Physiological features of *Saccharomyces cerevisiae* and alternative wine yeast species in relation to alcohol level reduction in wine

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Características fisiológicas de *Saccharomyces cerevisiae* y especies de levaduras enológicas alternativas en relación con la reducción del contenido alcohólico del vino

Memoria presentada por

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CERTIFICAN

Que la presente memoria titulada "**Physiological features of *Saccharomyces cerevisiae* and alternative wine yeast species in relation to alcohol level reduction in wine**" presentada por Dña. Alda João Sousa RODRIGUES para optar al grado de doctor por la Universidad de La Rioja ha sido realizada bajo nuestra dirección, autorizando su presentación para proseguir los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

Logroño, Diciembre de 2018

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Abstract

One of the major problems of the wine industry in warm climate countries is the increasing alcohol content in wines, experienced during the last decades; which is the result of increasing sugar content in grapes at harvest time. This problem is mainly related with global climate change, but it is also connected to the changing preferences of consumers for full-bodied wines and strong aroma. However, due to health and road safety considerations, as well as to tax policies in some importing countries, the market is also demanding for wines with lower ethanol content.

There are many points in the vine growing and winemaking workflow that can be targeted to reduce the alcohol content of the final wine. In this thesis I focused on the fermentation step, in which sugars are converted into ethanol by the activity of yeasts, mainly *Saccharomyces cerevisiae*. Previous work in this research group centered on respiration as the most promising yeast metabolic pathway that would have to be increased in order to divert carbon flow from ethanol production. Considering the Crabtree features of this yeast species, the use of non-*Saccharomyces* species was required.

One major problem found to implement this approach at the industrial level was acetic acid production by *S. cerevisiae*, which is greatly enhanced in the presence of oxygen. This does not only affect wine fermentations inoculated with *S. cerevisiae*, since some *S. cerevisiae* cells will be naturally present, and tend to dominate the process. This would result in increased volatile acidity for aerated fermentations, whether they have been inoculated with *S. cerevisiae* or with non-*Saccharomyces* starters.

In order to advance in the development of efficient yeast strains and fermentation procedures aiming to alcohol reduction in wine, while avoiding the drawbacks related with acetic acid production, my PhD work targeted both *S. cerevisiae* and non-*Saccharomyces* species. The focus of my work

on non-*Saccharomyces* yeast strains was on understanding the physiology of aerobic growth on grape must for these species, including factors that affect alcohol and acetate yields, and the impact of these growth conditions at the transcriptome level. In the case of *S. cerevisiae*, I tried to understand, by a combination of computational biology and genetic engineering approaches, the genetic determinants of excess acetate production when cultures are aerated.

The results obtained indicate that environmental factors that can be easily manipulated during wine fermentation have a huge impact on the yields of acetic acid and alcohol for all yeast species tested. In addition, I was able to identify several genes whose deletion results in reducing the problem of acetic acid production by aerated cultures of *S. cerevisiae*. These results will serve to guide the development of fermentation procedures using some non-*Saccharomyces* species, aiming to alcohol level reduction by respiration. On the other side, the information is serving to develop non-GMO *S. cerevisiae* derivatives that are improved for acetic acid production (reduced yield) and can be combined with non-*Saccharomyces* yeasts during the aerated step or used as pure cultures for alcohol level reduction.

Resumen

Uno de los principales problemas de la industria enológica en países de clima cálido es el incremento que se ha producido en las últimas décadas en el contenido alcohólico de los vinos, que deriva a su vez del mayor contenido en azúcares de las uvas en el momento de la vendimia. Este problema está principalmente relacionado con el cambio climático global, pero también con el cambio en las preferencias de los consumidores hacia vinos con mayor cuerpo y potencial aromático. Sin embargo, teniendo en cuenta consideraciones de salud y seguridad vial, así como las políticas de impuestos sobre bebidas alcohólicas en algunos países importadores, el mercado está demandando a su vez vinos con menor contenido alcohólico.

Hay varios puntos de la cadena de valor viña-vino que pueden ser objeto de mejora para reducir el contenido alcohólico de los vinos. En esta tesis me he concentrado sobre la etapa de fermentación, en la cual los azúcares son transformados en etanol debido a la actividad de las levaduras, especialmente *Saccharomyces cerevisiae*. El trabajo previo en este grupo de investigación identificó la respiración como la vía metabólica más prometedora que se podría fomentar para desviar el flujo de carbono de la producción de etanol. Teniendo en cuenta las características Crabtree de esta especie de levadura, este objetivo requiere el uso de levaduras no-*Saccharomyces*.

Uno de los principales problemas encontrados para implementar esta estrategia a escala industrial fue la producción de ácido acético por parte de *S. cerevisiae*, que se incrementa en gran manera en presencia de oxígeno. Dado que esta especie está presente en el mosto en prácticamente todas las ocasiones, incluso aunque no se inocule, y tiende a dominar el proceso al cabo de algunas horas de fermentación, proporcionar oxígeno al mosto natural, para favorecer la respiración de las levaduras non-*Saccharomyces*,

casi siempre acarrea un riesgo de dar lugar a un exceso de acidez volátil que impediría la comercialización del vino, a pesar de la reducción de alcohol.

Para avanzar en el desarrollo de levaduras y procesos de fermentación que permitan reducir el grado alcohólico, pero evitando el inconveniente del ácido acético, mi tesis trata tanto sobre *S. cerevisiae* como sobre otras especies de levaduras. En el caso de levaduras no-*Saccharomyces* he tratado de comprender su fisiología en mosto, en condiciones aeróbicas, incluyendo los factores que afectan al rendimiento en alcohol y acetato, y el impacto de estas condiciones sobre el transcriptoma. Para *S. cerevisiae* he utilizado una combinación de biología computacional e ingeniería genética para identificar los factores genéticos que influyen en la producción de acetato en cultivos aireados.

Los resultados de la tesis indican que hay factores ambientales, fácilmente controlables en enología, que pueden tener un gran impacto sobre los rendimientos de alcohol y ácido acético para todas las especies de levaduras analizadas. Además, he podido identificar varios genes cuya deleción permite reducir el problema del ácido acético en cultivos aireados de *S. cerevisiae*. Mis resultados pueden servir de guía para el desarrollo de procesos fermentativos con levaduras no-*Saccharomyces* que permitan reducir el contenido alcohólico del vino. Por otro lado, esta información está sirviendo para el desarrollo de cepas no recombinantes de *S. cerevisiae* mejoradas respecto al problema del exceso de acético en aerobiosis que se podrían utilizar solas o en combinación con otras especies de levaduras para reducir el grado alcohólico.

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Chapter 1.

Introduction

THE INCREASING ALCOHOL CONTENT OF WINE

In recent years there has been an increase in the alcohol content of wines, especially red wines. There are several reasons behind this problem and some possible solutions, based on the biotechnological control of the fermentation process, have been addressed in this thesis.

The largest impact on the amount of alcohol quantified in wines after fermentation (14%-16%, v/v) is attributed to climate change, as well as to the current consumer preferences for well-structured and full bodied wines which require late harvest in order to achieve proper phenolic and/or aromatic maturity, resulting in a noticeable increase in the sugar content of the berries (Mira de Orduña, 2010; Conibear, 2006; Olego et al., 2016).

Alcohol level in wine is mainly determined by the amount of sugar present in the grapes used in wine production. As the grapes mature, sugar levels rise, and acid levels decrease. This process is known as technological ripeness. Besides this, the accumulation of phenolic and aroma compounds, or its precursors, also take place in the grape berries during the ripening process, as well as changes in tannins and reduction in methoxypyrazine levels (Olego et al., 2016).

Climate has a strong impact on grape maturation (Jones and Webb, 2010), and climate changes can lead to modifications on the expression of varietal aroma compounds, wine chemical stability and sensory balance (Mira de Orduña, 2010; Holland and Smit, 2010). Temperature directly influences the ripening process, through photosynthetic effectiveness and consequent sugar accumulation in the grape. Altered precipitation patterns can equally result in grape dehydration and sugar build up in grape berries (Keller, 2010).

Vine management choices that normally seek a more complete general maturation, like vineyard site, soil composition irrigation strategies, rootstocks, grape varieties and clones (that naturally accumulate more sugars), virus free vines (which can photosynthesize more efficiently), and canopy management may also affect the grape's ability to accumulate more sugars.

High sugar content leads to elevated ethanol production during must fermentation. This has negative consequences for both the fermentation process itself and for wine marketing. Concerning the process, high ethanol level can lead to stuck or sluggish alcoholic fermentation (Bisson, 1999; Bisson and Butzke, 2000; Coulter et al., 2008). This in turns might result in problems like excess volatile acidity (Goldner, 2009; Costantini et al., 2009) or difficulties to complete malolactic fermentation (Lonvaud-Funel et al., 1988; Capucho and San Romao, 1994).

High levels of alcohol also compromise the concentration and perception of many volatiles present in the headspace of wine, since most of the aroma compounds are more soluble in ethanol than in water (Keller, 2015). Other disadvantages associated with the sensory perception of wines with high alcohol content include an increment in the perception of warmth or hotness (Li et al., 2017), astringency, bitterness and sourness (Martin and Pangborn, 1970; Fischer and Noble, 1994). At the same time, it decreases acidity sensations and masks the perception of some important aroma compounds such as higher alcohols, esters and monoterpenes (Robinson et al., 2009; Escudero et al., 2007). The effect of ethanol on the sensory properties of the wine is so relevant that relatively small changes in alcohol content could have a great influence on how the other components of the wine are perceived.

In addition, excess alcohol content in the final wine becomes a market issue. Decreasing alcohol consumption is a worldwide trend and lower alcohol consumption rates are associated with a number of potential social and health benefits for consumers (Schmidtke et al., 2012). From a social point of view, there has been a growing demand for wines with reduced alcohol content (9%-13%, v/v), associated to road safety and acceptable social behaviour. Consequently, high alcohol content on the labels potentially discourages health concerned consumers (Meillon et al., 2010; Saliba et al., 2013), on the ground of alcohol related diseases or calorie intake. In addition, some countries apply higher tax rates on wines with a higher alcoholic level (de Barros Lopes et al., 2003; Contreras et al., 2014).

Wines with reduced alcohol content have been commercially available for a long time, but alcohol reduction in wine can be difficult to achieve. The application of techniques to reduce ethanol content is time consuming, costly and may affect the quality of wine. Since early 1900s, several strategies and techniques have been developed and considered for the reduction of alcohol content in wine, taking into account their impact on flavour, aroma and colour, since these are critical elements for their consumption. Wines with reduced ethanol content have been classified according to their alcohol degree as dealcoholized or alcohol free (< 0.5 % v/v), low alcohol (0.5 to 1.2% v/v), reduced alcohol (1.2 to 5.5–6.5 % v/v) and lower alcohol wine (5.5 to 10.5 % v/v), even if these classifications vary between different countries (Pickering, 2000). However, most wine producers are interested in developing techniques that aim to reduce only two or three degrees of alcohol, in order to compensate the effects of global warming and to obtain better-balanced wines (Meillon et al., 2010; Gambutti et al., 2011).

Some of the approaches that enable to produce wines with reduced alcohol levels involve lowering the concentration of fermentable sugars in grape juice through proper ripening or delayed grape maturity. To achieve proper ripening there are different solutions that can be employed as can be basal leaf removal, early grape harvest, or double harvest and selection of vineyard location, soil composition or biotypes or clones within the same cultivated variety (Stoll et al., 2009; Whiting, 2010; Schmidtke et al., 2011; Ozturk and Anli, 2014). Adjusting vine leaf area with basal leaf removal has a positive effect on phenolic development while it allows increasing the rate of enzymatic activity, responsible for the synthesis of phenolic compounds and allows addressing the imbalance between carbohydrate accumulation and the development of other grape constituents (Schmidtke et al., 2011). However, it has different effects on harvest quality according to the time and the shoot position when they were carried out (Olego et al., 2016), and consequently there is still required significant research to determine the optimum timing and location of leaf removal in relation to fruit location and their long-term impact on vine physiology (Schmidtke et al., 2011). Early grape harvest also allows moderating the concentration of sugars in the grape. Nonetheless, this technique may lead to the production of wines that are organoleptically undeveloped due to reduced flavour precursor development in grapes prior to harvest or in wines that present organoleptic defects as owing herbaceous character and high acidity levels (Schmidtke et al., 2011; Ozturk and Anli, 2014). Double harvest requires the development of different experiences to determine the exact moment to make the two harvests, the amount of grapes that must be collected, and the form of preservation as must or wine (Martinez de Toda and Balda, 2013). Changes in clonal choice or vineyard location require the establishment of new

vineyards, planted with the new material. This type of change in viticultural exploitation is inherently costly and slow to implement.

Sugar concentration in must can be reduced, prior to fermentation, by different approaches. These include dilution of grape juice with water (which destroys future quality and is forbidden in most wine producing countries); use of glucose oxidase (GOX), proposed by some authors but not currently employed; the use of different membrane-based technologies (Mira, et al., 2017). Indeed, membrane technologies can be used for both sugar removal, before fermentation, and ethanol removal, after fermentation. In all cases, the challenge is selectivity, since many other molecules that contribute to the quality of wines can be lost during the process. This is usually deal with by a second separation process in which these other molecules are taken apart from ethanol or sugar and returned into wine. Depending on pore sizes, working pressure employed, or other features, these technologies are named as ultrafiltration, nanofiltration, reverse osmosis, osmotic distillation, or electro-membrane processes (Catarino and Mendes, 2011; Labanda et al., 2009; Gonçalves et al., 2013; Diban et al., 2008; Fedrizzi et al., 2014).

Ethanol can also be removed by distillation techniques. Similar to membrane-based techniques, distillation removes many other volatile compounds that must somehow be introduced back to wine. This extensive manipulation easily results in products that are not well balanced from a sensory perspective. Conventional distillation, at relatively high temperatures, is very harmful to wine quality (Rowe, 1989), but a number of alternatives exist, working at relatively low temperature. Some of them are, vacuum distillation, pervaporation, stripping with CO_2, and spinning cone columns (Aguera et al., 2010; Catarino and Mendes, 2011; Gray, 1993; Karlsson and Tragardh, 1996; Olego et al., 2016; Pickering, 2000; Pyle, 1994; Sykes, 1992; Villetaz,

1986) the latter being currently the most commonly used to produce low alcohol wines.

Biotechnological approaches appear as more respectful for the sensory attributes of wines and have the potential to positively contribute to wine quality. To attain a relevant alcohol level reduction in wine, yeast strains must show low alcohol yield on sugar under the fermentation conditions. This can be attained through yeast strain selection, combined with control of fermentation conditions, mostly oxygen availability. For this purpose, yeast strains might be either genetically improved *S. cerevisiae* wine yeast strains (either recombinant or not) or non-*Saccharomyces* natural wine yeast strains. *S. cerevisiae* has been shaped by thousands of years of evolution to quickly and efficiently produce ethanol from sugars under most environmental conditions, following the make-accumulate-consume life strategy (Piskur et al., 2006). Despite some natural variability can be found among wild strains, the distribution of ethanol yield values is rather narrow, and sharply results in the consumption of 17 g/L of sugar to produce 1% ethanol. The aim of genetic improvement has been redirecting metabolic flux away from ethanol fermentation, toward other products (Kutyna et al., 2010) However, it has a risk of unpredictable effects on metabolite generation (Ozturk and Anli, 2014) possibly leading to overproduction of undesirable compounds, from an organoleptic point of view that require proper management or additional genetic modification rounds (Cambon et al., 2006; Ehsani et al., 2009; Quirós et al., 2014; Remize et al., 1999; Kutyna et al., 2010; Varela et al., 2012). There are other limitations associated with genetic engineering approach, where the employment of genetically modified organisms (GMOs) is not allowed in every country, due to existing legislation, and the commercial use of genetically engineered wine yeasts does not

seem feasible in short term (Gonzalez et al., 2013). Nonetheless, some researchers are now using adaptive laboratory evolution to circumvent this limitation (Kutyna et al., 2012; Cadiere et al., 2011). This method mimics the natural evolution, using environmental or metabolic constrains to improve yeast strains for winemaking processes (Olego et al., 2016; Cadiere et al., 2011). One consideration that must always be taken into account is the metabolic sink for carbon from sugars, if not used for fermentation. The increase in concentration required to reach a relevant impact on wine final alcohol content (2-3 % v/v), would certainly compromise wine quality for most alternative metabolites. This holds true even for glycerol, one of the preferred targets for researchers in this field. Reduction of 2% v/v ethanol by diverting carbon flux towards glycerol production would result in more than 30 g/L extra glycerol (about five times the usual values). Almost any other chemical compound would also become unacceptable in wine at such elevated concentrations. Probably, carbon dioxide is the only compound that can be overproduced by yeast without a negative impact on wine quality.

This is the reason several researchers have turned towards respiration, as the preferred metabolic pathway to boost in order to reduce alcohol yield during fermentation (Gonzalez et al., 2013). Research is currently under development in this field, as well as with mixed cultures between non-*Saccharomyces* and *Saccharomyces* cerevisiae strains that emerge as a very promising alternative. The possibility of reducing ethanol yields by promoting respiration of sugars by *S. cerevisiae* or other yeast species was initially suggested by Smith (1995), and the idea has been independently recovered and developed to different levels in recent years (Erten and Campbell, 2001; Contreras et al., 2015; Morales et al., 2015).There are, however, two restrictions to make yeast cells respire sugars under standard

winemaking conditions, oxygen requirement and the Crabtree effect. Respiratory metabolism has a huge oxygen demand, but it is known to participate in many other chemical reactions that can be detrimental to wine quality. Proper management of dissolved oxygen during wine fermentation will be required to meet respiration requirements while preserving other wine compounds from excessive oxidation. On the other side, *S. cerevisiae* is the archetype Crabtree-positive yeast species. This metabolic feature strongly favours fermentative over respiratory metabolism, despite oxygen availability (Pronk et al., 1996). In this species aerobic fermentation involves above 98% of the sugars consumed in the presence of oxygen (de Deken, 1966). Fortunately, not all wine yeast species are Crabtree-positive. Common ethanol yields on sugar after complete grape juice fermentation are 90–95% of theoretical, with the remaining 5–10% being explained by biomass biosynthesis, ethanol stripping, and alternative metabolic pathways (Konig et al., 2009). This mainly reflects anaerobic carbon flux distribution in the predominant species, *S. cerevisiae*. However, non-*Saccharomyces* wine yeasts usually differ from *S. cerevisiae* in metabolic flux distribution during fermentation and, consequently, in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2000; Magyar and Toth, 2011; Milanovic et al., 2012; Tofalo et al., 2012). Under anaerobic conditions, the diversion of alcoholic fermentation and the abundant formation of secondary compounds may in part explain the low ethanol yield of some of these non-*Saccharomyces* yeast strains. However, the main quantitative difference in carbon flux between *S. cerevisiae* and some other yeast species is related to respiration. Researchers have found important reductions in ethanol yields, under winemaking conditions, for several wine yeast species (Quirós et al., 2014; Contreras et al., 2015), although important differences can be found among yeast strains belonging to the same species.

Fermentation processes based on the use of non-*Saccharomyces* strains, either aerobic or anaerobic, aiming to reduce ethanol content of wines have been developed by several authors (Sipiczki et al., 2005; Ciani and Ferraro, 1998; Morales et al., 2015). Development of such fermentation processes requires specific design, considering the specific features of the non-*Saccharomyces* strain employed, such as nutrient or oxygen requirements, optimal temperature, sulphite susceptibility, or compatibility among the non-*Saccharomyces* and the *S. cerevisiae* strains employed. This results in constrains concerning sulphite management, temperature management, yeast nutrition management, or inoculation timing (that can be either sequential or simultaneous). However, alcohol level reduction attained in the examples mentioned above, is generally rather limited. The strategy proposed by Gonzalez et al., (2013) will be used as the reference framework for the discussion concerning application of yeast strains and knowledge developed in this thesis (Figure 1.1).

A major issue of aeration of wine during fermentation is acetic acid production. Several authors have described a boost in acetic acid production by *S. cerevisiae* when fermenting under aerobic or micro-aerobic conditions (Giovanelli et al., 1996; Papini et al., 2012; Quirós et al., 2014; Contreras et al., 2015). Other yeast species have also been shown to negatively impact volatile acidity under aerated growth conditions in synthetic grape must (Quirós et al., 2014). But some yeast species produce very little volatile acidity even under oxygenated conditions. It is possible to manage oxygen supply in fermentation trials driven by simultaneous inoculation of *S. cerevisiae* and a non-*Saccharomyces* strain (Morales et al., 2015). However, the strict control of the process required under such growth conditions suggests that a better control of volatile acidity would be achieved by

inoculating *S. cerevisiae* only after oxygen supply has been arrested (i.e., by sequential inoculation).

