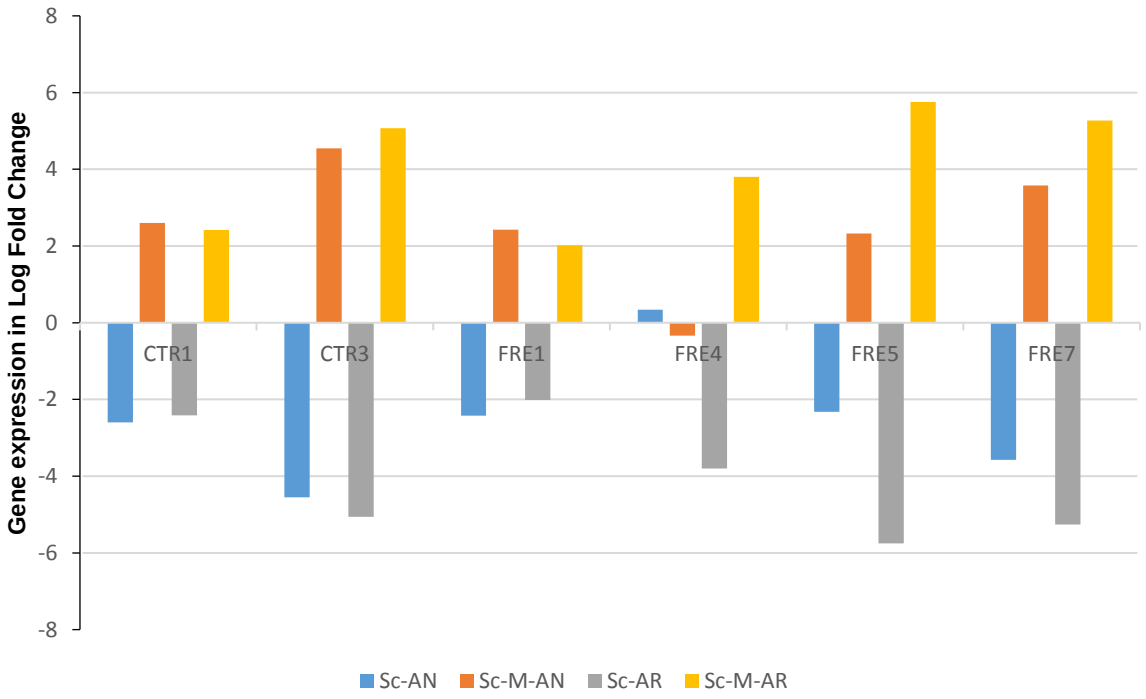

Figure 5.4: Differential expression of genes involved in metal utilization in Sc-Mix fermentation relative to single *S. cerevisiae* fermentations under anaerobic and aerobic conditions (in Log FC).

5.4.4 Response to cell wall integrity

In mixed culture fermentation compared to single culture fermentation, the GO analysis showed enrichment of genes that are involved in maintaining cell wall integrity. For instance, in comparison to single *S. cerevisiae*-AN fermentation, the Sc-M-AN mixed fermentation showed higher transcripts

for a series of seripauperin encoding (*PAU*) genes (*PAU5, PAU9, PAU12, PAU17, PAU24*), and *FIT2*, *FIT3* (Mannoproteins and Facilitators of Iron Transport). Similarly, the Sc-M-AR showed up-regulation of *FIT2, FIT3,* flocculation associated genes *FLO5* and *FLO9,* as well as other genes such *HPF1, UTR2, YDR134C* and *YPS6* involved in cell wall organization (Table 5.2). In contrast, under anaerobic conditions, *L. thermotolerans* in mixed fermentation (Lt-M-AN) showed up-regulation of *FLO1, FLO8, HSP12, SSA2* and *SSA3*; while under aerobic conditions Lt-M-AR showed *FLO5*, *HSP30, FIT2* genes that are involved in maintaining the cell wall integrity under stress conditions (Table 5.2). These results indicate that the both yeast responds in similar ways to mixing, however, a different set of genes are activated as a function of oxygen condition.

5.4.5 Differential expression of genes involved in sulfur assimilation

The transcriptome comparison of mixed fermentations and the single fermentations under anaerobic and aerobic conditions revealed that in mixed fermentation both yeasts, *S. cerevisiae* and *L. thermotolerans* showed higher abundance of transcripts of genes endoding enzymes involved in the sulfur assimilation pathway (including uptake of inorganic sulfur, sulfur containing amino acids, glutathione metabolism and synthesis of sulfur-iron containing proteins). For instance, Sc-M-AR fermentation showed up-regulation of genes that are responsible for sulfur uptake (*SUL1*), methionine (*MUP1, MMP1*) and the synthesis of sulfur-containing molecules *SAM3*, *YPR003C, MXR1* and *SDH7,* respectively. Moreover, genes that were involved in glutathione metabolism and its redox cycling (*GEX1*, *GEX2*) were also up-regulated in Sc-M-AR fermentation. Similarly, genes of sulfur assimilation pathway such as *SUL1, MET17* were up-regulated in Sc-M-AN relative to Sc-AN. Likewise, Lt-M-AN showed higher transcript levels of *MET6, MET22* (involved in sulfur uptake)*, CYS3* (uptake of cysteine)*, GTT3, HYR1, STR3* (sulfur assimilation pathway) while the Lt-M-AR showed enhanced transcripts for *SAH1, GEX1* (glutathione pathway) relative to their single culture fermentations (Table 5.2). From all the above-mentioned genes, we can say that the entire sulfur assimilation pathway was more active in mixed culture fermentation.

5.4.6 Thiamine metabolism

Our data revealed a high expression of genes involved in the utilization of thiamine mainly in *S. cerevisiae* when in mixed fermentation compared to monoculture fermentations. Overall, the whole thiamine synthesis pathway was enriched and most genes had an FDR $\geq$ 0.05 except for *THI73* which was up-regulated under aerobic conditions and *THI11* up-regulated under anaerobic conditions. *THI13* was up-regulated in Sc-Mixed fermentations under both conditions, while *THI12* and *THI17* were upregulated under anaerobic conditions, and *THI22* under aerobic conditions. In contrast, *L. thermotolerans THI4* was up-regulated under anaerobic conditions while *THI72* was up-regulated under aerobic conditions (Table 5.2).

Table 5.2 Differential expression of genes involved in cell wall integrity, sulfur assimilation and thiamine metabolism (values are presented in log fold change).

Gene Name	Sc-MIX-AR	Sc-MIX-AN	Lt-MIX-AR	Lt-MIX-AN
PAU5	-1.95	1.74		
PAU9	-1.92	2.99		
PAU12	-2.18	3.75		
PAU17	-0.9	1.79		
PAU24	-2.19	3.54		
FIT2	4.16	2.57	1.13	-7.79
FIT3	2.14	1.38	0.04	-4.80
FLO1	0.53	-0.45	0.18	1.40
FLO5	1.59	0.01	2.64	-1.27
FLO8	-0.15	0.47	-1.13	1.21
FLO9	2.33	0.49	-0.72	0.48
HPF1	4.39	1.61		
UTR2	1.81	0.15	0.56	-0.98
YDR134C	1.99	0.14		
HSP12	-0.43	-1.26	-1.51	1.25
SAA2	-0.68	1.12	-1.76	1.18
SAA3	-0.12	-0.29	-0.41	1.98
HSP30	2.87	-0.89	1.83	-0.37
SUL1	3.14	3.89		
MUP1	2.76	0.66	-0.34	0.18
MMP1	1.42	1.08		
SAM3	2.07	0.24		
YPR003C	1.78	0.71	0.38	0.51
MXR1	1.65	0.67	0.52	-1.15
SDH7	1.30	0.99		
GEX1	8.01	0.28	2.55	0.33
GEX2	4.11	-0.17		
MET6	-0.96	0.43	-0.03	1.09
MET22	-0.06	-0.15	0.72	1.20
MET17	-0.26	1.67	0.11	-0.66
CYS3	-0.69	0.18	-3.41	1.83
GTT3	-0.63	0.16	-1.36	1.54
HYR1	0.03	0.14	-1.37	1.51
STR3	-0.56	0.96	-1.44	1.08
SAH1	-0.22	0.48	1.77	0.49
THI4	-1.43	1.75	-1.58	1.05
THI11	1.03	1.74		
THI12	0.71	1.10		
THI13	1.13	1.57	-1.42	0.08
THI22	1.52	-0.01		
THI72	-0.65	-0.03	1.20	0.40
THI73	1.72	0.34	0.75	-0.90

* the blank columns indicate the absence of that gene in *L. thermotolerans*

5.4.7 Expression analysis for the genes involved in aroma compounds production

Co-inoculation of *L. thermotolerans* with *S. cerevisiae* showed a distinct metabolic profile of major volatile compounds in the resulting wine (Table 5.3). Mixed fermentation resulted in enhanced concentration of higher alcohols, esters, medium chain fatty acids and glycerol in anaerobic conditions, while in aerobic conditions high concentration of higher alcohols and acetate esters was observed (Table 5.3).

Table 5.3 Major volatile compounds profile in *S. cerevisiae*, *L. thermotolerans* single culture and mixed culture fermentations at 48 h.

	Sc+Lt-AN48	Sc+Lt-AB-48	Lt-AN-48	Lt-AB-48	Sc-AN-48	Sc-AB-48
Propanol	4.73±0.15	7.15±0.48	4.95±0.16	6.72±0.26	3.58±0.46	4.94±0.82
Isobutanol	8.22±0.57	15.34±0.61	9.54±0.52	11.37±0.59	5.27±0.44	6.27±0.84
Butanol	0.71±0.05	0.67±0.04	0.87±0.06	1.02±0.08	0.11±0.02	0.33±0.04
Isoamyl alcohol	17.28±2.23	25.7±1.97	25.08±2.65	33.4±1.07	11.52±0.91	16.70±1.27
Ethyl hexanoate	0.68±0.08	0.23±0.05	0.38±0.06	0.10±0.01	0.41±0.08	0.57±0.08
Pentanol	0.27±0.00	0.23±0.05	0.33±0.12	0.48±0.05	0.17±0.00	0.18±0.03
Hexanol	0.08±0.01	0.19±0.00	0.11±0.01	0.10±0.00	0.14±0.05	0.19±0.00
3-Ethoxy-1-propanol	0.71±0.12	1.66±0.43	2.75±0.23	3.38±0.35	0.50±0.46	0.84±0.13
2-Phenylethanol	8.62±1.87	11.78±1.19	13.84±0.61	18.11±0.55	4.51±0.49	5.89±0.84
Ethyl acetate	6.67±0.25	12.26±2.12	9.68±1.24	10.41±0.48	3.87±0.76	5.25±1.45
Ethyl butyrate	0.12±0.00	0.15±0.03	0.12±0.00	0.17±0.17	0.09±0.00	0.15±0.03
Ethyl lactate	1.49±0.49	2.10±0.23	1.43±0.12	1.34±0.27	0.61±0.30	0.97±0.25
Ethyl caprylate	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.12±0.01	0.15±0.00
Ethyl caprate	0.003±0.00	0.01±0.00	0.04±0.00	00.00±0.00	0.03±0.00	0.04±0.00
Isoamyl acetate	0.99±0.00	0.09±0.00	0.59±0.08	0.01±0.00	0.09±0.01	0.08±0.01
Ethyl phenylacetate	0.28±0.12	0.32±0.00	0.66±0.07	0.32±0.00	0.30±0.00	0.29±0.00
2-Phenylethyl acetate	0.75±0.07	0.54±0.03	0.44±0.04	0.29±0.01	0.65±0.04	0.68±0.00
Diethyl succinate	0.37±0.03	0.67±0.05	0.72±0.02	0.63±0.00	0.44±0.00	0.75±0.21
Propionic Acid	2.67±0.30	0.90±0.10	1.08±0.06	2.00±1.49	1.06±0.49	1.81±1.25
Isobutyric acid	1.11±0.20	1.33±0.27	2.63±0.19	1.79±0.26	1.59±0.01	0.98±0.21
Valeric Acid	0.82±0.12	1.07±0.02	1.22±0.06	1.01±0.02	1.28±0.00	1.13±0.05
Hexanoic Acid	0.65±0.03	0.59±0.00	0.75±0.05	0.50±0.00	0.90±0.00	0.97±0.36
Octanoic Acid	0.7±0.08	0.52±0.02	0.47±0.03	0.39±0.01	1.41±0.02	1.58±0.90
Decanoic Acid	0.35±0.02	0.21±0.04	0.23±0.01	0.23±0.05	0.89±0.11	1.03±0.76
Butyric Acid	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	1.69±0.19	2.10±0.01
Iso-valeric Acid	0.75±0.03	0.42±0.00	0.52±0.00	0.43±0.00	1.02±0.01	0.78±0.18

All the compounds are presented in mg L^{-1} and are average of two biological duplicates and their SD
*AN- Anaerobic, AB-Aerobic.

5.4.7.1 Higher alcohols

Both *S. cerevisiae* and *L. thermotolerans* revealed higher expression of genes encoding aromatic amino acid transaminases, alcohol dehydrogenases and amino acid permeases in mixed fermentation compared to their monocultures under both aerobic and anaerobic conditions. For instance, *BAT1, ARO8, ARO9, PDC5, AAD14, and SFA1* were up-regulated in *L. thermotolerans* under anaerobic conditions, while *ARO4, ARO7* and *ARO80,* were upregulated under aerobic conditions. In contrast, the amino acid permeases *MUP1* and *SFA1* were up-regulated in *S. cerevisiae* under anaerobic conditions, while *MUP1, BAP1, YCT1*, *ADH7* and the aryl-alcohol dehydrogenase *AAD3* were up-regulated under aerobic conditions (Fig. 5.5).

Amino acids

	Sc-M-AR	Sc-M-AN
BAP2	1.86	-0.93
MUP1	2.76	0.66
YCT1	2.36	0.66

Amino acids uptake

	Lt-M-AR	Lt-M-AN	Sc-M-AR	Sc-M-AN
ARO4	1.13	-0.31	-0.36	-0.50
ARO7	1.57	-0.70	0.53	-0.45
ARO8	0.16	2.90	-0.57	-0.22
ARO9	4.02	-0.61	-1.15	-1.36
ARO 80	1.18	-0.24	-0.18	-0.08
BAT1	-0.30	1.58	0.06	0.26

Keto-acids

	Lt-M-AR	Lt-M-AN	Sc-M-AR	Sc-M-AN
PDC5	-0.29	1.34	0.26	0.04

Aldehydes

	Lt-M-AR	Lt-M-AN	Sc-M-AR	Sc-M-AN
SFA1	0.16	1.99	-0.40	1.50
ADH7	-0.30	-0.10	2.94	0.30
AAD3			1.73	-0.09
AAD14	-0.54	1.36	-0.08	-0.30

Higher alcohols

Figure 5.5: Differential expression of genes involved in formation of higher alcohols in *S. cerevisiae* and *L. thermotolerans* mixed and single fermentation under anaerobic and aerobic conditions. (The green color shows up-regulation and red color shows down-regulation, while white cells show absence of that particular gene in *L. thermotolerans*)

5.4.7.2 Esters

In comparison to single fermentation, in mixed fermentation both yeasts showed higher transcripts for genes encoding acetyl transferase and acyl-CoA. For example, *L. thermotolerans* showed higher expression of *ATF1, EHT1, ACS1* (acetyl transferases, acetyl-CoA) in mixed anaerobic fermentation relative to its single *L. thermotolerans*-AN fermentation, while *S. cerevisiae* showed a higher number of transcripts for *EEB1* (ethyl ester biosynthesis) in Sc-M-AR fermentation compared to its Sc-AR single fermentation.

5.4.8 Genes down-regulated in mixed culture fermentation

Overall, both yeasts, in mixed fermentations showed down-regulation of genes which are involved in multi-cellar processes, cell division, mating, spliceosomal complex assembly, r-RNA processing. For instance, in comparison to *L. thermotolerans* single culture fermentations, the Lt-M-AR showed down-regulation of genes involved in mating type and cell division (*CDC28, HCM1, MCM1, FKH2, FUS3, CLN3, HOS3*); similarly, Lt-M-AN showed down-regulation of gene involved in meiotic cell cycle (*RIM4, SPS4, CHL4, SPO21, ADY4, RMI1, MND1, HOP1, RED1, AMA1, MAM1*). Likewise, in case of Sc-M-AR, genes involved in multi-organism process such as*: YDR034C-D, PRM1, YDR261W-B, MF(ALPHA)2, YOR343W-B, YDR210C-D, YOR192C-B, YDR098C-B, YBL100W-B, YLR410W-B* were down-regulated and Sc-M-AN showed down-regulation of genes participate in multi-organism process (*PRM1, SAG1, MF(ALPHA)2, FLO10, FLO11*). From all down-regulated genes in mixed culture fermentations, we can see that the functional category involved in reproduction or for multi-organism process is down-regulated. Top ten down-regulated genes in mixed fermentations are presented in Table S 5.8-5.11.

5.5 Discussion

The current study focused on evaluating the transcriptional signature of *L. thermotolerans* and *S. cerevisiae* mixed and single culture fermentation under anaerobic and aerobic conditions. The results indicate the metabolic interaction between *L. thermotolerans* and *S. cerevisiae* in mixed culture fermentation. Several studies have investigated direct and in-direct interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts in mixed culture fermentations (Taillandier et al., 2014; Wang et al., 2015, 2016). The results not surprisingly suggest the presence of metabolic interactions (competition for nutrients etc). More intriguingly, they also suggest that other interactions are related to factors such as direct physical contact between two species. However, the molecular nature of such interactions remains unknown.

Our study provides a better understanding of the metabolic interaction between *L. thermotolerans* and *S. cerevisiae* at molecular level under multistarter wine fermentations, while also providing some suggestions about the nature of other types of interactions.

5.5.1 Impact of dilution rates of growth *L. thermotolerans* and *S. cerevisiae*

The dilution rates at which both yeasts could be sustained in mixed anaerobic and aerobic fermentation after 24 hours of fermentation were 0.1 h^{-1} and 0.125 h^{-1} and generated a total biomass of 9.8 × 10^7 3.0 × 10^8 cfu mL^{-1}, respectively. In single culture, anaerobic fermentations, *L. thermotolerans* was able to grow with approximately similar viable cell count (2.1 × 10^8 cfu mL^{-1}) as in mixed anaerobic fermentation at dilution rate of 0.075 h^{-1}, while *S. cerevisiae* produced the approximate viable cell counts count (1.2 × 10^8 cfu mL^{-1}) at a dilution rate of 0.10 h^{-1}. The dilution rates at which both yeast species grow appears to be species dependent. For instance, in anaerobic single fermentation, *S. cerevisiae* generated similar biomass at a dilution rate of 0.1h^{-1}, while *L. thermotolerans* generated at a dilution rate of 0.075 h^{-1}. The differences in dilution rates for both yeasts could be attributed to the faster growth rate of *S. cerevisiae* than *L. thermotolerans* in anaerobic conditions. In anaerobic mixed culture fermentation, we used a dilution rate of 0.1 h^{-1}, at this dilution rate, *L. thermotolerans* showed a decline after 48 h, however, both yeasts stayed together till 96 h in this system. After 96 h *L. thermotolerans* showed a complete wash out; therefore, this system was monitored only for 96 h for all fermentation. In contrast, in aerobic mixed culture fermentation *L. thermotolerans* was not washed out after 96 h, however, the growth of *L. thermotolerans* was also showed a decline after at 96 h (results not presented after 96 h). In aerobic single culture fermentation, *L. thermotolerans* and *S. cerevisiae* both grew at a dilution rate of 0.125 h^{-1}. This equal dilution rate for both yeasts under aerobic fermentations further suggests a slow metabolism of *L. thermotolerans* in comparison to *S. cerevisiae* in this system with continuous flow of medium, as depending on the results we obtained in our last chapters, one would expect high growth rate of *L. thermotolerans* under aerobic conditions, while it stayed almost equal to *S. cerevisiae* even though *L. thermotolerans* was inoculated with higher cell counts than *S. cerevisiae*. In the current study, we did not evaluate growth limiting factor, however, the dilution rates which were used for growth of *L. thermotolerans* and *S. cerevisiae* were less than the growth rates in exponential phase (Table-5.1). Therefore, this suggests that there was some nutrient limitation.

The system described above is by no means a true representation of a chemostat. Nonetheless, it provided the best set-up in which equal population sizes of the two yeasts could be obtained, therefore avoiding interferences in RNAseq analysis. Related studies have employed strategies which only allowed for analysis of early transcriptional responses to mixed culture or analyses of *S. cerevisiae* transcriptional response only. For instance, Tronchoni et al., (2017) first pre-cultured *S. cerevisiae* and the *T. delbrueckii* in separate bioreactors and then mixed the two at equal volumes, followed by withdrawal of samples for RNAseq before the cells started proliferating and in the early exponential phase to avoid over-representation of only one species. In contrast, Barbosa et al. (2015) conducted mixed-culture fermentations of *S. cerevisiae* with *Hanseniaspora guilliermondii* in

a typical wine fermentation batch set-up, extracted RNA at different fermentation stages and only focused on the transcriptional response of *S. cerevisiae*.

5.5.2 Transcriptional responses in single and mixed culture fermentation

The main transcriptional responses of both yeasts to the presence of each other, appear to be through maintenance of cell wall integrity and competition for micro-nutrients during fermentation. The presence of both yeast in fermentation also influenced the expression patterns of genes involved in the production of various flavour-active compounds, which could explain the differences obtained on the aroma profiles of the wines especially between the single and mixed culture fermentations.

In the current study, it was evident that in mixed-culture fermentation, both *S. cerevisiae* and *L. thermotolerans* increased transcriptional activity of several genes whose expression is induced by low copper and iron levels. Under anaerobic and aerobic conditions, both yeasts show up-regulation of *CTR1* and *CTR3* which are Cu transporters as well as *FRE1* which is essential for the uptake of environmental Cu^{2+} and Fe^{3+}. Both *CTR1* and *FRE1* are induced by copper deficiency, while the expression of *CTR1* is also modulated by changes in iron status. Maximal activity of ferric reductase enzyme and high expression of *FRE1, FRE7* (which is also up-regulated in *S. cerevisiae*) genes is known to be induced by iron starvation (Dancis et al., 1990; Kaplan et al., 2006). The reduced copper is transported by Ctr1p and Ctr3p which are both high affinity transporters. In the current study, *FET4* which encodes a lower affinity and low specificity transporter with activity towards Cu^{2+} and Fe^{2+} is significantly up-regulated in Lt-M-AR, Lt-M-AN and Sc-M-AR, but the gene is also up-regulated in Sc-M-AN although only by the 1.6-fold change. Fet4p serves as an oxygen-independent low affinity Cu transporter. In *S. cerevisiae,* high expression of *CTR1* gene is reported to be induced when copper levels are below 10 µM (Azeha et al., 2000; De Freitas et al., 2004; Rustici et al., 2007; van Bakel et al., 2005). The standard synthetic grape juice medium used in the current study contains 0.157 µM copper. Therefore, the higher expression of these genes in both yeasts under both conditions (aerobic and anaerobic) seems as a result of copper deprivation in the medium. The data suggest that both yeasts rev-up copper uptake in order to capture the small amount available as quick as possible. Hodgins-Davis et al. (2012) also obtained similar results in different *S. cerevisiae* strains due to the minimal availability of copper in the medium. The authors also showed down-regulation of the *CTR1* and *CTR3* when there was a high concentration of copper in the medium (Hodgins-Davis et al., 2012; Gross et al., 2000; van Bakel et al., 2005). Copper is required for iron homeostasis in yeast and the link between copper and iron metabolism in *S. cerevisiae* is well recognized. The current data show that under aerobic conditions both yeasts employ two uptake systems to obtain iron from the environment: (i) the reductive iron uptake system which requires copper, (ii) the uptake of bound iron through different siderophore transporters e.g. Sit1p and Arn1p, which is facilitated by the cell wall mannoproteins (Fit2p and Fit3p in *S cerevisiae* and Fit3p in *L. thermotolerans*). Although the mechanism by which the *FIT* genes enhance uptake of iron is not

known, it may involve facilitating the passage of bulky iron-siderophore chelates through the cell wall or increasing the concentration of siderophores (Philpott et al., 2007). In addition to the genes common in both yeasts, *S. cerevisiae* also activated Fre4p (a siderophore iron reductase) and Fet3p (a multicopper ferroxidase) under aerobic conditions, as well as Enb1p (which confers enterobactin uptake) under both aerobic and anaerobic conditions. These data suggest that in mixed-culture fermentation, *S. cerevisiae* competes with *L. thermotolerans* by activating a full set of genes to acquire different forms of iron from the environment and to store it in the cells in bound-form (e.g. ferrichrome). Our results are in accordance with some other studies, showed high expression of *FRE* and *FIT* genes in low iron conditions (Dancis et al., 1990; Tronchoni et al., 2017).

Our data show a strong response to cell wall integrity in both yeasts in mixed fermentation compared to single fermentation. Indeed, both yeasts showed up-regulation of genes that are involved in cell wall integrity under stress conditions although different genes and processes are induced. For instance, *L. thermotolerans* showed an up-regulation of flocculation associated genes and genes encoding heat shock proteins such as *FLO1, FLO8, HSP12, SSA2* and *SSA3* under anaerobic conditions, and *FLO5*, *HSP30* under aerobic conditions, while *S. cerevisiae* mainly up-regulated *PAU* genes under anaerobic conditions and *FLO5* and *FLO9* genes under aerobic conditions. The gene products of *FLO1, FLO5* and *FLO9* promote cell-cell adhesion and formation of flocs while *FLO8* has been reported to be a transcriptional activator (García-Ríos et al., 2014; Goossens et al., 2015; Rossouw et al., 2015; Protchenko et al., 2001). Flocculation has been shown to play an important role in mating and survival in *S. cerevisiae* (Rossouw et al., 2015). Since the *FLO* genes are co-expressed with stress response genes in both yeasts, it is possible that both yeasts respond to nutrient competition (e.g. metal ion, vitamin and sulphur depletion) by forming flocs to ensure survival. In contrast, under anaerobic conditions *S. cerevisiae* seems to respond to the presence of *L. thermotolerans* through up-regulation of *PAU* genes that have been reported to play an important role in promoting fitness under anaerobic and fermentative condition as well as in yeast-yeast interactions (Luo et al., 2009; Rivero et al., 2015; Tronchoni et al., 2017). In particular, *PAU5* was shown to have the highest capacity to be induced by anaerobic conditions and wine fermentation conditions, but has also been shown to protect *S. cerevisiae* strains against killer toxins (Rivero et al., 2015; Tronchoni et al., 2017). An up-regulation of 5 *PAU* genes (*PAU5, PAU9, PAU12, PAU17, PAU24*) in *S. cerevisiae* could allude to the fact that in the presence of another yeast, *S. cerevisiae* mounts a protection mechanism that involves cell wall reinforcement with mannoproteins that shield the cell against toxic metabolites, in addition to anaerobiosis and ethanol. In contrast, *L. thermotolerans*, responds by forming flocs under both aerobic and anaerobic conditions. The high expression of these genes in mixed fermentation, might be interpreted as an adaptation in order to better compete for essential nutrients. However, this hypothesis needs to be proven with further experimental work.

Cellular requirements for sulfur can be fulfilled by the uptake of sulfur-containing amino acids, cysteine and methionine, or by the assimilation of inorganic sulfur into organic compounds such as cysteine and homocysteine, which are used for further biosynthesis of glutathione (GSH) and methionine, respectively (Boer et al., 2003; García-Ríos et al., 2014; Hébert et al., 2015). From the current dataset, we hypothesize that both yeasts perhaps compete for sulfur in the medium which is indeed essential for yeast in many ways. The up-regulation of the entire sulfur assimilation pathway which includes uptake to inorganic sulfur, glutathione uptake and biosynthesis, methionine and cysteine uptake, biosynthesis makes the hypothesis even stronger. Although the sulfur assimilation pathway was up-regulated under both conditions by both yeasts the response seems to species and condition dependent. Therefore, we speculate that the high expression of entire sulfur pathways in both yeasts could be due to completion between the both yeasts under anaerobic as well as aerobic condition.

In anaerobic mixed fermentation, *S. cerevisiae* showed higher expression *THI11*, *L. thermotolerans* showed higher expression of *THI4*. Both genes are involved in thiamine biosynthesis, however, our data indicates that the two yeasts employ different routes for thiamine synthesis, since in *S. cerevisiae* the genes up-regulated use pyridoxal 5- phosphate and L-histidine as a precursor, while *L. thermotolerans* seems to use the NAD L-glycine precursor route. Indeed, high expression of genes involved in thiamine biosynthesis and thiamine up-take (*THI73* and *THI72*) could be due to more requirement of thiamine, since thiamine plays a key role in the growth of yeast as well as fermentation activity (Barbosa et a., 2015; Nishimura et al., 1992). A study by Barbosa et al. (2015) also showed higher expression of *THI20* and *THI21* in *S. cerevisiae* due to mixing with *H. guilliermondii*. Also, depletion of thiamine in musts inoculated with *S. cerevisiae* and *Kloeckera apiculata* have been reported to lead to higher levels of glycerol in the final wines (Bataillon et al., 1996). Notably, our results are in line with these observations, as a higher amount of glycerol was produced in anaerobic mixed fermentation with higher expression of corresponding gene *GPP1* (Table 5.1).

The transcriptome data obtained from mixed culture fermentation compared to single culture fermentation also aligns very well with the exo-metabolome data obtained in the wine resulted from mixed fermentations. For instance, the aromatic amino acids transaminases are well reported for the production of higher alcohols, and in our study we saw the up-regulation of these genes in Lt-Mix fermentation in comparison to single *L. thermotolerans* fermentation (Lt-M-AN showed *ARO8, ARO9, SFA1, ADD14, BAT1* and Lt-M-AR showed *ARO1, ARO7, ARO80*), while we did not observe up-regulation of these genes in Sc-Mix fermentations in comparison to *S. cerevisiae* single culture fermentation. Therefore, we think high concentration of higher alcohols in mixed culture fermentation could be due to the response from *L. thermotolerans* in the presence of *S. cerevisiae*. These results are in agreement with those obtained by Barbosa et al. (2015) and Rossouw et al., (2008) who found

a modest correlation between the expression levels of these three genes and their corresponding higher alcohols. Similarly, higher expression of genes encoding acetyl transferases (*ATF1* and *EHT1*), was noticed in Lt-M-AN than its single fermentation of Lt-AN. Lastly, the *ACS1* gene (encoding an acetyl-coA synthetase isoform) codes for the enzyme responsible for the conversion of acetate to acetyl-coA, which is an intermediate or reactant in several of the aroma compound producing pathways (Rossouw et al., 2009; 2012) was also showed higher transcripts in *L. thermotolerans* mixed fermentations (anaerobic).

In conclusion, our study underlines the importance of such a global approach for the study of yeast-yeast interactions shedding light on the molecular basis of yeast growth during wine fermentation. This new information will be useful for the rational development of mixed-starter cultures to be used in the winemaking industry.

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Table S 5.1 The table provides details of number of reads per sample sequenced and after number of reads used to analyse the data after removing bad sequences and reads less than 35bp

Sample name	Total number of reads sequenced (Mb)	Total number of reads after trimming (Mb)
Lt-AN-1	6551879	6547636.00
Lt-AN-2	6345332	6341154.00
Lt-AR-1	6594078	6589708.75
Lt-AR-2	6629811	6625337.75
Sc-AN-1	6092286	6042448.25
Sc-AN-2	6383700	6368010.75
Sc-AR-1	6720850	6710827.25
Sc-AR-2	7321658	7309523.25
Sc+Lt-AN-1	7565930	7553464.25
Sc+Lt-AN-2	7050687	7039060.00
Sc+Lt-AR-1	7710553	7698741.50
Sc+Lt-AR-2	7458849	7443979.75

Table S 5.2 The commonly enriched processes with their corresponding genes by gene set analysis of up-regulated genes with Log fold change of 1.5 in *S. cerevisiae* mixed culture fermentation under anaerobic condition.

Enriched functional categories	Genes
Oxidation reduction processes	*PRX1 UGA2 ETR1 RFS1 ARA1 PDB1 MXR2 YCP4 GPD1 MDH3 NDE2 YDL124W YDL144C SFA1 DLD1 PST2 HEM13 MRP1 GMC1 MXR1 ARG5,6 RNR1 COX15 PDA1 LPD1 MET10 ERG4 OLE1 ARI1 ERG25 COQ6 AIM17 ERG11 SOD2 NCP1 GRE3 FMO1 GND1 ERG9 KGD1 YIR035C TDH1 YJR096W MET5 HOM6 OAR1 MDH1 YKL107W SDH1 MCR1 FAS1 MIA40 FMP46 ECM4 SDH2 JLP1 ERG3 XYL2 ERG27 AHP1 FRE1 TSA1 NDI1 YML131W ERG5 HFD1 ALD3 ALD2 YMR226C YMR315W IDH1 SPS19 ZWF1 GOR1 LYS9 ADH1 FRE7 CIR2 GDH1 FRE5 FDH1 IRC15 YPL113C FAS2 ERV2*
Metal Ion transport	*ATP1 FTH1 ATP16 MSC2 GMC1 FIT1 SIT1 PMC1 ZRT1 ARN1 YHK8 ATP2 ZRT3 ZRT2 FRE1 CTR3 ATP18 STV1 FET4 POR1 FRE7 ENB1 FIT2 FIT3 FRE5 ATP4 MMT2 ATP15 CTR1*
Carbohydrate metabolism	*YBR056W MAL32 GLK1 RBK1 GPD1 MDH3 EMI2 GLC3 PMI40 HXK1 SCW11 ALG13 AMS1 MIG2 HXK2 CRH1 XKS1 BGL2 SGA1 SUC2 IMA5 MDH1 XYL2 CRR1 CTS1 EXG1 TAL1 FBP1 PGM2 SCW10 GAS1 ZWF1 IMA2 ATH1 GPH1 GDB1*
Cell wall organization	*SSA1 PAU2, PAU5, PAU8, PAU9, PAU10, PAU24 SED1 FIT1 SCW11 CRH1 BGL2 DSE2 PRY3 HSP150 SSA2 CTS1 EXG1 CCW14 SCW10 GAS1 HPF1 TIR4 TIR2 FIT2 FIT3*
sulfur compound metabolic process	*MET3 HOM6 MET14 MET5 SAM2 LSC1 GTO3 THI11 LAP3 GRX2 MET10 GCG1 ACC1 MET17 ACS2 THI20 ECM38 MET30 THI5 PDB1 STR3 CIT1 LSC2 ISU1 TRX2 ECM4 LAT1 IRC7 GTT1*

Table S 5.3 The commonly enriched processes with their corresponding genes by gene set analysis of up-regulated genes with Log fold change of 1.5 in *S. cerevisiae* mixed culture fermentation under aerobic condition.

Enriched functional categories	Genes
Oxidation reduction	*ETR1 YCR102C ADH7 AAD3 COX9 YDL124W MRP1 RIP1 MXR1 COX15 FET5 AIM14 POX1 ADH4 ERV1 FMO1 IMD2 GUT2 RSM26 SOD1 YKL069W YKL070W YKL071W MDH1 MIA40 FOX2 SDH2 FRE6 HMX1 FRE1 COX8 IMD3 YLR460C CYB2 AIM33 FET3 NDE1 ALD3 ALD2 COX7 IDH1 COX5a SMM1 FRE4 YNR073C AIF1 FRE7 IDH2 CIR2 ALD4 FRE5 FDH1 GLR1 TYW1*
Metal Ion transport	*ATP1 ATP3 FTH1 GEX1 HSP30 ATP16 MSC2 CCC2 ATP5 TIM11 ATP17 SIT1 FTR1 FET5 AIM14 ZRT1 ARN1 YHK8 CTR2 POR2 ATP2 ATP7 ZRT3 GEX2 FRE6 ZRT2 FRE1 ATP14 CTR3 ATP18 FET3 ZRC1 FET4 ATX1 FRE4 ATP19 FRE7 ENB1 FIT2 FIT3 FRE5 ATP4 ATP15 ATP20 CTR1*
Cell wall organization	*FLO1 YAR062W YHR213W FLO10 FLO9 YAR066W TIP1 YDR134C UTR2 DSE2 FLO5 YPS6 HPF1 FIT2 FIT3*
Carbohydrate metabolic processes	*GAL7 GAL10 MDH3 UTR2 GUT1 SUC2 MDH1 CRR1 EXG1 GAL80 CAT8 MPA43 GAS4 IMA2 ATH1*
Sulfur compound transport	*MUP1 MMP1 GEX1 YPR003C VHT1 SUL1 YPR011C GEX2 YCT1 SAM3 THI7*

Table S 5.4 The commonly enriched processes with their corresponding genes by gene set analysis of up-regulated genes with Log fold change of 1.5 in *L. thermotolerans* mixed culture fermentation under anaerobic condition.

Enriched functional categories	Genes
Oxidation-reduction process	*ETR1 ARA1 IFA38 IDP1 MDH3 YFH1 SFA1 GLT1 PST2 HEM13 HOM2 YPR1 TPA1 ALD5 PDA1 LPD1 OLE1 MET13 ERG25 TDH3 ADE3 ERG11 SOD2 TDA3 GND1 AYR1 HYR1 RNR2 OSM1 LIA1 MDH1 SDH1 MCR1 ERG3 AHP1 IDP2 FRE1 DUS3 TSA1 HMG1 DUS1 ERO1 CCS1 YIM1 GCV2 SCS7 SPS19 ERG24 AAD14 ADH1 GCY1 DFR1 ALD4 MET12 FAS2*
Metal ion transport	*PTK2 SKY1 MDM32 HRK1 ATP1 VMA1 YDL206W ATP5 PMC1 PMA1 YKE4 KHA1 GEF1 NHA1 FRE1 ATP14 CTR3 FET4 POR1 SCO1, VMA3, PIC2, CTR2, CCS1*
Carbohydrate metabolic process	*GAL1 YBR056W MDH3 YDR109C GLC3 MIG1 ALG13 MIG2 CRH1 BGL2 GUT1 SOL3 PCL7 SGA1 GRR1 MDH1 EXG1 TAL1 YLR446W ATH1 GPH1 GDB1*
Cell wall organization	*SLA1 ACK1 ACT1 CRH1 BGL2 MHP1 KRE9 CWP1 CWP2 CCW12 YPS1 EXG1 PUN1 FLO1 FLO10*
Cellular amino acid biosynthesis	*CYS3 LYS21 GLT1 HOM2 MET6 STR3 CYS4 ADE3 ARG4 THR1 BAT1 MET30 MDE1 CPA2 TRP3 ILV2 ARG8 SER1 CPA1 MRI1*

Table S 5.5 The commonly enriched processes with their corresponding genes by gene set analysis of up-regulated genes with Log fold change of 1.5 in *L. thermotolerans* mixed culture fermentation under aerobic condition.

Enriched functional categories	Genes
Oxidation-reduction process	*ETR1 RFS1 LYS2 ARA1 IFA38 GPX2 FRM2 HBN1 HIS4 MXR2 AAD3 GPD1 MDH3 NDE2 SFA1 GLT1 HEM13 CBS2 PAM1 TRR1 YPR1 ARH1 TSA2 GMC1 FDC1 FRD1 DLD3 HEM14 PRO3 ALD5 SER3 AST2 ARI1 ADH4 ERG25 CTT1 GTO1 RNR4 TDH3 COQ6 GND2 ERG11 TDA3 PAN5 GRE3 TRR2 ERG9 IMD2 DOT5 RNR3 COX5b AYR1 IRC24 HYR1 RNR2 TDH1 TDH2 OSM1 JHD2 AAD10 GPX1 FRE2 SHB17 FMP46 CCP1 MET1 ECM4 MTD1 FRE6 JLP1 XYL2 AHP1 FRE1 DUS3 IMD3 HMG2 ALO1 AIM33 NDI1 PGA3 ADI1 CCS1 YIM1 ALD3 ALD2 SCS7 ADH2 IDP3 SPS19 GOR1 ERG24 AAD14 FRE4 GPD2 MDH2 FRE7 CYC2 CAT5 IDH2 FDH1 IRC15 OYE3 FAS2 ERV2 MET16*
Metal ion transport	*YRO2 VMA2 PCA1 VMA9 MRH1 ENA5 ENA1 MSC2 CCC2 ATP5 ATP17 ATO3 GMC1 FIT1 POR2 TRK1 ATP2 ZRT3 FRE2 TRK2 GEX2 FRE6 ZRT2 FRE1 CTR3 FET4 POR1 ATX1 BOR1 VNX1 ATO2 FRE4 FRE7 ENB1 PMA2 MMT2 ATP15 VMA13 CTR1 ANT1 BSD2 SMF2 SMF3 SCO1 FET5 CMC1 MAC1 FHT1*
Carbohydrate metabolic process	*MAL32 GLK1 RBK1 SOL2 GPD1 MDH3 GAL3 YDR109C EXG2 CTS2 EMI2 PMI40 PCL6 HXK1 MIG1 ALG13 MIG2 NQM1 CRH1 SOL4 BGL2 GUT1 SOL3 RPE1 IMA5 PGM1 SHB17 XYL2 ATG26 CRR1 CDA1 TAL1 GAS3 MPA43 GAS5 GPD2 MDH2 GAS4 IMA2 CAT5 SPR1 PCL8 GPH1*
Cell wall organization	*PRS4 RCR1 TIP1 SPS22 LRE1 BPH1 PST1 SED1 MKC7 EXG2 SBE2 HLR1 ACT1 MTL1 SKN1 CRH1 BGL2 KIC1 SBE22 SIM1 YPS6 BIT61 TAX4 CIS3 HSP150 KRE9 TOR1 CWP1 PIR3 PIR1 YPS3 CNA1 MYO5 KTR5 SLA2 WSC2 EMW1 KRE1 PRS5 ZEO1 HPF1 SLG1 SRL1 KTR6 CSR2*
	YHR112C STR2 GEX1 MET3 MET14 MET8 MET32 MET10 EFM2 GRX3 SAH1 HOM6

Table S 5.6 Genes of Venn diagram representations of comparisons between pairs of up-regulated genes in *L. thermotolerans* and *S. cerevisiae* in mixed culture fermentation under aerobic and anaerobic conditions. The number of genes identified common in a compared set of experiments is given in the intersection and number of genes that are unique to *L. thermotolerans* and *S. cerevisiae* are given in separate sections. is shown outside of the intersection region, along with the total number of genes identified for that individual experiment, shown in parentheses. (All DE genes are FDR≤0.05).

Names	Total	Elements (gene names common and different with in treatments)
Lt-M-AN-UP Lt-M-AR-UP Sc-M-AN-UP Sc-M-AR-UP	2	*CTR3 FRE1*
Lt-M-AN-UP Lt-M-AR-UP Sc-M-AR-UP	3	*FET4 VEL1 ZPS1*
Sc-M-AN-UP Sc-M-AR-UP	7	*FIT2 ENB1 FIT3 FRE7 CTR1 APT2 PET20*
Lt-M-AR-UP Sc-M-AN-UP	1	*AMS1*
Lt-M-AN-UP Sc-M-AR-UP	2	*GAC1 ICY2*
Lt-M-AN-UP Lt-M-AR-UP	4	*UGA3 HER2 PHO11 YLR225C*
Lt-M-AR-UP Sc-M-AR-UP	10	*GEX1 YPR010C-A QCR10 SFT1 CYB2 YJL218W SIT1 ARN1 FRE5 VHT1*
Sc-M-AN-UP	21	*MEF2 SNZ1 YKL036C PAU12 YPR145C-A YKR075C PAU5 HST2 XBP1 PAU17 PAU9 YSR3 PTP2 ECM29 TSL1 PAU24 GPG1 YAP6 ECM13 GOR1 PHM7*
Lt-M-AN-UP	309	*SSA2 GIP2 HRK1 RGS2 SKS1 CTF19 YMR196W MLS1 TDH3 YMR102C GPP2 KTR1 AMD2 PSP2 GLO3 MRPL10 NPL3 YBL095W TDA4 YDL129W LSC1 SEC31 IFA38 IPT1 FMP16 DEP1 YPR1 SFK1 HOS3 ECL1 YCR061W MDJ1 SFC1 YPL229W YMR295C FAS2 DFG5 FPR3 GLY1 PIS1 AYR1 SNZ3 HEM13 ELO3 MLF3 SFA1 NUS1 MIG2 CCS1 MRI1 USE1 YIM1 CBF1 ERG25 HSP12 YGR237C NOP12 ATP5 YGP1 KES1 TRM5 BAT1 ANP1 URA5 YGR111W ATP10 SPI1 AAD14 VPS33 TPO3 SKT5 ERG11 LAS17 CRH1 AMN1 MVD1 YLR072W ALD5 RXT3 TPI1 RNR2 LCP5 LIP5 HEM2 MIG1 PRC1 GUT1 ABF1 VRP1 ERG24 NOP15 TIF34 COQ9 PHO91 PPR1 YLR257W FCF1 STR3 SPS19 PRY2 CWP1 SEC17 YDL085C-A SUT1 KIP2 SIP5 RRI2 ERG12 LSC2 PUB1 HTS1 NPR1 ATG23 ASC1 GLN3 SIL1 ADO1 DER1 PEP4 SOL3 TAL1 PPQ1 GDI1 YMR074C BBC1 TRS23 NCE103 ISR1 UBC6 DUR3 ARO8 KRE9 RRD1 YJL016W FLO8 MAK21 GPH1 IZH1 YGR149W UGA4 PNC1 MIC27 YCH1 PIH1 DBF4 SMP1 HSF1 AHP1 FAT3 UTP11 DID2 SBA1 YIP5 CEM1 ETR1 IMP2' NSA2 RPA12 MCM16 UBC8 MSC1 YGR210C SBH2 YFR016C RIM101 TDA3 NAT4 GCR2 CYS3 YPR114W MIC60 DAT1 SCS7 RIB3 POC4 YNK1 ENT5 MCM1 YKL151C GDE1 YJL055W NMD4 SSA3 ALG13 BUD7 SED5 SAP1 SEC16 ISN1 SVF1 GLT1 BGL2 POL32 RCK2 SNF7 SEC22 PCT1 RAD23 YML018C HTZ1 DCS1 PEP5 TDA11 MGA1 YAP1801 MPC3 PEX13 INH1 YJR056C OM45 CDC34 OSM1 TAF9 BCY1 ECT1 THR1 ACS2 NEM1 YOR338W HYR1 CDC9 MTC1 NSL1 HOG1 SCS2 PLB1 ITR2 ATG19 PIC2 PRM2 PIL1 AAT2 ECM3 RPG1 MET6 POR1 ROX3 ACC1 SPT5 CPR3 FSH3 SAC3 FLO1 HTL1 DSE4 DSK2 GTT3 YDR109C TIF6 FMP48 MET22 SEH1 YCR016W STP2 POM33 YGR250C NAB3 ACT1 PIN3 RMT2 GYP5 PAA1 TRM1 ERG6 GGC1 YDL086W IRC3 PAC11 PTI1 YNL010W RHO5 RCO1 WSC4 NRG1 TRF5 HAP1 SLC1 GAD1 HEM15 MPT5 CBT1 SPO73 AAH1 CPR5 CDC28 PEX21 ARA1 DIF1 SCS3 YBR137W YPC1 SDS24 YHR022C MBF1 PIN4 RGC1 DUS3 CTI6 RCN2 PAN6 RTS3 PHO4 MDH3 VID24 IES4 TYE7 CUP9 CCT7 TDA7*

Sc-M-AR-UP	96	*HFM1 YCT1 ZIP1 YMR316C-B YCR101C COX5A PHO89 PNS1 YOR387C GEX2 ATP20 YDR134C EEB1 MIP6 AAD3 HPA2 UTR2 YGR266W YER053C-A YGL101W YGR035C GUP2 tL(CAA)L HXT1 QDR1 CMC2 FRE4 OSW5 YNR073C SYC1 snR4 ELO1 THI73 YTP1 COT1 YPS6 YOL131W BAP2 YBR201C-A YDR133C ARR2 YOL159C YCL074W YGR259C NCE102 SDH7 YTA6 YAR068W LSO1 YNR040W FLO5 COX7 HPF1 PXA2 AQY2 AIM33 PDH1 FLO9 PIG2 ZRT1 ADH7 PUT4 CCC2 FET3 RTC4 CYC1 MUP1 ALD2 YAR062W YHR180W --- AAC1 ACO1 ZRT3 TYW1 PDR12 ZRC1 MIX23 CIN5 YJL132W COX12 IMD1 FMO1 YGL072C AIF1 IMD2 FMP23 ZRT2 MIG3 YBL029W COA2 YFL021C-A CRR1 SAM3 YLL053C NDE1*
Lt-M-AR-UP	207	*YNL234W ADR1 SME1 HXT2 NRK1 COX16 GIT1 FYV6 RED1 YLR173W TRM112 YAL044W-A PEX15 MTQ1 THG1 PUT1 MIC19 YBR062C YOR020W-A TLG2 INO2 MHF2 YMR130W SWM2 RIM9 ARP10 IES5 YDL157C IGD1 TNA1 HOP1 SPG1 TEP1 YNL134C STF2 DSD1 URH1 CIN2 SPO1 YNL211C BNA7 SRN2 TDA5 YMC1 COA4 MCH4 GPI12 YIL024C AIM18 RIM13 OST5 IMA1 MRPL33 MDM30 SDC1 THI72 SPS4 BLI1 MVB12 IRC6 PSF1 SGF11 CHO2 NSE3 HIS7 YER152C NDL1 OPI1 ATG32 MOD5 SPC3 IPL1 YJR012C TRS65 AIM4 YJR112W-A PET191 YGL108C ARG3 SPO22 EST2 TRS33 RDR1 NIT1 HIS5 RHO2 YSY6 KIP3 ACM1 YPL168W ECM15 BNA1 ATG17 HMF1 SFG1 YDL121C FMP52 EMC6 PUT2 LOT6 LDB17 YFT2 GPI2 SAY1 POA1 NTR2 LSM3 SHG1 FMN1 YPT53 YGL041W-A SNN1 NNF1 RNY1 HEM4 APC9 PUG1 PEX10 SET6 YLR146W-A AIM1 BIO3 YLR283W KSH1 TFB6 MMS21 ARO80 REV7 LDB18 SPC34 DYN2 YIL166C YDR124W INA17 YHI9 CWC21 CSE4 CAP1 RDL1 ARG8 VMA7 DAD4 ATG12 BNA3 VMA21 DIT1 OXR1 RTG1 YDL144C BRR6 YOR289W DPP1 RRG8 SLX1 HAP2 YML108W POP7 CBP2 YML096W MCM22 FRA2 ELC1 TRP2 AGP2 YJR154W MTC3 HOT13 REE1 HTA1 YPR022C HAP5 IRC19 AMF1 RUB1 ECM19 POP4 UTR4 ARG7 YKU70 ATG38 PCC1 JNM1 MCM21 SCM3 YOR114W YHL018W TRI1 MOH1 ATG3 CHA4 YKL070W ATP18 YGL117W LYS5 JLP2 SPC24 PEX28 UGA2 MHF1 YDR338C GPI19 GAL4 SMX2 ATG29 TMT1 HIS2 AIM17*

Table S 5.7 Genes of Venn diagram representations of comparisons between pairs of down-regulated genes in *L. thermotolerans* and *S. cerevisiae* in mixed culture fermentation under aerobic and anaerobic conditions. The number of genes identified common in a compared set of experiments is given in the intersection and number of genes that are unique to *L. thermotolerans* and *S. cerevisiae* are given in separate sections. is shown outside of the intersection region, along with the total number of genes identified for that individual experiment, shown in parentheses. (All DE genes are FDR≤0.05).