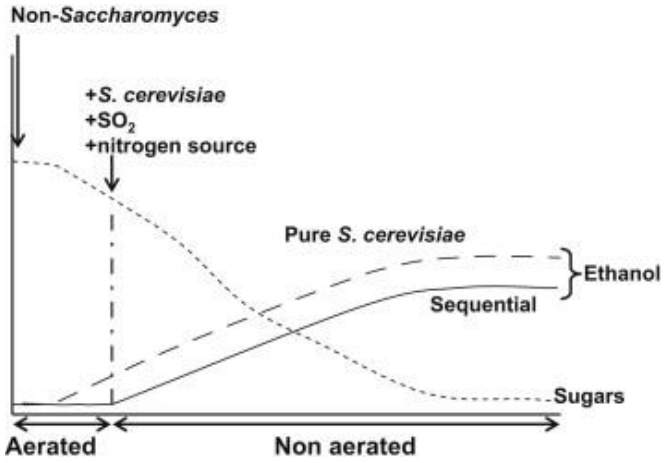

Figure 1.1. Idealized representation of the expected evolution of ethanol production during grape must fermentation in a sequential inoculation with a Crabtree-negative non-*Saccharomyces* yeast strain, followed by *S. cerevisiae* at the moment indicated (continuous line). Aeration would be restricted to the first stages of alcoholic fermentation, as indicated. The expected evolution of ethanol production for a pure *S. cerevisiae* starter in the same conditions is indicated by a dashed line. For simplicity, sugar consumption has been assumed to follow a similar pattern in both situations. Reproduced from Gonzalez et al. (2013) with permission.

MICROORGANISMS AS PROMOTERS OF THE TRANSFORMATION OF GRAPE MUST INTO WINE

Microbial ecology

There is a big diversity of microorganisms associated with winemaking. Grape must is not a sterile medium and the overall process of winemaking is the result of a complex ecological and biochemical interaction between many species of microorganisms (yeasts, bacteria, fungi and viruses) that may be present at different stages of fermentation or during post fermentation treatments of wine, and play a pivotal role in the final product (Bisson and Kunkee, 1991; Heard and Fleet, 1990). The study of the physiology and metabolism of the microorganisms involved in the process of winemaking is of special importance. It is proved that they may contribute to the overall organoleptic quality of the wine, as well as to the wine stability in either a positive or negative way, and to the equilibrium of the final product. These microorganisms can either be found in the vineyard, in the harvest or cellar equipment, and in instruments. Their presence is determined by several factors such as the amount of rainfall prior to the grape harvest, degree of physical damage to the berry, use of fungicides, and time between harvest, crushing, and fermentation (Heard and Fleet, 1990). In this topic will be presented the main microorganisms that are capable of metabolizing the sugars from the grape and/or have some metabolic activity through fermentation that impacts on the final composition of the wine, conferring increased perceived complexity as a consequence of the production of specific metabolic end products.

Organisms with oenological interest

Yeasts

Many species and genera of yeasts are found during wine production. The high content of sugars, low pH and the rapid formation of anaerobic conditions create the ideal environment to support the growth of yeasts over other microorganisms (Bisson, 1999). The microbiota naturally present of the grape includes ascomycete and basidiomycete yeasts. Nonetheless ascomycetes are those that primarily contribute to grape must transformation (Aranda et al., 2011; Ciani et al., 2010; Jolly et al., 2014).

The amount and diversity of yeast species is a key factor for establishing wine quality and composition, because the organoleptic properties of the wine result from several compounds produced as a consequence of yeast metabolism (Romano et al., 1997; Brandolini et al., 2002). Yeasts metabolize grape sugars into ethanol, carbon dioxide and other end-products such as higher alcohols, organic acids, esters, aldehydes, fatty acids, and sulphur compounds that contribute, as already mentioned, to the chemical composition and sensory quality of wine. According to the species of yeasts, their mode of growth and chemical changes associated with their metabolism the final product will acquire different properties.

Saccharomyces

The increment of ethanol levels combined with CO_2 saturation, and anoxic conditions leads to changes in must biodiversity, with the death of most of non-*Saccharomyces* strains and their replacement by *Saccharomyces* strains, better adapted to ethanol toxicity, anaerobiosis, presence of

sulphites, sugar concentration, and higher temperatures (Fleet and Heard, 1993).

The origin of *Saccharomyces* species is subject of controversy. Some authors argue that this species occurs naturally in fruits (Mortimer and Polsinelli, 1999), while others consider that their origin is recent and result from the hybridization of natural species that have been selected in artificial man-made environments (Martini, 1993), and the current isolates of this species are the result of the back-transportation carried out by insects between cellars and fields (Naumov., 1996). Nonetheless, the widespread use of whole-genome sequencing during the last decade has provided new insights into the biodiversity, population structure, phylogeography and evolutionary history of wine yeasts (Marsit and Dequin, 2015). Comparisons between *Saccharomyces* isolates from various origins have indicated a number of likely genomic signatures of domestication of a wild type, that include heterozygosity, nucleotide and structural variations, chromosomic rearrangements (Perez-Ortín et al., 2002; Almeida et al., 2017), copy number variation (Warringer et al., 2011; Almeida et al., 2017), horizontal gene transfer (Novo et al., 2009; Almeida et al., 2017) and introgressions and hybridization. In their totality, these mechanisms contribute to the genetic and phenotypic diversity of *S. cerevisiae* strains. *S. cerevisiae* is not domesticated as a whole and population genetics analysis of both domesticated and a growing number of wild isolates is continuously offering new insights into the ecological distribution, population structure and biogeography of this species (Marsit and Dequin, 2015). Recent work from Almeida et al., (2015), suggests that the closest natural relative of domesticated wine yeasts is a wild *S. cerevisiae* population associated with oaks, in the Mediterranean region. In general, there are evidences that oaks

and other trees of the Fagaceae are the most likely natural habitats of *S. cerevisiae* in temperate regions of the Northern Hemisphere (Naumov et al., 1998; Sampaio and Gonçalves 2008; Wang et al., 2012; Hyma and Fay, 2013). *Saccharomyces paradoxus* is the closest relative to *S. cerevisiae*, according to phylogenetic reconstructions (Rokas et al., 2003; Martinez de Toda et al., 2015). Strains of this species were isolated from natural environments, usually related to exudates from trees, leaves, or unidentified species of *Drosophila* (Naumov et al., 1997; Phaff and Demain 1956). There are evidences that oaks and other trees of the Fagaceae family are most likely the natural habitat of many *Saccharomyces* strains like *Saccharomyces kudriavzevii* and *Saccharomyces arboricolus*. *S. kudriavzevii* has been largely isolated from natural environments such as decaying leaves or oak barks. Like *S. paradoxus*, *Saccharomyces cariocanus* was also isolated from *Drosophila* sp. in Brazil. Other species such as *Saccharomyces mikatae* and *Saccharomyces eubayanus* were isolated from soil and fallen leaves in Japan and *Nothofagus* trees in Patagonia, respectively (Naumov et al., 2000; Libkind et al., 2011; Martinez de Toda et al., 2015).

The genus *Saccharomyces*, previously called *Saccharomyces sensu stricto*, includes the species *S. cerevisiae*, *S. paradoxus*, *Saccharomyces bayanus* (which includes the varieties *uvarum* and *bayanus*), *S. cariocanus*, *S. mikatae*, *S. kudriavzevii*, *Saccharomyces jurei*, *Saccharomyces arboricolus* and *S. eubayanus*. In addition to these species there are numerous documented cases of natural interspecific hybrid strains, as well as hybrid strains commercially produced that are used in industrial fermentations (Sipiczki, 2008; Borneman et al., 2011). *S. cerevisiae* is the main yeast species responsible for industrial scale fermentations such as baking, brewing, cider production, distilling, winemaking, as well as in different

traditional fermented beverages (Fleet and Heard, 1993; Fleet, 2003; Capozzi et al., 2015). *S. cerevisiae* show great genetic diversity and is constituted by numerous strains that offer a variety of technological properties like production of different secondary compounds that result in different amounts of fermentative by-products who can either influence the aromatic and flavour characteristics of the final product in a positive or negative way (Pretorius, 2000; Romano et al., 2003, Capece et al., 2012; Capozzi et al., 2015). Nonetheless, other species from the genus *Saccharomyces* and their interspecific hybrids can also be found at the end of wine, cider and lager beer fermentations. *S. paradoxus* can partially degrade malic acid, helping the biologic deacidification of the wine, besides presenting pectinolytic capacity, which may contribute to wine clarification and filterability (Martinez de Toda et al., 2015), while *S. kudriavzevii* is a cryotolerant yeast that grows well at low temperatures (10-15 ° C) (Belloch et al., 2008, Tronchoni et al., 2014).

Interspecific hybrids carry genomic contributions from both *S. cerevisiae* and other *Saccharomyces* species. Hybridization phenomena have probably been involved in the origin of novel yeast genotypes and phenotypes, adding genomic variation, enhancing genetic flexibility, and promoting adaptive change, which also occurs in other yeast genera of clinical (*Cryptococcus*) or industrial interest (*Zygosaccharomyces*) (Greig et al., 2002; Boekhout et. al., 2001). The conditions in which hybrids arose are still unknown, as well as the number of possible hybridization events that generated the whole set of natural hybrids described in the literature during recent years (Peris et al., 2012). These species have attracted significant interest in the last years due to their potential in solving the main challenges the winemaking industry faces, such as the enhancement of aroma (Tronchoni et al., 2017). The most

well-known hybrids are the lager yeast *Saccharomyces pastorianus*, which is an interspecific partial allotetraploid hybrid between *S. cerevisiae*, *S. uvarum* and *S. eubayanus* (Libkind et al., 2011; Martinez, 2016). In winemaking, hybrid strains are less prominent. However, there are still many documented cases of natural hybrid strains being isolated from wine fermentations (Gonzalez et al., 2006). Many of the hybrid strains that have been isolated from wine involve *S. cerevisiae* in addition to a *Saccharomyces bayanus* family member (*S. uvarum* x *S. eubayanus*) combining a bigger tolerance to ethanol and glucose (*S. cerevisiae*) with a major production of aromatic compounds and glycerol (*S. bayanus*) (Rainieri et al., 2006; Masneuf et al., 1998, Sipiczki, 2008). Interspecific hybrids formed between *S. cerevisiae* and *S. kudriavzevii* have also been isolated from both beer and wine fermentations (Gonzalez et al., 2006, Borneman et al., 2011). These hybrids are well adapted to ferment at low and intermediate conditions of temperature, giving intermediate or higher amounts of glycerol, less acetic acid, intermediate ethanol tolerance, and higher amounts of higher alcohols when compared to the parental strains (Gangl et al., 2009; González et al., 2007; Lopandic et al., 2007). Hybrids are less adapted than their parental strains to specific environmental conditions, but may be better adapted to intermediate fluctuating conditions, which provides them with a selective advantage. On the other hand, the hybrids acquire physiological properties of both parents (Pérez-Través et al., 2015). These physiological characteristics point to *Saccharomyces* hybrids as better adapted to respond the new winemakers trends, such as conducting wine fermentation at low temperatures, which causes wine aroma improvement (Lambrecht and Pretorius, 2000; Torija et al., 2003; Llauradó et al., 2002, Novo et al., 2003).

Non-*Saccharomyces*

As stated above, the organoleptic properties of wine result from the presence of different compounds, including higher alcohols, organic acids, esters, aldehydes, fatty acids and sulphur compounds. These by-products, normally resultant from the metabolism of the various species of yeast present in the must, directly affect the quality of the wine.

Non-*Saccharomyces* yeasts are part of the microbiota of ascomycetes or basidiomycetes naturally present in healthy grapes. These yeasts have vegetative states, which predominantly reproduce by budding or fission and do not form their sexual states within or on a fruiting body (Jolly et al., 2014). Currently there are 149 recognized yeast genera that include nearly 1500 species from which more than 40 have been isolated from grape must (Jolly et al., 2014; Ciani et al., 2010). Non-*Saccharomyces* reach the grapes through the dissemination of the wind and the insects that are present in the vineyards from the beginning of fruit maturity (Lafon-Lafourcade, 1983). 50 to 75% of the species present on the grape surface belong to the genera *Kloeckera* and *Hanseniaspora*, but other species belonging to the genus *Candida*, *Pichia*, *Metschnikowia, Cryptococcus*, *Rhodotorula*, *Kluyveromyces* and *Hansenula* can also be found (Fleet and Heard, 1993; Romano et al., 2003).

In spontaneous fermentations, there is a sequential succession of yeasts. During the first hours, species of *Hanseniaspora, Rhodotorula, Pichia, Candida, Metschnikowia* and *Cryptococcus* are found at low levels, in fresh must (Parish and Caroll, 1985; Bisson and Kunkee, 1991; Frezier and Dubourdieu, 1992; Granchi et al., 1998; Fleet et al., 2003; Combina et al., 2005; Jolly et al., 2014). At this stage, *H. uvarum* is usually present in the

highest numbers, followed by various *Candida spp.* This is usually more apparent in red must than white, possibly due to the higher pH of the first one. However, *Hanseniaspora* can also be absent or present at low levels (Van Zyl and Du Plessis, 1961; Parish and Caroll, 1985; Jolly et al., 2003; Jolly et al., 2006; Jolly et al., 2014).

Despite the sustained presence of certain non-*Saccharomyces* yeasts, most of them disappear during the early stages of a vigorous fermentation, normally due their slow growth and inhibition promoted by the combined effects of the specific environmental conditions as can be the nutrient limitation (caused by *S. cerevisiae* dominance), presence of SO_2, low pH, high ethanol and oxygen deficiency (Fleet et al., 1984; Heard and Fleet, 1988; Combina et al., 2005; Granchi et al., 1998, Henick-Kling et al., 1998; Jolly et al., 2014). The non-*Saccharomyces* strains that survive until the end of fermentation, usually have a higher tolerance to ethanol (Pina et al., 2004; Combina et al., 2005; Jolly et al., 2014). For example, *Zygosaccharomyces bailii* and *Pichia spp.* where reported throughout fermentation (Peynaud and Domercq, 1959; Bisson and Kunkee, 1991).

According to some authors, non-*Saccharomyces* yeasts found in grape must and during fermentation can be divided into three groups:

- Yeasts that are largely aerobic (*Pichia* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Candida* spp., *Cryptococcus albidus*).
- Apiculate yeasts with low fermentative activity (*Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Hanseniaspora occidentalis*.
- Yeasts with fermentative metabolism (*Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Z. bailii*).

The characteristics of the individual species will affect the extent to which they are present, and the contribution of non-*Saccharomyces* yeasts to wine flavour will depend on the concentration of metabolites formed. For a long time, non-*Saccharomyces* yeasts have been associated with problems in fermentation progression, as well as the formation of off-flavours and undesirable compounds such as acetate (*Zygosaccharomyces*) and biogenic amines (*Issatchenkia, Hanseniaspora*) and sulphur compounds (*Torulaspora, Candida*) (Capozzi et al., 2015). However, in this context we talk about non-*Saccharomyces* yeasts taking into account their oenological interest. Several studies carried out in different countries attribute an important contribution of the non-*Saccharomyces* to the growth of the fermentation dynamics developing the complexity of the chemical composition of wine (Pramafeftek et al., 2000; Capozzi et al., 2015). Many non-*Saccharomyces* strains are also able to produce extracellular enzymes that can liberate glycosidically bound aroma constituents that *S. cerevisiae* cannot (Whitener et al., 2017). A summary of the key features, positive or negative, of the main non-*Saccharomyces* wine yeast species is shown in Annex 1.1.

Recent studies using metagenomics techniques have allowed the study of the entire microbial population / community contributing to the identification of new species of yeast in the grapevine microbiome. Some examples include the genus *Cryptococcus* (*C. tephrensis, C. chernovii, C. stepposus*), *Filobasidium* (*F. floriforme*), *Hanseniaspora* (*H. thailandica*) *Rhodotorula* (*R. fujisanensis*), *Schizosaccharomyces* (*S. japonicus*), *Sclerostagonospora* (*S. opuntiae*) and *Sporobolomyces* (*S. coprosmae, S. oryzicola*) (Morgan et al., 2017). However, there is still insufficient information about its effect on

oenological properties, which requires specific studies to avoid any negative consequences and to explore the beneficial contributions.

Lactic acid bacteria

Lactic acid bacteria (LAB) consist of an ecologically diverse group of microorganisms that produce lactic acid as the primary metabolite of sugar metabolism (Lonvaud-Funel et al., 1999; Carr et al., 2002; Liu, 2002) and play a pivotal role in the secondary fermentation of wine through malolactic fermentation (MLF) (Wibowo et al., 1985; Kunkee et al., 1991; Henick-Kling et al., 1993; Lonvaud-Funel et al., 1995). LAB are gram-positive, catalase negative (although some strains can produce pseudocatalase), non-spore-forming, anaerobic or microaerophilic, aerotolerant, acid-tolerant and rod or coccus shaped.

Only a few species of LAB are able to grow and survive in grape must and wine due to low pH, lack of nutrients and presence of ethanol. These environmental conditions determine the native LAB populations and the succession of species and strains before, during and after alcoholic fermentation (Fleet et al., 1984). LAB isolated from grape musts or wine belong to two families that represent four genera. Lactobacillaceae are represented by the genera *Lactobacillus*, and *Pediococcus* and the Leuconostocaceae are represented by the genera *Leuconostoc* and *Oenococcus*. They can be divided into three metabolic categories. Homofermentative lactic acid bacteria ferment hexoses through the Embden-Meyerhof Parnas (EMP) pathway producing two moles of lactate and adenosine triphosphate (ATP) per mole of hexose (Muñoz et al., 2011). On the other hand, heterofermentative and facultative homofermentative bacteria ferment hexoses and pentoses through pentose phosphate and

phosphoketolase pathways producing one mole of lactate, ethanol, CO_2 and ATP per mole of hexose. These bacteria can also use fructose as an electron acceptor that is reduced to mannitol. Consequently, the acetyl phosphate formed during this hexose fermentation is converted to acetate instead of ethanol, originating an extra molecule of ATP. Heterofermentative LAB can also use oxygen and pyruvate as electron acceptor, also leading to the production of acetate and additional ATP (Muñoz et al., 2011). The proportion of these reactions depends on the redox balance and the energy sources present (Binati et al., 2015)

In the first stages of winemaking (must and beginning of fermentation) there are between 10^3-10^4 ufc/ml of LAB from different species, usually homofermentative. The most abundant are *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus hilgardii*, *Leuconostoc mesenteroides*, *Pediococcus damnosus* and to a lesser extent *Oenococcus oeni* and *Lactobacillus brevis*. Their number and proportion vary depending on the state of grape maturity and harvest. However, as the yeast transformation takes place and the microbiota is reduced, the number and diversity of LAB is also reduced, leaving only the most resistant to alcohol and low pH.

LAB are responsible for the production of many acids, being lactic acid the most relevant one. In MLF, mostly *O. oeni*, and *L. plantarum* species are responsible for an enzymatic reaction by which L-malic acid is decarboxylated into lactic acid and CO_2. MLF allows reducing the total acidity of wine, but also induces a change in his organoleptic quality, since the astringent taste of malic acid is replaced by that of lactic acid, which leads to a marked softness in the wine at tasting. During malolactic fermentation the wine also acquires a new aromatic profile due to the elevation of esters (ethyl lactate, diethyl succinate, etc.) and diacetyl ketone cycle metabolites. Other

bacterial metabolites can achieve an equally favourable effect with the reduction of herbaceous aromas, bitterness and astringency. This is often desired in the production of red wines and certain white and sparkling wine styles (Bartowsky et al., 2015; Lepe and Leal, 2004; Plessis et al., 2016).

Other microorganisms

Spoilage microorganisms comprise those filamentous fungi, yeast and bacteria that produce off-flavours. There are three stages at which microorganisms can affect the quality of the final product. The first stage of spoilage involves the raw material and the grape itself. Not all the grapes delivered in the winery are on a healthy state, and when they get in contact with the equipment and machinery that should be properly sanitized, they will also serve as inoculant of the grape juice. The second stage of spoilage may occur during fermentation. At this point the composition of the grape juice (low pH, high sugar and acid content) and the addiction of sulphur dioxide to the juice exert selective pressure on the development of yeasts and bacteria during fermentation (Du Toit and Pretorius, 2000). The third stage in which the product may be subject to change is after fermentation. In this case, spoilage may occur at the time of bottling or storage in barrels since, at this point, the wine may be attacked by yeasts or altering bacteria (Sponholz et al., 1993; Heard and Fleet, 1998; Boulton et al., 1996; Du Toit and Pretorius, 2000). During this stage, contamination through fungus present in corks or oak barrels may also occur (Du Toit and Pretorius, 2000). In this section, we will analyse some characteristics and effects inherent to the metabolism of these altering microorganisms.