Names	Total	Elements (gene names common and different with in treatments)
Down-Sc-M-AN Down-Sc-M-AR	11	*KTR2 CRS5 MEP1 PRM5 MRS3 PHO5 MRH1 MF(ALPHA)2 PRM1 FLO11 YBR032W*
Down-Lt-M-AR Down-Sc-M-AN	3	*SUT1 PTR2 YBL029W*
Down-Lt-M-AN Down-Sc-M-AR	6	*IGD1 YPR010C-A MND1 ADH5 YBR071W LEU1*
Down-Lt-M-AN Down-Lt-M-AR	4	*COX26 GAS1 ADH6 RIM4*
Down-Lt-M-AR Down-Sc-M-AR	26	*RGS2 GPP2 ECL1 HEM13 YGP1 CRH1 ALD5 RNR2 YMR122W-A VRP1 YLR257W PRY2 PRM10 YGR149W CWP2 IMP2 YKL151C CAR2 PLB1 YNL200C ERG6 GIS3 PRM4 SDS24 RCN2 OSW2*
Down-Sc-M-AN	17	*YNL058C MEP2 SPG1 YKL044W RRT5 MTD1 YFR020W snR24 SPO19 YGL188C-A OPT2 FLO10 FAA3 AFB1 YDL241W IMD1 SAG1*
Down-Lt-M-AN	170	*YCT1 FRE3 SME1 AMA1 COX9 PSD1 CLD1 HSP10 RED1 RMI1 PKP1 SPT14 TRM112 COX15 MXR1 THG1 YBR062C INO2 MHF2 YSF3 SMD1 SWM2 ARP10 IES5 FIT2 HMX1 MRP17 HOP1 TEP1 RPL29 YHB1 YNL211C TDA5 ARC1 YAR064W NCS2 TAH11 MGE1 ADY4 OAC1 YGR016W RPB8 CMC2 HNM1 SAD1 YMR087W YAL037W MRPL33 QCR10 SDC1 SPS4 CAX4 FLD1 RPC10 CHO2 YCR075W-A MGR2 TOM22 SPO21 MSN1 MBA1 YPL041C MAM1 RCF1 BAP2 RSA3 BDH2 PEX22 PEX17 AIM4 SFT1 SWS2 POX1 YJR112W-A ERP2 YGL108C HEM12 MRPL49 CYB2 RFU1 CRG1 RIP1 YDL177C MTQ2 FTR1 OGG1 HMF1 CTP1 AVO2 FIT3 MAK3 ARL3 DOS2 COX7 LDB17 GPI2 SYG1 PXA2 YBR242W SNN1 PDE1 NNF1 PXA1 TOD6 BBP1 TGL1 LSB6 MCP1 SSP2 GAL7 DAD3 FET3 IPK1 LEU2 PES4 TIM8 REV7 DPB11 CYC1 RSM19 ERP3 CWC21 YOL114C CSE4 YIP3 JID1 YAR062W RPL19B YNL122C YLL056C YAT1 DAD4 DIT1 QCR9 POP7 YVH1 RPC11 BLS1 CHL4 HTA1 COX23 PRP11 TIM13 POP8 ARN1 COX19 DIP5 ECM19 YAL068C POP4 FRE5 GRX8 UTR4 YAR023C RPL38 STE14 ATG38 PCC1 EFM2 AIM11 FMP23 YCR090C JLP2 CSI2 YOR097C APS2 GPI19 GAL4 KAR5 POT1*
Down-Sc-M-AR	174	*YDR210C-D YJL144W PGM2 GYP7 SVS1 ADH1 SRL3 HXT4 FRT2 RPA14 CUP2 YMR317W SHR5 ADH2 SOL2 BDH1 CSR1 HUG1 SCM4 CLN1 NCP1 YPR145C-A GTT1 IKS1 HUA1 DAN4 YPL067C SED1 YJL133C-A YNL134C YPS3 YOR192C-B STF2 ERG25 YDR391C RCR1 HES1 MSC7 YMR315W PUN1 UBP5 PAU2 ERG11 YHR097C YPL014W UIP3 DIA1 IRC15 RIM8 CMK2 ERG3 ERG24 YET2 MTH1 YDR261W-B PRB1 YER084W SIP5 ATF2 YER152C SET4 GSC2 CIT2 BBC1 YHR138C HXK1 PEP12 SSE2 PAU5 FMS1 FMP45 RRD1 PAN1 YJL016W YOR385W GPH1 PNC1 MAE1 UPC2 SMP1 YDR034C-D CSE2 PBI1 ADD37 XBP1 PCL2 TIR4 YBL100W-B RTC3 CPA2 PPM1 YPT53 YOR343W-B YBR056W SSK1 CIS3 ABP1 YSR3 TIR3 TSL1 PAU24 ADE3 RTA1 YOR062C AKL1 SKG3 YBR287W PGM1 HMS1 YIR007W TPO2 YLR194C YNL092W YDR098C-B ATG5 HSP42 YNL208W PDR11 EMI2 ORM2 DUF1 AFR1 HAC1 YNR034W-A TPS2 TOS3 ECM3 TIR2 NQM1 FMP46 WTM1 YBR056W-A DDR2 AMS1 ARE1 ICT1 DAN1 CAF120 CHS1 PHM8 YLR410W-B PYC1 MSB2 OCH1 RCN1 YPS1 TMA10 PIN3 EDC2 RNR3 YLR042C DAL80 SAF1 GLK1 FLC1 FHN1 GCY1 YLR149C TIR1 BAG7 YNR014W ARO10 ILV6 TSA2 INO1 RTS3 CSR2 ERG1 GDB1 KRE6 SRO77 PAU23 PLB3 AIM17*

Table S 5.8 Top20 of the genes differently expressed in *S. cerevisiae* mixed anaerobic (Sc-M-AN) fermentation

ORF	Gene name	Function	Fold change
YLR411W	*CTR3*	High affinity copper transporter of the plasma membrane	4.55
YBR294W	*SUL1*	High affinity sulfate permease of the SulP anion transporter family	3.89
YOL152W	*FRE7*	Putative ferric reductase; expression induced by low copper levels	3.57
YBR301W	*PAU24*	Cell wall mannoprotein; expressed under anaerobic conditions, completely repressed during aerobic growth	3.54
YKL036C	unknown	unknown	3.45
YBL108C-A	*PAU9*	Protein of unknown function; member of the seripauperin multigene family	2.99
YPR124W	*CRT1*	High-affinity copper transporter of the plasma membrane; mediates nearly all copper uptake under low copper conditions	2.60
YOR382W	*FIT7*	Mannoprotein that is incorporated into the cell wall; incorporated via a glycosylphosphatidylinositol (GPI) anchor	2.57
YLR214W	*FRE1*	Ferric reductase and cupric reductase; expression induced by low copper and iron levels	2.42
YIL101C]	*XBP1*	Transcriptional repressor; expression is induced by stress	2.33
YPR194C	*OPT2*	Oligopeptide transporter; localized to peroxisomes and affects glutathione redox homeostasis	-5.17
YGL089C	*MF(ALPHA)2*	Mating pheromone alpha-factor, made by alpha cells	-4.47
YGR236C	*SPG1*	Protein required for high temperature survival during stationary phase	-3.49
YAR073W	*IMD1*	Non-functional protein with homology to IMP dehydrogenase	-2.79
YPL130W	*SPO19*	Meiosis-specific prospore protein; required to produce bending force necessary for proper assembly of the prospore membrane during sporulation	-2.66
YFR032C	*RRT5*	Putative protein of unknown function	-2.36
YDR033W	*MRH1*	Protein that localizes primarily to the plasma membrane	-2.43
YKR093W	*PTR2*	Integral membrane peptide transporter	-2.90
YBR032W	unknown	Unknown	-2.24
YIR019C	*FLO11*	*FLO11* GPI-anchored cell surface glycoprotein (flocculin)	-2.14

Table S 5.9 Top20 of the genes differently expressed in *S. cerevisiae* mixed aerobic (Sc-M-AR) fermentation.

ORF	Gene name	Function	Fold change
YCL073C	*GEX1*	imports glutathione from the vacuole and exports it through the plasma membrane	8.00
YOR384W	*FRE5*	Putative ferric; expression induced by low iron levels	5.75
YOL159C	Unknown	Unknown	5.53
YGL258W	*VEL1*	Protein of unknown function; highly induced in zinc-depleted conditions	5.39
YOR387C	Unknown	Putative protein of unknown function; regulated by the metal-responsive Aft1p transcription factor; highly inducible in zinc-depleted conditions	7.76
YLR411W	*CTR3*	High-affinity copper transporter of the plasma membrane	5.06
YOL152W	*FRE7*	Putative ferric reductase; expression induced by low copper levels	5.26
YCL074W	Unknown	Unknown	5.44
YOL155C	*HPF1*	Haze-protective mannoprotein; reduces the particle size of aggregated proteins in white wines	4.39
YFL021C-A	Unknown	Dubious open reading frame; unlikely to encode a functional protein	4.26
YJR150C	*DAN1*	Cell wall mannoprotein; expressed under anaerobic conditions, completely repressed during aerobic growth	-7.27
YER011W	*TIR1*	Cell wall mannoprotein; expression is downregulated at acidic pH and induced by cold shock and anaerobiosis	-5.25
YNL160W	*YGP1*	Cell wall-related secretory glycoprotein; induced by nutrient deprivation-associated growth arrest and upon entry into stationary phase	-4.18
YOR237W	*HES1*	Protein implicated in the regulation of ergosterol biosynthesis	-5.50
YMR317W	Unknown	Putative protein of unknown function	-4.90
YPL272C	*PBI1*	Putative protein of unknown function; gene expression induced in response to ketoconazole	-4.66
YPR145C-A	Unknown	Unknown	-3.53
YIL011W	*TIR3*	Cell wall mannoprotein; expressed under anaerobic conditions and required for anaerobic growth	-3.29
YKR053C	*YSR3*	Dihydrosphingosine 1-phosphate phosphatase; membrane protein involved in sphingolipid metabolism	-4.84
YGR131W	*FHN1*	Protein of unknown function; induced by ketoconazole	-4.05

Table S 5.10 11 Top20 of the genes differently expressed in *L. thermotolerans* mixed anaerobic fermentation (Lt-M-AN).

ORF of *L. thermotolerans*	ORF	Gene name	Function	Fold change
KLTH0C00220g	YIR042C	Unknown	Putative protein of unknown function	5.95
KLTH0C00264g	YGL263W	*COS12*	Endosomal protein involved in turnover of plasma membrane proteins	3.95
KLTH0H13552g	YMR319C	*FET4*	Low-affinity Fe(II) transporter of the plasma membrane	4.79
KLTH0B10472g	YMR293C	*HER2*	Subunit of the trimeric GatFAB AmidoTransferase(AdT) complex; involved in the formation of Q-tRNAQ; required for remodeling of ER caused by Hmg2p overexpression	3.50
KLTH0D15004g	YLR411W	*CTR3*	High-affinity copper transporter of the plasma membrane	8.92
KLTH0H16390g	YGL258W	*VEL1*	Protein of unknown function; highly induced in zinc-depleted conditions	7.65
KLTH0G04026g	YGL117W	Unknown	Putative protein of unknown function	3.87
KLTH0B00176g	YER152C	Unknown	Protein with 2-aminoadipate transaminase activity; shares amino acid similarity with the aminotransferases Aro8p and Aro9p	4.01
KLTH0B00154g	YBR132C	*AGP2*	Plasma membrane regulator of polyamine and carnitine transport	3.15
KLTH0C00396g	YAR071W	*PHO11*	One of three repressible acid phosphatases; glycoprotein that is transported to the cell surface by the secretory pathway	3.37
KLTH0D10384g	YOR220W	*RCN2*	Protein of unknown function	-4.34
KLTH0E04114g	YOR298C-A	*MBF1*	Transcriptional coactivator; protein abundance increases in response to DNA replication stress	-4.03
KLTH0A01210g	YOL117W	*RRI2*	Subunit of the COP9 signalosome (CSN) complex; plays a role in the mating pheromone response	-5.82
KLTH0C04906g	YNR067C	*DSE4*	Daughter cell-specific secreted protein with similarity to glucanases; degrades cell wall from the daughter side causing daughter to separate from mother	-5.62
KLTH0C10648g	YOL012C	*HTZ1*	Histone variant H2AZ; exchanged for histone H2A in nucleosomes by the SWR1 complex; involved in transcriptional regulation through prevention of the spread of silent heterochromatin	-4.77
KLTH0A01584g	YNL289W	*PCL1*	Cyclin, interacts with cyclin-dependent kinase Pho85p; member of the Pcl1,2-like subfamily, involved in the regulation of polarized growth and morphogenesis and progression through the cell cycle	-4.71
KLTH0B06380g	YNL160W	*YGP1*	Cell wall-related secretory glycoprotein	-4.33
KLTH0D07018g	YMR074C	Unknown	Protein with homology to human PDCD5; PDCD5 is involved in programmed cell death	-4.88
KLTH0D08272g	YLR044C	*PDC1*	Major of three pyruvate decarboxylase isozymes; key enzyme in alcoholic fermentation	-6.75
KLTH0A03146g	YLR054C	*OSW2*	Protein of unknown function reputedly involved in spore wall assembly	-4.50

Table S 5.11 Top20 of the genes differently expressed in *L. thermotolerans* mixed aerobic fermentation (Lt-M-AR).

ORF of *L. thermotolerans*	ORF	Gene name	Function	Fold change
KLTH0D15004g	YLR411W	*CTR3*	High affinity copper transporter of the plasma membrane	4.98
KLTH0C00396g	YAR071W	*PHO11*	One of three repressible acid phosphatases; glycoprotein that is transported to the cell surface by the secretory pathway	3.56
KLTH0H11484g	YFR016C	Unknown	Putative protein of unknown function	3.11
KLTH0H11506g	YLR257W	Unknown	Protein of unknown function; protein abundance increases in response to DNA replication stress	3.29
KLTH0G10120g	YNL036W	*NCE103*	Carbonic anhydrase; metalloenzyme that catalyses CO2 hydration to bicarbonate, which is an important metabolic substrate, and protons	3.11
KLTH0B06380g	YNL160W	*YGP1*	Cell wall-related secretory glycoprotein	3.37
KLTH0G09526g	YPL231W	*FAS2*	Alpha subunit of fatty acid synthetase; complex catalyses the synthesis of long-chain saturated fatty acids	3.00
KLTH0G07172g	YHR022C	Unknown	Putative protein of unknown function	3.06
KLTH0E14080g	YMR250W	*GAD1*	Glutamate decarboxylase; converts glutamate into gamma-aminobutyric acid (GABA) during glutamate catabolism	2.75
KLTH0E07678g	YGL202W	*ARO8*	Aromatic aminotransferase I; expression is regulated by general control of amino acid biosynthesis	2.89
KLTH0C06446g	YER106W	*MAM1*	Monopolin; kinetochore associated protein	-3.06
KLTH0E16632g	YOR382W	*FIT2*	Mannoprotein that is incorporated into the cell wall	-7.78
KLTH0E16654g	YOR383C	*FIT3*	Mannoprotein that is incorporated into the cell wall	-4.80
KLTH0E16676g	YOR384W	*FRE5*	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels	-4.44
KLTH0D18150g	YHL040C	*ARN1*	ARN family transporter for siderophore-iron chelates; responsible for uptake of iron bound to ferrirubin	-2.86
KLTH0C01848g	YGR225W	*AMA1*	Activator of meiotic anaphase promoting complex (APC/C); Cdc20p family member; required for initiation of spore wall assembly	-2.76
KLTH0G13442g	YDR119W-A	*COX26*	Putative protein of unknown function; co-purifies with respiratory chain super complexes composed of Complex III and Complex IV	-2.18
KLTH0H09460g	YHR209W	*CRG1*	S-AdoMet-dependent methyltransferase involved in lipid homeostasis	-2.16
KLTH0C01056g	YOR381W	*FRE3*	Ferric reductase; reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels	-2.83
KLTH0E06468g	YGL192W	*IME4*	mRNA N6-adenosine methyltransferase required for entry into meiosis	-3.38

Chapter 6

Research results IV

Two-dimensional proteomics analysis of mixed and single culture fermentation under aerobic and anaerobic conditions

Two-dimensional proteomics analysis of mixed and single culture fermentation under aerobic and anaerobic conditions

6.1 Abstract

Although wine yeast gene expression has been thoroughly investigated only a few data are available on the protein profile of mixed and single fermentation. This work aimed at specifying the change in proteome of mixed and single fermentation and to assess its connection with transcriptome. We have previously analyzed the transcriptomes of *Lachancea thermotolerans* and *Saccharomyces cerevisiae* wine yeast strains in single and co-culture fermentation under anaerobic and 5% oxygen. Herein, we extend the comparative approach to include 2-Dimentional-based proteomic analysis of these fermentations in synthetic wine must. The data show that functional categories of proteins profile of mixed and single fermentation align with the functional categories of transcriptomics data. We identified 14 and 12 different spots in anaerobic aerobic mixed fermentations, respectively, in comparison to their respective single fermentations. The data in particular suggest that glycolysis and stress management metabolism were the most represented functional categories in a mixed fermentation. The data also allow the generation of hypotheses regarding the possible impact of mixed fermentation on the central carbon metabolism of *S. cerevisiae* as well as *L. thermotolerans* and role of glycolytic enzymes in stress management during mixed culture fermentations.

6.2 Introduction

The use of ‘omics’ technologies has become more popular recently including in wine environment. The transcriptome profile of mixed and single alcoholic fermentations has been investigated intensively by microarray and RT-PCR (Barbosa et al., 2015; Mendes et al., 2013). However, the transcriptomics analysis alone is not sufficient to describe a biological system as mRNA is not the final product and does not determine all the regulation mechanisms. Therefore, for system biology, proteome and transcriptome can be integrated for a better understanding of mechanisms. In this context, two-dimensional gel electrophoresis is a powerful technique which facilitates the identifications of hundreds of proteins in one analysis (Gygi et al., 2000; Lim et al., 2003). This technique has been widely used to visualize yeast proteins under different conditions. In wine fermentation, 2-D analysis has been used to study the proteomic response of *Saccharomyces cerevisiae* to different carbon, nitrogen sources and under different oxygen provision (Bruckmann et al., 2009; Kolkman et al., 2004; Zhao et al., 2014). However, use of the 2-D approach to investigate the protein profile in mixed alcoholic fermentation is still needs to be investigated. Some untargeted proteomic analysis of single and mixed culture fermentations has been to understand the mechanism

of interactions at the protein level and studies showed the importance of non-*Saccharomyces* yeasts in wine fermentation at the protein level (Mostert and Divol, 2014).

Incorporation of oxygen has been shown to improve the performance of non-*Saccharomyces* yeasts, which may result in changes in yeast to yeast interactions. However, more comprehensive investigations into proteins released in presence and absence of oxygen need to be done to understand the survival and interaction better. Therefore, the aim of this chapter is to observe the behaviour of *S. cerevisiae* and *L. thermotolerans* in mixed fermentations at a protein level using 2-D gel electrophoresis and assess its connection with transcriptomics profile obtained in the previous chapter.

6.3 Material and Methods

6.3.1 Yeast Strains, media and fermentations

For current chapter yeast strains, media and fermentations were the same as described in chapter 3. The sample used to extract the proteins were also same as mentioned in chapter 3 to extract RNA.

6.3.2 Proteome extraction and 2-D gel electrophoresis

Protein extraction, two-dimensional gel electrophoresis, statistical analyses, in-gel tryptic digestion and mass spectrometry were done as described before (Bruckmann et al., 2007). Briefly, total protein extracts were prepared from the cells re-suspended in Lysis buffer called Y-PER™ (Protein Extraction Reagent). The cells were vortexed for 30 minutes in Lysis buffer to extract the protein. For one-dimensional gel electrophoresis, a total of 15 µg of samples were loaded to see the differences in banding patterns of different fermentations. For two-dimensional gel electrophoresis protein samples (500 µg of protein) were loaded onto 17cm IPG strips pH 3-10 (Bio-Rad). The strips were rehydrated for 16 h using the Bio-Rad rehydration buffer, followed by isoelectric focussing on the Protean® IEF Cell under following conditions: 20 min at 250 V, 2.5 hrs at 10,000 V, again 10,000 V for 40,000 Vhrs (rapid). Later, subsequent treatment was done with equilibration buffer I and II (dithiothreitol and iodoacetamide) for 15 min each. After this, strips were then loaded onto 12.5% SDS-polyacrylamide gels followed by electrophoresis in a Protean® Plus Dodeca Cell (Bio-Rad) at 12.5 mA per gel. Gels were stained overnight in Coomassie stain and de-stained for 1h with destaining solution (10% methanol/7% acetic acid). Gels were stored in water until they were scanned using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences). Spot detection, matching, quantification and statistical analysis were performed using the PDQuest™ software version 7.3.1 (Bio-Rad, USA).

6.3.3 In-gel digest and peptide extraction

Briefly, the differential spots were manually excised from 2-D gels and washed twice in distilled water for 10 min. The gel pieces were then immersed in a destaining solution (50% acetonitrile and 25 mM ammonium biocarbonate) and sonication for 3-5 min, followed by dehydration with 2 × 10 min washes in 50% acetonitrile (ACN). After dehydration the gel pieces were digested overnight with 50 ng of sequencing grade trypsin (Promega) at 37°C according to the manufacturer's guide. Peptides were then extracted with a 10 µl solution of 30% ACN and 0.1% trifluoroacetic acid (TFA) (Sigma) for 30 min at room temperature and stored at 4°C until analysis.

Proteins present in the excised protein spots were digested using 50 ng of modified trypsin (Promega). For LC-MS analysis, samples were injected onto a nano-LC system (Ultimate, Dionex/LC Packings, Amsterdam, The Netherlands) equipped with a peptide trap column (Pepmap 100, 0.3 i.d. × 1 mm, Dionex/LC Packings, Amsterdam, The Netherlands) and an analytical column (Pepmap 100, 0.075 i.d × 150 mm, Dionex/LC Packings). The mobile phases consisted of (A) 0.04% formic acid/0.4% acetonitrile and (B) 0.04% formic acid/90% acetonitrile. A 45 min linear gradient from 0 to 60% B was applied at a flow rate of 0.2 µl/min. The outlet of the LC system was coupled to an HCT ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) using a nano-electrospray ionisation source. Eluting peptides were analyzed in the data dependent MS/MS mode over a 400–1600 m/z range. The five most abundant fragments in each MS spectrum were selected for MS/MS analysis by collision-induced dissociation. Mass spectra were evaluated using the DataAnalysis 3.1 software package (Bruker Daltonics). The spectra obtained from MS/MS were further used to searched against the *S. cerevisiae* and *L. thermotolerans* database using the Mascot search algorithm (Matrix Science Ltd., London, UK) allowing one missed cleavage site. The protein identification was made by highly identical homology with *S. cerevisiae* and *L. thermotolerans* proteins. Methionine oxidation was considered as a variable modification while carbamidomethyl cysteine was taken as a fixed modification. When a protein was identified with an almost identical homolog in the *S. cerevisiae* and *L. thermotolerans* proteome, the mass spectra were screened for selective peptides to enable discrimination between homologs.

6.4 Results

To investigate the protein profile of mixed fermentation and single fermentation of *S. cerevisiae* and *L. thermotolerans*, a comparative proteome analysis was performed using 2-DGE, under aerobic and anaerobic conditions, respectively.

In Fig. 6.1 shows a typical 1-D gel electrophoresis image of mixed and single fermentations in aerobic and anaerobic fermentation. The 1-D gel did not show any difference in banding pattern of mixed and single fermentation, even the protein profile of *S. cerevisiae* and *L. thermotolerans* resulted

almost similar. However, the protein yield in the single fermentation of *L. thermotolerans* was significantly less than the single fermentation of *S. cerevisiae* and mixed fermentations in aerobic as well as anaerobic.

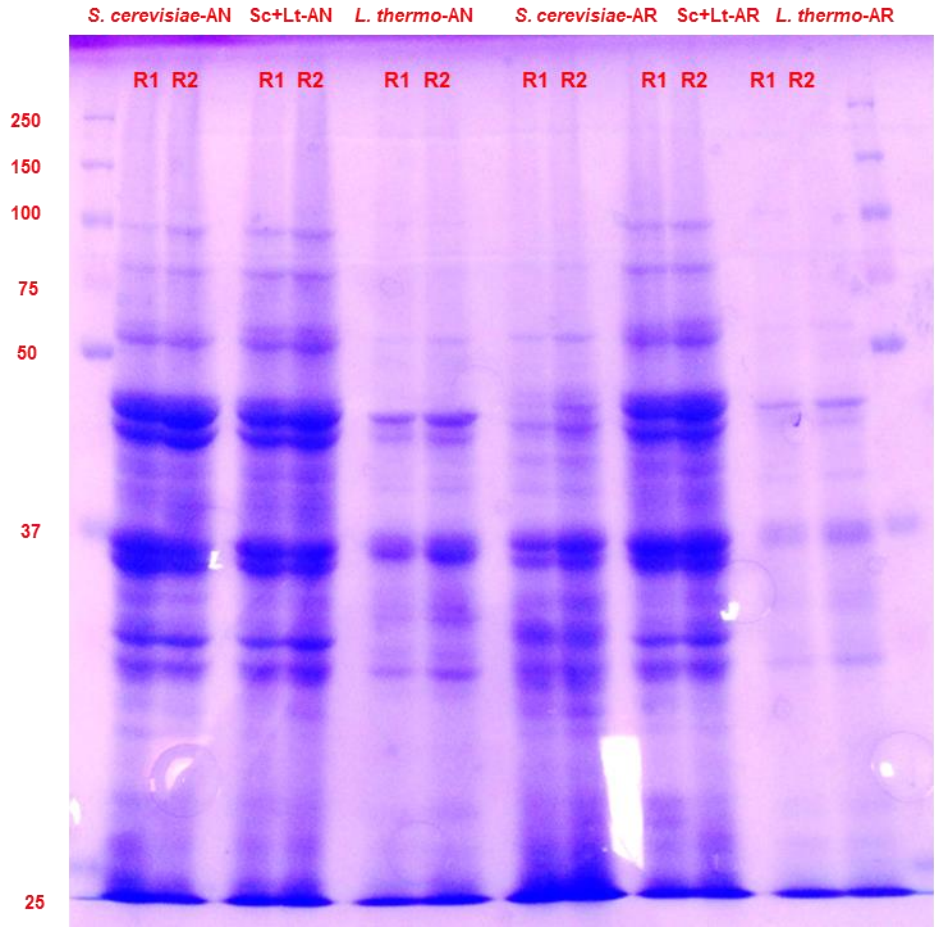

Figure 6.1 One-dimensional gel electrophoresis of mixed and single fermentation of *S. cerevisiae* and *L. thermotolerans* in anaerobic and aerobic conditions

The 2-D gel analysis was performed in duplicate for each biological repeat. Approximately 150 spots were detected in single *S. cerevisiae* and mixed fermentation gels, while in *L. thermotolerans*, the 2-D gel was obtained only for anaerobic fermentation with approximately 40 spots.