Filamentous fungi

Filamentous fungi are usually associated with the wine spoilage because of the interaction between them and the grape, so that contamination of the grape by filament fungi before harvest can be disastrous for wine quality, if not controlled.

In general, filamentous fungi can affect the quality of the wine through losses in juice yield; slippery nature of infected grapes who prolongs the pressing process; alteration of the chemical composition of the wine through the production of gluconic acid, high levels of glycerol and oxidation of phenolic compounds; secretion of β-glucan negatively affecting clarification; production of off-flavours such as acetic acid; stimulation of the growth of altering yeasts and bacteria (Pearson and Goheen, 1994).

Filamentous fungi present in the grape include species of the genus *Alternaria, Aspergillus, Botrytis, Cladosporum, Mucor, Oidium, Penincillum, Plasmopara, Rhizopus and Uncinula* (McGrew 19882; Pearson et al., 1990; Doneche 1993; Fugelsang, et al.,1997, Fleet, 1998; Du Toit and Pretorius, 2000). The species that stands out from this list is *Botrytis cinerea.* This fungus removes all the nutrients necessary for the growth of the berry cells, causing large and important changes in grape composition which include reduction of sugars content such as glucose and fructose and accumulation of metabolites (glycerol and gluconic acid) and enzymes that catalyse the oxidation of the phenolic compounds (Cantoral and Collado, 2011). These wines are also not recommended for aging, as they are susceptible to bacterial oxidation and contamination (Bulit and Dubos, 1988; Coley-Smith, 1980). Although *B. cinerea* causes severe damages in the production of wines, under certain circumstances it can also give rise to wines of excellent

quality in which, through noble rot, acids are consumed in bigger extent than sugars which leading to wines that are more soft, sweet, full bodied and with a pleasant bouquet of aromas (Coley-Smith, 1980).

In an infected vineyard, while the fungus is not exposed, i.e., while it is underneath the grape, it degrades little amounts of sugar due to lack of oxygen. However, as it leaves the grape and has unlimited supply of oxygen, it begins to degrade the monosaccharides in gluconic acid, which cannot be degraded by the yeast during fermentation. This environment allows the growth of non-*Saccharomyces* yeasts such as *Kloeckera apiculata* and *Candida stellata* over *Saccharomyces cerevisiae*, normally producing a large amount of undesirable volatile compounds that affect the aroma and wine taste (Fleet, 1993). In addition, fungi of the genera *Aspergillus* and *Penicillium* can produce mycotoxins such as aflatoxins, patulin and ochratoxin A (Scott, 1977; Boulton et al., 1996; Du Toit and Pretorius, 2000). However, it seems that the winemaking/fermentation processes inactivate these mycotoxins, since they could not be detected in wines produced from infected grapes. Other studies indicate a high incidence of acetic acid bacteria in fermentation of botrytized wines (Bokulich et al., 2012; Pinto et al., 2015; Morgan et al., 2017) that quickly lead to wine spoilage.

As previously reported, in relation to non-*Saccharomyces* yeasts, recent metagenomics studies on fungal communities associated with grapevine reveal the presence of filamentous fungi belonging to the genera *Albugo, Ascochyta, Aspergillus, Alternaria, Penicillium, Cladosporium, Cadophora, Catelunostroma, Chloroscypha*, *Cytospora*, *Didymella*, *Gigaspora*, *Glomium, Haplographium, Holtermannia, Hypholoma, Kabatiella, Mycosphaerella, Pandora, Peniosphora, Piptoporus, Puccinia, Sarocladium, Sclerotinia, Sebacina, Sphaeropsis, Stephanonectaria, Sydowia, Lewia, Davidiella,*

Erysiphe, Veluticeps, Vuilleminia, Zoophthora, Botrytis and yeast like fungus *Aureobasidium pullulans*. (Morgan et al., 2017) recompiles the species of filamentous fungi detected by next generation sequence (NGS) monitoring program in the various stages of wine production. However, there is still insufficient information about the contribution of these species to rot and to the features of the final product.

Lactic acid bacteria

Apart from their positive role of some species during malolactic fermentation, LAB may act as spoilage agents due to their ability to significantly increase the acidity of wines. LAB act in detriment of wine quality when the proliferation of these occurs at the wrong time during the winemaking process.

Acetic acid bacteria

Acetic acid bacteria (AAB) are a group of Gram-negative aerobic bacilli included in the Acetobacteraceae family, characterized by their ability to oxidize alcohol in acetic acid. These are specialized in the rapid oxidation of sugars or alcohols and oxygen plays an essential role in its growth and activity. As the grapes ripen, the amount of sugars increases, as well as the possibility of developing AAB. In healthy grapes, the predominant species is *Gluconobacter oxydans*, although some *Acetobacter* species can also be found in small quantities (Du Toit 2002; Gonzalez et al., 2004; Prieto et al., 2007). On the other hand, damaged grapes, which may be partially fermented, contain high AAB populations favoured by ethanol availability (Barbe, 2001). Under these conditions, most AAB belong to the genus *Acetobacter* (*Acetobacter aceti* and *Acetobacter pasteurianus*) and

Gluconacetobacter. Species of the genus *Gluconobacter* differ from the species of the genus *Acetobacter* because of their tolerance to acidity and inability to completely oxidize the alcohols, with the latter being able to oxidize acetic acid to form CO_2. A property of this type of microorganism is its high tolerance to acidity. Although its optimum pH is 5.5-6.3, these can survive and grow at pH 3.0-4.0 as in wine (Du Toit and Pretorius 2002).

In contrast with LAB acetic acid bacteria are only linked to wine spoilage processes. Acetic acid, acetaldehyde and ethyl acetate formed through the oxidative metabolism of sugars and alcohols are the main spoilage compounds produced by AAB. In addition, through their metabolism AAB can also produce other end products as ethyl acetate and dihydroxyacetone (Bartowsky et al., 2008). Besides that, AAB are able to oxidize glucose to gluconic acid (Fleet, 1993) galactose to galactonic acid and arabinose to arabinonic acid. Therefore, excessive growth of these bacteria on grapes alters the chemical composition of the grape juice, which can affect the growth of yeasts during alcoholic fermentation. Besides that, they are responsible for spoilage phenomena producing acetaldehyde, acetic acid and ethyl acetate.

Endo-spore-forming bacteria

The bacteria of the genus *Bacillus* are Gram positive, catalase negative and endo-spore-forming rods. Gini and Vaughn (1962) found, for the first time, *B. subtilis*, *B. circulans* and *B. coagulans* in spoiled wines. Later, Murrell and Rankine (1979) reported the growth of *Bacillus megaterium* on altered brandy bottles. Lee et al., (1984) grew Bacillus spp. isolated from wine corks. Kunkee (1991) also isolated *Bacillus* from wines of Eastern Europe and noted that the growth of these bacteria in bottled wine did not affect flavour

properties and sediment or haze formation was also limited (Kunkee, 1996). Fleet (1993) reported that growth of *Bacillus* species is accompanied by significant increases in volatile and total acidity. Consequently, they are considered primarily spoilage organisms because volatile acidity is associated with aroma and flavour faults in wine. *B. thuringiensis* was also isolated from fermenting grape juice in a commercial winery. According to Bae et al., (2004), this bacterium could apparently remain viable in wine, however growth and multiplication was inhibited. Campisano et al., (2014) reported the presence of *Bacillus* as part of the endophilic community of grape berries.

The presence of *Clostridium* in altered wines has rarely been reported due to the acidic nature of wines (pH <4.0). These bacteria are obligate anaerobic, spore forming and develop in wines with high pH (pH> 4.0) (Sponholz et al., 1993). For this reason, Clostridia are only formed in heavily de-acidified juices or in juices with low acid content. Therefore, they are likely to constitute a bigger problem due to hot weather where grapes reach maximum ripeness and retain low acid content. Clostridia ferment sugars, producing n-butyric acid, which gives undesirable taint of rancidness, acetic acid, carbon dioxide, hydrogen peroxide and depending on the species, different amounts of butanol, acetone and propanol (Sponholz et al.,1993; Du Toit and Pretorius, 2000).

Although *Bacillus* and *Clostridium* are rare, they should be taken into account, since under appropriate conditions they have the potential to reduce the quality of wine.

Other bacterial species

More recently, high-throughput sequencing techniques have been employed to evaluate the bacterial communities associated with the vineyard. Morgan et al., (2017) reviewed the most abundant phyla found in vineyard soils, flowers and grape berry surfaces (Annex 1.2), detected through high-throughput analysis. According to this author, the relative abundances of the groups vary, depending on the plant tissue or organ. Nevertheless, their possible impact in wine fermentation and wine quality still requires further research.

Starter/Inoculum cultures and sluggish or stuck fermentation

Wine production may proceed either by natural (uninoculated / spontaneous) fermentation or by inoculation with a starter culture. Spontaneous fermentations involve a succession of different yeast species throughout the fermentation (Torija et al., 2002). In the absence of spoilage microorganisms or hurdles for fermentation progression, spontaneous fermentation can add complexity to the wine, contributing to its sensorial characteristics. However, it may also be responsible for a decrease in its quality and spontaneous fermentations often suffer from fermentation stuck and arrests, which leads to more instable wines due to the high levels of residual sugars which make them more prone to contamination, and to the production of undesired metabolites as acetic acid (Capozzi et al., 2011; Capozzi et al., 2015; Mira de Orduña, 2010). Wine quality is tightly associated with changes in the ecosystem such as soil composition, climatic conditions, variety and quality of the grape, as well as with the spontaneous microbiota. During the first two or three days of spontaneous fermentation there is an increase of a large variety of non-*Saccharomyces* yeasts, whose metabolic activities are

responsible for the production of compounds that give a touch of distinction to the final product (Fleet and Heard 1993; Dequin et al., 2001).

At the present, the usual strategy to carry out wine production involves the inoculation of the yeast from selected yeasts in the form of industrial active dry yeast rehydrated or a portion of wine that is already in full fermentation (Boulton et al., 2002). This procedure simplifies the microbiological process of alcoholic fermentation and minimizes the influence of wild yeasts on the quality of the wine. Active dry yeasts represent the easiest and most efficient way of using selected yeasts, especially for large industries, since they allow the availability of large amounts of inoculum without need of preparation prior to harvesting, as in the case of traditional “pie-de-cube”. Active dry yeasts can be inoculated into any amount of grape must by a rehydration process that lasts about 30 to 40 minutes (Suárez-Lepe, 1997). The practice of yeast starter inoculation allows a decrease in the lag phase, a significant reduction in the influence of naturally occurring yeasts, improving biological stability, rapid and complete fermentation of the grape must, a complete utilization of fermentable sugars, increased alcohol production and controlled formation of acetic acid and acetaldehyde and clearer clarification (Zuzuarregui and Olmo, 2004; Dorneles et al., 2003). In addition, the use of a starter culture ensures predominance of *Saccharomyces* during the fermentation. With selected yeasts it is possible to obtain a higher degree of reproducibility in wines from different harvest sessions.

Over the years, yeasts have been the object of research and selection, according to criteria that improve the quality of the wine or to control the technologies that allow obtaining products with regional characteristics with little variability between harvests. The main objective is to choose yeast strains suitable for the grape must that will be fermented and endowed with

oenological properties that are of interest. In this sense, the research and later selection of yeasts try to identify strains of high yield in ethanol that do not produce volatile acidity, with good fermentative kinetics, and resistant to sulphur dioxide, among other characteristics of technological interest (Suárez-Lepe and Iñigo-Leal, 2004). By using selected yeasts, it is possible to avoid certain drawbacks such as slow fermentation due to the presence of agrochemical residues and sulphites, poor presence of yeasts in the fermentation, excessive formation of volatile acids, formation of high and persistent foam, and development of microorganisms not desirable, that directly influence the quality of wine (Zambonelli et al., 1998).

Nonetheless, the use of starter cultures is only justified whenever the selection of the cultures is carefully done, since there are cases in which the fermentation with starter cultures stopped earlier, when compared to other spontaneous cultures under the same conditions, perhaps because they are intolerant to some agent in the medium. Since inoculated cultures impose themselves from the beginning, it becomes impossible for them to be replaced by other yeasts that can continue the process. The starter cultures can dominate the process because they are added at high concentrations, prevailing over the indigenous microbiota. However, several studies show that the transient microbiota plays an important role in the process (de Barros-Lopes et al., 1996). Traditional wineries, in special, continue to use spontaneous alcoholic fermentation because they believe that it provides greater complexity to their wines (Zamora, 2009). Conventionally, wine yeasts have been selected from among the natural yeasts of the grape microbiota, due to the relationship vineyard / yeast in the wine-growing regions. The use of strains isolated from certain regions is an interesting factor. In recent years, there has been an increase in the demand for

autochthonous yeast starters, since these strains present high adaptation to the climatic conditions, and to a defined grape must reflecting the biodiversity of a particular area, promoting the characteristics of wines, in order to overcome the apparent problem of homogeneous or industrial wines; and contributing to the richness of the organoleptic properties of the wine produced through the expression of the aromatic precursor compounds, providing consumers with a wider choice of wine tastes and styles (Ugliano et al., 2009; Bokulich et al., 2014; Capozzi et al., 2015). Apart from the expected contribution to intrinsic wine quality, marketing considerations seem to also play a role in the decision by different companies of using locally isolated wine yeasts.

During the last years, several oenological properties of non-*Saccharomyces* have been reported highlighting their role in winemaking (Rojas et al., 2001; Rojas et al., 2003; Zohre and Erten, 2002; Fleet et al., 2003; Jolly et al., 2003; Ciani and Picciotti 1995; Capozzi et al., 2015). Nowadays, it is accepted that selected non-*Saccharomyces* strains can positively impact on the winemaking process, since they enhance the composition and aroma profile of the wine, as well as they secrete enzymes, produce secondary metabolites as glycerol, release mannoproteins or contribute to colour stability (Padilla et al., 2016).

Nonetheless, it is still recognized that non-*Saccharomyces* present a small fermentation capacity since they are not able to metabolize all the sugars present in the grape must (Ribereau-Gayon et al., 2000, Fleet and Heard, 1993). Accordingly, the design of mixed starters including selected non-*Saccharomyces* and *S. cerevisiae* strains to ensure a complete fermentation has become one important target for biotechnological development in winemaking.

ENERGY METABOLISM OF YEASTS

Respiration, fermentation and regulatory phenomena in yeast

The physiology of *S. cerevisiae* under fermentative, respiratory and respire-fermentative conditions has always attracted attention, considering it is one of the few eukaryotic organisms that can grow under strictly anaerobic conditions, as well as because of its industrial importance for the production of ethanol, proteins, biomass and other by-products.

Yeasts can be characterized according to their process of energy production and to the fate of pyruvate producing by glycolysis. Under respiratory conditions, glucose can be fully oxidized to biomass and CO_2, while under respire-fermentative conditions it will be primarily oxidized to CO_2 and ethanol according to the following metabolic equations.

$$C_6H_{12}O_6 + 6O_6 \rightarrow 6CO_2 + 6H_2O + 36ATP + biomass\ (cellular\ respiration)$$

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_6O + 2ATP\ (alcoholic\ fermentation)$$

Anaerobically, pyruvate is decarboxylated through pyruvate decarboxylase (PDC) to give acetaldehyde. This last one is reduced to ethanol by alcohol dehydrogenase, completing the conversion of sugar to alcohol. Under aerobic conditions, tricarboxylic acid cycle (TCA) is the responsible for the oxidation of nutrients. Carbohydrate, amino acid and lipid derivatives are oxidized to CO_2 and water. When coupled to the respiratory chain, they act as the main energy producers of the yeast cell. In terms of energy recovery, anaerobic processes are much less efficient than aerobic ones. The aerobic ATP yield is higher than what can be theoretically obtained through ethanol fermentation (Bakker et al., 2001). The respiratory pathway yields more than

10 ATP per molecule of glucose, which can support a biomass yield five folds higher (0.5 g) per gram of glucose (Van Dijken et al., 2000) and the fermentation of glucose to ethanol and CO_2 brings a biomass yield 0.1 g biomass per gram of glucose. To respond to a lower ATP yield under fermentative conditions the cell increases glycolytic fluxes.

Some yeasts, including *S. cerevisiae* make use of the fermentation pathway, repressing respiration whether or not oxygen is available, even in the presence of moderate levels of glucose (van Hoek et al., 1998). This is known as the Crabtree effect. This effect will be reviewed in detail below.

In what concerns to sugar metabolism, yeasts can be divided into non-, facultative or obligate fermentative (Van Dijken and Scheffers, 1986). All three types of yeasts are likely to be found in grape juice at some stage during its progression into wine. Non-fermentative yeasts have an exclusively respiratory metabolism, through which they are not able to use glucose in the absence of oxygen, and pyruvate is channelled into TCA cycle to be oxidized. This group includes all species of *Rhodotorula*, *Saccharomycopsis* and *Sporobolomyces*. Some species of *Torulopsis*, *Pichia* and *Hansensula* also belong to this group (Smith et al., 1995). Obligate fermentative yeasts are only able to metabolize glucose through alcoholic fermentation. Under exclusively anaerobic conditions, the only source of ATP generation is substrate-level phosphorylation through fermentative processes that require specific hypoxic genes, since no oxygen is present (Kwast, et al., 1998). On the other hand, in fully fermentative metabolism, ethanol is the main product found, however formation of glycerol, carbon dioxide and smaller amounts of other by-products as acetate and pyruvate are also observed. *Candida sloofii* is a good example of an obligate fermentative species. Most yeasts are facultative fermentative (Boulton et a., 1996). In anaerobic conditions, these

yeasts metabolize glucose to ethanol through alcoholic fermentation, while during aerobic growth both fermentation and respiration may contribute to glucose catabolism. Most yeasts identified as facultative fermentative may exhibit a fully respiratory metabolism, fully fermentative or respirofermentative metabolism, depending on the type and level of carbohydrate concentration of the medium and/or O_2 availability.

Crabtree effect

Several regulatory phenomena are present in yeast species as a response to environmental changes, such as the presence of oxygen or glucose. Table 1.1 summarizes the mechanisms that regulate these responses to certain conditions.

In the nineteenth century, Louis Pasteur observed that yeasts like *S. cerevisiae* showed a bigger consumption of sugars such as glucose, in the absence of O_2 than in their presence. This metabolic response came to be known as Pasteur Effect. This phenomenon is only observed under special experimental conditions, like in continuous cultures with a specific slow growth rate, restrictive glucose levels or in steady state cells.

Table 1.1. Main regulatory phenomena affecting carbon source utilization described for yeasts.

Effect	Observations and remarks
Pasteur effect (1876)	Decrease of fermentation affinity by presence of oxygen (Wyman et al., 2000), associated with a reduced affinity for sugar uptake under aerobic conditions (Lagunas, 1981). Insignificant during growth on glucose, mannose and galactose. Resting cells of *S. cerevisiae* show limited Pasteur effect.
Crabtree effect (1929)	Suppression of respiration by high glucose concentration Initially observed in tumour cells.
Kluyver effect (1940)	Exclusively anaerobic fermentation of glucose, probably due to the slower uptake of sugars in the absence of oxygen; Maltose, lactose and sucrose cannot be fermented (Kaliterna, et al., 1995); Present in yeasts such as *Candida utilis*, *Kluyveromyces wickerhamii* and *Debaryomyces yamadae*.
Custer effect (1966)	Transient inhibition of fermentation by anaerobiosis (Scheffer.et al., 1966); Oxygen stimulates ethanol production due to a lack of intracellular NAD+ (Walker et al., 1998) Not observed in *S. cerevisiae;* Yeasts from the species *Dekkera* and *Brettanomyces* ferment glucose to ethanol faster under aerobic conditions.

Of the listed effects, the Crabtree effect has received special attention from several research teams. It was initially believed that alcoholic fermentation of sugars only occurred during the anaerobic growth of yeasts. However, in 1929, Herbert Crabtree discovered the suppression of respiration in tumour cells, when they were subjected to high concentrations of glucose. This

author observed that glucose was acting as an inhibitor of the respiratory metabolism of tumour cells. In 1948 Swason and Clifton showed that *S. cerevisiae* catabolizes glucose mainly by fermentative processes. Nevertheless, it was not until 1966 that De Deken recognizes this mechanism in *S. cerevisiae*, describing the Crabtree effect as “the phenotypic expression of a regulatory system involved in the synthesis of cytochromes”. In that work, de Deken detected an excess of CO_2 and ethanol formation during the aerobic growth of *S. cerevisiae*. This author also noted that at high glucose concentration, the fermentation rate increased. On the contrary, at low glucose concentrations the rate of respiratory increased and the fermentation rate was reduced. de Deken attributed the Crabtree effect to the inhibition of the synthesis or activity of respiratory enzymes (Alexander and Jeffries, 1990; De Deken, 1966). Until the 1980s, glucose repression and Crabtree where considered synonyms. The Crabtree effect was then divided into long-term and short-term effects, based on continuous cultures where, under steady-state conditions, the growth rate could be experimentally manipulated (Petrik et al., 1983). The long-term Crabtree effect characterizes the respirofermentative metabolism observed in batch cultivations or continuous cultures above critical dilution rates and is attributed to an insufficient respiratory capacity due to the repression of respiratory genes (Postma, 1989). The short-term Crabtree effect is defined as the ability of triggering alcoholic fermentation upon transition from glucose limitation to glucose excess (Van Urk et al., 1988). The Crabtree effect is currently defined as the occurrence of alcoholic fermentation under aerobic conditions (Pronk et al., 1996). Different hypotheses have been suggested in order to explain this phenomenon (Beck and Von Meyenburg, 1968; Polakis and Bartley, 1965; Sonnleiter and Kappeli, 1986), being overflow at the pyruvate metabolic branching point the most accepted one.

Facultative fermentative yeasts can be divided according to the Crabtree effect. As described above *S. cerevisiae* is a Crabtree positive yeast, able to perform alcoholic fermentation of glucose when growing aerobically (Papini, 2012). Nonetheless, the separation of facultative fermentative yeasts in Crabtree-positive and Crabtree-negative is not strict. Some Crabtree negative yeasts can undergo a deregulation of the metabolism that results in the formation of ethanol and other metabolites, under strictly aerobic conditions. In dense cultures of *Kluyveromyces marxianus*, for example, aerobic production of ethanol and organic acids can occur under some cultivation conditions. Likewise, *Kluyveromyces lactis* can grow in the absence of oxygen (Gonzalez-Siso, et al., 1996). Table 1.2 summarizes the main species categorized as Crabtree positive or Crabtree negative.

Table 1.2. Classification of several yeast species according to their Crabtree features.

Crabtree positive	Crabtree negative
Saccharomyces cerevisiae	*Hanseniaspora uvarum*
Zygosaccharomyces bailii	*Pichia anomala*
Brettanomyces intermedius	*Candida utilis*
Torulopsis glabrata	*Hansenula neofermentans*
Schizosaccharomyces pombe	*Kluyveromyces marxianus*
Hanseniaspora guilliermondii	*Debaryomyces hansenii*
Candida stellata	*Torulasporula delbrueckii*

Carbon catabolite repression

Carbon catabolite repression (CCR) (Ronne, 1995, Carlson, 1998, Gancedo, 1998) is a phenomenon present in *S. cerevisiae* and many other free-living microorganisms by which the presence of glucose triggers the repression of enzymatic activities, essential for the growth on other carbon sources (Santangelo et al, 2006, Papini, 2012). There are at least two pathways for CCR in *S. cerevisiae*, which include the main pathway in which the Mig1 protein takes part, and the cAMP-protein kinase A (cAMP-PKA) pathway. Transcriptional repressors such as Mig1, Cat8, and Cyc8/Tup1 complex prevents the transcription of glucose-repressed genes, such as those implicated in gluconeogenesis and metabolism of alternative carbon sources. The activity of these repressors is mediated by kinases and phosphatases such as Snf1 and Glc7/Reg1, respectively (Lane et al., 2017).

The activity of Snf1 is regulated by the availability of glucose (inhibited by glucose and activated when it is limiting) (Rolland et al., 2000). This protein reacts to the decline of glucose promoting respiratory metabolism, glycogen accumulation, gluconeogenesis, glyoxylate cycle, autophagy, peroxisome biogenesis, as well as regulating acetyl-CoA homeostasis and histone acetylation (recently reviewed by Conrad et al., 2014). Snf1 also represses anabolic processes, such as biosynthesis of fatty acids and amino acids. Snf1 plays a central role in the shift from fermentation to respiration, by regulating a series of repressors and suppressors. Snf1 interacts with the Mig1 transcriptional repressor that binds to DNA. The repressing operation through Mig1 depends on the intracellular localization of this protein, which depends on phosphorylation and the active or inactive state of the Snf1 protein (Ostling and Ronne, 1998; Treitel et al., 1998). Snf1 works as part of a heterotrimetric protein complex composed of Snf1 as catalytic kinase

subunit, a γ-like regulatory subunit – Snf4 and a β-subunit encoded by *GAL83*, *SIP1* or *SIP2* (Jiang and Carlson 1997; Conrad et al., 2014). The state of activation of the Snf1 protein depends on a number of factors: interaction with the Snf4 protein (Cat3); interaction of the catalytic and regulatory regions of the Snf1 protein itself; physical binding to Sip1, Sip2 and Gal83 proteins (Gancedo 1998, Ludin et al., 1998; Vincent and Carlson et al., 1999). During growth on optimal glucose levels, Snf1 is inactive and excluded from the nucleus (Kayikci and Nielsen, 2015). This allows Mig1 to localize into the nucleus where it binds to the promoters of glucose-repressible genes (Rolland et al., 2010). Upon glucose depletion and increase in ADP levels, ADP binds to the weaker site of Snf4-activating subunit, inducing a conformational change that protects the active Snf1, counteracting its auto-inhibitory activity (Rolland et al., 2000; Wilson et al., 1996; Mayer et al., 2011); thus, allowing Mig1 phosphorylation and translocation to the cytoplasm (Rolland et al., 2010).

Mig1 is responsible for the recognition of a specific sequence in the promoter region of many of the glucose-repressed genes. Mig1 protein recognition sequences are rich in GC (Nehlin and Ronne, 1990) with the consensus region (G/C)(C/T)GGGG and a A-T-rich region 5' to the GC box (Gancedo 1998). Mig1 recruits the activity of Tup1 and Cyc8 (Ssn6) which repress transcription by preventing binding of RNA polymerase (Wu and Trumbly et al., 1998; Younge et al., 2003; Roth et al., 2004; Kayikci and Nielsen, 2015). Mig1 also interacts with Hxk2, a moonlighting protein acting as hexokinase in the glycolytic pathway, and as transcription factor in the nucleus. When glucose is abundant, Hxk2 interacts with Mig1, preventing Snf1 phosphorylation and removal of Mig1 from the nucleus. Data suggest that Hxk2 is phosphorylated and dephosphorylated by Snf1 and Reg1/Glc7

complex, respectively. This prevents its nuclear localization and interaction with transcription factors (Kayikci and Nielsen, 2015).

Cat8 is another transcription factor regulated by Snf1 activation, required for gluconeogenesis, and survival on alternate carbon sources. While the removal of Mig1 repression by Snf1 allows the upregulation of *CAT8* expression, Cat8 phosphorylation by Snf1 depletes its activation (Kayikci and Nielsen, 2015). Cat8 is also regulated by the protein Hap2. Depletion of *MIG1* or *HAP2* reduces the repression of *CAT8* by glucose. In addition to being repressed by glucose, the expression of genes involved in gluconeogenesis, glyoxylate cycle, and utilization of non-fermentable carbon sources depends on the induction of a pathway involving Cat8. Cat8 is also required for the repression of genes under non-fermentative growth conditions.

Snf1 also activates transcription factors responsible for induction of gluconeogenesis, such as Sip4 and Rsd2 (Vincent and Carlson, 1999; Roth et al., 2004: Conrad et al., 2014). Besides that, it triggers stress response genes through phosphorylation of transcription factors such as Hsf1 and Msn2, and induces genes involved in the β-oxidation of fatty acids and ethanol.

Another element that forms part of the glycolysis repression is the transcription activator Hap4. This forms a complex with the Hap2, Hap3 and Hap5 proteins, targeting them to the promoter regions of genes involved in the TCA cycle and the respiratory chain, activating their transcription (Rosenkrantz et al., 1994). Hap4 is repressed by glucose and regulated by Snf1.

S. cerevisiae also adjust diverse cellular activities in accordance to the extra and intracellular amounts of glucose present in the media, through the mobilization of different hexose transporters in conformity to sugar levels. These hexose transporters play an important role in glucose repression as they act on delivering glucose to the glycolytic pathway, contributing to determine the extent of fermentation and respiration (Ye, et al., 1999, Papini, 2012), being thus closely related to the Crabtree effect.

Transport and sugar phosphorylation are subject to complex regulation by glucose, through different mechanisms of signal transduction. There is a link between glycolytic flux and the membrane composition of sugar transporters. Among the large number of hexoses (*HXT* genes) transporters found in *S. cerevisiae*, there are two genes that are expressed at a lower level compared to most HXTs that play a specific regulatory role. Snf3 and Rgt2 are glucose membrane sensors that internalize information about extracellular glucose concentration and induce the expression of other HXTs. Snf3 is repressed by high glucose concentrations, while Rgt2 is constitutively expressed. Besides playing an important role regulating yeast growth rate, the low affinity of glucose to Rgt2, and the high affinity of Snf3, initiates a signal cascade that ends in the regulation of the glucose transporters (Lane et al., 2018). This allows the cell to feel and respond to high glucose levels, through the expression of low-affinity glucose transporters. In response to high glucose levels, the sugar transporters Hxt2, Hxt6 and Hxt7 undergo endocytosis and are degraded in the vacuole due to tight regulation on yeast membrane. In contrast, Hxt1 and Hxt3 are integrated into the cell after induction by the Rgt2 sensor.

The second signal transduction pathway for glucose repression is the cAMP-protein kinase A pathway (cAMP-PKA), or via Ras-cAMP. In *S. cerevisiae*,

this pathway controls a wide variety of cellular functions, in correlation with fermentation and cell proliferation, and plays a key role in metabolic balance, and tolerance to stress. The Ras protein serves as a mediator of intracellular glucose sensing for the activation of cAMP synthesis, along with a G-protein-coupled-receptor (GPCR) system for extracellular glucose sensing (Conrad et al., 2014).

The enzyme adenylate cyclase (AC) catalyses the synthesis of cAMP from ATP. It activates cAMP-dependent protein kinase A (PKA), when binding to its regulatory two subunits (encoded by *BCY1*), which promotes de dissociation of the catalytic protein kinase subunits (Tpk1, Tpk2, Tpk3) and activation of PKA.

In cells growing on non-fermentable carbon sources or in stationary phase, glucose addition triggers a rapid and transient increase in cAMP levels, which initiates the PKA phosphorylation cascade. Extracellular glucose signal is transmitted through two systems: through GPCR system, which involves the proteins Gpr1 and its associated Gα protein Gpa2, and controls the glucose-induced activation of cAMP synthesis (Rolland et al., 2002); and through an intracellular system dependent on glucose uptake and hexokinase mediated phosphorylation that activates the Ras protein (Rolland et al., 2000). AC is controlled by two G proteins (Ras 1 and Ras 2) that mediate the two branches of the glucose-sensing pathway. GPCR system that senses extracellular glucose is unable to activate AC if the latter is not made responsive by the activation of the Ras proteins (Rolland et al., 2000). The activation of the Ras proteins requires glucose transport and phosphorylation. The activation of AC is suppressed by glucose. This seems to confine the physiological role of this pathway to a short period of transition between the repressed and

derepressed states through the cAMP triggered protein phosphorylation cascade.

Levels of metabolism regulation in the cell

The activation of pathways contributing to sugar metabolism in yeasts depends on environmental factors, such as the type and amount of carbon source available, and the presence or absence of oxygen. Metabolic pathways are up- or down-regulated by the cell to meet nutrient requirements in changing environments, and to maintain homeostasis, ensuring sufficient intracellular metabolite pools. The metabolic regulation of the cell can occur at different level through several mechanisms:

- Gene level. Steady state mRNA abundance depends on both transcription rates and mRNA degradation, which define the mRNA turnover for each gene in a given environmental condition and metabolic state. In addition, the rate of mRNA translation can be regulated.

- Enzyme level. Enzyme regulation depends on mechanisms as feedback inhibition and allosteric activation, as well as co-factor availability, and allows quick adaptation to changing metabolic states.

- Compartment level. The localization of a certain protein in a compartment (mitochondria, peroxisome, glyoxysome, cytoplasm, nucleus, etc.) is crucial for its activity. Changing the intracellular localization of enzymes or transcription factors is also a quick way of metabolic adjustment (Papini et al., 2012).

Metabolic regulation

Regulation of the glycolytic pathway

Glycolysis is the pathway responsible for splitting a molecule of glycolysis into two pyruvates, producing one molecule of ATP and two molecules of NADH. Additionally, it is also responsible for generating the biomass precursors (3PG and PEP). Different glycolytic enzymes are subject to allosteric regulation. The presence of ATP inhibits the activity of Hxk2, which catalyses the first step of glycolysis, controlling the glycolytic flow (Larsson et al., 2000). The presence of glycolytic metabolites also affects the transcription of the genes involved in this pathway, and consequently the fluxes. In Crabtree positive yeasts, low concentrations of G6P and F6P stimulate respiratory flow, whereas F1-6bP inhibits respiration (Diaz-Ruiz et al., 2008). Strains with altered hexose transporters show reduced glycolytic flux and decreased ethanol production (Elbing et al., 2004).

At the end of glycolysis, pyruvate represents an important branch point in yeast central carbon metabolism. Pyruvate can follow three metabolic fates, depending on the yeast type and the environmental conditions to which the cell is subject (Pronk et al., 1996). It can be directly converted to acetyl-CoA by the mitochondrial pyruvate dehydrogenase (PDH) multienzyme complex after its transport to the mitochondria. Acetyl-CoA is metabolized in the TCA cycle to generate reducing equivalents used in mitochondrial respiration. However, when the cell is in a preferably fermentative mode, pyruvate remains in the cytoplasm where it can be converted to acetyl-CoA through the production of acetaldehyde by pyruvate decarboxylase (PDC), codified by *PDC1*, *PDC5*, *PDC6*, and successively acetate by acetaldehyde dehydrogenase (ALD). Acetyl-CoA synthase (ACS) converts acetate to

acetyl-CoA by the PDH bypass pathway (Pronk et al., 1996). Pyruvate can also be converted to acetaldehyde by PDC and successively reduced to ethanol by alcohol dehydrogenase (ADH) with reoxidation of NADH. After diauxic shift, ethanol can be metabolized by the respiratory pathway leading to the coupled formation of ATP.

The PDH bypass requires the activity of 3 distinct enzymes: PDC (whose activity is regulated by the intracellular levels of different metabolic derivates generated during glycolysis), ALD and ACS. At low glycolytic concentrations, the mitochondrial PDH multienzyme complex has more affinity for pyruvate, which will cause more pyruvate to flow into it. However, when the glucose concentration increases, the glycolytic rate also merges, causing an increment in the formation of pyruvate, which leads to a saturation of the PDH bypass and a shift of carbon flow to the ethanol production and beginning of the fermentation (Steensma et al., 1997).

Redox balance regulation

Redox balance plays a central role in yeast metabolism. The reoxidation of NADH occurs in the compartments where it is generated, since the internal mitochondrial membrane is impermeable to pyridine nucleotide coenzymes. Therefore, the turnover of NADH occurs at high rates, both in the cytoplasm and in the mitochondrial matrix. In contrast, NADPH turnover occurs predominantly in the cytoplasm, through the pentose phosphate pathway (PPP) (Albers et al., 1996). In general, it is accepted that the two coenzymes disclose different functions. NADH participates mainly in dissimilatory processes on aerobic conditions and is an intermediary for the production of

ATP. On aerobic conditions, NADPH also functions as a dissimilatory coenzyme to produce ethanol. On the other hand, NADPH is predominantly implicated in assimilatory reactions, along with biosynthesis and energy precursors (ATP) (Figure 1.2)

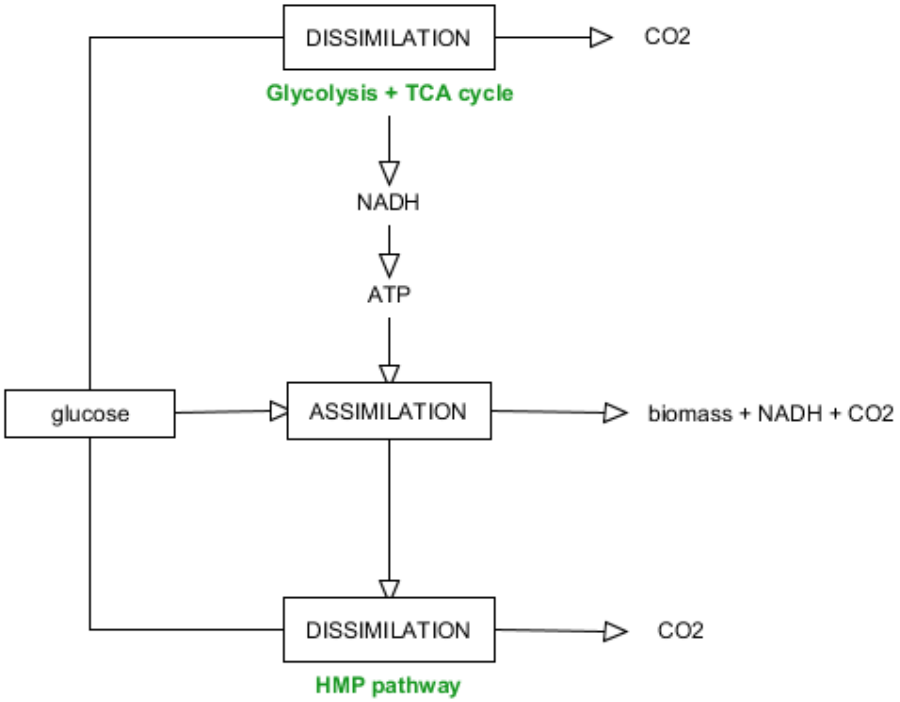

Figure 1.2. Schematic representation of glucose metabolism in yeast. Assimilation of glucose requires energy (ATP) and reducing power (NADPH). From Bruinenberg et al., (1986).

When *S. cerevisiae* grows in glucose as carbon source, the major source of NADH production, in the cytosol, is the glycolytic pathway. NADH is formed during the conversion of glyceraldehyde-3-phosphate 3PG to 1-3

bisphosphoglycerate 1,3BPG. When the glycolysis intermediates are required to server as biomass precursors, an excess of NADH is formed in the cytosol (van Dijken and Scheffers, 1986). Cells must re-cycle the NADH, since NAD+ is essential for the progression of glycolysis, otherwise the glycolytic flow is reduced, potentially leading to the depletion of ATP, which could be lethal to the cell (Verduyn et al., 1990).

Reduction of NAD+ may also occur in the mitochondria by the action of the mitochondrial PDH multienzyme complex PDH and dehydrogenases from the TCA cycle (Pronk et al., 1996). Under aerobic conditions, the formation of intermediates in the TCA cycle allows the cyclic regeneration of NADH to be used in energy production. The succinate dehydrogenase complex is directly coupled to the respiratory chain as the $FADH_2$ produced during oxidation of succinate to fumarate acts as an electron donor for ubiquinone (Cimini et al., 2009).

Acetic acid formation may also play a minor role in the formation of cytosolic NADH on aerobic conditions (Nissen et al., 1997; van Dijken and Scheffers, 1986).

Aerobically, there are different systems to guide the excess of cytosolic NADH to the mitochondria, contributing to the ubiquinone pool of the respiratory chain. Yeast mitochondria contain an internal mitochondrial NADH dehydrogenase (Ndi1), as well as two external (to the mitochondrial matrix) mitochondrial isoenzymes (encoded by *NDE1* and *NDE2*). These isoenzymes catalyse the transfer of cytosolic NADH to the respiratory chain. Studies performed by Bakker et al., (2000) in ndi1Δ strains showed the existence of an ethanol-acetaldehyde shuttle that transfers redox equivalents from mitochondria to the cytosol. In this shuttle, the acetaldehyde formed in

cytosol diffuses to the mitochondria where it is converted into ethanol by Adh3, consuming NADH. The formed NAD+ is once again used for the operation of the TCA cycle and the formed ethanol can diffuse into the cytosol where it is converted to acetaldehyde by the action of Adh2, instead of being secreted by the cell. This latter reaction consumes a molecule of NAD+ leading to the formation of NADH which is thus channelled into the respiratory chain through the NADH external dehydrogenase (Bakker 2001).