To analyse the 2-D gels, the gels were laid over on each other to compare the difference between mixed and single fermentations with and without oxygen. In anaerobic condition, the mixed fermentation gels were compared with *S. cerevisiae* as well as *L. thermotolerans* single anaerobic fermentation gels. However, with aerobic fermentation, the mixed fermentation gels were compared only with single *S. cerevisiae* gel (single *L. thermotolerans* aerobic fermentation gels were unsuccessful). Based on this analysis we could classify the proteins into four different categories (i) proteins that were detected only in mixed fermentation (with and without oxygen) (ii) proteins that were present only in aerobic S*. cerevisiae* single fermentation in comparison to single anaerobic fermentation (iii) proteins which were common in mixed fermentation *S. cerevisiae* but not in *L.*

thermotolerans anaerobic fermentation (iv) proteins that were present in all fermentations (Table 6.1-6.3).

Table 6.1 Total number of different proteins identified in mixed anaerobic gel in comparison to single anaerobic *S. cerevisiae* and *L. thermotolerans* gels (red colour shows spots those were not identified confidently)

Spot No.	Protein name	Number of peptides matched with *S. cerevisiae*	Number of peptides matched with *L. thermotolerans*
4	Pyk2	21	4
5	Pyk2	3	1
9	Thi13	12	5
10	Fba1	3	2
30	Dug1	10	3
31	Gdh1	16	4
46	Ilv5	15	7
56	Gpm1	3	17
57	Fba1	7	2
58	Pyk2	16	8
59	Pyk2	5	19
60	Ade17	13	5

In mixed anaerobic fermentation, a total of 14 different spots were excised (Fig. 6.2a- Mixed anaerobic, Fig. 6.2b- Mixed aerobic). However, only 12 could be identified (Table 6.1). Among them 8 spots originated from *S. cerevisiae*, (Pky2, Thi12, Dug1, Gdh1, Ilv5, Fba1, Ade17) while two were from *L. thermotolerans* (Gpm1, Pyk2) and another 2 spots could not be confidently assigned to either of the yeasts or seems to originate from both yeasts (Fba1, Pyk2). From the mixed aerobic fermentations 11 spots were selected. All were found originated from *S. cerevisiae*. The proteins were identified as, Hxk2, Sam1, Ssa2, Hsp72, Tdh3, Fba1, Tdh3, Sod1, Fba1, Bmh1, Oye2 (Table 6.1-6.2).

Table 6.2 Total number of different proteins identified in the mixed aerobic gel in comparison to single aerobic *S. cerevisiae* gel.

Spot No.	Protein name	Number of peptides matched with *S. cerevisiae*	Number of peptides matched with *L. thermotolerans*
5	Hxk2	12	0
6	Sam1	11	3
10	Ssa2	3	2
11	Hsp72	5	4
15	Tdh3	3	0
16	Fba1	7	0
17	Tdh3	7	6
19	Sod1	8	0
23	Fba1	9	3
39	Bmh1	8	0
47	Oye2	7	0

Clearly, a maximum number of highly expressed proteins identified in mixed fermentation comes from glycolytic pathway (Table 6.1, 6.2), namely, Hxk2, Fba1 and Tdh3 in mixed aerobic fermentation, while Fba1, Pyk2, Gpm1 were detected in anaerobic mixed fermentation. The expression of Hxk2 glycolytic enzymes also aligns with corresponding genes *HXK2* in transcriptomics data. In aerobic mixed fermentation *S. cerevisiae* also showed higher transcripts for *HXK2* (1.5-fold change). The high expression of Pyk2 and Gpm1p coming from *L. thermotolerans* also links with the transcriptomics data of *L. thermotolerans*, where *L. thermotolerans* showed higher gene expression of *PYK2* and *GPM1* (1.59, 1.17-fold change) in anaerobic mixed fermentation. The possible reason for the higher abundance of these proteins in mixed fermentation could be due to the fact that presence of both yeasts perturbs the central carbon metabolism.

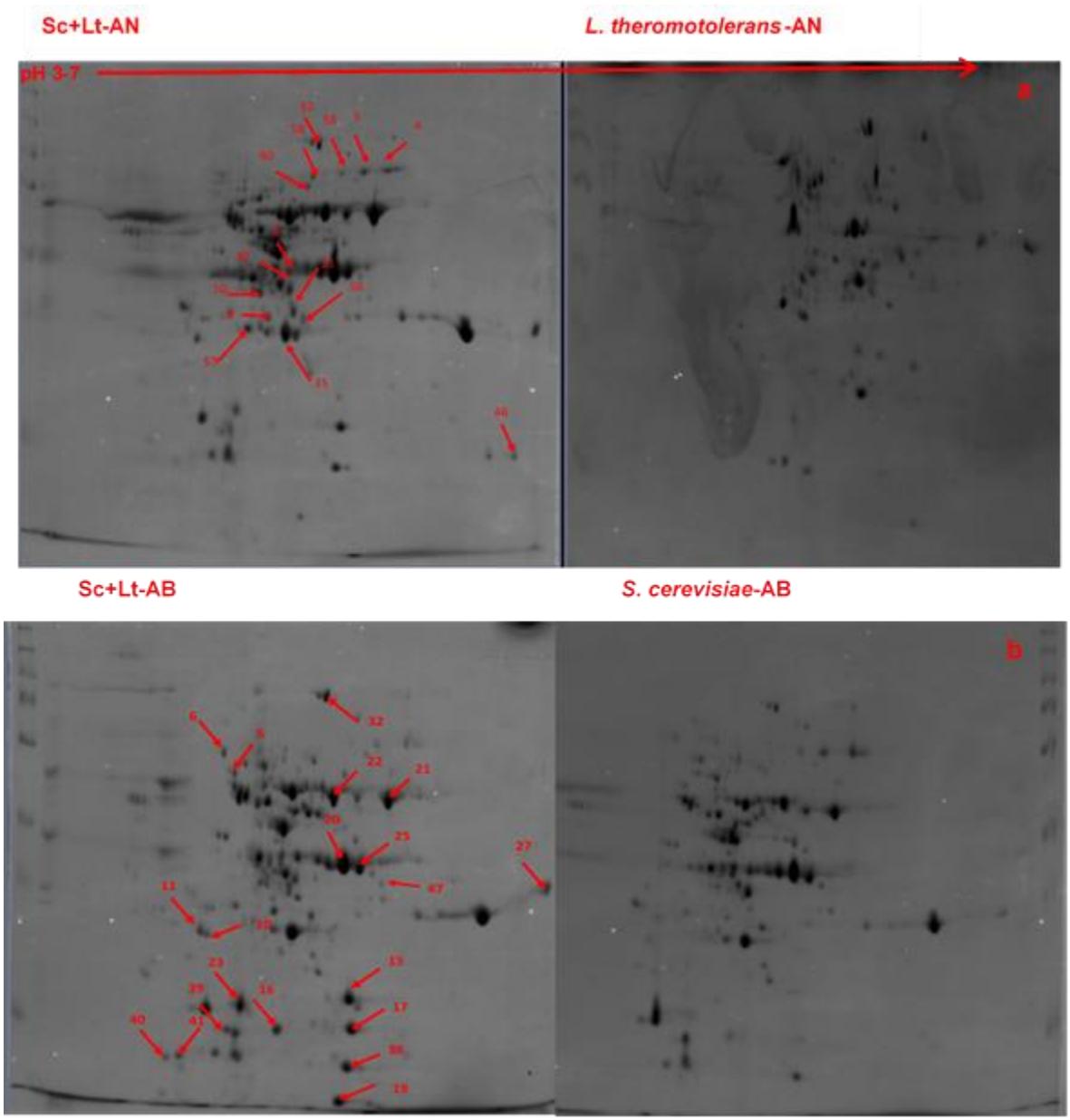

Figure 6.2 Comparison of two-dimensional gel electrophoresis of mixed and single fermentation of *S. cerevisiae*, *L. thermotolerans* in anaerobic and aerobic conditions (a, b).

Furthermore, we also found proteins that are related to stress response. For instance, in mixed aerobic fermentation, protein of Hsp70 family, Ssa2p (in two spots), proteins that are involved in oxidative stress such as Sam1, Sod1p, Oye2p, Bmh1 were up-regulated. The up-regulation of these proteins also aligns with the transcriptomics data by showing higher transcripts for gene *SAM1* (1.6-fold change), *SOD1* (1.9-fold change) and *SSA2* (1.5-fold change) in *S. cerevisiae* under aerobic mixed fermentation conditions. The high expression of proteins involved in redox balance also aligns with oxidation reduction category of transcriptomics data (Table 5.2 in chapter-5).

In mixed anaerobic fermentation proteins involved in redox balance were also identified such as, Gpm1p (produced by *L. thermotolerans*), Gdh1p (produced by S. cerevisiae). In *S. cerevisiae,* anaerobic fermentation showed high expression of *GDH1* gene was also observed with a fold

change of 1.5, while higher transcripts were observed for *GPM1* gene in *L. thermotolerans* mixed anaerobic fermentation with a fold change of 1.17.

In a comparison of *S. cerevisiae* single fermentations under aerobic and anaerobic conditions five spots present in the aerobic fermentation but absent under anaerobic conditions were selected. These proteins were, namely, Yhb1, Hsp77, Adh1, Hxk2, Ahp1 (Fig. 6.3).

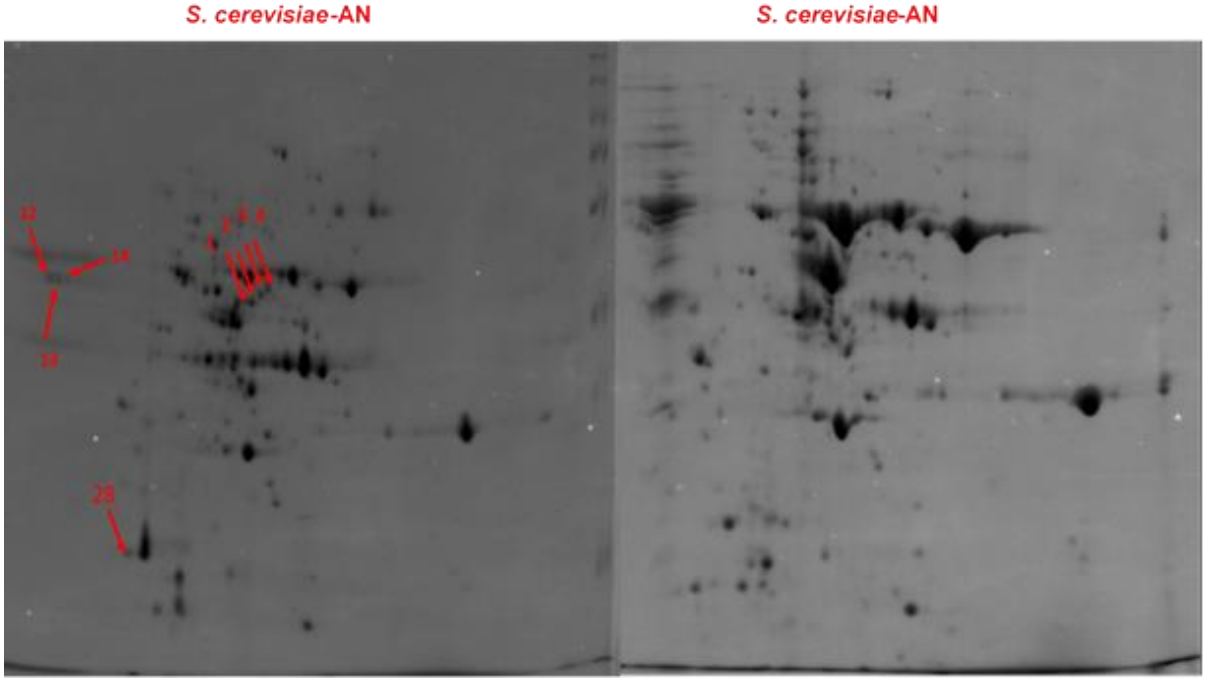

Figure A 2.3 Comparison of two-dimensional gel electrophoresis of single fermentation of *S. cerevisiae* gels under aerobic and anaerobic conditions.

We also analyzed common spots. Among them 5 were common in all gels (a single *S. cerevisiae*, *L. thermotolerans* and mixed). However, 5 spots were only present in mixed and single *S. cerevisiae* gels and were not present in *L. thermotolerans* single anaerobic fermentation. These common detected proteins were namely, three spots with Pep1p, Tef1p, Tps1p, all these five spots were present in all mixed as well as single S*. cerevisiae* fermentation, while 5 common proteins were detected in all gels including *L. thermotolerans*, namely, Cpr1p, Tdh3p, Pgk1p, Eno1p, Tdh2p, Met6p.

From the MS protein identification of different spots, we found some proteins that were detected in different spots, of approximately the same Mr but with slight different pI. For example, Pep1p was detected in three different spots with a slight difference in their pI. Similarly, we also found different spots with a same protein of the same pI with different Mr. For instance, Fba1p was detected in different spots of mixed anaerobic gel with almost same pI. Thd3p was detected in to three different spots and one of was detected only in mixed aerobic fermentation.

Table 6.3 Common proteins identified in mixed and single fermentations of *S. cerevisiae* and *L. thermotolerans* gels

Spot No.	Protein name	All Mixed and *S. cerevisiae* gels	*L. thermotolerans* gel (only anaerobic)
12	Pep4	Present	Absent
13	Pep4	Present	Absent
14	Pep4	Present	Absent
18	Cpr1	Present	Present
20	Tdh3	Present	Present
21	Pgk1	Present	Present
22	Eno1	Present	Present
25	Tdh2	Present	Present
27	Tef1	Present	Absent
32	Met6	Present	Present
35	Tps1	Present	Absent

Overall, these identified proteins play a role in functions other than in the central carbon metabolism, such as amino acid synthesis (Ilv5p), and nucleotide metabolism (Ade17p), and proteins redox balance and with antioxidant properties (Oye2p, Gpm1p, Sod1p).

6.5 Comparison with transcriptome data

The functional categories of proteins up-regulated in mixed fermentations such as glycolysis, stress and oxidation-reduction process, sulfur metabolism are in alignment with our transcriptomics data. These categories align with transcriptome by up-regulation their transcripts such as *HXK2*, *SSA2*, *SOD1*, *SAM1* (*S. cerevisiae* mixed aerobic), *GDH1*(*S. cerevisiae* mixed anaerobic), *PYK2*, *GMP1* (*L. thermotolerans* in mixed anaerobic fermentation). However, we must consider that the study was indeed biased towards proteins with higher concentrations. Moreover, small number of proteins were excised for MS analysis, hence, the study also gave us very few spots to compare the data with the transcriptome profile.

6.6. Discussion

The current chapter enables the analysis of relative protein expression under a mixed and single populated fermentation under two different oxygen conditions. The data shows changes in protein expression profile mainly involved in glycolytic pathway, oxidation-reduction process and stress related proteins. To emphasize more on function of each protein and their possible role in different

fermentation condition, below we have further discussed in detail the outcome of our proteome analysis and link with transcriptomics data.

6.6.1 Glycolysis proteins

Glycolytic enzymes that are responsible to convert glucose into pyruvate, were significantly more abundant in the mixed fermentations (Table 6.1-6.2). These proteins were namely, Hxk2, Fba1 and Tdh3 in mixed aerobic fermentation. While Fba1, Pyk2, Gpm1 were detected in anaerobic mixed fermentation. The expression of some of these glycolytic enzymes aligns with their corresponding genes in transcriptomics data. For instance, in aerobic mixed fermentation *S. cerevisiae* also showed higher transcripts for *HXK2* (1.5-fold change). The high expression of Pyk2 and Gpm1p coming from *L. thermotolerans* also links with the transcriptomics data of *L. thermotolerans*, where *L. thermotolerans* showed higher gene expression of *PYK2* and *GMP1* in anaerobic mixed fermentation. The similar results were also obtained by Mostert and Divol (2014), where higher expression of proteins involved in glycolysis pathway were detected in mixed fermentation of *S. cerevisiae* with *M. pulcherrima* and *L. thermotolerans*. Moreover, the high expression of glycolytic isozymes such as Hxk2, Tdh2, Tdh3 is also linked to notable feature of the stress response, indicating the divergence for optimal reactions in different conditions, and possible general and specific regulations in response to stresses (Grant et al. 1999; Kim et a. 2013; Postmus et al. 2012; Trabalzini et al. 2003). Studies by Cheng et al. and Kim et al. (2008, 2013) also showed high expression of Fba1 and Hxk2 as a response to oxidative stress generated in fermentation conditions. From all available literature the data suggests that the possible reason for higher abundance of these proteins in mixed fermentation could be due to the fact that presence of both yeast perturbs the central carbon metabolism.

6.6.2 Proteins involved in stress response and redox balance

Among proteins that are involved in stress response, in mixed aerobic fermentation, protein of Hsp70 family, Ssa2p (in two spots), proteins that are involved in oxidative stress such as Sod1p, Oye2p, Bmh1 were up-regulated (Kumar and Srivastava 2016; Lawrence et al. 2003; Lottersberger et al. 2004; Trabalzini et al. 2003). The up-regulation of these proteins also aligns with the transcriptomics data to some extent by showing high gene expression of *SOD1* (1.9-fold change) and *SAA2* (1.5-fold change) in *S. cerevisiae* under aerobic mixed fermentation conditions. The high expression of these stress induced proteins could be due to either formation of reactive oxygen species or could be due to handle with mixed population environment.

Furthermore, the protein analysis data showed differences in fermentations as high expression of proteins involved in maintaining redox balance. The high expression of proteins involved in redox balance also aligns with oxidation reduction category of transcriptomics data. In aerobic mixed

fermentation, high expression of Sam1p, Oye2 protein suggests that *S. cerevisiae* adapts the metabolism by high expression of these proteins to maintain the redox balance (Gudipati et al. 2014; Thomas and Surdin-Kerjan 1990; Tehlivets et al. 2013), this was also in-line with high expression of *SAM1* (1.6-fold change) gene in *S. cerevisiae* aerobic mixed fermentation. In mixed anaerobic fermentation proteins involved in redox balance were also identified such as, Gpm1p, Gdh1p. The data indicates that maintaining redox balance is an important feature for yeast and higher expression of these proteins in mixed fermentation could help both yeasts to adjust the metabolism according to different conditions.

6.7 Conclusion

In conclusion, the analysis and comparison of these two levels of regulation, transcript and protein, provided the biological support to our transcriptomics data to some extent. Although not all the identified proteins showed up-regulation of their respective genes in transcriptomics data, the representative proteins were up-regulated from GO categories enriched from transcriptomics data such as stress and glycolysis pathway. Keeping in mind both data set, we reach to the conclusion that in mixed fermentations both yeasts show more perturbance in central carbon metabolism and experience more stress in comparison single culture fermentations. In addition to the molecular-based information on mixed and single fermentations provided by this study, the two-dimensional protein map reported here could be used as a reference for future studies.

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Chapter 7

General discussion and conclusions

7.1. General discussion and conclusion

The main aims of this project were (i) to investigate the impact of oxygen on the persistence of non-*Saccharomyces* yeasts and (ii) the impact of oxygen and changed population dynamics on the aroma profile of wine and on metabolic and other interactions between two yeast species in mixed culture fermentation. Three non-*Saccharomyces* yeast species were characterized for their survival as a function of oxygen in mixed culture fermentation with *S. cerevisiae* and compared regarding their fermentation capacity and physiology in synthetic grape juice. One of these non-*Saccharomyces* yeasts (*L. thermotolerans*) was further characterized in real grape must to check the reproducibility under real wine-making conditions. A comparative transcriptomics analysis was performed between the two yeasts to understand the impact of oxygen provision on the physiology of *L. thermotolerans* and *S. cerevisiae*. The mixed culture fermentations of *L. thermotolerans* and *S. cerevisiae* were further subjected to a transcriptomic analysis comparing the transcriptomic signature of single species and mixed species fermentations in aerobic and anaerobic conditions to understand the ecological interactions. The transcriptomics data were further validated through 2-D gel electrophoresis-based proteomics.

Characterization of the three non-*Saccharomyces* yeast species in mixed culture fermentation with *S. cerevisiae* with different levels of dissolved oxygen showed that there were significant differences regarding their persistence and the volatile aroma compounds produced by these strains. The data make a significant contribution and provide information about non-*Saccharomyces* yeasts.

Firstly, the data show that carefully managed oxygen inputs can enhance the persistence of non-*Saccharomyces* yeasts and ultimately their contribution to wine aroma. The levels of oxygen provided are dependent on the respiratory quotient of the individual strains. Oxygen provision supports the growth of non-*Saccharomyces* yeasts even at ethanol levels above 10% v/v. This observation, confirmed that for yeasts such as *L. thermotolerans* and *T. delbrueckii*, the dominance of advantage of *S. cerevisiae* over these two yeasts is not via ethanol production but rather via oxygen depletion (Williams et al., 2015). Furthermore, the data show that a similar strategy applies to *M. pulcherrima*. This yeast is known to be an oxidative yeast with a higher oxygen requirement than *S. cerevisiae*, *L. thermotolerans* and *T. delbrueckii* (Quirós et al., 2014). Indeed, the data in the current study show that this yeast required a continuous input of 21% dissolved oxygen before it could dominate *S. cerevisiae* in wine fermentation. Furthermore, the current study revealed that oxygen inputs similar to punch-downs and pump-overs could provide enough oxygen to enhance the persistence of *L. thermotolerans*. However, it was evident that in the absence of regular agitation, these types of interventions might not provide enough oxygen. Therefore, a higher oxygenation strategy such as transfer from tank to tank might be required when mixed culture fermentations are employed. This is the first study to highlight this major challenge in the application of non-

Saccharomyces yeasts in fermentations. Incidentally, strains of the three-species used in the current study are already available as commercial active dry yeasts and are used as co-inoculants with *S. cerevisiae*. In fact, *T. delbrueckii* and *L. thermotolerans* are also available as a blend e.g. Viniflora®MELODY™ (CHR Hansen). The current study shows that in order to benefit from the contribution of these strains in wine fermentation, a combination of regular agitation and effective oxygen input is necessary. Of course, the oxygen requirements are also strain dependent (Quiros et al., 2014). Therefore, it is important that further studies should consider testing multiple strains.

Although the higher amount of oxygen leads to better performance of non-*Saccharomyces*, the detrimental effect of oxygen also led higher concentration of acetic acid and acetaldehyde. Moreover, maintaining levels of oxygen is not a realistic strategy in a commercial cellar, therefore, we further confirmed the results in Chardonnay grape juice with different, but commercially feasible oxygenation regimes. The results showed a trend similar to those obtained in synthetic grape juice, with a positive effect of oxygen on the relative performance of *L. thermotolerans*. Data also suggests that the lower level of oxygen seems beneficial by increasing more concentration of higher alcohols, diethyl succinate, propionic acids, ethyl phenylacetate and decreased the concentration of ethanol. In our study, use of non-*Saccharomyces* yeasts with controlled regimes of oxygen has agreed with some of the recent studies (Ciani et al., 2016: Morales et al., 2015). Our study shows that the different response of *L. thermotolerans* and *S. cerevisiae* to oxygen could be due to different adaptation mechanisms of both yeasts to oxygen availability, probably due to the evolution of the two yeasts. Transcriptomic analysis showed differences in the expression of genes that are involved in cellular processes, namely the ergosterol biosynthesis pathway, the central carbon metabolism, the mechanism of autophagy and other stress related responses. This might be related to different strategies for cell survival in the different environment. One of the important stresses that yeasts have to deal with is the accumulation of ethanol. Yeast ethanol tolerance has been correlated to the ability of yeasts to modulate their membrane lipid composition to respond to the disruptive nature of ethanol. Indeed, studies performed with *S. cerevisiae* have shown that the yeast adapts to self-produced ethanol by increasing ergosterol levels in the membrane with higher ratios of phosphatidylinositol-to-phosphatidylcholine and larger amounts of $C_{18:0}$ fatty acids relative to $C_{16:0}$ fatty acids (Arneborg et al., 1995). Furthermore, studies on *Kluyveromyces marxianus* have shown that this yeast adapts to ethanol by up-regulating autophagy related genes (Gao et al., 2015). Similar results are obtained in *L. thermotolerans* under anaerobic condition and these results suggest that perhaps *L. thermotolerans* uses autophagy mechanism in order to adapt with ethanol or anaerobic condition. Moreover, *L. thermotolerans* also exhibited higher expression of *ERG* genes, suggesting, a different cell wall modulating mechanism in *L. thermotolerans* than *S. cerevisiae*. The results provide a unique insight into the physiology of *L. thermotolerans* and how if differs one from *S. cerevisiae*, from which new strategies for further investigation could be derived for non-*Saccharomyces* yeasts under enological conditions. Similar results were also obtained for *Pichia pastoris* and *S. cerevisiae* (Baumann et al., 2011).

Furthermore, the gene expression profiles of the *L. thermotolerans* and *S. cerevisiae* was examined in mixed and single culture fermentations under aerobic and anaerobic conditions. Previous studies have been mostly carried out in a batch system, where the continuously changing fermentation conditions result in continuously shifting transcriptomes, making it difficult to differentiate between responses to environmental changes from those that are due to a specific response to the presence of another species. For this reason, we adopted an approach based on the use of a chemostat, providing a controlled system to attain an equal population of two yeasts species at the same stage to assess yeast-yeast interactions and fermentations patterns under aerobic and anaerobic conditions. Our data indicates a competitive interaction between the two yeasts in mixed fermentation for nutrients such as copper, sulfur and thiamine by up-regulating genes potentially linked to these nutrients. For instance, in mixed fermentation, both yeasts showed higher expression of genes involved in copper uptake *CTR1* and *CTR3*. Studies by Hodgins-Davis et al., (2012) have shown that lower concentration of copper is responsible for higher expression of *CTR1* and *CTR3*. Further, in the mixed culture fermentation high expression of genes which are linked to and cell wall integrity such as *PAU* and *FLO* genes. On the basis of literature, the higher expression of *PAU* genes in *S. cerevisiae* seems to respond to the presence of *L. thermotolerans,* it has been reported that *PAU* genes play an important role in promoting fitness under anaerobic and fermentative condition as well as in yeast-yeast interactions (Luo et al., 2009; Rivero et al., 2015). Moreover, the higher expression of genes involved in aroma profile of wine also nicely aligns with our metabolomic data and suggests a metabolic interaction between both yeasts. For example, higher expression of *BAT1, ARO8, ARO9, AAD14, SFA1* in *L. thermotolerans* mixed fermentation clearly suggest a response to the presence of *S. cerevisiae* and justifies the reason behind the accelerated concentration of higher alcohols in mixed culture fermentation than single fermentation (Rossouw et al., 2008).