The glycerol 3-P shuttle is another indirect method that allows the oxidation of cytosolic NADH (Larsson et al., 1998). Through Gpd1 and Gpd2, dihydroxyacetone-phosphate (DHAP) formed during glycolysis is converted to 3PG using NADH as a cofactor. 3PG enters mitochondria and is converted to DHAP again by Gut2, using FAD+ as a cofactor. The $FADH_2$ formed in this reaction enters the electron transport chain and DHAP returns to the cytosol, giving continuity to the cycle (Larsson et al., 1998). The glycerol 3-P shuttle seems to be more efficient when the energy is limited.

Glucose repression of *NDI1*, *NDE1*, *NDE2* and *GUT2* suggests a minor contribution to cytosolic redox balance by these mitochondrial-based mechanisms during growth on high glucose concentrations and that cellular-based systems, other than NADH dehydrogenases participate in the oxidation of NADH (Bakker et al., 2001; Larsson et al., 1998; Kutyna et al., 2010).

With increasing glucose concentration under aerobic conditions, respiration is only partially suppressed. This inactivates the glycerol 3-P shuttle, since Gut2 is suppressed by glucose. On high sugar concentrations, the cytosolic redox balance is restored by the production of metabolic by-products, such as glycerol, ethanol and acetic acid. In the absence of oxygen, NADH surplus

is generated by the formation of biomass, as well as acids or glycerol (Gancedo 1968), in order to maintain a closed cytosolic redox balance. Thus, the activity of NADH external dehydrogenase may explain the reduction of glycerol formation under aerobic conditions when compared to cultures in anaerobiosis.

Alcoholic fermentation of glucose is used by yeast to re-oxidize excess of cytosolic NADH (Overkamp et al., 2000). When *S. cerevisiae* is supplied with an alternative heterologous oxidase, reduced aerobic ethanol fermentation is observed (Vemuri et al., 2007), thus suggesting that, in *S. cerevisiae*, the onset of fermentation is a consequence of the limited respiratory capacity (Papini et al., 2012)

During sugar fermentation, the role of NADPH is limited, since the higher alcohol dehydrogenase is NAD+ dependent. *K. lactis* uses PPP for glucose dissimilation when glycolysis is blocked, which means that in these species, the oxidation of cytosolic NADPH generated in PPP can be efficiently coupled with mitochondrial respiration by dispersing excess NADPH. However, this coupling mechanism is absent in *S. cerevisiae*. It was shown that *S. cerevisiae* have low catabolic fluxes through the PPP (Blank and Sauer, 2004). This phenomenon is accompanied by an increase in the production of CO_2 and the secretion, in smaller quantities, of other metabolites such as acetate, pyruvic acid (van Dijken et al., 1993) and glycerol, needed to maintain redox balance (Walker, 1998). These observations indicate that PPP, in Crabtree positive yeasts, is predominantly used for NADPH production for biosynthesis, but not for catabolic reactions, or as a pathway for NADPH dissimilation.

The distribution of carbon flux between glycolysis and the PPP seems to play a more important role in glucose dissimilation in Crabtree negative yeasts than in Crabtree positive ones (Bruinenberg et al., 1983; González-Siso et al., 2000) (Figure 1.3).

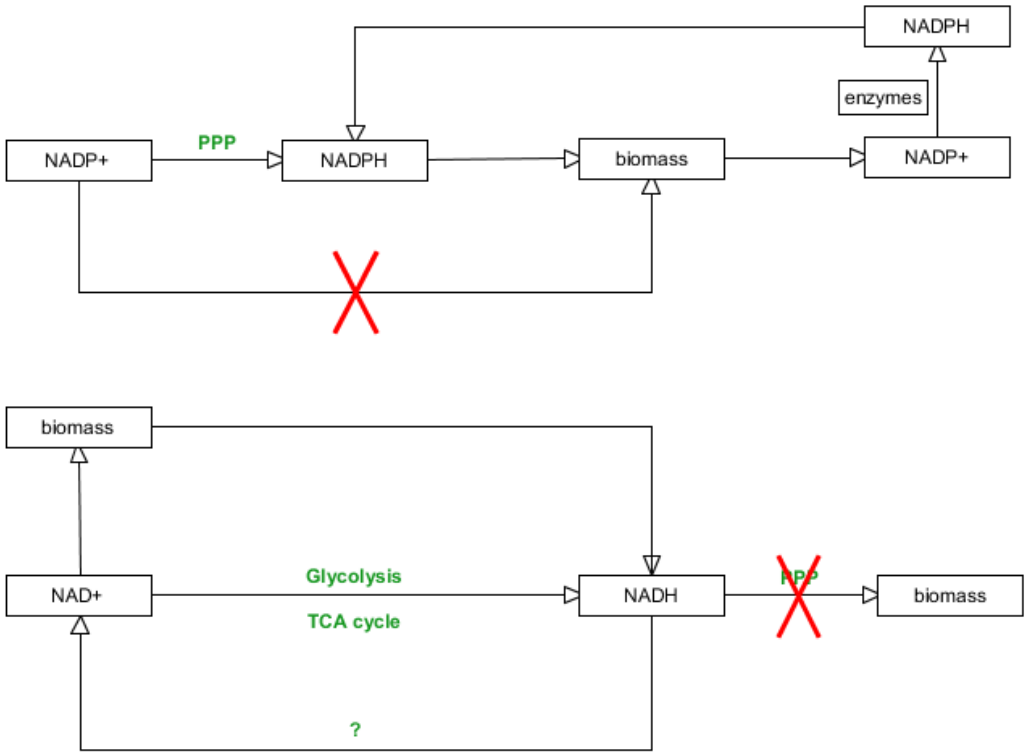

Figure 1.3. Carbon flux distribution in Crabtree-positive yeasts according to the use of the PPP an glycolysis/TCA pathways.

TCA cycle regulation

The TCA cycle is repressed by glucose at the transcriptional level (Liu and Butow, 1999). High glucose concentrations cause transcriptional repression of the genes from the TCA cycle and respiration. Citrate synthase (CIT) catalyses the formation of citrate from acetyl-coA and oxaloacetate. This enzyme suffers allosteric inhibition when the levels of ATP/ADP and NADH/NAD+ are elevated. This enzyme can also be activated by citrate and succinyl-coA. Only mRNAs corresponding to *CIT2* and NADP-specific isocitrate dehydrogenase (IDP1) have been reported to be over-expressed at high glucose concentrations (Yin et al., 2003; Papini et al., 2012).

Regulation of the mitochondrial respiration

Mitochondrial respiration allows, through oxidative phosphorylation, to generate energy (ATP) by the reoxidation of reduced cofactors under aerobic conditions and generation of a protonmotive force used by mitochondrial ATP synthase (Rosenfeld and Beauvoit, 2003). During this process, NADH produced in the mitochondrial matrix is oxidized to NAD+ (Bakker et al, 2001). Nevertheless, yeasts can also oxidize the cytosolic NADH through an external NADH dehydrogenase, as well as through the glycerol-3-phosphate shuttle, coupling the oxidation of cytosolic NADH to the respiratory chain. All NADH oxidation pathways converge to mitochondria, more properly to the pool of ubiquinone donating electrodes to cytochrome c, whose oxidation is catalysed by cytochrome c oxidase, through the bc1 complex (de Vries and Marres, 1987). Some of these genes (e.g. *CYC1*) exhibit reduced expression

in response to specific environmental conditions such as anaerobiosis or high concentration of glucose (Marykwas and Fox, 1989).

METABOLIC ENGINEERING

Metabolic engineering (ME) has been defined as the rational modification of the metabolism of the cell, using recombinant DNA technology, as well as other techniques of cell biology (Lee et al 2009; Nielsen et al., 1998). It also provided a valid alternative to traditional methods of strain improvement through genetic engineering techniques like random mutagenesis, which despite being successful, are time consuming and generate mutations that are often not beneficial, neither contribute to the desired characteristics. ME strategies seek to improve cellular activities by modifying metabolic pathways or cell-specific functions, such as enzymatic activities, transporters and regulatory functions, with the aim of producing new metabolites, heterologous peptides, as well as optimize and improved new and existing processes in order to increase the production of substances at lower cost (Blazeck and Alper, 2010, Golovleva and Golovlev, 2000, Steensels et al., 2014). ME is a systematic approach that differs from other biotechnological strategies because it focuses on understanding the cell's largest metabolic networks. This approach considers detailed information on both the regulatory and the intracellular reaction networks, allowing the identification of gene targets that may not be intuitive when relying only on extracellular or activity measurements.

Classical ME strategies are based on the identification of rate-limiting steps in a given pathway to alleviate their regulation by overexpression of genes that encode specific enzymes, recruit heterologous activities or overexpress genes in the production host. This can be achieved through the use of recombinant DNA technologies, as well as other cell biology techniques for the introduction, deletion and modification of metabolic pathways (Lee et al., 2009; Nielsen, 1998; Bailey et al., 1991). ME has allowed great advances in understanding the operation of thousands of genes in various organisms, as well as in the development of theoretical and experimental tools to identify bottlenecks and to determine the flow of metabolites through different pathways (Papoutsakis, 2000; Varma and Palsson, 1994). It has also provided clues about many potential cellular targets whose modification may lead to the production of phenotypes of interest for research or industry that present a metabolism with improved properties and optimized for a given process as may be increasing the production of high-value products in living cells.

Metabolic engineering has been updated to the systems level by the introduction of global technologies of systems biology and synthetic biology. Several techniques, including high throughput screening, in silico modelling and simulation, genomics, transcriptomics, proteomics, metabolomics, fluxomics, gene synthesis, synthesis of regulatory circuits, engineered enzymes and pathways have been applied for metabolic engineering (Jang et al., 2012; Park et al., 2008; Park et al., 2009).

Systems biology

Systems biology (SB) is a highly interdisciplinary field of research that encompasses multiple techniques, in order to analyse relevant data and properties. The main objective of SB is to reveal the fundamental principles of biological systems to enable the understanding of biological pathways as a complex, and furnish a better understanding of the operational cell. In order to reach this more integrated view of the functioning of the cell, SB needs to be able to deal with different sources of information and knowledge on biotechnological processes (heuristic and hidden in raw data knowledge), and the integration of large amounts of generated data.

The omics reversed the way we study any biological system by allowing us to analyse and understand whole groups of macromolecules like DNA, RNA proteins and metabolites as a whole. The genome sequence only represents a fragment of the capabilities of an organism, providing few indications of other crucial aspects related to the life cycle of the cell. Taking this into consideration, other levels of knowledge must be taken into account (Fondi and Lió, 2015)

Genomics can be defined as the systematic comprehensive study of the genome of organisms, in order to understand the expression of the genes and their interactions (McKunsick, 1997). It can be applied to study genome sequence information and gene function, allowing understanding differences between strains and differences between metabolic pathways associated with stress response and environmental changes, for example.

Transcriptomics, on the other hand, seeks to identify changes in gene expression at the whole genome level. It addresses mRNA of a cell or

organism. It provides functional insights since many functional genes that belong to the same metabolic pathway have proved to be co-expressed. Transcriptomic data are commonly generated through hybridization arrays, or mRNA reverse transcription and sequencing (RNAseq). Transcriptomics is one of the most commonly used omics for data integration in genome scale models due to the relative easiness of generating data and the fact that they are genome wide, unlike most omics data (Sanchez and Nielsen, 2015; Fondi and Lió, 2015).

Proteomics refers to the study of the structure, function and abundance of the entire set of proteins throughout the cell. In a certain condition, it allows to identify and characterize complete protein sets, as well as possible interactions who may occur between them that dynamically represent genes and their environment. Proteomic data are usually combined with complete transcription data in order to establish a complete cell map of pathways and networks that allows understanding the functional relevance of proteins. It can be applied for the identification of function of proteins, protein-protein interaction and activation by phosphorylation. There are several methods available to conduct proteomic studies that allow quantification of the level of expression of cellular proteins. Some examples are 2D-gel electrophoresis, gas or liquid chromatography couples to mass spectrometry analysis, iTRAQS and Networks, glycoproteomics pipelines (Hitchen and Dell, 2006), phosphoproteomics approaches (Lin et al., 2010), protein-protein interaction. Proteomics is the last omic to be introduced in genome scale models due to its intrinsic complexity. Another disadvantage lies in the fact that enzymes are the worst annotated elements of the genome scale models, since only a fraction of EC numbers are present and high order relationships between

enzymes and reactions are often incomplete or non-existent (Sanchez et al., 2015).

Metabolomics provides the metabolic profiling of a given organism in the form of an instantaneous snapshot of the physiology of that cell. Metabolomics measures all metabolites (intra and extracellular) present in the cell in a certain moment (Villas-Boas et al., 2005; Villas-Boas et al., 2006; Mappelli et al.). The metabolome is the final product of genetic transcription. Changes in the metabolome are magnified relatively to changes in the transcriptome and proteome due to developmental responses, genetics, or environmental stimuli. Metabolomics allows to identify and quantify metabolites as key compounds to elucidate a certain metabolic behaviour. It has special relevance for the characterization of the consequences of genetic variation in wine-making processes, since wine represents the combined metabolome of the grape, yeasts and bacteria present during fermentation.

Different quenching and extraction methods are available for intra and extracellular metabolome analysis, but as for proteomics, mass spectrometry plays an important role in metabolomics, being the main method used in identification of analytes, in conjunction with high performance liquid chromatography. Due to the large amount of data generated in these studies and the amalgam of metabolites originated it was necessary to develop sophisticated bioinformatic tools. Metabolomic information can be automatically computed starting from genome annotations. Derived metabolic reconstructions (models) can be exploited for *in silico* metabolic modelling and simulation, using constraints-based methods, as for example Flux Balance Analysis (FBA), that are currently widely adopted (mainly because they do not require detailed information on the chemical equations of the studied system) (Fondi and Lió, 2015)

Fluxomics seeks to integrate metabolite levels with their fluxes, providing information about the activity of pathways and topology of the metabolic network (Nielsen et al., 2003). Through measurements of the ensemble of metabolic fluxes active in the cell, under a certain condition, it provides a model of cellular reaction networks (Wiechert, 2001, Witmann et al., 2007). Often determination of flux distribution is based on ^{13}C labelling and analysis of enrichment patterns of proteinogenic amino acids and is often used in genome scale modelling since it is easy to integrate with FBA modelling.

Interomics is the last level of Systems Biology. It aims to compare and integrate all previous levels and essential to create a final view of the entire system.

Metabolic systems engineering/Genome-scale metabolic models

Metabolic systems engineering can be considered as a broader version of ME that, in conjunction with SB, provides strategies that allow elucidate specific problems on strains physiology that otherwise would get unnoticed (Park et al., 2008).

Mathematical models thus emerge as approximate and standardized representations of knowledge of elementary processes, composed of a series of components, interaction rates, and physical laws that regulate the reactions in which they are involved (Durot et al., 2009; Fondi and Lió, 2015).

Metabolic models can be used to explain or to predict the behaviour of a system, since they allow analysing and simulating cellular metabolism in response to different stimuli, under various environmental and genetic

conditions (Kim et al., 2018; Liu et al., 2010). They allow the design of desirable strains, through systematic test and prediction of manipulations, such as gene deletion or expression modulation that generate suboptimal phenotypes for a specific application as, for example, the overproduction of industrial compounds (Burgard et al., 2003; Patil et al., 2005). Good models can be used to supplement or even substitute in vivo or in vitro experiments, aiding in the hypothesis and interpretation of biological phenomena (Voit, 2000)

There are several ways to approach cell modelling. The objective of modelling and employing the appropriate measures depends on the intention of the model, that is, on the definition of the problem related to the identification of the biological question to answer. For the mathematical representation of biological processes, one can consider several types of models that can belong to more than one class consecutively. According to different criteria, the models can be classified in:

- Continuous (used to model continuous systems where the variances undergo slight changes) or Discrete (used to model discrete systems where changes in a variable occur in discrete steps).
- Deterministic (for a fixed set of initial conditions, always produces the same output) or Stochastic (for a given input, the model takes randomness or probability distributions into account, and the outcome takes a range of possible values).
- Dynamic (their behaviour varies with time, so time works as an independent variable (differential equation) and allow to study small-scale biological processes through detailed kinetic parameters) or static

(their behaviour is constant and does not vary with time (mass-balance equations).

- Quantitative (designed to study time dependent behaviour) or Qualitative (used to identify high-level properties, such as structure and global functions of biological systems).

Dynamic models rely on stochastic or deterministic kinetic modelling and allow studying small-scale biological processes through detailed kinetic parameters. These models usually predict reaction fluxes, component concentration and regulatory states, but time demonstrated that the mechanistic dynamic order of these models was inefficient (Brien et al., 2015), since they are computationally intensive, difficult to parametrize and challenging when there is the need to model multiple timescales. Nevertheless, its dynamic structure motivated the development of interaction-based models that comprises graph-based representations based on biological data that encompasses most cellular processes (protein-protein and genetic interaction networks), and constraint-based models (CBM) that typically allow to predict metabolic flux states and gene essentiality with no need for kinetic information. There are other methods that allow to model and analyse high-throughput data that include:

- Logical, Boolean or rule-based formalisms (used to model signalling networks and transcriptional regulatory networks predicting global activity states and on-off states of genes).
- Bayesian approaches (used to model gene regulatory networks and signalling networks, and predict probability distribution score).

- Pathway enrichment analysis (used to model metabolic and signalling networks, predicting enriched pathways).

The advances in high-throughput sequencing techniques and genetic annotation methods allowed the construction of Genome-scale metabolic models (GSMM) for hundreds of organisms (Machado et al., 2011).

These models are reconstructed based on information of the biological sequence and biochemical detailed and updated information (Liu et al 2010; Osterlund and Nielsen 2012). The reconstruction of models of cellular metabolism, that describe the formation and consumption of each metabolite based on mass-balance constraints, provides a simplified quantitative and mathematical representation of the biological system or components. Simplistically, the cell can be represented by a black box that receives the substrates, such as carbon, energy sources, nitrogen, and oxygen. From these substrates, the cell manufactures biomass, water and carbon dioxide, as well as products of interest.

GSMMs are reconstructed on a bottom-up perspective where any information on metabolism is collected and used to determine the stoichiometry of all possible metabolic reactions that occur in the studied cell. To obtain the high quality GSMM refinements and modifications must be made using intensive simulations and computational tools (Osterlund and Nielsen, 2012). These models are typically constructed in iterative cycles of experiments and refinements by multidisciplinary research teams that include biologists, engineers and computational scientists (Machado et al., 2011).

The concept of GSMM plays a very important role since it allows the direct integration of experimental data with high yield mathematical modelling.

GSMM networks represent a valuable resource, since they contain curated highly noted information about genes, proteins, enzymes, reactions and metabolites training, degradation, transport, cellular information and further references, for which there are extended databases and literature (Bordbar et al., 2014; Nielsen, 2009; Maertens and Vanrolleguem, 2010), and describe the mechanistic connection between genotype and phenotype, predicting the response to genetic and environmental disturbances.

GSMM's have been relevant in the study of inaccessible network properties, such as the existence of loops, optimal pathway use, connectivity metabolites and pathways redundancy (Oberhardt and Palsson, 2009). The applications of GSMM can be divided into some broad categories (Bassel et al., 2012; Osterlund and Nielsen, 2012):

- Guide for metabolic engineering (rational strain improvement).
- Interpretation and biological discovery.
- Application of new computing platforms.
- Evolutionary clarification.
- Scaffold for integration, visualization and interpretation of omics data.

From the earliest stages of metabolic modelling, stoichiometric metabolic networks have been used to facilitate the choice about where to intervene genetically and identify targets for metabolic engineering (Fondi and Lió, 2015). Constraint-based stoichiometric models are used to automatically compute feasible and optimal physiological states, as for example, the resulting balance of all the chemical reactions predicted to be active in the cell to bridge the gap between knowledge of the metabolic network structure and observed metabolic processes (Varma and Palsson, 1994; Brien et al.,

2015). These models include stoichiometric relations between reactions substrates and products, as well as information on reaction directions or flux constraints (Jouhten, 2012)

A clear description of the distribution of the metabolic fluxes of the system is fundamental to understand metabolic regulation. The base of the knowledge required for modelling (structural modelling) is the system stoichiometry. This can be used to predict the growth phenotype of mutants such that misguided predictions can guide iterative improvements of the system.