The protein profile of mixed fermentation also aligns to transcriptomics data, however, due to the short number to spots, it is quite hard to compare the two data sets completely. The proteomics data of mixed fermentations under anaerobic and aerobic conditions agrees with previous studies with higher expression of glycolytic proteins in mixed culture fermentation and suggests perturbation in central carbon metabolism of *S. cerevisiae* due presence of two yeasts together (Mostert and Divol, 2016).

In conclusion, our results show the role of oxygen in regulating the succession of yeasts during wine fermentations and its impact on yeasts physiology. Our study has shed light on some of the underlying facts of molecular mechanism yeasts may follow to adapt under different oxygen provisions. By comparing the transcriptomic datasets of *L. thermotolerans* and *S. cerevisiae* in mixed and single fermentations we could identify numerous gene/genes set that could be linked with relevant aspects of yeast performance under multi-species wine fermentation. For instance, genes that are related to cell wall integrity or stress management, nutrients, and metabolism. Such study

provides an understanding of metabolic changes that occur during fermentation under wine-making conditions.

7.2 References

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Appendix I

A comparative analysis of *Lachancea theromotolerans* and *Saccharomyces cerevisiae* transcriptome in response to oxygen

A comparative analysis of *Lachancea theromotolerans* and *Saccharomyces cerevisiae* transcriptome in response to oxygen

A 1.1 Abstract

Lachancea thermotolerans is an important non-*Saccharomyces* yeast which contributes many positive characters in mixed wine fermentation with *Saccharomyces cerevisiae.* This yeast however, has a higher oxygen demand than *S. cerevisiae* and therefore tends to decline rapidly with the development of anaerobic conditions during fermentation. Indeed, previous studies have shown that oxygen supports the fermentative performance of *L. thermotolerans*, and leads to higher growth and longer persistence of this yeast even in the presence of *S. cerevisiae*. The impact of oxygen on yeast physiology has been studied primarily in *S. cerevisiae* and very few studies have assessed the molecular response of other yeast species. In this work, we compared the transcriptome of *L. thermotolerans* and *S. cerevisiae* in the anaerobic and aerobic condition in chemostat cultures with 0 and 5% of dissolved oxygen. The data show that the two yeast respond differently to oxygen availability. Overall, oxygen availability had a more significant impact on the transcriptome in *L. thermotolerans* than in *S. cerevisiae*. The data also highlight the significant impact of oxygen supply on genes encoding proteins involved in ergosterol biosynthesis, autophagy, central carbon metabolism and other stress responses. This comparative transcriptomic study provides novel insights into the different adaptive responses of *L. thermotolerans* and *S. cerevisiae* to oxygen availability.

Keywords: non-*Saccharomyces*, aerobic, anaerobic, transcriptome, ergosterol, autophagy

A 1.2 Introduction

The importance of non-*Saccharomyces* in wine fermentation has been emphasized in the past decade, and some of these yeast species are now commercially available, in most cases as co-starter cultures to be combined with *S. cerevisiae* strains (Padilla et al., 2016). However, the physiology of these yeast species has received limited attention. The available literature nevertheless sheds some light on the response of some non-*Saccharomyces* yeasts to a range of wine-relevant factors such as ethanol, temperature, pH, cell-cell interaction, killer toxins and oxygen (Hansen et al., 2001; Morales et al., 2015; Nissen et al., 2003; Pérez-Nevado et al., 2006; Wang et al., 2016). Among such factors, the role of oxygen in the persistence of non-*Saccharomyces* yeasts has recently been emphasized. In particular, incorporation of oxygen enhances the persistence of non-*Saccharomyces* yeasts in wine fermentation (Morales et al., 2015; Quirós et al., 2014). During the wine making process, oxygen may be discretely added to avoid stuck fermentations and to increase the yeast biomass (Aceituno et al., 2012). Incorporation of oxygen is generally achieved

through pump-overs, where the concentration of dissolved oxygen could rapidly reach up to 100 µM (Rosenfeld et al., 2003). Addition of a range of dissolved oxygen concentrations has been shown to have a profound impact on the physiology of *S. cerevisiae*, suggesting in particular major impacts on cellular functions such as sterol biosynthesis, proline uptake, and unsaturated lipid biosynthesis (Aceituno et al., 2012; Rintala et al., Rosenfeld et al., 2003). To the best of our knowledge, no study has focused on elucidating the molecular response to oxygen in any wine related non-*Saccharomyces* Crabtree positive yeast.

The full genome sequence of *Lachancea thermotolerans* has recently become available, allowing the application of genome-wide studies of this non-*Saccharomyces* yeast, which has also been gaining high valuable relevance in the wine industry in the recent years.

Our data show that the two yeast respond differently to oxygen availability. The data highlight the significant impact of oxygen supply on genes encoding proteins involved in ergosterol biosynthesis, autophagy, central carbon metabolism and stress responses.

A 1.3 Material and Methods

The Yeast strains, fermentations and transcriptomics data analysis for current Appendix has been already explained in Materials and method section of Chapter-5 for single culture fermentation.

A 1.4 Results and Discussion

A 1.4.1 Fermentation and sampling

The expression analysis of *L. thermotolerans* and *S. cerevisiae* fermentation was performed with samples collected at 48 h of continuous fermentation, when the population of *L. theromotolerans* and *S. cerevisiae* was approximately similar (between 1.0×10^8 mL^{-1} to 2.5×10^8 mL^{-1}) (Fig. A 1.1). In order to attain equal population dynamics and sugar concentration, different dilution rates were used in anaerobic and aerobic single fermentations as mentioned in Table A 1.1.

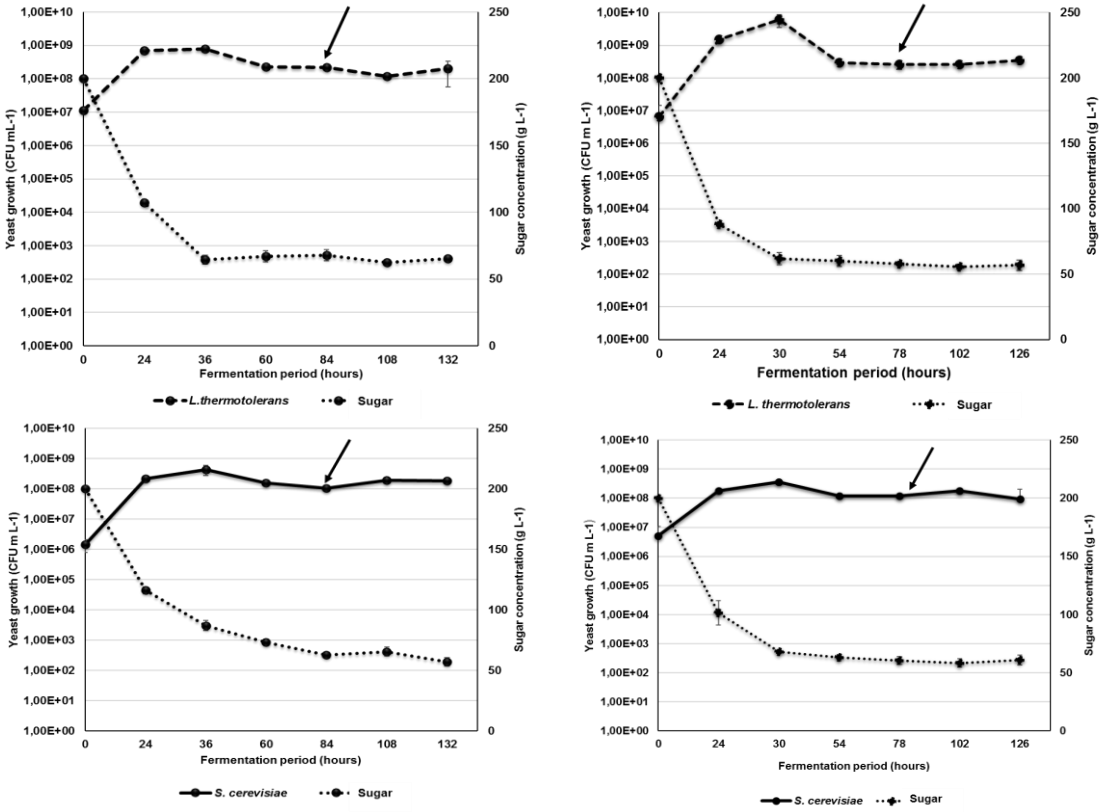


Figure A 1.1 Fermentation kinetics and population dynamics of *S. cerevisiae* and *L. thermotolerans* under anaerobic and aerobic continuous fermentation conditions.
*The arrows indicate the sampling points in each graph

Table A 1.1. Population dynamics and sugar concentration of *L. thermotolerans* and *S. cerevisiae* at 48 hours of sampling for RNA-sequencing with different dilution rates under aerobic and anaerobic conditions.

Fermentations	Dilution rate h^{-1}	CFU mL^{-1} at 48 h	Sugar concentration at 48 h (g L^{-1})
L. thermotolerans-AN	0.075	2.1E+08	68.0
S. cerevisiae-AN	0.10	1.2E+08	62.5
S. cerevisiae-AR	0.125	1.0E+08	60.0
L. thermotolerans-AR	0.125	2.5E+08	58.0

A 1.4.2 Identification and statistical analysis of differentially expressed genes

Gene expression levels were normalized using fragments per kilobase of exon per million mapped reads (FPKM). A negative binomial generalized linear model (GLM) was fitted against the normalized counts using the EdgeR 3.4.0 package of Bioconductor (Robinson et al., 2007). Differential expression was tested for with a GLM likelihood ratio test, also implemented in the EdgeR package. The resulting p-values were corrected for multiple testing with Benjamini-Hochberg to control the

false discovery rate (Benjamini & Hochberg 1995). The gene lists were analyzed for enrichment of functional categories using the Gorillila gene ontology program (Eran et al., 2007; 2009).

A 1.4.3 General overview of the transcriptional analysis

After statistical analysis of transcriptomic data from *L. thermotolerans* and *S. cerevisiae* grown under anaerobic (0%) and aerobic conditions (5% dissolved oxygen) using Benjamini and Hochberg method to control the false discovery rate. Our data show significant differential expression (log2 fold change threshold; FDR-value ≤ 0.05) of 899 genes for *L. thermotolerans* and 414 genes for *S. cerevisiae*. Both yeasts shared a total of 58 differentially expressed genes, among them 46 were up-regulated in aerobic condition, while 12 genes were down-regulated (or up-regulated in anaerobic conditions). *L. thermotolerans* showed 492 genes upregulated and 407 were down-regulated when oxygen was supplied, while *S. cerevisiae* showed 260 genes up-regulated and 154 genes down regulated under same conditions (Fig. A 1.2). The gene lists were analyzed for enrichment of functional categories using the Gorillila, gene ontology program (Eran et al., 2007; 2009).

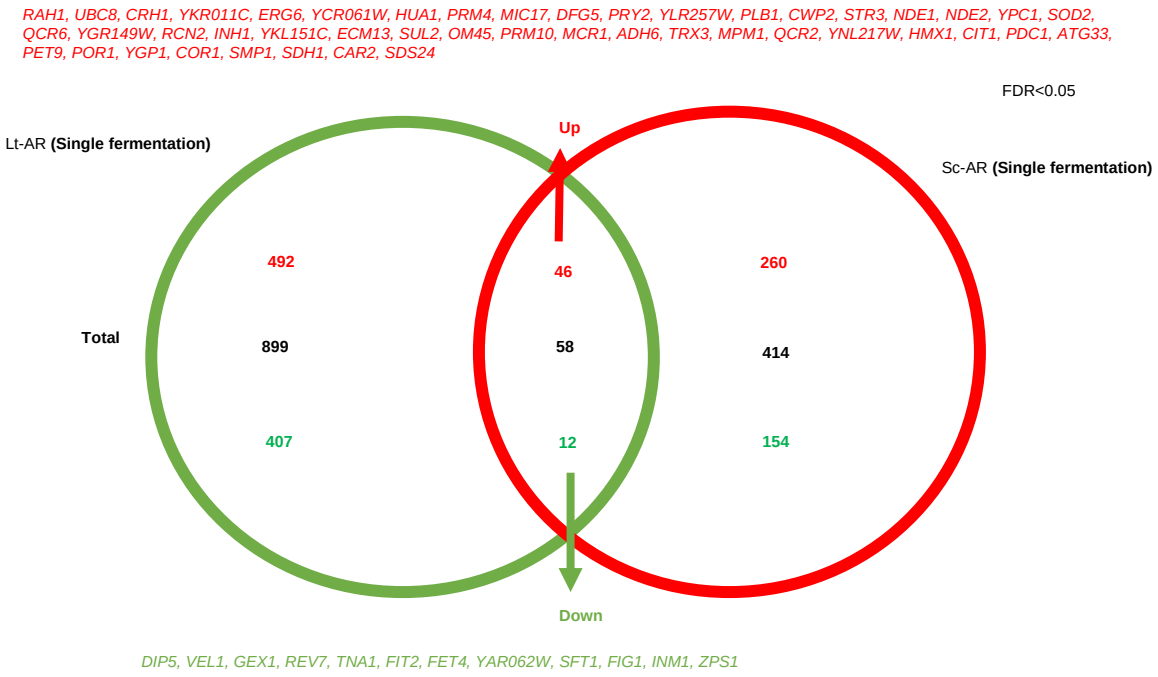


Figure A 1.2 A Venn figure presentation of genes differentially expression in *L. thermotolerans* and *S. cerevisiae* under aerobic and anaerobic conditions.

The GO analysis of the up-regulated genes in *S. cerevisiae* under aerobic condition, showed enrichment for: respiratory electron transport chain, ergosterol biosynthesis and oxidation-reduction process. In contrast, under same conditions, *L. thermotolerans* showed enrichment for positive regulation of transcription from RNA polymerase II promoter, alpha-amino acid metabolic process, regulation of filamentous growth of a population of unicellular organisms, oxidation-reduction process.

The GO analysis of the down-regulated genes in *L. thermotolerans* under aerobic conditions. Showed enrichment for: ergosterol biosynthesis process, meiotic cell cycle checkpoint, organic acid catabolic process, secondary alcohol biosynthetic process. In contrast, *S. cerevisiae* showed enrichment for: iron coordination entity transport, transmembrane transport, multi-organism process, iron chelate transport, siderophore transport, kynurenine metabolic process.

A 1.4.4 Effect of oxygen on central carbon metabolism of *S. cerevisiae* and *L. thermotolerans*

Under aerobic condition, and as expected, both yeasts showed higher transcript levels of genes encoding enzymes involved in mitochondrial phosphorylation, TCA cycle and glycolytic pathway. However, the yeasts differed in which specific genes were most affected in these functional categories.

Of the electron transport chain both yeasts showed higher expression of *NDE1, NDE2, QCR2, QCR6, COR1* (genes mentioned in yellow color in Fig. A 1.3a). Furthermore, *S. cerevisiae*, showed higher expression for; *QCR8, QCR9, QCR10* (Complex III of electron transport chain, green color genes in Fig. A 1.3 a); *CYC1* (Part of Complex I); *COX4, COX5a, COX7, COX13, COX20* (Complex IV); *ATP1* (Complex V). In contrast, in *L. thermotolerans*, the expression of *QCR9, QCR10* remained unchanged in *L. thermotolerans* (*QCR8* is absent in *L. thermotolerans* genome); *CYC1* down-regulate; *COX4, COX5a, COX13, COX20* remained unchanged in *L. thermotolerans*. Furthermore, *L. thermotolerans* showed higher expression for *COX11* (Complex IV, which remained unchanged in *S. cerevisiae*), *ATP3, ATP5, ATP10, ATP14* (Complex V, remained unchanged in *S. cerevisiae*) (Fig. A 1.3 a, b).

Moreover, in comparison to *S. cerevisiae*, more genes of *L. thermotolerans* were up-regulated involved in encoding enzymes for TCA cycle. For instance, *S. cerevisiae* showed higher expression of *CIT1*, *SDH3* and *SDH4*. In contract, *L. thermotolerans* showed higher expression of more genes such as *CIT1*, *SDH3, SDH4, ICL1, ACO1, KGD1, LSC1* (Fig. A 1.3 a). Unlike *L. thermotolerans*, *S. cerevisiae*, showed down-regulation for *ICL1, ACO1* and *LSC1*, while expression of *KGD1* remained unchanged (Fig. A 1.3, a-b). The higher expression of genes encoding enzymes involved in of TCA cycle and Complex V of electron transport chain in *L. thermotolerans* than *S. cerevisiae*, suggests that *L. theromotolerans* perhaps use more of the carbon in respiration under aerobic condition than *S. cerevisiae*.

Additionally, under aerobic condition, both yeasts also showed higher transcripts for *PDC1* (Pyruvate decarboxylase, a key enzyme for alcoholic fermentation) in the glycolytic pathway and *L. thermotolerans* also showed higher expression of *ALD4* and *ALD5* (Aldehyde dehydrogenase, responsible for the conversion of acetaldehyde to acetate) (Mukhopadhyay et al., 2013). As reported in the literature and observed in our previous research chapters that both yeasts are Crabtree

positive. Therefore, higher expression of genes encoding enzymes involved in alcoholic fermentation under aerobic condition, indicates that both yeasts possibly switched the metabolism to respiro-fermentation. Higher expression of *PDC1* under aerobic condition has also been reported in *Pichia anamola* (Fredlund et al., 2006).

Under anaerobic condition, both yeasts showed higher expression for genes involved in fermentation metabolism. For instance, *L. thermotolerans* up-regulated *ADH3* and *ADH5* while *S. cerevisiae* showed up-regulation of *ADH4* and *ADH7* (Fig. A 1.3 a).

Studies have shown different oxygen requirement of *L. thermotolerans* and *S. cerevisiae* (Nissen et al., 2004; Hanl et al., 2005). Therefore, this variation in gene expression in response to oxygen could be due to different oxygen requirement of both yeasts.

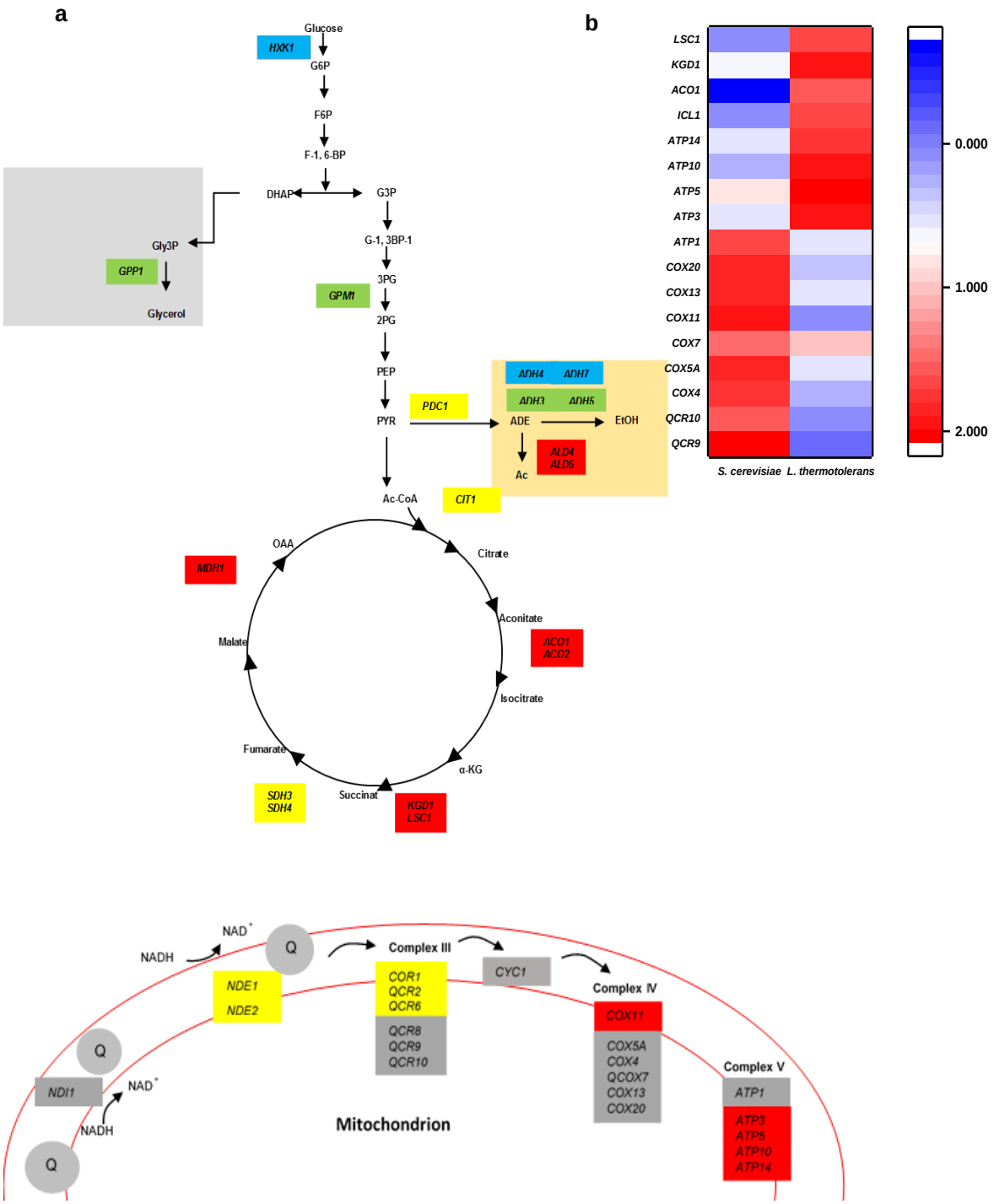


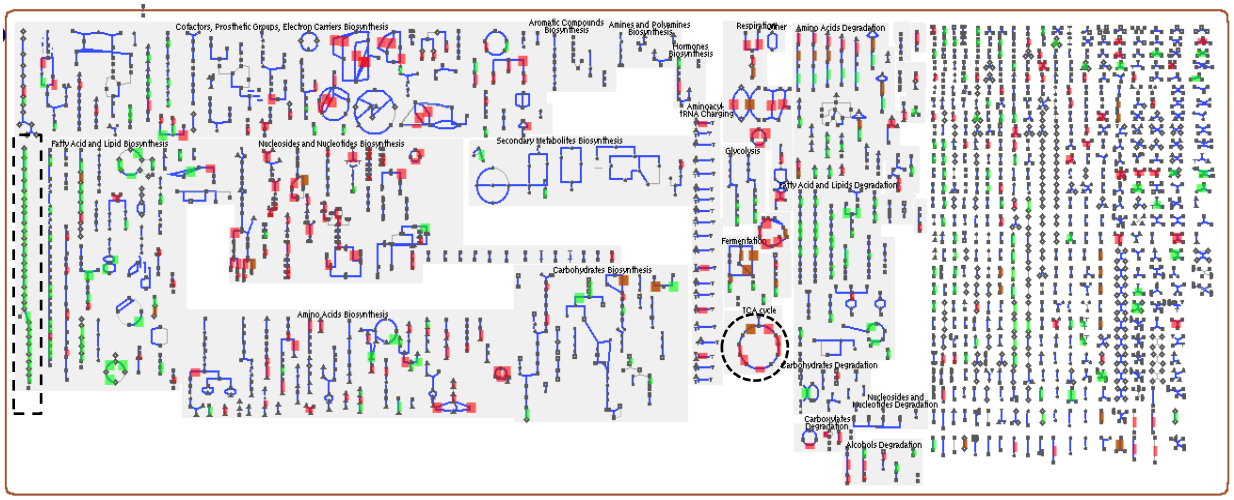
Figure A 1.3 Schematic presentation of the effect of oxygen provision on central carbon metabolism of *L. thermotolerans* and *S. cerevisiae*. (a) The red color shows, higher gene expression of *L. thermotolerans* under aerobic condition, while green shows under anaerobic conditions. The yellow color shows genes those were up-regulated in *L. thermotolerans* as well as *S. cerevisiae* under aerobic condition, grey color indicates genes those were up-regulated in *S. cerevisiae* under aerobic condition, while blue color showed genes up-regulated in *S. cerevisiae* under anaerobic conditions. (b) Differential expression in log fold change for genes involved in TCA and mitochondrial phosphorylation in *L thermotolerans* and *S. cerevisiae*.

A 1.4.5 Effect of oxygen on Ergosterol biosynthesis pathway

The transcriptional analysis showed a different and opposite response for genes encoding enzymes involved in ergosterol biosynthesis. Ergosterol is an important nutrient to maintain the yeast viability in fermentation, and is a critical component of the plasma membrane, regulating the fluidity and permeability. It impacts on many functions in cellular processes such as plasma membrane fusion, pheromone signaling or protein sorting (Bagnat et al., 2000; Jin et al., 2008; Proszynski et al., 2005). Furthermore, ergosterol biosynthesis is an oxygen-dependent process. The transcriptomic comparison of *S. cerevisiae* and *L. thermotolerans* under aerobic conditions revealed opposite behaviour of both yeasts in response to oxygen. Under aerobic conditions and as expected, *S. cerevisiae* showed higher expression of genes that encode for enzymes involved in the ergosterol biosynthesis pathway such as *ERG2, ERG3, ERG5, ERG6, ERG9, ERG10, ERG11, ERG13, ERG20*. In contrast, under same conditions *L. thermotolerans* exhibited down-regulation of these genes (*ERG2, ERG3, ERG13, ERG26, ERG27* and *ERG28*) and were strongly up--regulated in anaerobic condition, only *ERG6* showed higher transcript numbers in aerobic conditions (Fig. A 4, a-b, the cellular overview shows differences in both yeasts for ergosterol biosynthesis pathway, highlighted in box). We believe that, in *L. thermotolerans*, the higher expression of genes which are involved in ergosterol synthesis, could be due to the formation of an intermediate or end product which is provoked by the anaerobic condition. Similar results were also obtained by Baumann et al. (2011) for *P. pastoris*, which showed higher expression of *ERG1, ERG3, ERG11* and *ERG25* genes in oxygen limited conditions. Moreover, the ergosterol content was shown to be reduced under hypoxic conditions in a parallel study (Carnicer et al., 2009). Therefore, our data suggest that oxygen concentration could directly influence the transcriptional regulation of these genes. Bunn and Poyton (1996) demonstrated that different genes responding to the presence of oxygen have different thresholds for activation/deactivation of their transcription.

In this view, it could be possible that both yeasts have a different mechanism to sense the oxygen availability, and the *ERG* genes could be involved in the formation intermediates which are necessary for anaerobic growth.

a



b

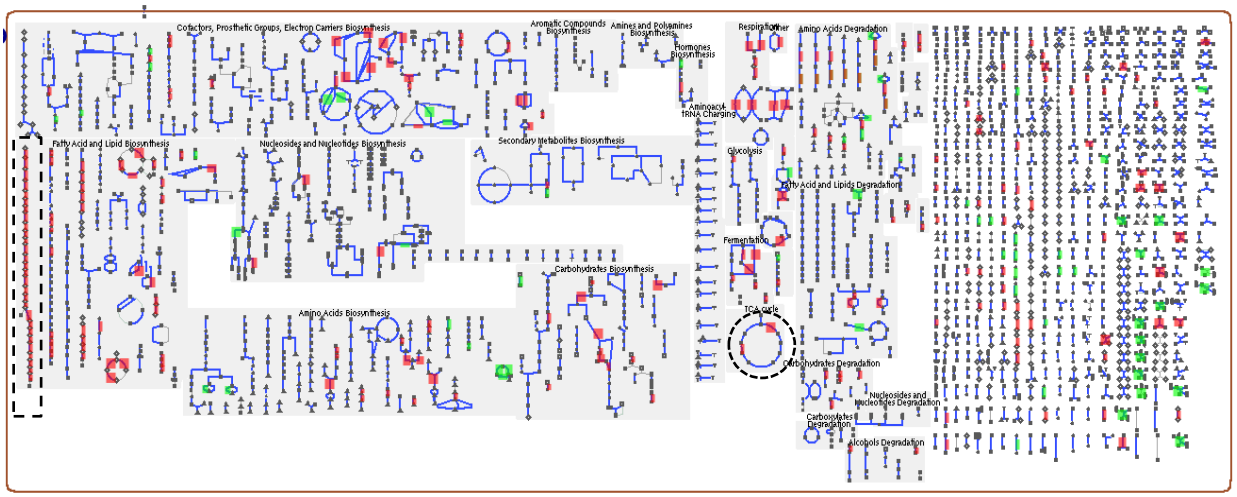


Figure A 1.4 Overlay of transcriptome data of *S. cerevisiae* and *L. thermotolerans* on the *S. cerevisiae* S288c metabolic map. Transcriptome data of the pairwise comparison aerobic vs. anaerobic of *L. thermotolerans* (a) and *S. cerevisiae* (b) strain are overlapped with the metabolic map of *S. cerevisiae* (MetaCyc, BioCyc collection of pathways and genome database; Caspi et al., 2014). Each node in the diagram represents a single metabolite, and each line represents a single bioreaction. In the right part of the diagram the small molecule metabolism is represented (for a complete description of the map see https://biocyc.org). Reaction lines are colour-coded (three colour bins) according to the up and down-regulation: red for up-regulated genes/pathways in aerobic condition, green color for genes/Pathways up-regulated in anaerobic conditions, and brown for genes/pathways that were partially up-regulated in both conditions.

A 1.4.6 Effect of oxygen on Autophagy pathway of *S. cerevisiae* and *L. thermotolerans*

The transcriptome comparison of both yeasts under two different physiological conditions revealed differences in the process of autophagy in both yeasts. In *L. theromotolerans*, the comparison showed up-regulation of genes involved in autophagy under anaerobic conditions, while in the case of *S. cerevisiae* this was observed under aerobic conditions. The up-regulated genes in *L. thermotolerans* under anaerobic conditions were, *ATG8, ATG12, ATG31, ATG32, ATG17, ATG29*, while in *S. cerevisiae* under aerobic conditions were, *ATG3 ATG5 ATG8, ATG9 ATG29*. Autophagy

is a process by which cells recycle the cytoplasm and defective organelles and plays a vital role in maintaining cell survival. Autophagy is known to be induced under stress conditions such as oxidative stress, starvation and toxic compounds. Recently the role of autophagy in is also discovered for keeping the cell viability of *Kluyveromyces marxianus* in ethanol concentration of 98 g L^{-1}. Authors showed a co-relation between the higher expression of *ATG* genes with the higher viability of *K. marxianus* in high ethanol concentration. Hence, the expression of *ATG* genes might be involved in regulating and maintaining viability in *L. thermotolerans* at higher ethanol concentrations. Also, autophagy is well known to overcome the oxidative stress in yeast, the accumulation of reactive oxygen species (ROS), in yeast, generally comes from internal metabolic processes associated with respiration, but can also be triggered by environmental stress stimuli (Jamieson,1998; Perrone et al.,2008; Speldewinde et al., 2015). Therefore, the high expression of the autophagy genes in case of *S. cerevisiae* under aerobic conditions could be due to coping with oxidative stress.

A 1.4.7 Response linked to stress related stimulus

Under anaerobic condition, both yeasts showed up-regulation of genes linked to stress stimulus. *L. thermotolerans* showed abundant transcripts for DNA repair or DNA damage stimulus, such as *SAW1, HTA2, NHP6b, MHF2, HTA1, NSE3, MAG1, MMS2, SHU1, RRM3, REV7, EAF6, UBI4, NEJ1, RAD33, YIM1, HHT2, MHF1, HNT3*. It is well known that the integrity of genomic information plays a crucial role in the survival and propagation of any cellular organisms (Elledge, 1996; Gasch et al., 2001; Hartwell and Weinert, 1989; Hartwell et al., 1994;). The up-regulation of genes involved in DNA damage stimulus or repair could be a result of environmental stresses that occur during the growth under anaerobic conditions. Thus, to cope with such conditions *L. thermotolerans* could have evolved surveillance mechanisms to repair the DNA damage and monitor genomic integrity in response to DNA damage. Under the same conditions *S. cerevisiae* also showed high expression of genes which are involved in managing stress conditions such as *TIP1, PAU3, TIR1, ASG1, DAN1, TIR2, TIR4*. All these genes have a role to play in the metabolism of long chain fatty acids and are essential for anaerobic growth and cell membrane integrity. The role of *TIR1,2* and *TIR 4* has been reported as a signature response by *S. cerevisiae* to anaerobic conditions (Brink et al., 2008). Therefore, this comparative transcriptome data analysis indicates that both yeasts adapt to the fermentation environment at the molecular level by evolving different ways to manage stress (Abramova et al., 2001; Richidi et al., 2000).

A 1.4.8 Response linked to amino acid metabolism

The transcriptomic data revealed that both yeasts showed the difference in gene expression that encodes for enzymes involved in amino acid metabolism. In response to oxygen incorporation, *L. thermotolerans* up-regulated genes whose enzymes are involved in aromatic amino acid metabolism, such as: tryptophan, isoleucine and leucine *TRP3, BAT1, ILV3*; aspartate *HOM2*; serine *SER1, SHM2*; threonine *THR1*; while *S. cerevisiae* showed down-regulation of these genes (Fig. A

1.5). The exo-metabolic data has shown increased concentration of higher alcohols, especially in *L. thermotolerans* (data showed in Chapter-3, Table-5.3). Therefore, we believe that the oxygen addition enhances the aromatic amino acid metabolism which could be responsible for the higher concentration of higher alcohols under aerobic condition in comparison to anaerobic conditions.

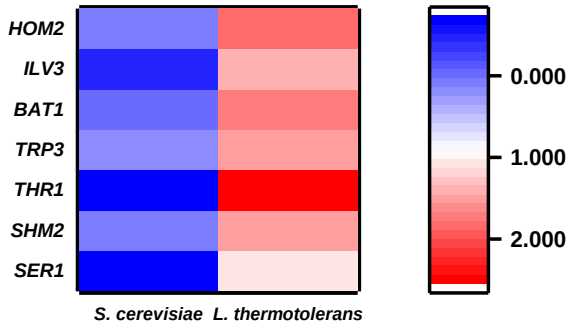


Figure A 1.5 Differentially expressed genes involved in aromatic amino acid metabolism in S. *cerevisiae* and *L. thermotolerans* single fermentation in anaerobic and aerobic conditions (log fold change). Red bars denote an increase in expression while blue bars indicate a decrease in expression for a given gene

A 1.5 Conclusion

The effect of oxygen provision on the transcriptome of *L. thermotolerans* and *S. cerevisiae* revealed a very distinct transcriptome profile, indicating different adaptation mechanism to oxygen availability, probably due to the evolution of the two yeasts. The most significantly affected processes included ergosterol biosynthesis, central carbon metabolism, autophagy and several other stress related responses. The data suggest that the two yeast employ different strategies to adjust to changes in oxygen concentration and provide novel insights into the physiology of *L. thermotolerans*. Such data are essential for our understanding of the contribution of non-*Saccharomyces* yeasts to fermentation-related processes such as wine making.

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Appendix II

Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*

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Author's contribution

Ms. Kirti Shekhawat, Dr. Evodia Setati and Prof. Florian Bauer conceived the project. Ms. Kirti Shekhawat conducted the experiments and analyzed the data. Dr. Evodia Setati, Prof. Florian Bauer supervised the experimental design, execution of experiments and analysis of the data. Ms. Kirti Shekhawat wrote the manuscript, Dr. Evodia Setati and Prof. Florian Bauer provided their inputs and edited the manuscript. All authors approved the final version of the manuscript.

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Impact of oxygenation on the performance of three non-*Saccharomyces* yeasts in co-fermentation with *Saccharomyces cerevisiae*

Kirti Shekhawat[1] · **Florian F. Bauer**[1] · **Mathabatha E. Setati**[1]

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Abstract The sequential or co-inoculation of grape must with non-*Saccharomyces* yeast species and *Saccharomyces cerevisiae* wine yeast strains has recently become a common practice in winemaking. The procedure intends to enhance unique aroma and flavor profiles of wine. The extent of the impact of non-*Saccharomyces* strains depends on their ability to produce biomass and to remain metabolically active for a sufficiently long period. However, mixed-culture wine fermentations tend to become rapidly dominated by *S. cerevisiae*, reducing or eliminating the non-*Saccharomyces* yeast contribution. For an efficient application of these yeasts, it is therefore essential to understand the environmental factors that modulate the population dynamics of such ecosystems. Several environmental parameters have been shown to influence population dynamics, but their specific effect remains largely uncharacterized. In this study, the population dynamics in co-fermentations of *S. cerevisiae* and three non-*Saccharomyces* yeast species: *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima*, was investigated as a function of oxygen availability. In all cases, oxygen availability strongly influenced population dynamics, but clear species-dependent differences were observed. Our data show that *L. thermotolerans* required the least oxygen, followed by *T. delbrueckii* and *M. pulcherrima*. Distinct species-specific chemical volatile profiles correlated in all cases with increased persistence of non-*Saccharomyces* yeasts, in particular increases in some higher alcohols and medium chain fatty acids. The results highlight the role of oxygen in regulating the succession of yeasts during wine fermentations and suggests that more stringent aeration strategies would be necessary to support the persistence of non-*Saccharomyces* yeasts in real must fermentations.

Keywords Non-*Saccharomyces* yeast · Dissolved oxygen · Yeast dynamics · Mixed-culture fermentation · Wine fermentation

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✉ Mathabatha E. Setati
setati@sun.ac.za

[1] Institute for Wine Biotechnology, Department of Viticulture and Oenology, Stellenbosch, Stellenbosch, Western Cape, South Africa

Introduction

The majority of commercial wine fermentations are performed by inoculating *Saccharomyces cerevisiae* active dry yeast starter cultures. The advantages of inoculation include more predictable fermentation properties and aromatic profiles (Ciani et al. 2006, 2010; Comitini et al. 2011; Gobbi et al. 2013; Sadoudi et al. 2012; Soden et al. 2000). However, anecdotal evidence suggests that the extensive use of single strains, inoculated at high cell density and therefore dominating the natural microbiota from the start, may reduce the sensorial complexity of the finished wine in comparison with spontaneously fermented wines where multiple yeast species may contribute significantly to the final aromatic features. Consequently, the last decade has seen a re-evaluation of the role of non-*Saccharomyces* yeast species in wine fermentation with the aim of identifying alternative starter cultures to be used in mixed fermentation regimes (Ciani et al. 2010). The desirable attributes of such yeast species may include increasing the fruitiness and complexity of wine, reducing ethanol and acetic acid content, or alleviating sluggish/stuck fermentation of high sugar musts (Ciani et al. 2006; Comitini et al.

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2011; Gobbi et al. 2013; Sadoudi et al. 2012). The contribution of these yeasts to the final organoleptic characteristics of wine will primarily depend on their ability to be metabolically active and to maintain a high cellular concentration during a significant part of the fermentation process (Ciani et al. 2006; Zuzuarregui et al. 2006). However, data regarding the impact of fermentation conditions on the relative performance of these species when competing with *S. cerevisiae* are limited. It is well established that in wine fermentation, whether spontaneous or inoculated, strains of *S. cerevisiae* tend to dominate the later stages of fermentation. This pattern also persists in multi-starter fermentations, even when non-*Saccharomyces* yeast species are inoculated at higher concentrations prior to *S. cerevisiae* to ensure a significant contribution (Andorrà et al., 2010).

The relative decline of non-*Saccharomyces* yeast species during wine fermentation has been attributed to various factors including low ethanol tolerance, absence or low levels of oxygen, cell-to-cell contact inhibition, and presence of proteinaceous antifungal compounds and killer toxins (Hansen et al. 2001; Hanl et al. 2005; Nissen et al. 2003; Panon 1997; Pérez-Nevado et al. 2006; Visser et al. 1990). Recently, studies demonstrated that oxygen limitation in particular exerts a strong selective pressure during wine fermentation, and that the growth and persistence of non-*Saccharomyces* yeast species such as *Lachancea thermotolerans*, *Torulaspora delbrueckii*, and *Metschnikowia pulcherrima* are strongly dependent on oxygen availability (Hansen et al. 2001; Hanl et al. 2005; Morales et al. 2015; Pérez-Nevado et al. 2006). Some studies have evaluated the impact of dissolved oxygen and have demonstrated the positive influence of oxygen addition on the cell physiology of *S. cerevisiae* and *T. delbrueckii* during fermentation (Aceituno et al. 2012; Brandam et al. 2013; Rintala et al. 2009; Varela et al. 2012). In addition to affecting population dynamics, oxygen also affects the production of major wine volatile compounds especially the ratio of esters to higher alcohols (Valero et al. 2002), and oxygenation of mixed starter fermentations employing *M. pulcherrima* and *S. cerevisiae* reduced the final ethanol levels in wine (Morales et al. 2015). However, the research regarding the impact of oxygen on population dynamics is still in its infancy and our understanding of the influence of oxygen on the overall yeast dynamics and contribution of non-*Saccharomyces* yeast species to the organoleptic properties of wine remains limited. In particular, data regarding the response of mixed fermentation to different levels of oxygenation is limited. Therefore, the present study aimed to evaluate the effect of three different levels of dissolved oxygen on the growth and fermentation dynamics of *T. delbrueckii*, *L. thermotolerans*, and *M. pulcherrima* during co-fermentation with *S. cerevisiae*. We also investigated the influence of these conditions on the volatile chemical profiles derived from these fermentations. Our study clearly suggests the potential of oxygen manipulation strategies to steer yeast population dynamics and ensure a desirable contribution to wine sensorial signatures by different non-*Saccharomyces* yeast species.

Materials and methods

Yeast strains and media

S. cerevisiae (Cross evolution-285) and *T. delbrueckii* (Biodiva) are commercial strains from Lallemand SAS (Blagnac, France), while *M. pulcherrima* (IWBT-Y1337) and *L. thermotolerans* (IWBT-Y1240) were obtained from the culture collection of the Institute for Wine Biotechnology (Stellenbosch University). The selection criteria for these particular non-*Saccharomyces* yeasts were on the basis of their positive contribution reported in pervious literature and their commercialization (Ciani et al. 2006, 2010; Comitini et al. 2011; Gobbi et al. 2013; Sadoudi et al. 2012; Soden et al. 2000). Cryogenically maintained (−80 °C) strains were reactivated by streaking out on YPD agar plates containing 10 g yeast extract, 20 g peptone, and 20 g glucose per liter. Cultures were stored at 4 °C for short-term use.

Fermentations and sampling

Fermentations were performed in synthetic grape juice (pH 3.5) containing (per liter) 100 g glucose, 100 g fructose, 1 g yeast extract (Oxoid; Thermo Fisher Scientific, Hampshire, United Kingdom), 2 g $(NH_4)_2SO_4$, 0.3 g citric acid, 5 g L-malic acid, 5 g L-tartaric acid, 0.4 g $MgSO_4$, 5 g KH_2PO_4, 0.2 g NaCl, 0.05 g $MnSO_4$, and anaerobic factors (ergosterol 10 mg L^{-1}, Tween 80 0.5 mL L^{-1}) (Henschke and Jiranek 1993; Ough et al. 1989). Fermentations were conducted in 1.3 L BioFlo 110 bench top bioreactors (New Brunswick, NJ, USA) using 900 mL of final working volume, a temperature of 25 °C, and an agitation speed of 200 rpm. Fermentations were carried out anaerobically and with three different levels of oxygenation corresponding to 1% (0.08 mg L^{-1}), 5% (0.41 mg L^{-1}), and 21% (1.71 mg L^{-1}) of dissolved oxygen (DO). The anaerobic conditions were created by initially sparging N_2 to bring down the DO level to 0%, and then to minimize diffusion of atmospheric oxygen into the cultures, the entire fermentation setup was equipped with Norprene tubing. For aerobic fermentation, the DO probe was calibrated by adding oxygen to the medium as compressed air, using a peristaltic pump and with air flow rate of 1 vvm (volume per volume per minute). The three different DO levels were maintained through supplementary addition of gas mixture (CO_2, N_2, O_2, and compressed air at 1 vvm) from which O_2 was introduced into the fermentation whenever required, using an automated gas flow controller. To

minimize the gas variability in each vessel, the same gas mixture module was used for all the vessels and experiments were performed in duplicate at the same time. The dissolved-oxygen concentration in the cultures was monitored with an oxygen electrode. Samples were collected at 24-h intervals to monitor growth and fermentation progress. In all experimental conditions, both non-*Saccharomyces* and *S. cerevisiae* were inoculated simultaneously with cell number 10^7:10^6 (non-*Saccharomyces*/*S. cerevisiae*). All fermentations were conducted in duplicate.

Analysis of population dynamics and dry biomass

Serial dilutions of the cell suspensions were performed with 0.9% (*w*/*v*) NaCl. One-hundred-microliter samples were spread on YPD agar and incubated at 30 °C for 2–3 days. For yeast enumeration in mixed-culture fermentations, the individual species were distinguished based on colony morphology (the pictures illustrating colony morphologies are provided in Supplementary Fig. S1). Colony counts were performed on plates with 30–300 colonies.

The dry-weight biomass was determined by separating the cells from the liquid by centrifugation at 5000×*g* (4 mL of volume in triplicate) in tubes. The empty tubes were preweighed and then kept at 90 °C. After reaching a constant weight, the dry biomass was obtained by subtracting the weight of empty tubes.

Analytical methods

Cell-free supernatants were obtained by centrifuging cell suspensions at 5000×*g* for 5 min. Glucose, fructose, glycerol, acetic acid, and acetaldehyde were measured using specific enzymatic kits, Enytec™ *Fluid* D-glucose, fructose, acetic acid (Thermo Fisher Scientific Oy, Helsinki, Finland), Boehringer Mannheim/R-Biopharm-acetaldehyde (R-Biopharm AG, Darmstadt, Germany), and analyzed using an Arena 20XT photometric analyzer (Thermo Electron Oy, Helsinki, Finland) (Schnierda et al. 2014). Ethanol was analyzed by high-performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Agilent RID and UV detectors were used in tandem for peak detection and quantification. Final analysis was done using the HPChemstation software (Rossouw et al. 2012). Liquid-liquid extraction method was used for volatile compound analysis using GC-FID, where a 5-mL sample of synthetic must was added with internal standard 4-methyl-2-pentanol (final concentration 5 mg L^{-1}). To perform liquid-liquid extraction, 1 mL diethyl ether was added to each sample and sonicated for 5 min. The wine/ether mixture was then centrifuged at 4000×*g* for 5 min and the ether layer (supernatant) removed and dried on Na_2SO_4 to remove excess water. For gas chromatography (GC), a DB-FFAP capillary column (Agilent, Little Falls, Wilmington, USA) with dimensions 60 m length × 0.32 mm i.d. × 0.5 μm film thickness and a Hewlett Packard 6890 Plus GC instrument (Little Falls, USA) equipped with a split/splitless injector and a flame ionization detector (FID) was used. The initial oven temperature was 33 °C, held for 17 min, after which the temperature was increased by 12 °C min^{-1} to 240 °C, and held for 5 min. Three microliters of the diethyl-ether extract was injected at 200 °C in split mode. The split ratio was 15:1 and the split flow rate 49.5 mL min^{-1}. The column flow rate was 3.3 mL min^{-1} using hydrogen as carrier gas. The detector temperature was 250 °C (Louw et al. 2010).

Statistical analysis

All chemical analyses were performed in duplicate technical repeats on two independent fermentations, and all the values were expressed as means ± SD. Differences between measurements within different treatments were determined using analysis of variance (a least-significant-difference [LSD] test) with the statistical software Statistica version 13.0 (StatSoft Inc., Tulsa, Oklahoma, USA), and differences were considered significant when p values were less than 0.05. For multivariate data analysis, principal component analysis (PCA) was constructed using SIMCA-P software version 14.0 (Umetrics, Umea, Sweden).

Results

Impact of aeration on yeast population dynamics

The fermentation kinetics and growth dynamics of *S. cerevisiae*, *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima* in single (at anaerobic and 21% DO) or in mixed cultures (at anaerobic, 1%, 5%, and 21% DO) were evaluated. In single species anaerobic fermentations, *S. cerevisiae* completed the fermentation (sugar levels <2 g L^{-1}) in 96 h reaching 7.6 × 10^9 CFU mL^{-1}. The non-*Saccharomyces* yeast *L. thermotolerans* and *T. delbrueckii* completed fermentation in 120 h, reaching cell counts of 1.5 × 10^6 and 7.0 × 10^7 CFU mL^{-1}, respectively, while the fermentations with *M. pulcherrima* became stuck with 55 g L^{-1} residual sugar and reached 2.19 × 10^5 CFU mL^{-1} (Fig. 1). Under aerobic condition at 21% DO level, all single species culture completed the fermentation faster than in anaerobic conditions and reached higher cell counts. *S. cerevisiae* completed fermentation within 48 h and a viable cell count of 7.5 × 10^{11} CFU mL^{-1}, followed by *L. thermotolerans* and *T. delbrueckii* within 72 h, reaching CFUs of 3.8 × 10^{10} and 6.7 × 10^{10} mL^{-1}, while

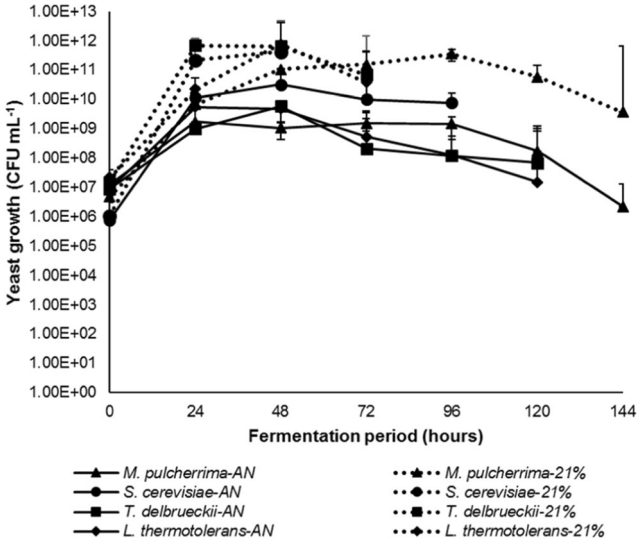

Fig. 1 Population kinetics of three non-*Saccharomyces* and *S. cerevisiae* single-culture fermentations in anaerobic and 21% DO level

M. pulcherrima achieved dryness after 144 h and reached viable cell count of 3.75×10^9 CFU mL^{-1} (Fig. 1).

The population dynamics of mixed fermentations showed species-specific differences in response to different oxygen conditions. Under anaerobic conditions, all mixed fermentations were completed in 120 h, and throughout the fermentation *S. cerevisiae* established itself rapidly as the dominant yeast, maintaining viable cell counts of 10^9 CFU mL^{-1}. However, significant differences were observed regarding the ability of the non-*Saccharomyces* species to grow and persist in these conditions: *L. thermotolerans* (Fig. 2a) and *T. delbrueckii* (Fig. 3a) persisted until the end of fermentation at maximum cell densities of 2.5×10^9 and 6.5×10^9 CFU mL^{-1}, respectively. In contrast, *M. pulcherrima* grew in the first 24 h reaching 1.25×10^8 CFU mL^{-1} but could no longer be detected after 48 h of fermentation (Fig. 4a).