Stoichiometric reconstructions represent the chemical transformations that can occur in a cell and form the basis of whole cell models. The stoichiometry of all reactions can be specified in a matrix where the columns represent reactions and the rows represent the various metabolites (Nielsen, 2009).

Assuming that the different flows into and leaving, different metabolite pools are balanced, i.e., in steady state, it is possible to establish material balance for each metabolite in the model (Nielsen, 2009) according to the equation (1) where s_{ij} is the stoichiometric coefficient associated with flux r_j and b_i the net transport flux of metabolite x_i. Under pseudo steady-state conditions Equation (1) will reduce to Equation (2) that can be rewritten in matrix notation (3) where S is the stoichiometric matrix, R is the vector of metabolic fluxes, and b is the vector representing m transport fluxes over the cell membrane (Maertens and Vanrolleghem, 2010).

$$\frac{dx_i}{dt} = \sum_j s_{ij} r_j - b_i \quad \textbf{(1)}$$

$$0 \cong \sum_j s_{ij} r_j - b_i \quad \textbf{(2)}$$

$$\begin{bmatrix} 0 \\ b \end{bmatrix} \cong S \times R \quad \textbf{(3)}$$

One of the most widely adapted computational methods for predicting gene targets to be engineered is constraints-based flux analysis (Lee et al., 2009). Constraint-based flux analysis has been applied to biochemical reaction networks for more than 25 years (Bordbar et al., 2014). As already explained, constraint-based modelling includes stoichiometric, thermodynamic and flux capacity constraints to model the fluxes of metabolites (Bassel et al., 2012). It has been used in metabolic engineering strategies for the determination of flux distributions (metabolic flux analysis (Wiechert, 2001; Alper et al. 2005; Burgard et al., 2003), flux balance analysis (Kauffman et al., 2003), prediction of outcomes of gene deletions (minimization of metabolic adjustment (Segrè et al., 2002), regulatory on/off minimization (Shlomi et al., 2005), drug-target identification (Raman et al., 2005) or to enumerate all possible pathways (extreme pathways (Schilling et al., 2000), elementary flux modes (Schuster et al., 1999). Although their main application has been on metabolic networks, there are efforts towards application on gene regulatory and signalling networks (Covert et al., 2004; Daniel et al., 2011; Gianchandani et al., 2009; Papin et al., 2005; Lee et al., 2008).

Constraints on network operation are used to predict functional states of the cell and can be divided into four categories (Raman and Chandra, 2009).

- physico-chemical constraints.
- spatial or topological constraints.
- condition dependent environmental constraints.
- regulatory constraints.

The simplest constrains (condition dependent environmental constraints) include setting the input and output ranges based on uptake and secretion

of metabolites. These is made by defining lower and upper bounds for the fluxes of metabolites within the network according to physiological data, based on the knowledge of cellular thermodynamics or actual measurements on particular exchange fluxes. Other techniques include modifying reaction bounds based on nutrient uptake rates, consumption of ATP, phosphate/oxygen ratio, mRNA and protein expression data.

The measurement of all the fluxes of a network is a tedious process. It is possible to solve the distribution of fluxes of a network assuming that the indeterminate metabolic network is optimized for a certain objective function (OF), establishing the indeterminate system as a problem of optimization (Raman and Chandra, 2009). OF is generally defined to maximize the growth rate, deriving from the idea that over time the fastest growing organism is the one that eventually dominates the population, so that the model must direct the flows in order to allow to growth fast. Other objective functions such as maximizing production of energy or of some desired product have also been used.

Since objective functions can be used to explore the capabilities and limitations of biochemical networks and to analyse their robustness, (Deutscher et al. 2006; Edwards and Palsson, 2000; Kauffman et al. 2003) identifying an appropriate objective function is a critical step in capturing the biochemical goal of the system itself. It is important to take into account that the choice of OF cannot be independent of the conditions of the simulation and is influenced by the purpose of the study, since the environmental conditions and adjustments applied to disturbances will determine the success of an approach.

A wide range of objective functions have been used in flux balance models (Raman and Chandra, 2009). Nonetheless, the optimum cell behaviour does not always coincide with maximizing growth (Maertens and Vanrolleghem, 2010). Objective functions such as maximizing/minimizing ATP production also proved to be a good predictable source, when used to determine conditions of optimal metabolic energy efficiency (Liu et al., 2010; Nielsen, 2009; Raman and Chandra, 2009). Other OFs include minimization of nutrient uptake, maximization of metabolite production, particularly to determine production capabilities of a particular cell, maximization of biomass and metabolite production, and optimal metabolite channelling, by minimization of the absolute norm of the flux vector, or the Euclidean norm (quadratic objective function). Sometimes, multiple optimal solutions may be obtained for a system. These alternate optimal solutions can be analysed to identify redundancies in the metabolic network (Burgert et al., 2003).

Systems biology in the context of the thesis

Fluxomics

The expression of the metabolic fluxes of a cell represents the ultimate outcome of cellular regulation, in response to certain conditions. Metabolic fluxes can be seen as a fundamental parameter in cellular physiology studies, since they show in a quantitative way the contribution of the various pathways to the general cellular functions.

Fluxomics involves quantification of the rate of turnover of metabolites through metabolic pathways. With this information, comprehensive characterization of metabolic networks (control and functional regulation)

and, subsequently, the phenotype of an organism can be assessed (Kim et al., 2012). Fluxomics is widely used in metabolic engineering as it provides a direct view on how the carbon fluxes are distributing throughout the metabolic network. So, it is possible to readout the impact of genetic modifications on the global physiological behaviour of an organism (Kim et al., 2012).

A number of algorithms have been developed to assist the study of several pathways. Once the model is constructed, optimization techniques are applied to predict the distribution of flows through the network of reactions. According to the desired purpose, methods of optimization algorithms can be divided into (Liu et al., 2010):

- Linear programming;
- Quadratic programming;
- Mixed integer linear programming;
- Evolutionary programming.

Flux balance analysis (FBA) is the most common CBM application. It uses linear programming, with the defined objective function, to simulate the distribution of steady-state flows, giving an overview of the metabolic capabilities of the system (Altafini and Facchetti, 2015; Raman and Chandra, 2009; Vasilakou et al., 2016). Additional details as regulatory effects may introduce nonlinearities or on / off decisions, requiring nonlinear programming (NLP) or mixed integer programming (MILP / MINLP) to solve it.

FBA allows identifying targets for metabolic engineering, which are not intuitively recognized by the simple study of network maps (Jouhten, 2012). This technique makes possible to simulate systems on different variant conditions, as well as to analyse the lethality of single or double knockouts, to identify pairs of essential genes or to determine and analyse synthetic genetic interactions (Raman and Chandra, 2009).

There are four steps involved in FBA, in order to identify an optimal flux distribution within the feasible space. The first step requires the definition of the system. For that purpose, the individual reactions of the model are listed in detail, in what concerns to metabolites, genes and catalytic enzymes, as well as to compartmentalization and reversibility. This phase also identifies transport reactions and external metabolites that will be exchanged with the rest of the system, like carbon sources required for growth, co-factors that are ubiquitous, end products from a pathway, or components of the biomass. The second step involves obtaining stoichiometry from the reactions relating products and substrates.

The set of linear equations can be represented in the form of a matrix Sv = 0, where "S" is the matrix of stoichiometric coefficients and "v" is the vector of the associated reactions fluxes. To the set of reactions that represent the system is joined a growth equation or biomass equation that represents the final set of metabolites necessary for cell replication. In addition, equations that represent uptake of nutrients and excretion of certain molecules into the extracellular space may also be included. Taken together, these equations make it possible to constraint the flow of metabolites through the metabolic network. In the third phase, a biologically relevant objective function is defined, based on the assumption that nature has optimized life towards some evolutionary goal (Buescher et al., 2015). The maximization of the rate

of biomass production is often used as OF, as well as the maximization of ATP, growth rate production, or minimization of nutrient utilization, on the premise that the selective plan during optimization guides the system towards optimality (Vasilakou et al., 2016; Raman and Chandra, 2009) If growth maximization is used as an objective function of an optimization problem, then the set of flows obtained will constitute a plausible representation of the distribution of flows of the organism (Altafini and Fachetti, 2015). Once the objective function is fixed, the system of equations can be solved to obtain the distribution of flows, assuming that the internal metabolites will be at a pseudo steady state, being produced and consumed at approximately the same rate This leads to the last step involved in FBA - the optimization.

Extensions of FBA and other in silico methods based on stoichiometric models have successfully been applied to identify metabolic engineering targets in genome-wide networks (Jouhten, 2012). The most common perturbation studied through FBA is the deletion of one or more genes from the system. The effect of gene knockouts in the metabolic flux distribution of the cell can be analysed by constraining the reaction fluxes of the corresponding genes (and corresponding proteins) to zero, during the simulation (Blazeck and Arper, 2010; Lee et al., 2009).

The two main criteria named in the literature for describing the metabolic response of the system to a given disorder are Minimization of Metabolic Adjustment (MoMA) and Regulatory On / Off Minimization (ROOM) (Altafini and Facchetti, 2015).

While evolutionary pressure eventually directs an organism's metabolism to a particular optimum, metabolic engineering allows the metabolic network to

be altered without the need to wait for the required number of generations for the organism to readapt. MoMA reflects this adopting a quadratic optimization function to minimize the variation in flux distribution relative to the wild type. MoMA exhibits the same stoichiometric constraints as FBA, but reduces optimum growth for mutants, looking for an approximate solution to a suboptimal growth state, with a flux distribution close to that found in the unperturbed or wild type state (Segre et al., 2002).

MoMA does not assume optimality of growth or any other metabolic function, allowing circumventing objective functions for optimization that may not reflect in a very accurate way the physiological situation, giving more accurate predictions for knockout strains, in relation to FBA.

ROOM in another way attempts to minimize the number of significant flux changes from the wild-type flux distribution, requiring the solution of a Mixed Integer Linear Programming (MILP) problem. ROOM showed better predictions than MoMA in studies of lethality predictions on *S. cerevisiae*, for example (Raman and Chandra, 2009).

Analysis of intracellular metabolic fluxes allows obtaining meaningful information about how these fluxes are distributed in the cell, as a result of regulatory phenomena (Frick and Wittmann, 2005). Initially these studies were done based on the measurement of exchange fluxes render a determined equation system, through stoichiometric metabolic flux analysis (MFA). However, this method was not precise and accurate. The most common mode of flow measurement currently depends on carbon labelling (Wiechert, 2001). During this process cells are grown in labelled substrate (^{13}C glucose) until they reach a constant growth rate, where all carbon has been integrated. The labelling is consequently distributed throughout the

metabolic network, allowing the measurement of proteinogenic amino acid enrichment patterns through nuclear magnetic radiation or mass spectrometry. When combined with the former MFA approach, this is often referred as metabolic network analysis (MNA) (Christensen and Nielsen, 2000; Klapa et al., 2003) Although this technique is extremely powerful and provides realistic information about the metabolic state of the organism over a particular condition, it is not widely used, perhaps because of the complexity of the calculations needed to estimate the fluxes from the enrichment patterns.

In contrast to these approaches, instead of looking for a single solution to the model, is possible to analyse the topology of the metabolic network using convex analysis and enumerate all possible steady state solutions through Elementary Flux Modes or Extreme Pathways analysis (Schuster et al., 2000; Schilling et al., 2000).

Due to the incomplete nature of annotation of the proteins in the genome, metabolic gaps, and inconsistencies with experimental data can be observed during simulations. Nonetheless, these gaps can help to refine the networks and thereby improve knowledge about an organism's metabolism and elucidate possible new regulatory mechanisms.

Transcriptomics

There has always been an interest in understanding what determines the shift from oxidative to fermentative metabolism, as well as in characterize the details of the metabolic states of *S. cerevisiae.* These metabolic changes have been investigated through different methods, from traditional

physiological studies to sequencing and transcriptome analysis, as well as through metabolome analysis and flow distribution.

Sequencing and transcriptome analysis are essential for validation of genomic data and discovery of variants, determination of alternative splicing, and development of DNA markers. Differential expression analysis of genes allows inferring the mechanisms of gene regulation, even for those organisms in which the genome is not available in public data banks.

In order to know and quantify the transcriptome, a series of technologies have been developed, which are mainly based on the hybridization and sequencing of cDNA. Hybridization-based technologies (e.g. microarrays) generally involve the incubation of cDNA derived from fluorescently labelled biological samples, with a series of DNA probes immobilized on a solid matrix. These techniques allow the simultaneous analysis of the expression of thousands of genes in a single assay. However, hybridization-based technologies also have a number of limitations, since they depend on previous knowledge of the genomic sequence, cross-hybridization may occur, and the technique presents a limited detection scale associated with uncertainty related to signal intensity (Wang et al., 2009).

Sequence-based techniques directly determine the cDNA sequence. In the past, Sanger sequencing of cDNA or ESTs (expressed sequence tag) was used to obtain cDNA sequences, but this methodology presents relatively limited, costly, and generally non-quantitative performance (Wang et al., 2009).

To overcome these limitations, high performance methods have been developed to provide accurate digital quantification of gene expression

levels. These are based on small sequence tags, which include serial analysis of gene expression (SAGE), cap analysis of gene expression (CAGE), and massively parallel signature sequencing (MPSS) (Wang et al., 2009).

The development of deep-sequencing technologies as the NGS has allowed sequencing, mapping and quantifying transcripts through RNA sequencing (RNAseq) or whole transcriptome sequencing (WTSS). This high-performance method outweighs the limitations of other existing methodologies. The low costs, time and volume of data produced make this a highly accessible methodology that is currently commercially available on six platforms, which are classified into two groups (Wang et al., 2009).

The first group encompasses techniques in which cDNA amplification must be performed by PCR prior to sequencing, while the latter encompasses technologies that are based on the sequencing of a single molecule, and therefore does not require pre-sequencing amplification. Table 1.3 summarizes the major sequencing platforms.

Table 1.3. Features of the major NGS platforms relevant for transcriptomic analysis.

Group	Sequencing platform	Amplification method	Read length (bp)	Maximum Output per run	Accuracy (%)
1	Roche 454[1]	Emulsion PCR	400-700	700 Mbp	99.9
	Illumina[2]	Bridge PCR	100-300	600 Gbp	99.9
	SOLiD[3]	Emulsion PCR	75-85	80-360 Gbp	99.99
	Ion Torrent[4]	Emulsion PCR	100-400	100 Mbp-64 Gbp	99
2	HeliScope[5]	No amplification Single molecule	25-55	35 Gbp	97
	PacBio RS[6]	No amplification Single molecule real-time (or SMRT)	4000-5000	200 Mbp-1Gbp	95

[1]Roche Diagnostics Corp., Branford, CT, USA
[2]Illumina Inc., San Diego, CA, USA
[3]Life Technologies Corp., Carlsbad, CA, USA
[4]Life Technologies, South San Francisco, CA, USA
[5]Helicos BioScience Corp.,Cambridge, MA, USA
[6]Pacific Biosciences, Menlo Park, CA, USA

The Roche 454 platform generates the largest size readings that are the most appropriate when in required to do "de novo assembly". The Illumina platform offers lower costs; however, the generated readings are smaller than those generated by Roche 454. Illumina also has the highest sequencing capacity, lower cost and time, as well as good accuracy (Liu et al., 2012). For high depths of coverage, yield and accuracy are prioritized, rather than the size of the readings (Barba et al., 2014). These conditions have positioned Illumina as the dominating platform in the market.

Illumina has developed a series of sequencing platforms, including HiSeq 2500, HiSeq 2000, HiSeq 1500 and HiSeq 1000 that differ between them in accordance to their performance (Barba et al., 2014).

RNAseq data processing incudes a set of computational techniques that allow to estimate and compare the abundance of RNA transcripts in different biological samples, at a certain stage of development, and / or on a certain physiological condition (Korpelain et al., 2018; Wang et al., 2009). Although there are commercial and free bioinformatics tools that have graphical interfaces that allow the processing of RNAseq data, it is advisable to use tools based on robust and efficient algorithms, when large amounts of data need to be processed. In general, computational processing of RNAseq data uses pipelines that involve mapping or assembling of the readings, identification, annotation, and quantification of genes and/or transcripts. Depending on the objectives of the work, it may be necessary to do "de novo assembly", if there is no reference genome for the species of interest and/or when there are polymorphisms, nucleotides or haplotypes that can be lost by comparison with the reference genome. It may also be necessary to assemble with a reference transcriptome, if the sequences are very short (<50 base pairs), or to assemble using a reference genome, when none of the above cases applies.

There are several software that can be applied in the process of mapping and assembling of the readings that vary according to the strategy used. For the mapping with the reference genome, there are three important tools: TopHat (Trapnell et al., 2009), Star (Dobin et al., 2013), and HiSat (Kim et al., 2015). Regarding the efficiency of these assemblers it is observed that TopHat consumes many hours in the assembly of the data, when compared to the Star that spends the least time in this stage. Nevertheless, TopHat

does not require much memory unlike Star. Still, HiSat is the best current choice to assemble RNAseq data, since it uses less processing time and requires less memory.

After mapping the readings, it is necessary to perform a differential expression analysis to quantify and statistically compare the abundance levels of genes and transcripts. There are several methods that count the number of sequenced readings that belong to a given gene or transcript as measure of abundance or level of expression. These can be divided into two categories: union exon, that include the featureCounts programs (Liao et al., 2014), and HTSeq-count (Anders, et al., 2015); and transcript-based than englobe the Cufflinks tools (Trapnell et al., 2012 (Brayet et al., 1991), BitSeq (Glaus et al., 2012), RSEM (Li et al., 2011), Sailfish (Patro et al., 2014), RapMap (Srivastava et al., 2016), Kallisto and Salmon (Patro et al., 2017).

The transcript-based approach to measure gene abundance is intrinsically more difficult, since different isoforms of the same gene have a high proportion of genomic overlap, but it is also the most relevant method, biologically speaking, since genes are expressed in one or more isoforms (Zhang et al., 2017; Zhao et al., 2015).

To conduct the differential expression analysis of the gene abundance data, there is a series of programs that use parametric statistical methods, which allow to predict an unknown value from the observation of a model and its parameters, as well as non-parametric methods that can capture more details related to data distribution, since these models are not limited by a rigid model, and the distribution of the data is not defined by a finite set of parameters (Costa-Silva et al., 2017).

In the range of available programs, edgeR (Robinson et al., 2010), BaySeq (Hardcastle et al., 2010), and DESeq (Anders, Huber, 2010) use parametric methods such as the negative binomial distribution to analyse the differential expression of genes, while tools such as NOIseq (Tarazona et al., 2015) and SAMseq (Li and Tibshirani, 2013) adopt non-parametric methods. Other programs such as EBSeq (Leng et al., 2013) and Cuffdiff (Trapnell et al., 2013) are based on methods that are more appropriate for the quantification and analysis of expression at the level of transcripts or isoforms, preferably. There is no consensus regarding the most appropriate methodology to validate the results of differential analysis of abundances in terms of accuracy, robustness and reproducibility (Costa-Silva et al., 2017; Jia et al., 2015). DESeq, edgeR, and Cuffdiff are the three most commonly used analysis tools. DESeq and edgeR are known to have a better performance in the control of false positives, whereas Cuffdiff is less conservative, allowing finding a higher number of true positives. Nonetheless, it is also possible to find a bigger number of false positives in the final result (Jia et al., 2015; Trapnell et al., 2013; Zhang et al. 2014).

Trapnell et al., (2012) developed a pipeline formed by TopHat and Cufflinks (includes Cuddiff) tools, which together solve the mapping of reference genome readings, annotation of transcripts, and quantification and analysis of differential expression of genes and transcripts. TopHat aligns the readings in the genome and discovers splice sites, while the Cufflinks contrasts this map with the genome to assemble the readings in transcripts. Cuffdiff analyses the aligned readings and reports genes and transcripts that are differentially expressed in two or more conditions.

Objectives

The general objective of this PhD work is the understanding of metabolic and genetic traits related to ethanol and acetic acid production by several species of wine yeasts, in the context of developing yeast strains and fermentation conditions that would help solve the problem of increasing alcohol content of wines. Several specific objectives have been addressed in order to attain this main objective.