Increasing levels of DO favored growth and persistence of the non-*Saccharomyces* yeast species to varying degrees. As expected, in comparison to anaerobic mixed fermentations, all aerobic mixed fermentations generated higher total CFU counts, mainly due to increased CFU counts of the non-*Saccharomyces* yeasts. Among the three species assessed here, *L. thermotolerans* achieved the highest CFU counts, exceeding the cellular concentrations of *S. cerevisiae* at all three levels of oxygenation, reaching 5.5×10^9, 9.8×10^9, and 2.8×10^{10} CFU mL^{-1} at 1%, 5%, and 21% DO, respectively (Fig. 2b–d). This numerical dominance of *L. thermotolerans* over *S. cerevisiae* was maintained until the end of fermentation. *T. delbrueckii*, on the other hand, was outcompeted by *S. cerevisiae* at 1% DO (Fig. 3b), but achieved higher cell counts than *S. cerevisiae* at 5% and 21% DO and reached a maximum cell density of 1.06×10^{10} and 1.86×10^{10} CFU mL^{-1}, respectively (Fig. 3c, d). Similarly, *M. pulcherrima* showed rapid growth in the first 24 h at 1%, 5%, and 21% DO levels and generated a viable cell count of 9.8×10^9, 4.73×10^{10}, and 9.1×10^{10}, respectively (Fig. 4b–d). These levels were 10-fold higher than *S. cerevisiae* and were maintained at all the DO levels for 72 h. However, at 1% DO, the population of *M. pulcherrima* declined steadily after 72 h reaching 2×10^5 CFU mL^{-1} at the end of fermentation, while at 5%, a decline was only observed after 96 h (Fig. 4b, c). In contrast, at 21% DO, *M. pulcherrima* displayed higher cell counts than

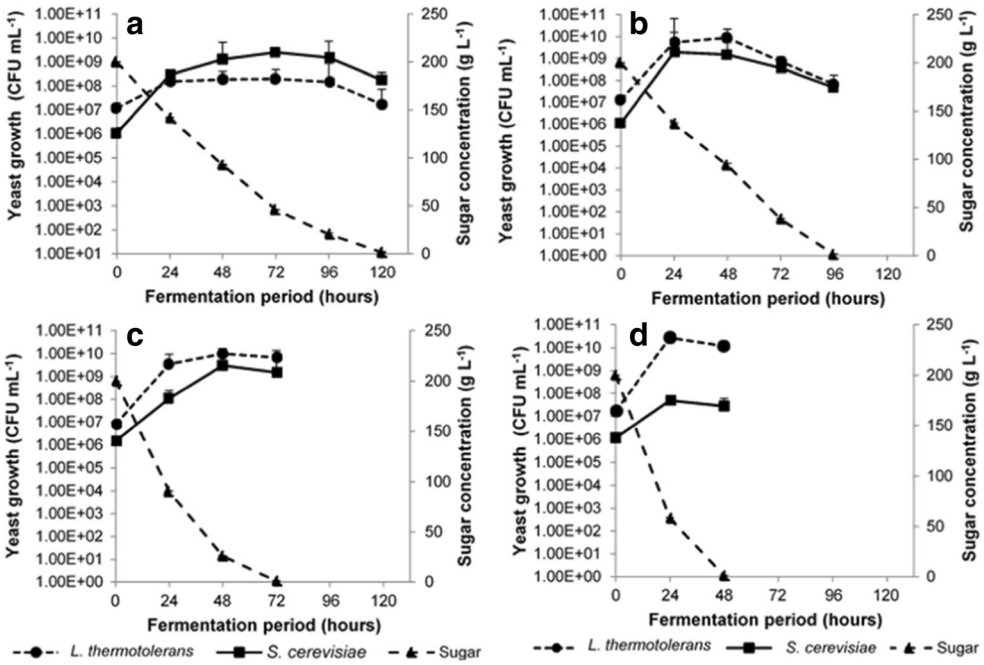

Fig. 2. Population dynamics in mixed cultures of *L. thermotolerans* (*round*) and *S. cerevisiae* (*square*) in anaerobic (**a**), 1% (**b**), 5% (**c**), and 21% (**d**) level of dissolved oxygen conditions. Secondary *y*-axis indicates utilization of sugar (*triangle*) in grams per liter

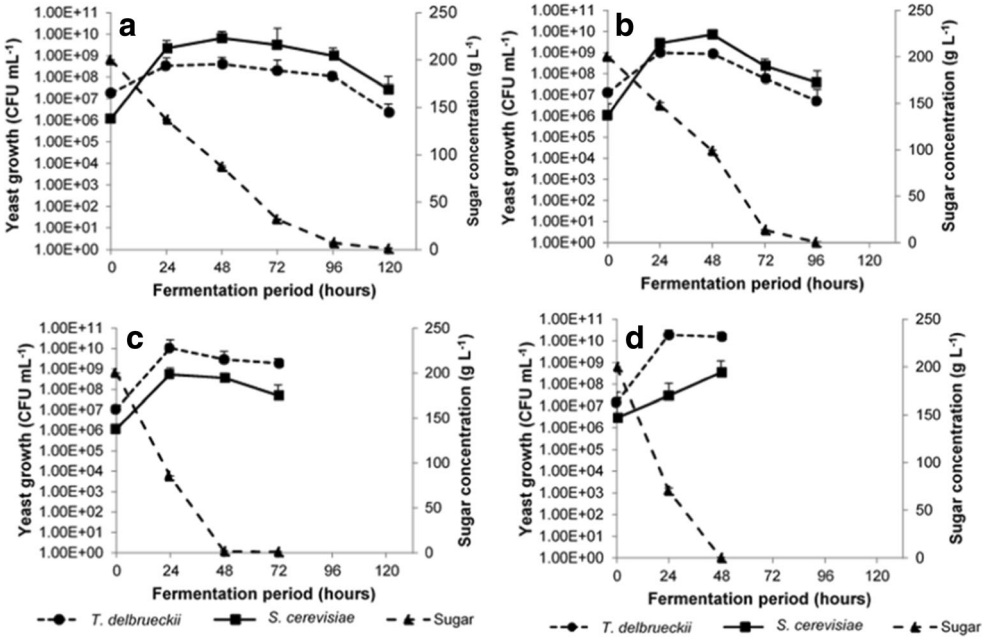

Fig. 3 Population dynamics of *T. delbrueckii* (*round*) and *S. cerevisiae* (*square*) in anaerobic (**a**), 1% (**b**), 5% (**c**), and 21% (**d**) level of dissolved oxygen conditions. Secondary *y*-axis indicates utilization of sugar (*triangle*) in grams per liter

S. cerevisiae, reaching 9.1×10^{10} CFU mL^{-1} in the middle of fermentation and maintaining this numeric dominance until the end of fermentation (Fig. 4d).

The effect of aeration on biomass generation was also evaluated by measuring the dry biomass of samples from fermentations under anaerobic conditions and at 5% DO. Overall, the supply of oxygen at 5% DO resulted in approximately a 2-fold increase in biomass production compared to fermentation under anaerobic conditions (Fig. 5). The anaerobic fermentations with *S. cerevisiae*, *S. cerevisiae*/*L. thermotolerans*, *S. cerevisiae*/*T. delbrueckii*, and *S. cerevisiae*/*M. pulcherrima* generated 6.1, 5.1, 6.1, and 6.0 g L^{-1}, respectively, while at 5% level of DO, the biomass was 11.0, 10.73, and 11.73 g L^{-1}, respectively (Fig. 5).

Production of metabolites under anaerobic and aerobic fermentation conditions

Regarding the primary products of fermentative metabolism, in comparison to anaerobic fermentations, oxygenation at all three DO levels resulted in ethanol and glycerol reduction (Table 1). In the *S. cerevisiae* single culture fermentation, the ethanol yield decreased from 0.50 (under anaerobic conditions) to 0.36 (at 21% DO). Similarly, the

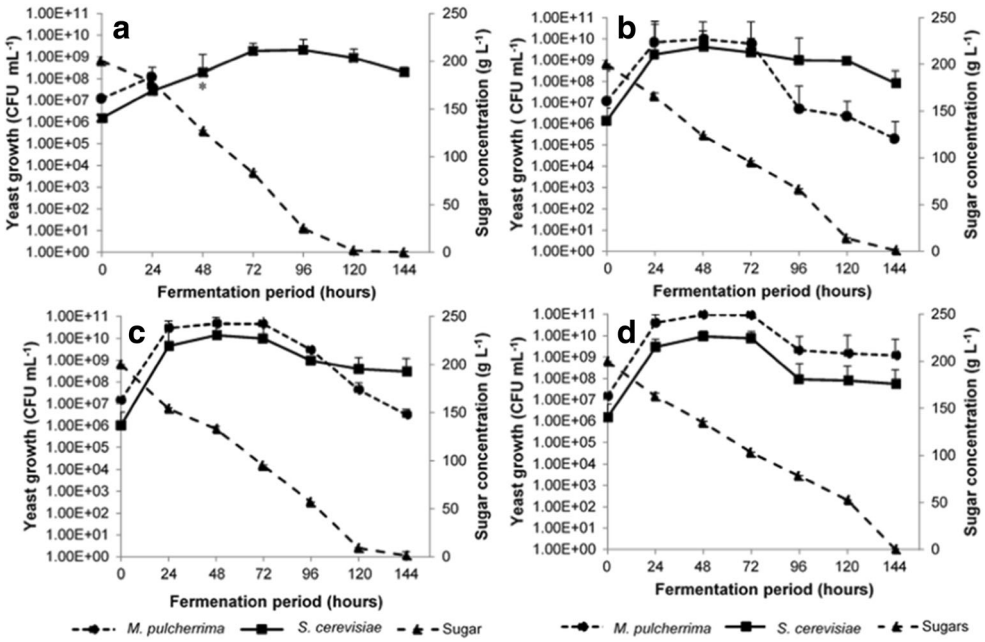

Fig. 4 Population kinetics of *M. pulcherrima* (*round*) and *S. cerevisiae* (*square*) in anaerobic (**a**), 1% (**b**), 5% (**c**), and 21% (**d**) level of dissolved oxygen conditions. Secondary *y*-axis indicates utilization of sugar (*triangle*) in grams per liter. In anaerobic condition, * indicates that *M. pulcherrima* could not be detected

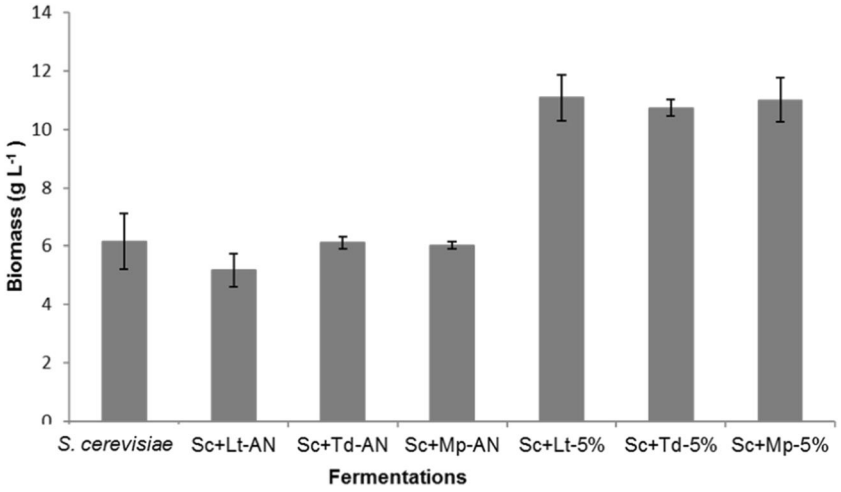

Fig. 5 Dry mass produced by control *S. cerevisiae* and mixed fermentation of three non-*Saccharomyces* and *S. cerevisiae* in anaerobic and 5% OD level aerobic fermentation. Values are in grams per liter

anaerobic mixed-culture fermentation of *S. cerevisiae*/*L. thermotolerans* resulted in an ethanol yield of 0.49, which was reduced to 0.44, 0.40, and 0.29 at 1%, 5%, and 21% DO levels, respectively (Table 1). In the case of *S. cerevisiae*/*T. delbrueckii* co-fermentations, the ethanol yield decreased from 0.49 under anaerobic conditions to 0.46 at 1% and 5% DO, and 0.23 at 21% DO. The *S. cerevisiae*/*M. pulcherrima* mixed fermentations displayed a similar trend, resulting in a reduction in ethanol yield from 0.50 under anaerobic conditions to 0.44, 0.39, and 0.23 at 1%, 5%, and 21% DO, respectively (Table 1). A general decrease in glycerol levels was evident in mixed-culture fermentations with a 6-fold reduction in *S. cerevisiae*/*T. delbrueckii* fermentations under 21% DO compared to anaerobic conditions, while in *S. cerevisiae*/*L. thermotolerans* and *S. cerevisiae*/*M. pulcherrima* fermentations, a 1.6-fold reduction in glycerol concentrations was observed (Table 1). In comparison to the *S. cerevisiae* fermentation, all anaerobic mixed fermentation had lower acetic acid. The mixed fermentation with *M. pulcherrima* produced the lowest acetic acid concentration followed by the *L. thermotolerans* and *T. delbrueckii* mixed fermentation (Table 1). The concentration of acetic acid and acetaldehyde gradually increased from anaerobic to 1%, 5%, and 21% DO level, and a more than 2-fold increase was observed at 21% DO for all fermentations (Table 1).

Table 1 Ethanol, acetic acid, acetaldehyde, and glycerol concentrations in non-*Saccharomyces* anaerobic individual control *S. cerevisiae* and their mixed fermentations in anaerobic and three aerobic fermentations for mixed culture and 21% for control *S. cerevisiae*

Fermentations	Ethanol yield (g ethanol/g sugar)	Ethanol (g L^{-1})	Acetic acid (g L^{-1})	Acetaldehyde (mg L^{-1})	Glycerol (g L^{-1})
S. cerevisiae-AN	0.50	100.23 ± 0.06	1.06 ± 0.041	49 ± 4.42	4.36 ± 1.83
S. cerevisiae-21%	0.36	72.00 ± 0.03	1.70 ± 0.012	85 ± 7.07	4.86 ± 0.92
L. thermotolerans-AN	0.49	98.00 ± 0.08	0.63 ± 0.32	30 ± 2.43	7.3 ± 0.37
Sc+Lt-AN	0.49	98.79 ± 0.10	0.94 ± 0.06	41 ± 7.07	7.05 ± 0.39
Sc+Lt-1%	0.44	89.35 ± 0.04	0.91 ± 0.051	84 ± 8.48	6.80 ± 0.728
Sc+Lt-5%	0.40	81.87 ± 0.06	2.03 ± 0.031	285 ± 7.70	4.12 ± 0.59
Sc+Lt-21%	0.29	59.08 ± 0.04	3.84 ± 0.04	369 ± 5.65	4.57 ± 1.04
T. delbrueckii-AN	0.47	94.16 ± 0.09	0.89 ± 0.11	42 ± 1.42	6.79 ± 0.93
Sc+Td-AN	0.49	99.64 ± 0.08	0.79 ± 0.17	54 ± 2.82	6.84 ± 0.45
Sc+Td-1%	0.46	92.88 ± 0.13	0.71 ± 0.014	70 ± 2.82	6.46 ± 1.41
Sc+Td-5%	0.40	80.12 ± 0.09	1.03 ± 0.05	399 ± 7.10	1.74 ± 1.02
Sc+Td-21%	0.23	46.91 ± 0.13	2.06 ± 0.10	551 ± 8.84	1.09 ± 1.62
M. pulcherrima-AN	0.38	56.19 ± 0.20	0.24 ± 0.61	28 ± 1.09	7.1 ± 1.45
Sc+Mp-AN	0.50	100.22 ± 0.03	0.69 ± 0.19	37 ± 7.07	7.93 ± 2.01
Sc+Mp-1%	0.44	88.04 ± 0.08	1.44 ± 0.072	39 ± 16.90	5.53 ± 1.73
Sc+Mp-5%	0.39	78.75 ± 0.06	2.06 ± 0.02	117 ± 12.70	4.70 ± 1.40
Sc+Mp-21%	0.23	46.96 ± 0.04	2.05 ± 0.78	471 ± 6.91	4.41 ± 0.63

All the compounds are the average of two biological duplicates ± SD

Non-*Saccharomyces* and oxygenation-derived changes in volatile compounds profile

Volatile compounds produced during the fermentations were measured at the end of the process. Significant differences were observed for the different yeast combinations and for different oxygen levels. The non-*Saccharomyces* single species fermentations generally exhibited a high production of higher alcohols (mainly 2-phenylethanol, isoamyl alcohol, and isobutanol) in anaerobic fermentations (Tables 2, 3, and 4). In addition, *L. thermotolerans* produced significantly high levels of 3-ethoxy-1-propanol and isobutyric acid (Table 2), and *T. delbrueckii* contributed higher levels of propionic acid (Table 3), while *M. pulcherrima* contributed high levels of ethyl acetate, diethyl succinate, and ethyl lactate in both mono- and mixed-culture fermentations (Table 4). The *S. cerevisiae* single-culture fermentation generally showed higher levels of MCFAs (medium-chain fatty acids) than the non-*Saccharomyces* species single fermentations.

The metabolic profile of the *S. cerevisiae* single-species anaerobic fermentations differed significantly from its mixed anaerobic fermentations. Anaerobic mixed fermentations with *S. cerevisiae*/*L. thermotolerans* and *S. cerevisiae*/*T. delbrueckii* showed higher concentration of the higher alcohols, MCFAs, and esters (2-phenylethyl acetate, diethyl succinate, 2-isoamyl-acetate, ethyl-hexanoate, ethyl-caprylate, and ethyl-phenylacetate). For the *S. cerevisiae*/*M. pulcherrima* fermentation, the concentration of MCFAs reduced while that of isoamyl alcohol, 2-phenylethanol, isobutanol, and esters (2-phenylethyl acetate, diethyl

Table 2 Major volatile compounds detected at end of the fermentation in *S. cerevisiae*/*L. thermotolerans* single culture and their mixed cultures

Major volatiles	*S. cerevisiae* (AN)	*L. thermotolerans* (AN)	Sc+Lt (AN)	Sc+Lt-1%	Sc+Lt-5%	Sc+Lt-21%
2-Phenylethanol	6.30 ± 1.24^{d}	39.84 ± 9.31^{c}	33 ± 3.02^{c}	92 ± 3.48^{b}	104 ± 4.14^{b}	105 ± 4.65^{a}
Isoamyl alcohol	50.8 ± 8.75^{d}	86.49 ± 0.10^{d}	120.5 ± 2.81^{c}	203 ± 4.42^{b}	215 ± 1.96^{b}	322 ± 1.12^{a}
Isobutanol	12.5 ± 2.85^{e}	20.79 ± 2.80^{d}	33.57 ± 0.24^{c}	125 ± 2.82^{bc}	139 ± 3.02^{b}	197 ± 2.21^{a}
Propanol	31.18 ± 5.48^{a}	17.59 ± 1.41^{b}	0.0 ± 0.00^{a}	0.0 ± 0.01^{a}	25.53 ± 4.37^{ab}	0.0 ± 0.00^{a}
Butanol	0.0 ± 0.00^{c}	3.34 ± 0.25^{a}	0.98 ± 0.03^{bc}	0.65 ± 0.08^{bc}	1.05 ± 0.07^{bc}	1.40 ± 0.69^{b}
Pentanol	1.56 ± 0.00^{a}	0.96 ± 0.01^{b}	0.0 ± 0.00^{d}	0.90 ± 0.01^{c}	0.96 ± 0.02^{b}	0.0 ± 0.00^{d}
Hexanol	0.89 ± 0.03^{b}	0.59 ± 0.02^{b}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	10.55 ± 0.090^{a}	0.0 ± 0.00^{c}
3-Ethoxy-1-propanol	1.15 ± 0.40^{d}	28.51 ± 1.91^{a}	4.28 ± 0.23^{bc}	6.56 ± 0.11^{cd}	8.27 ± 0.45^{c}	9.87 ± 0.12^{c}
3-Methyl-1-pentanol	Nd	Nd	Nd	Nd	Nd	Nd
Propionic acid	1.69 ± 0.12^{c}	4.55 ± 0.20^{b}	2.03 ± 0.09^{c}	7.28 ± 0.67^{a}	9.46 ± 1.27^{a}	1.84 ± 0.16^{c}
Isobutyric acid	1.57 ± 0.55^{b}	3.19 ± 0.30^{b}	1.44 ± 0.05^{b}	8.84 ± 0.61^{a}	9.70 ± 0.27^{a}	10.87 ± 1.06^{a}
Butyric acid	1.30 ± 0.04^{b}	0.98 ± 0.11^{c}	0.98 ± 0.03^{c}	1.80 ± 0.04^{a}	1.77 ± 0.10^{a}	0.92 ± 0.01^{c}
Isovaleric acid	1.07 ± 0.00^{a}	0.79 ± 0.27^{b}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	0.67 ± 0.05^{b}
Valeric acid	0.66 ± 0.06	1.42 ± 0.08^{a}	0.70 ± 0.04^{b}	0.80 ± 0.00^{b}	0.66 ± 0.07^{b}	0.85 ± 0.01^{b}
Hexanoic acid	1.96 ± 0.20^{b}	0.83 ± 0.02^{c}	2.16 ± 0.01^{a}	1.03 ± 0.01^{d}	0.71 ± 0.02^{d}	0.28 ± 0.09^{e}
Octanoic acid	2.64 ± 0.03^{b}	1.49 ± 0.12^{c}	3.29 ± 0.74^{a}	$1.02 \pm .020^{ab}$	1.06 ± 0.05^{ab}	0.93 ± 0.63^{d}
Decanoic acid	3.76 ± 0.35^{b}	2.19 ± 0.05^{c}	8.34 ± 0.34^{a}	1.27 ± 0.04^{d}	1.24 ± 0.06^{d}	0.90 ± 0.96^{d}
2-Phenylethyl acetate	1.07 ± 0.03^{bc}	1.4 ± 0.81^{b}	2.25 ± 0.07^{a}	0.96 ± 0.11^{c}	0.0 ± 0.00^{c}	0.79 ± 0.80^{c}
2-Isoamyl acetate	0.80 ± 0.01^{a}	0.49 ± 0.03^{abc}	0.78 ± 0.00^{a}	0.15 ± 0.22^{c}	0.30 ± 0.00^{bc}	0.55 ± 0.06^{ab}
Hexyl acetate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl-hexanoate	1.05 ± 0.23^{a}	0.15 ± 0.01^{abc}	0.69 ± 0.02^{bc}	0.25 ± 0.36^{ab}	0.0 ± 0.00^{c}	0.54 ± 0.01^{abc}
Ethyl-caprylate	0.34 ± 0.03^{a}	0.51 ± 0.07^{a}	0.26 ± 0.01^{a}	0.29 ± 0.28^{a}	0.0 ± 0.00^{a}	0.14 ± 0.03^{a}
Ethyl acetate	24.15 ± 2.04^{b}	23.61 ± 1.35^{b}	39.85 ± 1.44^{a}	36.64 ± 1.82^{a}	34.12 ± 3.14^{ab}	30.94 ± 4.89^{ab}
Ethyl butyrate	0.75 ± 0.27^{a}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}
Ethyl lactate	0.0 ± 0.00^{b}	0.51 ± 0.07^{a}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}
Ethyl-3-hydroxybutanoate	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	1.80 ± 0.05^{a}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}
Ethyl caprate	0.51 ± 0.04^{a}	0.15 ± 0.05^{b}	1.14 ± 0.03^{a}	0.80 ± 0.02^{ab}	0.0 ± 0.00^{c}	0.19 ± 0.10^{c}
Ethyl-phenylacetate	0.0 ± 0.00^{d}	1.55 ± 0.03^{b}	1.15 ± 0.00^{c}	1.22 ± 0.00^{c}	1.75 ± 0.02^{a}	1.21 ± 0.00^{c}
Diethyl succinate	0.0 ± 0.00^{d}	1.05 ± 0.08^{a}	1.06 ± 0.02^{a}	3.02 ± 0.61^{a}	1.87 ± 0.12^{b}	1.05 ± 0.03^{c}

Mean values bearing differing superscript letters showed significant differences and mean values bearing the same letter were statistically similar. All the compounds are presented in milligrams per liter and are the average of two biological duplicates ± SD

AN anaerobic conditions, *Nd* not detected

Table 3 Major volatile compounds detected at the end of the fermentation in *S. cerevisiae*/*T. delbrueckii* single culture and their mixed cultures

Major volatiles	*S. cerevisiae* (AN)	*T. delbrueckii* (AN)	Sc+Td (AN)	Sc+Td-1%	Sc+Td-5%	Sc+Td-21%
2-Phenylethanol	6.30 ± 1.24^{c}	18.29 ± 7.21^{c}	36 ± 0.40^{cb}	54 ± 0.02^{b}	213 ± 0.54^{a}	222 ± 18.00^{a}
Isoamyl alcohol	50.8 ± 8.75^{c}	87.82 ± 0.58^{c}	119 ± 0.85^{b}	132 ± 2.50^{a}	198 ± 2.26^{a}	208 ± 16.40^{a}
Isobutanol	12.5 ± 2.85^{d}	16.17 ± 2.09^{d}	26.2 ± 2.75^{d}	87 ± 0.26^{c}	128 ± 2.70^{b}	169 ± 1.34^{a}
Propanol	31.18 ± 5.48^{ab}	20.65 ± 4.51^{b}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	37.87 ± 2.17^{a}	0.0 ± 0.00^{c}
Butanol	0.0 ± 0.00^{b}	0.69 ± 0.09^{b}	0.71 ± 0.00^{b}	$0.52 \pm .07^{b}$	3.56 ± 0.56^{a}	0.73 ± 0.03^{b}
Pentanol	1.56 ± 0.00^{a}	0.48 ± 0.13^{c}	0.0 ± 0.00^{d}	0.88 ± 0.02^{b}	1.11 ± 0.01^{b}	0.0 ± 0.00^{d}
Hexanol	0.89 ± 0.00^{b}	0.04 ± 0.02^{c}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	10.32 ± 0.01^{a}	0.0 ± 0.00^{c}
3-Ethoxy-1-propanol	1.15 ± 0.40^{c}	16.89 ± 4.44^{ab}	8.68 ± 0.44^{bc}	6.41 ± 0.08^{bc}	25.07 ± 3.43^{a}	8.04 ± 0.76^{bc}
3-Methyl-1-pentanol	0.0 ± 0.00^{c}	0.48 ± 0.00^{b}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}	1.02 ± 0.00^{a}	0.0 ± 0.00^{c}
Propionic acid	1.69 ± 0.12^{b}	2.96 ± 0.55^{b}	2.10 ± 0.06^{b}	4.2 ± 1.34^{b}	7.6 ± 1.15^{a}	7.4 ± 0.50^{a}
Isobutyric acid	1.57 ± 0.55^{b}	1.58 ± 0.33^{b}	2.08 ± 0.09^{b}	7.8 ± 0.54^{a}	5.8 ± 1.20^{a}	2.08 ± 0.08^{b}
Butyric acid	1.30 ± 0.04^{a}	1.38 ± 0.32^{a}	1.24 ± 0.02^{a}	1.91 ± 0.04^{a}	2.08 ± 0.57^{a}	2.12 ± 0.13^{a}
Isovaleric acid	1.07 ± 0.00^{a}	0.91 ± 0.08^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.33 ± 0.47^{bc}	0.0 ± 0.00^{b}
Valeric acid	0.66 ± 0.06^{a}	0.46 ± 0.05^{a}	0.56 ± 0.00^{a}	0.68 ± 0.01^{a}	0.82 ± 0.47^{a}	0.57 ± 0.00^{a}
Hexanoic acid	1.96 ± 0.20^{a}	0.68 ± 0.03^{b}	2.28 ± 0.04^{a}	0.92 ± 0.07^{b}	1.05 ± 0.02^{b}	0.38 ± 0.54^{b}
Octanoic acid	2.64 ± 0.03^{b}	0.88 ± 0.22^{c}	3.18 ± 0.01^{a}	0.96 ± 0.02^{b}	0.97 ± 0.08^{b}	0.45 ± 0.01^{c}
Decanoic acid	3.76 ± 0.35^{b}	2.02 ± 0.02^{c}	6.33 ± 0.02^{a}	1.19 ± 0.03^{a}	1.22 ± 0.14^{d}	1.00 ± 0.02^{d}
2-Phenylethyl acetate	1.07 ± 0.03^{b}	0.89 ± 0.03^{b}	2.47 ± 0.11^{a}	0.78 ± 0.26^{b}	1.13 ± 0.10^{b}	1.32 ± 0.32^{b}
2-Isoamyl acetate	0.80 ± 0.01^{a}	0.43 ± 0.07^{c}	0.59 ± 0.03^{bc}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.61 ± 0.06^{b}
Hexyl acetate	0.0 ± 0.00^{b}	0.66 ± 0.03^{a}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}
Ethyl-hexanoate	1.05 ± 0.23^{a}	0.49 ± 0.00^{c}	0.27 ± 0.03^{c}	0.0 ± 0.00^{a}	0.09 ± 0.01^{a}	0.63 ± 0.06^{b}
Ethyl-caprylate	0.34 ± 0.03^{b}	2.07 ± 0.63^{a}	0.63 ± 0.01^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.20 ± 0.02^{b}
Ethyl acetate	24.15 ± 2.04^{bc}	20.71 ± 1.73^{c}	26.43 ± 3.36^{bc}	22.43 ± 0.22^{bc}	28.18 ± 1.35^{ab}	33.79 ± 0.36^{a}
Ethyl butyrate	0.75 ± 0.27^{a}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}
Ethyl lactate	0.0 ± 0.00^{a}	0.71 ± 0.17^{a}	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	0.29 ± 0.42^{a}	0.0 ± 0.00^{a}
Ethyl-3-hydroxybutanoate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl caprate	0.51 ± 0.04^{ab}	0.29 ± 0.01^{ab}	0.96 ± 0.09^{a}	0.0 ± 0.00^{b}	0.14 ± 0.04^{ab}	0.53 ± 0.51^{ab}
Ethyl-phenylacetate	0.0 ± 0.00^{b}	1.37 ± 0.03^{a}	1.15 ± 0.00^{ab}	1.17 ± 0.00^{ab}	1.40 ± 0.08^{ab}	0.57 ± 0.81^{ab}
Diethyl succinate	0.0 ± 0.00^{c}	1.05 ± 0.00^{b}	1.29 ± 0.02^{b}	2.70 ± 0.08^{a}	1.52 ± 0.26^{b}	1.40 ± 0.17^{b}

Mean values bearing differing superscript letters showed significant differences and mean values bearing the same letter were statistically similar. All the compounds are presented in milligrams per liter and are the average of two biological duplicates ± SD

AN anaerobic conditions, *Nd* not detected

succinate, ethyl acetate and ethyl lactate, 2-isoamyl-acetate, ethyl-hexanoate, ethyl-caprylate, ethyl-phenylacetate) increased (Tables 2, 3, and 4).