- Study the impact of environmental factors on the physiology of several non-*Saccharomyces* strains and their relevance for alcohol level reduction.
- Analyse at the transcription level the influence of aerobic fermentation conditions on the physiology of non-*Saccharomyces*, Crabtree negative yeasts, using *Kluyveromyces lactis* as a model.
- Computational modelling of *Saccharomyces cerevisiae* under aerobic growth conditions in order to identify key genes involved in aerobic acetate yield
- Construction of recombinant *S. cerevisiae* wine yeast strains showing reduced acetate yield, as a guide to further genetic improvement by conventional genetic methods.

Chapter 2.

Environmental factors influencing the efficacy of different yeast strains for alcohol level reduction in wine by respiration

BACKGROUND

The steady increase in alcohol levels in wine is one of the main challenges faced by the oenological industry in recent decades. The problem is related in part to global warming, which results in faster grape ripening, as well as an imbalance between sugar accumulation and the phenolic maturity of berries (Jones et al., 2005; Mira de Orduña, 2010). This trend to increased sugar content in grape must is also driven by current consumer preferences for well-structured and full-bodied wines, which require late harvest in order to warrant proper aromatic and phenolic maturity. However, high sugar content leads to elevated ethanol production during must fermentation, and contribute to stuck or sluggish alcoholic or malolactic fermentation, due to alcohol toxicity on microbial cells. On the commercial side, excess ethanol might impair wine sensory quality; discourage consumers, due to health and road safety considerations; or become a hurdle in the global market, due to regulations and taxes associated to the alcohol content of beverages. The goal of reducing alcohol content of wines is being addressed by researchers involved in all the stages of wine production, from vine clone selection to partial dealcoholisation of the finished wine (Teissedre, 2013).

Development of *S. cerevisiae* yeast strains showing reduced alcohol yield during the fermentation of grape must has been a recurrent topic in wine biotechnology during the last thirty years. It was initially explored by genetic engineering approaches (Michnick et al., 1997; Heux et al., 2006; Rossouw et al., 2013; Varela et al., 2012); and more recently by evolutionary engineering (Cadière et al., 2011; Tilloy, 2014). However, fine-tuning of *S. cerevisiae* metabolism to low ethanol yield is still a tough work.

Despite *S. cerevisiae* is the main yeast species responsible of transforming grape must into wine, many other yeast species participate in the initial stages of the process (Ciani et al., 2010; Cordero-Bueso et al., 2013; Fleet, 2003; Guadalupe-Medina et al., 2013; Rojas et al., 2003; Sadoudi et al., 2012). Our research group recently proposed using the respiratory metabolism of some of these non-*Saccharomyces* yeasts as a tool for reducing the alcohol content of wine (Gonzalez et al., 2013). The proposed procedure involves the use of a non-*Saccharomyces* yeast strains and controlled oxygenation during the first two to four days of fermentation. The final steps of the fermentation would be carried out under standard conditions and driven by *S. cerevisiae* (Morales, et al., 2015). Several yeast strains, including *Metschnikowia pulcherrima*, *Kluyveromyces lactis*, and *Candida sake* isolates were found to be good candidates to develop fermentation procedures aiming at reducing alcohol content in wine by respiration (Quirós et al., 2014). Results of previous work also indicated that, besides the ability to respire sugars under aerated winemaking conditions, production of volatile acidity under such conditions had to be taken into account. Differences of up to one order of magnitude in acetic acid yield were found among the different yeast strains studied (Quirós et al., 2014).

However, our current knowledge of the metabolic features of these alternative yeast species is limited, including the two main parameters to be considered for an effective alcohol content reduction in wine, alcohol and acetic acid yields on sugar. In order to help optimization of alcohol level reduction with these yeast species we have addressed the impact of three different and easily controllable environmental factors (fermentation temperature, nitrogen source availability, and oxygen supply) on sugar consumption, as well as ethanol, acetate and glycerol production, in a model

wine fermentation system, for four yeast strains belonging to different species and selected according to previous (Quirós et al., 2014).

METHODS

Strains

A commercial *S. cerevisiae* wine yeast strain, EC1118 (Lallemand Inc., Ontario, Canada), and three non-*Saccharomyces* strains, *Metschnikowia pulcherrima* CECT 12898 (labelled as IFI1459 in previous works), *Candida sake* CBS 5093, and *Kluyveromyces lactis* CECT 10669, all of them selected from a previous study (Quirós et al., 2014), were used in this work. The strains were grown at 25 ºC, and maintained at 4 °C on YPD plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar), as well as in glycerol stocks at -80 °C.

Batch cultures

Batch cultures were performed in a defined medium containing 200 g/L glucose, 6 g/L citric acid, 1.7 g/L YNB without amino acids and $(NH_4)_2SO_4$ (Difco™, Becton Dickinson, New Jersey, USA), 0.018 g/L myo-inositol and NH_4Cl. The amounts of NH_4Cl used to get different YAN levels (Yeast Assimilable Nitrogen in mg N/L) were: 0.573 g/L for 150 YAN, 0.764 g/L for 200 YAN, and 0.955 g/L for 250 YAN. The pH of medium was adjusted to 3.5 with NaOH.

Yeast inocula were grown in YPD broth for 48 h, at 25ºC and 250 rpm and washed twice with water before use. The medium was inoculated to 0.2 initial optical density at 600 nm (OD_{600}). Experiments were performed in a DASGIP parallel fermentation platform (DASGIP AG, Jülich, Germany) equipped with four SR0400SS vessels. Bioreactors were filled with 200 mL of culture medium and 200 µL (aprox.) of antifoam 204 (Sigma-Aldrich) were added. Agitation was maintained at 250 rpm and the temperature kept at 15, 20 or 25 ºC with a recirculating chiller. The pH of the medium was kept at 3.5 by the automated addition of 2N NaOH. The cultures were sparged with either pure air or mixtures of air, O_2 and N_2, to get 10 %, 21 % (pure air) and 50 % oxygen content in the in gas, at a gas flow of 1.0 L/h. In order to standardize ethanol stripping, we chose stablishing the different aeration regimes by keeping the gas flow constant and varying oxygen content with gas mixtures. Gas flow was controlled with gas flow controllers (MFC17, Aalborg), whose calibration was regularly verified with a soap bubble flow meter.

Samples for determination of metabolite concentrations and OD_{600} were withdrawn twice a day for 7 days. Exhaust gas was cooled in a condenser and the instant concentrations of O_2 and CO_2 recorded with a GA4 gas analyser (DASGIP AG). For technical reasons gas exchanges were only determined for fermentation experiments sparged with pure air, but not for gas mixtures.

Analytic methods for extracellular metabolites determination

The concentrations of glucose, glycerol, ethanol, and acetic acid were determined in duplicate by HPLC using a Surveyor Plus Liquid

chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively); and a HyperREZ^{TM} XP Carbohydrate H+ (8 µm particle size) column and guard (Thermo Fisher Scientific). The column was maintained at 50 ºC and 1.5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/ min. Prior to injection the samples were filtered through 0.45 µm pore size nylon filters (Fisher Scientific, Madrid, Spain) and diluted 20-fold in MiliQ water.

Two calculations were performed with analytical data from the 72 h samples. Alcohol level reduction was calculated as the difference between the expected increase in ethanol content, according to sugar consumed up to this time point (17 g/L of sugar consumed for an increase of 1% alcohol by volume; ABV), and the actually measured ethanol content. Even though this alcohol level reduction value is useful for comparison between conditions and strains, it is probably overestimated, since part of the ethanol produced will have been lost by stripping and must be taken with caution outside this context. In addition, an integrative parameter, Efficacy (efficacy for alcohol level reduction) was designed to simplify comparisons between strains or growth conditions. It was calculated as follows: Efficacy= AR x2 x(0.5-AA). Were AR is the alcohol level reduction (expressed as % ABV), and AA is acetic acid content (g/L), all values referred to 72h. The 0.5 g/L value was chosen as a maximum tolerable content on acetic acid at this time point. Higher values would result in excess volatile acidity by the end of the process. The 2x factor allows for a better comparison with the alcohol level reduction value (Efficacy equals alcohol level reduction when no acetic acid is produced).

Oxygen consumption and CO_2 production were determined by taking into account the in and out gas flows, and their respective concentrations in air and in the off gas. Instant values were integrated over time. Respiration quotient (RQ) was calculated as que quotient between CO_2 production and oxygen consumption.

Experimental Design and statistical analysis

An orthogonal design was used to get the best combination of factors with the minimal number of experiments. Orthogonal design and boxplot figures were calculated and drawn with the IBM SPSS Statistics v19 software. Table 2.1 shows the values for each variable in the design obtained.

Results were analysed by means of the IBM SPSS Conjoint v19 software. Importance values obtained in this analysis (0 to 100) provide a measure of how important each factor was to determine the overall result for each parameter and strain.

Table 2.1. Summary of the nine combinations of the three parameters evaluated resulting from an orthogonal design, performed with IBM SPSS conjoint program.

Condition	Aeration (%O_2)	YAN (mg N/L)	Temperature (ºC)
1	21	150	20
2	10	150	25
3	50	250	20
4	21	250	25
5	50	150	15
6	21	200	15
7	50	200	25
8	10	250	15
9	10	200	20

RESULTS AND DISCUSSION

Three non-*Saccharomyces* yeast strains, belonging to three different yeast species were selected according to results from a previous work (Quirós et al., 2014) in order to explore the potential of different yeast species to contribute to ethanol content reduction by respiration during the transformation of grape must into wine. *S. cerevisiae* EC1118 was also included in the study for comparison. We addressed the impact on yeast metabolism of three easily manageable factors, fermentation temperature, nitrogen nutrient availability (YAN), and aeration regime. Nine combinations of these three factors at three different levels were assayed for each strain,

as stablished in an orthogonal design (Table 2.1), and results were further analysed by IBM SPSS Conjoint program. Fermentation experiments were run for six days, and samples were withdrawn twice a day (Annex 2.1). Data from the 72 h sample were chosen for conjoint analysis. This choice was based on three considerations: I) according to previous results (Morales et al., 2015), three days was considered as a suitable oxygenation interval in order to reach the desired ethanol content reduction (indeed the average alcohol level reduction by 72 h was 2.1 % ABV); ii) in most fermentation experiments sugar consumption was sufficient after 72 h in order to calculate metabolic yields with acceptable precision; iii) none of the fermentation experiments was stuck at this time point. Values for the main metabolic parameters at this time point are summarized in Annex 2.2, and Figure 2.1.

Boxplot for sugar consumption, considering all nine fermentation conditions, shows different median values but overlapping distributions and similar boundaries for all four yeast strains (Figure 2.1A). The only evident extreme value was obtained for *S. cerevisiae* under condition 4, showing the highest sugar consumption after 72 h. This result would be in agreement with the adaptation of *S. cerevisiae* to growth in high sugar content substrates. Indeed, *S. cerevisiae* shows the highest consumption values among the four strains for six out of the nine growth conditions studied (Annex 2.2). Also noticeably, condition 4, oxygenated with pure air (i.e. 21% O2), and involving the highest YAN and temperature values (250 mg N/L and 25ºC), results in the highest sugar consumption for all the strains used in this study (ex aequo with condition 9 in the case of *K. lactis*).

The most striking differences between the four strains were found for ethanol yield values (Figure 2.1B). The distribution for *S. cerevisiae* is narrower, and values, including median, clearly higher than for any other strain. Actually, *S.*

cerevisiae showed the highest ethanol yield in all but one of the growth conditions assayed (Annex 2.2). This trend towards high ethanol production under most culture conditions is in agreement with the well-described metabolic features and the evolutionary history of this species (Piškur et al., 2006), including the Crabtree effect (Pronk et al., 1996), which favours fermentative over respiratory metabolism despite oxygen availability. Another confirmation of the specific adaptation of *S. cerevisiae* to fermentative metabolism is that it was unable to grow under hyper-oxygenated conditions (sparging with 100% O2), in contrast to all the other yeast strains tested (data not shown). Low tolerance to oxygen might be due to high sensitivity of specific metabolic pathways or cell components to oxidative stress in this species, or to higher intracellular stationary levels of molecular oxygen due to low oxygen consumption by respiration. This would be in agreement with the higher dissolved oxygen levels generally appreciated in *S. cerevisiae* cultures, as compared to the other strains (Annex 2.1).

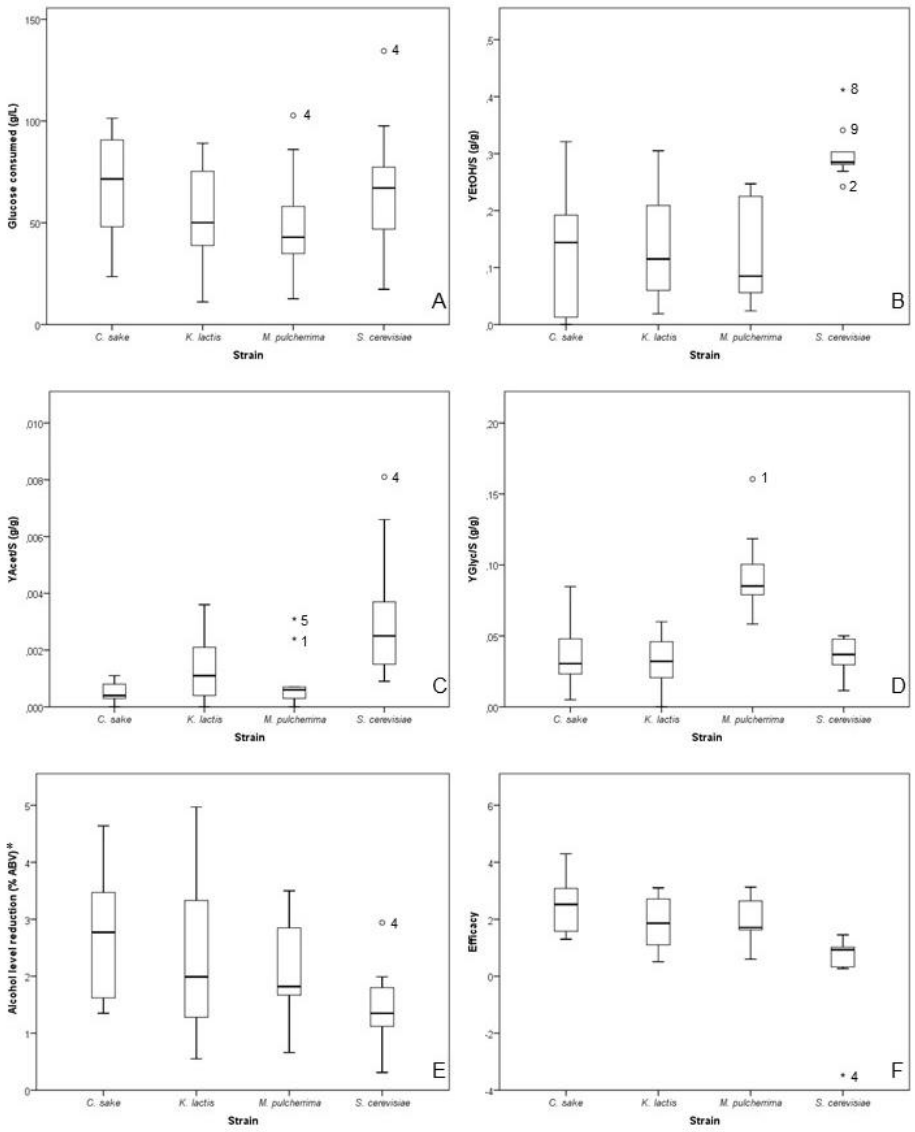

Figure 2.1. Boxplots of the mean sugar consumption (A), ethanol yield on glucose (B), acetic acid yield on glucose (C), glycerol yield on glucose (D), alcohol level reduction (E), and Efficacy (F), by the strains analysed in all conditions tested. Numbers indicate growth conditions for outliers. *Alcohol level reduction values cannot be taken as absolute, they can be overestimated by ethanol stripping, but they are useful for comparative purposes.

RQ was calculated for all the experiments sparged with pure air. The results are in line with ethanol yields, with RQ values for *S. cerevisiae* ranging from 4.2 to 11.9, while the range of RQ values observed for all the other strains fell between 1.3 and 3.5 (Annex 2.2). These values indicate the portion of carbon source metabolized by respiration was 3 % to 9 % for *S. cerevisiae*, and 12 % to 53 % for the other strains. These data confirm respiratory metabolism as one major determinant of ethanol yields under these fermentation conditions. The RQ values obtained for *S. cerevisiae* are in agreement with our previous results (Quirós et al., 2014), as well as other authors, depending on the strain and growth conditions, RQ values ranging from 2.8 to ∞ have been described for *S. cerevisiae* under aerated glucose rich conditions (de Deken, 1966; Franzen, 2003; Aceituno et al., 2012). It is worth noting that oxygen consumed after 72 h in these cultures ranged from 1.9 g/L to 19.4 g/L (Annex 2.2), far apart from the microgram or milligram range used in other oenological applications. Any trials aiming to scale up the process of alcohol level reduction in wine by respiration should take into account the relatively strong aeration conditions required.

Concerning acetic acid yield, the highest median value and overall distribution were observed for *S. cerevisiae* (Figure 2.1C). This species was the highest acetic acid producer in eight out of the nine growth conditions tested (Annex 2.2), with all acetic acid production values above the overall median of the experiment (considering all strains and growth conditions). This is in agreement with some of our previous results with this *S. cerevisiae* strain (Morales et al., 2015); and confirm volatile acidity production as the major drawback of using *S. cerevisiae* under aerated winemaking conditions. In contrast, the low median and distribution of values observed for this parameter in *C. sake* (Figure 2.1C) suggest this species might be an

interesting option for alcohol level reduction. Glycerol yield showed similar distributions for most strains apart for *M. pulcherrima*, which showed both the highest median and extreme values (Figure 2.1D). This strain showed also the highest glycerol yields for all the growth conditions tested (Annex 2.2). It would be a very interesting option for wine styles where high glycerol content is perceived as a positive quality trait.

Results of the conjoint analysis are summarized in Table 2.2. Glucose consumption is favoured both by increasing nitrogen availability (YAN) and increasing fermentation temperature, with quite similar relative impact for all the strains in the study. However, a clear effect of increasing oxygen supply on glucose consumption was only revealed for *C. sake*, being the most important factor governing glucose consumption for this strain.

Surprisingly, YAN shows the highest relative impact on ethanol yield for all the strains apart *K. lactis*. In all four cases, the correlation of YAN and ethanol yield was direct (Table 2.2). This suggests that nitrogen source availability stimulates sugar uptake, resulting in a higher rate of fermentation over respiration. This might be due to limitations in the oxygen transfer rate or to overflow metabolism, as described for Crabtree effect in *S. cerevisiae* (Pronk et al., 1996). On the other side, increasing oxygen supply shows a negative impact on ethanol yield, also compatible with the expected impact of oxygen availability on respiratory metabolism, for all strains but *C. sake*. It seems that the stimulation of sugar uptake induced by oxygen in this later species might go beyond its respiratory capacity, resulting in glycolytic overflow and increased ethanol yields. On the other side, *K. lactis* is the only yeast strain for which oxygen supply appears as the most relevant factor affecting ethanol yield (Table 2.2). According to this analysis, in order to reduce ethanol yield by respiration with *S. cerevisiae*, *M. pulcherrima*, or *C. sake*, either YAN or

fermentation temperature would be better targets for process optimization than oxygen supply (always considering the range of values used in this work).

As a rule, we found a direct correlation between oxygen supply and acetic acid production (Table 2.2). This is, indeed, the most important factor influencing volatile acidity production by *C. sake*. However, considering this strain showed very low acetic acid yields under all growth conditions tested (Figure 2.1C), the technological relevance of acetic acid production would be negligible for this species. On the other side, both YAN and fermentation temperature showed clearly different effects for each of the strains (Table 2.2). YAN is negatively correlated with acetic acid production for all strains but *C. sake*; while the correlation of acetic acid production with temperature is negative for all of them but *S. cerevisiae*. The opposite impact of YAN and temperature on acetic acid production, depending on the yeast strain, should be taken into account for the development of fermentation processes based on mixed cultures. In contrast to acetic acid, a negative correlation was found for glycerol yield with oxygen supply for all the strains assayed (Table 2.2). Oxygen supply is the most important factor affecting glycerol yield for all strains but *M. pulcherrima*. In contrast, YAN showed the strongest impact on glycerol yield for this strain (with a negative correlation).

Table 2.2. Importance values, calculated by Conjoint analysis, of the effect of the three different environmental parameters (O_2 supplied – O_2; Nitrogen availability – YAN; Temperature – TEMP) on different fermentation parameters for different yeast species.