Oxygenation of both single and mixed-culture fermentations resulted in a general increase in higher alcohols, particularly in isoamyl alcohol, 2-phenylethanol, and isobutanol, and a decrease was observed in MCFAs and 2-phenylethyl acetate. Moreover, the incorporation of oxygen enhanced the production of 3-ethoxy-1-propanol and isobutyric acid in *S. cerevisiae*/*L. thermotolerans* fermentation (Table 2), while it increased butyric acid and propionic acids in the mixed fermentation with *T. delbrueckii* (Table 3). Both the *S. cerevisiae*/*L. thermotolerans* and *S. cerevisiae*/*T. delbrueckii* fermentations overall increase in the yield of the alcohols and volatile acids, and there was a concomitant decrease in MCFAs and their corresponding esters (Supplementary Table S1 and S2) in response to oxygenation. The yield of 2-phenyl ethanol, isobutanol, propanol, pentanol, and hexanol was also further enhanced by higher biomass production, while isoamyl alcohol decreased with the increase in biomass (Supplementary Table S4). In contrast, the oxygenation in *S. cerevisiae*/*M. pulcherrima* mixed fermentation enhanced the production of 2-phenyl ethanol, isoamyl alcohol, isobutanol, 3-ethoxy-1-propanol, diethyl succinate, ethyl acetate, and ethyl lactate, while the levels of isoamyl-acetate, ethyl-hexanoate, ethyl caprate, and ethyl-phenyl acetate were reduced (Table 4). The yield of butanol, pentanol, hexanol, and 3-methyl-1-pentanol decreased with the increase in oxygenation (Supplementary Table S3) and also with the increase in biomass (Supplementary Table S4). Furthermore, in this

Table 4 Major volatile compounds detected at the end of the fermentation in *S. cerevisiae*/*M. pulcherrima* single culture and their mixed cultures

Major volatiles	*S. cerevisiae* (AN)	*M. pulcherrima* (AN)	Sc+Mp (AN)	Sc+Mp-1%	Sc+Mp-5%	Sc+Mp-21%
2-Phenylethanol	6.30 ± 1.24^{b}	24.37 ± 1.89^{b}	27 ± 2.25^{b}	133 ± 0.42^{a}	119 ± 14.00^{a}	141 ± 7.70^{a}
Isoamyl alcohol	50.8 ± 8.75^{d}	137.43 ± 0.56^{c}	79 ± 7.57^{a}	152 ± 3.80^{bc}	196 ± 8.56^{b}	276 ± 29.00^{a}
Isobutanol	12.5 ± 2.85^{e}	227.44 ± 5.16^{a}	124 ± 1.45^{d}	122 ± 2.56^{d}	145 ± 4.26^{c}	167 ± 8.60^{b}
Propanol	31.1 ± 5.48^{c}	22.3 ± 0.00^{c}	51 ± 0.33^{c}	63 ± 6.30^{bc}	77 ± 1.52^{b}	107 ± 2.25^{a}
Butanol	0.00 ± 0.00^{a}	0.51 ± 0.00^{b}	0.83 ± 0.07^{c}	2.07 ± 0.13^{d}	0.67 ± 0.03^{bc}	0.69 ± 0.40^{bc}
Pentanol	1.56 ± 0.00^{b}	0.37 ± 0.00^{a}	1.62 ± 0.03^{b}	0.98 ± 0.00^{c}	0.94 ± 0.01^{c}	0.97 ± 0.00^{c}
Hexanol	0.89 ± 0.00^{a}	0.41 ± 0.00^{a}	0.84 ± 0.06^{a}	10.84 ± 0.09^{b}	0.71 ± 0.24^{a}	0.22 ± 0.31^{a}
3-Ethoxy-1-propanol	1.15 ± 0.40^{ab}	00.0 ± 0.00^{a}	2.28 ± 0.11^{abc}	4.5 ± 0.00^{a}	4.6 ± 1.80^{a}	4.67 ± 0.00^{a}
3-Methoxy-1-pentanol	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	1.56 ± 0.01^{a}	0.0 ± 0.00^{c}	0.97 ± 0.00^{b}	0.97 ± 0.00^{b}
Propionic acid	1.69 ± 0.12^{ab}	0.78 ± 0.02^{a}	1.25 ± 0.58^{ab}	1.49 ± 0.81^{ab}	1.42 ± 0.11^{ab}	2.62 ± 0.34^{b}
Isobutyric acid	1.57 ± 0.55^{ab}	0.72 ± 0.01^{a}	1.35 ± 0.19^{ab}	1.47 ± 0.04^{ab}	0.95 ± 0.01^{a}	2.30 ± 0.02^{a}
Butyric acid	1.30 ± 0.04^{c}	0.89 ± 0.01^{c}	1.50 ± 0.12^{c}	3.39 ± 0.33^{a}	1.06 ± 0.13^{b}	2.23 ± 0.17^{a}
Isovaleric acid	1.0 ± 0.09^{a}	0.00 ± 0.04^{d}	1.05 ± 0.05^{a}	0.73 ± 0.02^{b}	0.63 ± 0.00^{c}	0.75 ± 0.01^{d}
Valeric acid	0.62 ± 0.06^{a}	0.41 ± 0.03^{c}	0.64 ± 0.04^{a}	0.54 ± 0.03^{ab}	0.40 ± 0.00^{c}	0.42 ± 0.00^{bc}
Hexanoic acid	1.96 ± 0.20^{a}	0.61 ± 0.06^{b}	1.55 ± 0.22^{a}	1.52 ± 0.20^{a}	1.62 ± 0.31^{a}	1.58 ± 0.28^{a}
Octanoic acid	2.64 ± 0.03^{a}	0.73 ± 0.00^{b}	1.67 ± 0.05^{ab}	1.72 ± 0.34^{ab}	1.16 ± 0.98^{ab}	1.63 ± 0.46^{ab}
Decanoic acid	3.76 ± 0.35^{a}	1.96 ± 0.01^{b}	2.27 ± 0.03^{b}	2.27 ± 0.32^{ab}	2.34 ± 0.58^{b}	2.28 ± 0.40^{b}
2-Phenylethyl acetate	1.07 ± 0.03^{a}	0.95 ± 0.00^{c}	0.93 ± 0.05^{a}	0.47 ± 0.00^{a}	1.07 ± 0.09^{c}	0.47 ± 0.25^{a}
2-Isoamyl acetate	0.80 ± 0.01^{a}	0.03 ± 0.00^{a}	0.77 ± 0.04^{b}	0.0 ± 0.00^{c}	0.39 ± 0.12^{b}	0.2 ± 0.04^{ab}
Hexyl acetate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl-hexanoate	1.05 ± 0.23^{a}	0.00 ± 0.00^{e}	0.80 ± 0.01^{b}	0.25 ± 0.03^{b}	0.15 ± 0.01^{c}	0.09 ± 0.00^{d}
Ethyl-caprylate	0.34 ± 0.03^{a}	0.00 ± 0.01^{c}	0.12 ± 0.02^{b}	0.08 ± 0.00^{b}	0.0 ± 0.00^{c}	0.0 ± 0.00^{c}
Ethyl acetate	24 ± 2.04^{f}	157.16 ± 7.21^{d}	84 ± 0.90^{e}	310 ± 7.00^{b}	265 ± 6.34^{c}	366 ± 1.67^{a}
Ethyl butyrate	0.64 ± 0.02^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.10 ± 0.14^{c}
Ethyl lactate	0.0 ± 0.00d	0.64 ± 0.30^{d}	11 ± 2.51^{b}	12.4 ± 0.88^{c}	15 ± 1.40^{cd}	18 ± 1.14^{a}
Ethyl-3-hydroxybutanoate	Nd	Nd	Nd	Nd	Nd	Nd
Ethyl caprate	0.51 ± 0.04^{a}	0.00 ± 0.02^{c}	0.16 ± 0.01^{b}	0.0 ± 0.00^{c}	0.05 ± 0.00^{c}	0.04 ± 0.00^{c}
Ethyl-phenylacetate	0.0 ± 0.00^{c}	0.00 ± 0.03^{c}	1.56 ± 0.03^{a}	1.20 ± 0.01^{b}	0.47 ± 0.09^{b}	1.07 ± 0.25^{b}
Diethyl succinate	0.0 ± 0.00^{e}	0.60 ± 0.00^{cb}	1.26 ± 0.03^{bc}	2.3 ± 0.14^{cd}	2.22 ± 0.73^{cd}	2.7 ± 0.05^{a}

Mean values bearing differing superscript letters showed significant differences and mean values bearing the same letter were statistically similar. All the compounds are presented in milligrams per liter and are the average of two biological duplicates ± SD

AN anaerobic conditions, *Nd* not detected

fermentation, there was a marked increase in ethyl acetate in response to oxygenation (Supplementary Table S3) and a clear positive correlation with biomass (Supplementary Table S4).

Principal component analysis (PCA) biplot showed that the first two principal components explain 41% of the variability shown in the fermentations studied (Fig. 6a, b). PC1 differentiates the fermentations according to the yeast dominance profiles, resulting in the *S. cerevisiae*- and *M. pulcherrima*-dominated fermentations forming distinct groups separate from the *L. thermotolerans*- and *T. delbrueckii*-dominated fermentations. The fermentations are further separated along PC2, which explains 18% of the variance and separates according to levels of aeration. The metabolic profile of the anaerobic *S. cerevisiae*/*L. thermotolerans* and *S. cerevisiae*/*T. delbrueckii* is close to the *S. cerevisiae* single-culture fermentation, while their aerated mixed cultures exhibit a distinct chemical profile from the anaerobic mixed fermentations, however, similar to the aerated *L. thermotolerans*/*T. delbrueckii* single fermentations. The fermentation profiles of *L. thermotolerans*/*T. delbrueckii* mixed fermentation with *S. cerevisiae* could not show a clear separation on the basis of oxygenation levels. In contrast, the *S. cerevisiae*/*M. pulcherrima* mixed fermentations showed a clear separation between the 1%, 5%, and 21% DO treatments. The separation of the *S. cerevisiae*, *L. thermotolerans*, and *T. delbrueckii* single anaerobic fermentations and their mixed cultures along PC1 was mostly driven by the production of medium-chain fatty acids, while the separation of the aerated *L. thermotolerans*, *T. delbrueckii*, *S. cerevisiae*/*L. thermotolerans*, and *S. cerevisiae*/*T. delbrueckii* from the

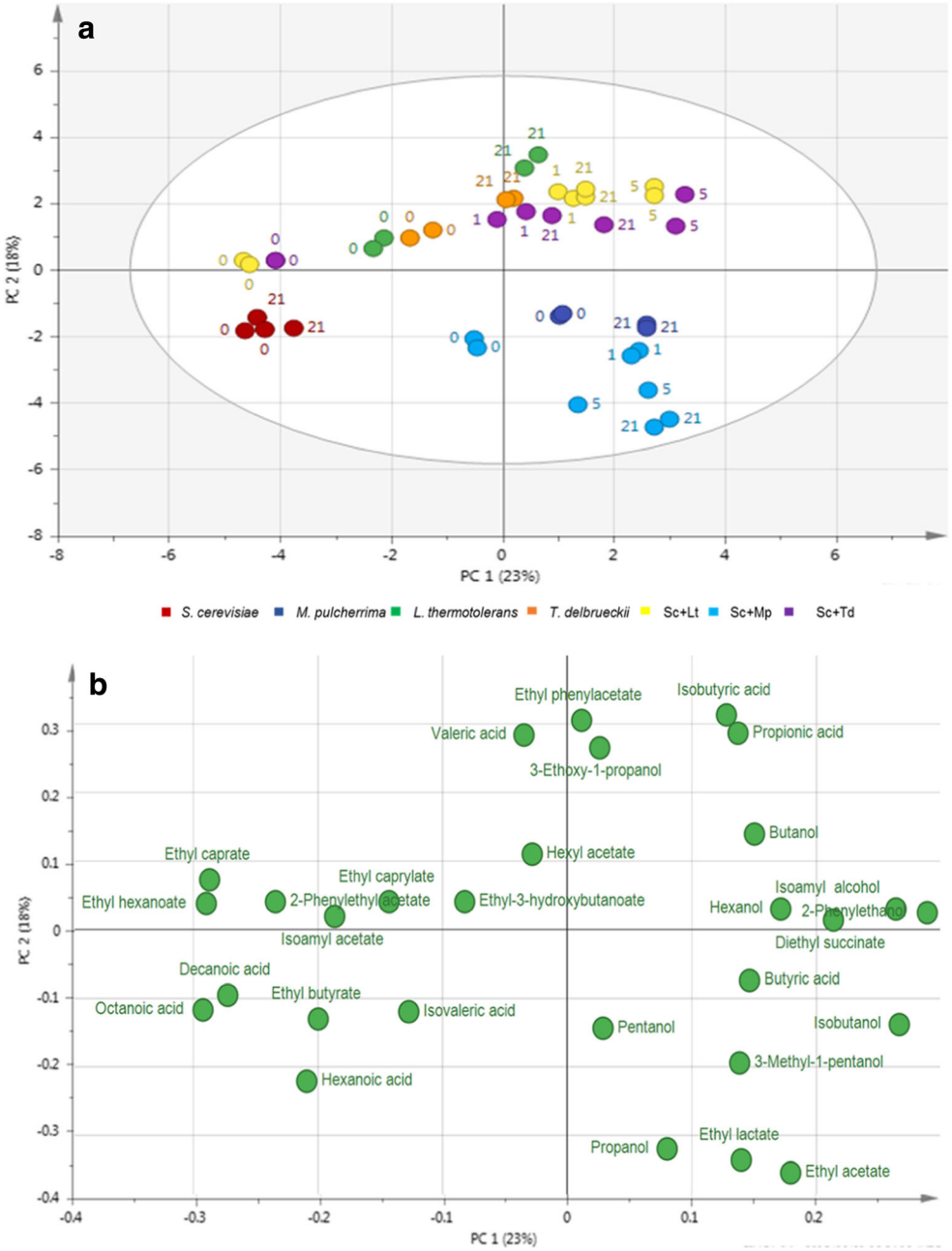

Fig. 6 PCA score plot (**a**) and loading plot (**b**) of the first principal components showing major volatiles produced by different single species and mixed fermentations with and without oxygen. The *numbers* (0, 1%, 5%, and 21%) indicate the DO levels in the fermentations

anaerobic cultures was strongly associated with the accumulation of higher alcohols. In contrast, the separation of the aerated *S. cerevisiae*/*M. pulcherrima* fermentation was mainly driven by acetate esters and higher alcohols. For a more clear understanding, PC3 and PC4 were also performed; however, again no clear separation was seen from there.

Discussion

The current study evaluated the effect of three different levels of oxygen on yeast dynamics and volatile compound production by applying a co-fermentation strategy with *S. cerevisiae* with either *T. delbrueckii*, *L. thermotolerans*, or *M. pulcherrima*. Our data show that oxygenation had a positive effect on yeast population dynamics especially on the growth and persistence of non-*Saccharomyces* yeasts. However, all three non-*Saccharomyces* yeasts responded very differently to oxygen availability, perhaps due to their different oxygen requirement. Of the three, *M. pulcherrima* displayed the strongest dependence on oxygen, and its ability to contribute significantly to the outcome of the fermentation strongly depended on the amount of oxygen supplied. Indeed, in anaerobic conditions, this yeast could only be detected in the first 24 to 48 h, and its contribution to the final aroma compound levels was insignificant. However, at 1%, 5%,

and 21% DO, this yeast displayed protracted persistence with viable cell count reaching up to 10^{10} CFU mL^{-1}. In contrast, *L. thermotolerans* and *T. delbrueckii* could grow and persist in anaerobic conditions albeit at relatively low cell numbers of 10^7 and 10^6 CFU mL^{-1}, respectively. The growth of both yeasts was significantly enhanced under oxygenation, resulting in cell numbers reaching up to 10^9 and 10^{10} CFU mL^{-1} in *T. delbrueckii* and *L. thermotolerans*, respectively, at 1% DO and 10^{10} CFU mL^{-1} in both yeasts at 5% and 21% DO. The difference in response to oxygen in the three yeasts can be explained by the lifestyle of these yeasts since *M. pulcherrima* is an obligate aerobe while *T. delbrueckii* and *L. thermotolerans* are facultative anaerobes. Indeed, previous studies have shown that *M. pulcherrima* displays a fully respiratory glucose metabolism, with respiratory quotient (RQ) values of 1.04–1.26 (Contreras et al. 2014; Morales et al. 2015; Quirós et al. 2014).

The three non-*Saccharomyces* yeasts not only responded differently to oxygenation but also influenced the growth of *S. cerevisiae* in different ways. In the conditions used here (taking into consideration a 1:10 *S. cerevisiae*/non-*Saccharomyces* inoculation ratio), the non-*Saccharomyces* yeasts were able to numerically dominate the fermentations for extended periods of time. For instance, the growth rate of *S. cerevisiae* at 5% DO was slower in the presence of *L. thermotolerans* such that the maximum cell concentration of 10^9 CFU mL^{-1} was only achieved after 48 h, while in the presence of *T. delbrueckii* a similar effect only becomes apparent at 21% DO. Both *L. thermotolerans* and *T. delbrueckii* displayed a competitive growth advantage over *S. cerevisiae* at 21% DO as *S. cerevisiae* only managed to grow to 10^7 and 10^8 CFU mL^{-1} in co-fermentation with *L. thermotolerans* and *T. delbrueckii*, respectively. In contrast, *S. cerevisiae* reached similar maximum growth levels of 10^9 CFU mL^{-1} under anaerobic and aerobic conditions (at all DO levels) in the presence of *M. pulcherrima*. Although both *L. thermotolerans* and *T. delbrueckii* are Crabtree-positive and facultative anaerobes like *S. cerevisiae*, it is clear that under oxygenated conditions they display a greater intrinsic growth rate than *S. cerevisiae*. The higher cell counts of non-*Saccharomyces* yeasts in the presence of oxygen is likely a consequence of the greater proportion of carbon flow through respiratory metabolism in these strains (Brandam et al. 2013; Morales et al. 2015; Visser et al. 1990). Indeed, our data show that at 21% DO, *S. cerevisiae* in monoculture generated 72 g L^{-1} ethanol while in the presence of *L. thermotolerans*, *T. delbrueckii*, and *M. pulcherrima*, only 59.08, 46.91, and 46.96 g L^{-1} ethanol was produced, suggesting that in the mixed cultures, most of the sugar is respired. This finding is congruent with previous studies which showed that under oxygenated conditions, *S. cerevisiae* only respires 25% of the sugar while most non-*Saccharomyces* yeasts such as *T. delbrueckii* can respire 40–100% of the sugar without concomitant production of ethanol. Overall, our data show that the ethanol yield decreases with the increase in aeration and that most of the carbon flux is channeled towards biomass and acetic acid production. However, it is also possible that minor levels of ethanol could escape, although for the current experimental setup this was minimized by fitment of a condenser (maintained at −4 °C) and through low gas flow rate, standardized for all fermentations.

A further look at the primary metabolites shows that the three non-*Saccharomyces* yeasts have very distinct metabolic responses to oxygenation. For instance, the *S. cerevisiae*/*M. pulcherrima* fermentations generated excessive amounts of acetic acid (>1200 mg L^{-1}) at all DO levels, followed by the *S. cerevisiae*/*L. thermotolerans* fermentation which at 5% and 21% DO also produced undesirable levels. In contrast, the *S. cerevisiae*/*T. delbrueckii* fermentations only produced high acetic acid levels at 21% DO, while at 1% and 5% DO, the levels were lower, and in fact lower than even the *S. cerevisiae* monoculture under anaerobic conditions. In addition, our data show that *T. delbrueckii*, which is often described as a low acetic acid producer under standard winemaking conditions, maintains this trait even under continuous oxygen supply. Regular punch-downs and pump-overs, which are standard practices in red wine fermentations, can incorporate varying amounts of DO up to 5.6 mg L^{-1} into grape must depending on the stage of fermentation (Moenne et al. 2014). Hence, the 1% DO (0.08 mg L^{-1}), which is favorable for the all three non-*Saccharomyces* yeasts, can be used during winemaking to sustain their growth and reduce the ethanol levels in wine without negative influence on quality, except for *M. pulcherrima* for which lower levels might be preferable to keep the acetic acid level lower.

In mixed-culture fermentations, the chemical compositions of the synthetic wines at the end of fermentation clearly showed the contribution of each non-*Saccharomyces* yeasts. *L. thermotolerans* and *T. delbrueckii* showed similar behavior and resulted in higher production of 2-phenylethanol, isoamyl alcohol, isobutanol, hexanoic, decanoic, and octanoic acids, while *M. pulcherrima* also affected ethyl lactate and ethyl acetate. The higher production of these compounds by *M. pulcherrima* has been attributed to high cell density ratios between non-*Saccharomyces* and *S. cerevisiae* yeasts in co-inoculation (Contreras et al. 2014; Sadoudi et al. 2012). In addition, it is important to note that *M. pulcherrima* generates high levels of ethyl acetate (>300 mg L^{-1}) in all aerobic fermentations, which could suggest that this yeast mainly uses ethyl acetate production as a detoxification mechanism to remove ethanol and acetate from cells. Ethyl acetate at levels above 100 mg L^{-1} contributes a solvent, balsamic aroma and is not desirable at high levels in wine.

The incorporation of oxygen in both mixed and single fermentations showed a significant increase in higher alcohols (particularly 2-phenylethanol, isoamyl alcohol, and

isobutanol), revealing a positive correlation between the production of higher alcohols, the growth of non-*Saccharomyces* yeasts, and oxygen levels. The yield data of volatile compounds normalized with biomass at 5% DO show an increase in higher alcohols (isoamyl alcohol, 2-phenylethanol, and isobutanol) as well as propionic acid and butyric acid (*S. cerevisiae*/*L. thermotolerans* and *S. cerevisiae*/*T. delbrueckii*), while a decrease in MCFA and esters was observed. This increase in the yield of these compounds can be in part due to increased biomass (Supplementary Table S1–S4) under aerobic conditions, but also due to increased uptake of branched chain amino acids such as leucine, isoleucine, and valine. Indeed, the expression of *BAP2*, which encodes branched chain amino acid permeases, is upregulated under aerobic conditions (Verbelen et al. 2009). Evidently, the total sum of the three higher alcohols (isoamyl alcohol, 2-phenylethanol, and isobutanol) accumulated at 5% and 21% levels ranged between 300 and 500 mg L^{-1} depending on the DO levels. At such high levels, these alcohols are known to impart harsh, spirituous, nail polish-like aroma, which are not desirable in wine (Panon 1997; Sun et al. 2014). The impact of oxygenation in all aerobic fermentations resulted in a decrease in MCFAs, reflecting the incorporation of fatty acids into long-chain fatty acid biosynthesis through the acetyl-CoA carboxylase and fatty acid synthetase activity (Lambrechts and Pretorius 2000; Sumper 1974). Overall, our data clearly show that the impact of non-*Saccharomyces* yeast on wine fermentation and aroma can be managed through controlled oxygen supply, and that the level of oxygen will largely determine the degree of impact of the non-*Saccharomyces* yeast including on the aromatic contribution of these strains. The impact is significant already at relatively low levels of oxygen supply, and such DO level can be managed within a winery through various strategies such as micro-oxygenation or regular pump-overs in red winemaking.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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