		*S. cerevisiae**		*M. pulcherrima**		*K. lactis**		*C. sake**	
Consumed Glucose	O_2	2	+	5	+	11	-	44	+
	YAN	45	+	45	+	49	+	29	+
	TEMP	53	+	50	+	40	+	27	+
Ethanol Yield	O_2	26	-	31	-	51	-	19	+
	YAN	44	+	52	+	34	+	62	+
	TEMP	30	-	17	+	15	+	19	+
Acetic Acid Yield	O_2	37	+	33	+	38	+	70	+
	YAN	15	-	38	-	36	-	24	+
	TEMP	48	+	29	-	26	-	6	-
Glycerol Yield	O_2	79	-	22	-	62	-	75	-
	YAN	2	+	71	-	3	-	12	-
	TEMP	19	+	7	+	35	+	13	-
Alcohol reduction	O_2	12	+	21	+	7	+	47	+
	YAN	27	+	30	+	47	+	35	-
	TEMP	61	+	49	+	46	+	18	+
Efficacy	O_2	19	+	18	+	13	+	44	+
	YAN	46	-	32	+	45	+	41	-
	TEMP	35	-	50	+	42	+	15	+

* Symbols indicate direct (+) or inverse (-) correlation. Importance values can vary from 0 to 100.

In the context of developing procedures for alcohol level reduction in wine by sugar respiration it would be difficult to take decisions based on a single parameter, like sugar consumption, ethanol production, or acetate production. For this reason, we decided to integrate the most relevant information for this purpose in a single parameter, Efficacy (see Methods). This parameter takes into account sugar consumption, as well as ethanol and acetate production. *C. sake* showed the best distribution of Efficacy values among the strains tested in this study (Figure 2.1F). In turn, *S. cerevisiae* showed the lowest Efficacy values in all but one of the growth conditions analysed (Figure 2.1F, Annex 2.2). The conjoint analysis showed a positive correlation of Efficacy with oxygen supply, for all the strains tested (Table 2.2). However, the relative impact is different for each strain, with *C. sake* sowing the strongest dependence of Efficacy on oxygen supply, while *K. lactis* showed very limited impact. Concerning Efficacy, YAN is a relevant variable to take into account for all yeast strains tested (Table 2.2). However, the direction of the correlation depends on the strain; it is positive for *M. pulcherrima* and *K lactis*, and negative for the other two strains. Similarly, Efficacy is affected by fermentation temperature in opposite directions depending on the strain, negatively for *S. cerevisiae* and positively for the other yeasts (Table 2.2). The lowest impact of temperature on Efficacy was observed for *C. sake*.

In summary, we have analysed the impact of three easily manageable environmental factors on the production of the main fermentation metabolites by three non-*Saccharomyces* yeast strains during the fermentation of synthetic grape must (and compared results with *S. cerevisiae*). The different levels for each environmental factor were hence chosen in a reasonable range for process optimization, considering alcohol level reduction trough

sugar respiration by yeast cells (Gonzalez et al., 2013). We must however consider that conclusions of this analysis cannot be extrapolated beyond the range of values assayed. For example, it is obvious that respiration would not take place in fully anaerobic cultures. Similarly, as mentioned above, 100% oxygen seems to be toxic for *S. cerevisiae*. This means including a 0% or 100% oxygenation level would change the result of the analysis, but it would be less relevant for practical purposes. Surprisingly, apart from glycerol production, oxygen supply is not the main driver of the differences observed for most parameters and strains. The different impact of the variations in the three environmental factors on the metabolism of each yeast strain is also appreciated by taking Efficacy as an integrative parameter. Both the relevance of each factor, and the direction they affect Efficacy, are different for each strain.

Concerning future process optimization, the main conclusions of this work would be that increasing oxygen supply (up to the upper levels assayed in this work) would positively contribute to alcohol level reduction and Efficacy of the process (despite the negative impact on acetic acid yields). In addition, *C. sake* CBS 5093 appears as the most promising strain among those tested. Using *C. sake* for alcohol level reduction by respiration would avoid most of the problems associated to volatile acidity (Figure 2.1C), while alcohol level reduction and Efficacy would be high. According to Table 2.2, optimal conditions for this strain would probably involve initial YAN close to 150 mg N/L, 25ºC, and high oxygenation levels.

Chapter 3.

Hypoxia and iron requirements are the main drivers in transcriptional adaptation of *Kluyveromyces lactis* during wine aerobic fermentation

BACKGROUND

Despite *Saccharomyces cerevisiae* is the main yeast species responsible for wine fermentation, other yeasts, including *Hanseniaspora*/*Kloeckera*, *Pichia*, *Candida* or *Metschnikowia* strains, develop during the first stages of grape must fermentation (Fleet, 1993). The metabolic footprint of these alternative yeast species is now recognized as a relevant contribution to wine aromatic complexity and sensory quality (Jolly et al., 2014). However, an uncontrolled fermentation process driven by non-*Saccharomyces* yeasts and bacteria would often result in wine spoilage. Historically, the use of *S. cerevisiae* starter cultures constituted an inflection point in the microbiological control of wine fermentation (Santiago et al., 2011), but it has been blamed for wine aromatic standardization and lack of complexity. In order to recover some of the positive contribution of "wild yeasts" to wine quality, while keeping a reasonable control of the fermentation process, wine microbiologists have been suggesting the use of non-*Saccharomyces* yeasts in mixed starter fermentations with *S. cerevisiae*, either by simultaneous or sequential inoculation (Jolly et al., 2014). Indeed, several non-*Saccharomyces* yeast starters are currently available in the market and their use by winemakers is growing. Most of these new starters are selected according to their impact on wine aromatic profile, glycerol or mannoprotein content, volatile acidity, or colour stability (Gonzalez et al., 2016).

Crabtree-negative non-*Saccharomyces* yeasts have been suggested in the context of climate change as an instrument to reduce ethanol content of wines (Gonzalez et al., 2013). The proposal for alcohol level reduction with these alternative yeast species is based on their capacity to respire sugars from grape must under aerated fermentation conditions (Quirós et al., 2014). Under oxygen sufficient conditions, Crabtree-negative yeasts consume all

sugar by respiration, while most of the carbon flux goes to ethanol production in the case of Crabtree positive species, like *S. cerevisiae*. Our research group has shown the usefulness of several non-*Saccharomyces* yeasts, including *Kluyveromyces lactis*, for this purpose (Morales et al., 2015, Chapter 2). These works identified the overproduction of acetic acid, above commercially accepted levels, as a critical point for the interest of this approach. The increase in acetic acid production occurred either under aerobic conditions or after growing in aerobic conditions and then switching to anaerobic conditions. A tight control of the dissolved oxygen levels reduced the production of acetic acid. In addition to oxygen supply, other environmental factors, namely temperature and nitrogen source availability have also been shown to affect the output of aerated fermentation experiments with different non-*Saccharomyces* yeasts (Chapter 2).

Reducing ethanol content of wines by the strategies mentioned above involves the use of yeast species usually poorly characterized as well as growth conditions (aerated wine fermentation) that are unexplored from a biotechnological viewpoint. In order to make advances in the development of fermentation procedures based on respiratory metabolism of non-*Saccharomyces* yeasts, it was judged interesting to know the transcriptional changes experienced by yeast cells under these previously unexplored growth conditions. *K. lactis* was chosen because of its good properties for alcohol level reduction by respiration (Chapter 2). In addition, the genome of this species is well sequenced and annotated (Dujon et al., 2004; Sherman et al., 2004). The aim of this work was hence using *K. lactis* as a model system to study changes, at the transcription level, relevant for our understanding of yeast physiology under aerated fermentation conditions, as those employed for alcohol level reduction in wine.

METHODS

Strains and media

Three yeast strains were used in this work, *S. cerevisiae* EC1118, a widely used industrial wine yeast strain; *K. lactis* type strain, CBS2359; and *K. lactis* AQ2166, a natural oak isolate from Hungary. In order to mimic industrial wine fermentations, we used a synthetic grape must recipe based on the Herwig complex synthetic media (Herwig et al., 2001) with some modifications as follows (per liter): glucose 200 g; $(NH_4)_2SO_4$ 5 g, KH_2PO_4 3 g, $MgSO_4$ $7H_2O$ 0.5 g, trace element solution 2.67 mL, vitamin solution 2.67 mL, anti-foam (PPG P2000) 0.1 mL. The vitamin solution, stored in refrigerator and sterilized by filtration, contained per liter: biotine 0.05 g, Ca-D(+)pantothenate 1 g, nicotinic acid 1 g, myo-inositol 25 g, thiamine hydrochloride 1 g, pyridoxal hydrochloride 1 g, para-amino benzoic acid 0.2 g. The autoclaved trace element solution contained per liter: EDTA 15 g, $ZnSO_4 \cdot 7H_2O$ 45 g, $MnCl_2$ $4H_2O$ 1 g, $CoCl_2 \cdot 6H_2O$ 0.3 g, $CuSO_4 \cdot 5H_2O$ 0.3 g, $Na_2MoO_4 \cdot 2H_2O$ 0.4 g, $CaCl_2 \cdot 2H_2O$ 4.5 g, $FeSO_4 \cdot 7H_2O$ 3 g, H_3BO_3 1 g, KI 0.1 g. Fermentations in real grape must were previously performed to ensure that our results were comparable to synthetic grape must (Figure 3.1).

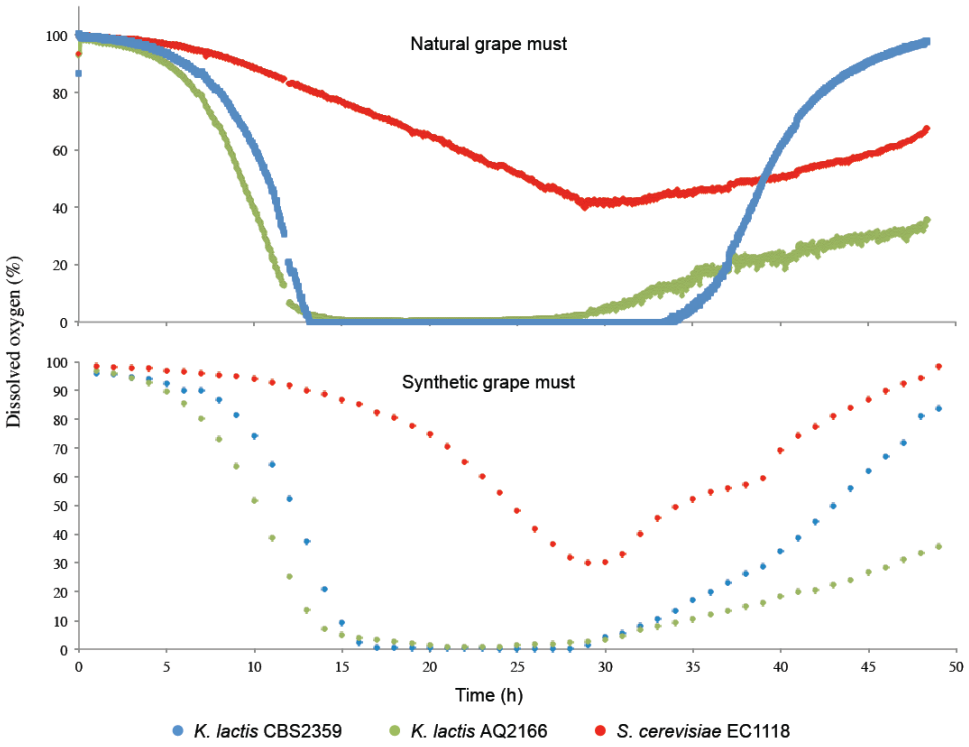

Figure 3.1. Evolution of dissolved oxygen levels under aerated conditions in synthetic or natural grape must.

Synthetic must fermentations under controlled aerobic conditions and RQ determination

Fermentation experiments were performed in triplicate in small bioreactors MiniBio (Applikon Biotechnology B.V., Delft, The Netherlands) coupled to BlueInOne Cell gas analyzer units (BlueSens, Germany). This setup allows

monitoring different parameters: temperature, pH and dissolved oxygen (DO) in the media, as well as O_2 and CO_2 from the output gas.

Seed cultures were grown in YPD broth for 48 h, at 25 °C and 250 rpm. Bioreactors were filled in with 150 mL of synthetic grape must. Temperature was set to 25 °C, stirring to 1000 rpm, pH to 3.5, and inoculation to approximately 0.2 initial optical density at 600 nm (OD_{600}). The cultures were sparged with air at 25 ml/min (10 gas volumes/culture volume/h (vvh)). Gas flow was controlled with MFC17 mass flow controllers (Aalborg Instruments and Controls, Inc., Orangeburg, NY), whose calibration was regularly verified with an electronic flowmeter (Agilent Technologies, Santa Clara, CA).

CO_2 and O_2 readings from the gas analyzer were recorded every minute and used to calculate RQ in three steps. First the contribution of gas exchanges to changes in gas volume was taken into account to calculate actual amount (per minute) of CO_2 and O_2 coming out of the bioreactor. Then, CO_2 release and O_2 consumption rates were calculated as the difference between in and out values (per minute). Finally, RQ was estimated as the ratio between CO_2 production and O_2 consumption rates for each time point.

Analytical methods

Production and consumption of the main metabolites, glucose, fructose, glycerol, and ethanol, were determined in duplicate using a Surveyor Plus Liquid Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively) on a 300 × 7.7 mm HyperREZ™ XP Carbohydrate H+ (8 µm particle size) column and guard (Thermo Fisher

Scientific). The column was maintained at 50 °C, and 1.5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Prior to injection in duplicate, the samples were filtered through 0.22 µm pore size nylon filters (Micron Analitica, Madrid, Spain) and diluted 10-fold in MilliQ water.

RNA sequencing and data analysis

Total RNA from biological triplicates was extracted using RNeasy® mini kit (QIAGEN) and subjected to DNAase treatment using the Ambion DNA-free™ kit according to the manufacturers' instructions. Concentration, purity and integrity of RNA samples were determined by spectrophotometric analysis considering the absorbance ratio at 260/280 nm and at 230/260 nm. Library preparation and sequencing of RNA was performed at Institute of Biomedicine & Biotechnology of Cantabria (Santander, Spain). After poly-A filtering, libraries were generated for the different time points and conditions. From these libraries, 50-bp single-end sequence reads were produced with Illumina HiSeq 2000. All raw RNAseq data have been deposited in NCBI under Sequence Read Archive SRP064945 (BioProject PRJNA298965) accession number.

Alignment of reads to the S288c *S. cerevisiae* yeast reference genome assembly or to CBS2359 *K. lactis* reference genome assembly was carried out using TopHat2 v.2.0.13 (Kim et al., 2013). Only uniquely mapped single copy, ≤1 polymorphism per 25bp reads with quality ≥20 were kept for further analysis. The htseq-count tool (v.0.5.4p5) from HTSeq (Anders and Huber, 2015) was used to estimate unambiguous read count per genome assembly annotated transcript. Normalization following the trimmed mean of M-values

(TMM) method (Robinson and Oshlack, 2010), as well as a time-points DEGs searches (adjusted Benjamini–Hochberg P≤0.05 and ≥2-fold change) were performed in edgeR v.2.2.6 (Robinson et al., 2010). Finally, fragments per kb of exon per million fragments mapped (RPKM) was calculated using Cuffdiff v.2.2.1 (Trapnell et al., 2013) and low-expressed transcripts were filtered out when RPKM was <1 in both samples.

Real-time quantitative PCR

RNA was prepared as described above, and quantification was run in triplicate. All the reactions were run in a LightCycler® 480 Real-Time PCR System. The gene expression levels are shown as the changes in the concentration of the studied gene as compared to the control sample and were normalized with the concentration of the housekeeping *ACT1* gene and *PCA1* gene, with similar results (Teste et al., 2009) (only *ACT1* is shown in the figures) using the ΔΔCt method. Primers used in this study are listed in Annex 3.1.

Statistical analysis

Principal component analysis of the normalized RNAseq data transcripts per million (TPM) was done using MeV software (4.8v10.2). The remaining statistical analyses were done using STATA-SE. Venn diagram was drawn by using Venny 2.1 on-line tool software (Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams:

http://bioinfogp.cnb.csic.es/tools/venny/index.html). GO (gene ontology) term analysis was performed using YeastMine (Balakrishnan et al., 2012). The *p-values* were corrected for multiple testing by the Bonferroni test for functional associations and GO analyses. The statistical level of significance was set at p≤0.05. Then, GO terms were grouped in biomodules by GO-Module (Yang et al., 2011) to prioritize Gene Ontology terms.

RESULTS AND DISCUSSION

Fermentation profile under aerated conditions

Fermentation assays of synthetic grape must were performed with two different *K. lactis* yeast strains, the type strain CBS 2359, with complete annotated genome sequence available (Dujon et al., 2004; Sherman et al., 2004) and *K. lactis* AQ2166. This second *K. lactis* strain was selected as a more suitable yeast strain for winemaking applications, especially considering acetic acid production under aerated conditions (Figure 3.2). An industrial *S. cerevisiae* wine yeast strain, EC1118, was also included in the experiments in order to illustrate the impact of aerated fermentation methodology in an industrial wine yeast background (Novo et al., 2009). The experimental conditions, including aeration regime, were chosen according to previous results (Morales et al., 2015; Chapter 2). A validation of the aeration regime and synthetic grape must composition was done by comparing dissolved oxygen (DO) profiles in fermentations carried out in natural grape must. Similar DO profiles were obtained in natural or synthetic grape must (Figure 3.1). Under these experimental conditions total sugar

consumption was achieved in a relatively short time for all the strains assayed, 50 hours for both *K. lactis* strains, and slightly faster for the industrial *S. cerevisiae*, 42 hours (Figure 3.3).

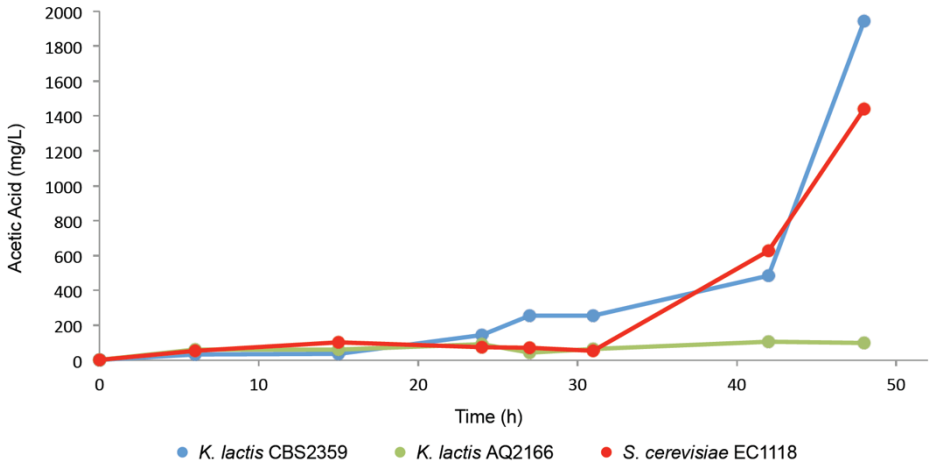

Figure 3.2. Acetic acid production under aerated conditions.

Ethanol production under these aerated conditions was low as compared to regular (anaerobic) fermentation conditions; and lower for both strains of *K. lactis* (7 to 8.30 % v/v ethanol) than for *S. cerevisiae* (10% v/v ethanol). This is in agreement with previous results by us and other authors, under both standard laboratory conditions (Quirós et al., 2014; Contreras et al., 2015) and in natural grape must (Morales et al. 2015). Also, as shown previously (Morales et al. 2015), oxygenation results in increased acetic acid production

for *S. cerevisiae* and the type strain of *K. lactis* CBS2359, from 30 hours. Although acetic acid is the primary volatile acid in wine, its production over detectable levels remembers vinegar and is considered as wine spoilage (Santiago et al., 2011). However, the second *K. lactis* strain showed very low acetic acid production (Figure 3.2).

Aeration regime, as mentioned above was based on previous results, with the main goal of inducing a quick oxygen consumption by *K. lactis* strains, that will protect grape must components against oxidation. Accordingly, the two *K. lactis* strains showed a high consumption rate after the initial 5 hours, reaching DO values below 10% after 15 hours. The decline in DO was slightly faster for the *K. lactis* strain AQ2166. On the other hand, *S. cerevisiae*'s oxygen consumption was noticeable after around 12 hours with a much slower decay, and never fell below 30% dissolved oxygen (Figure 3.4).

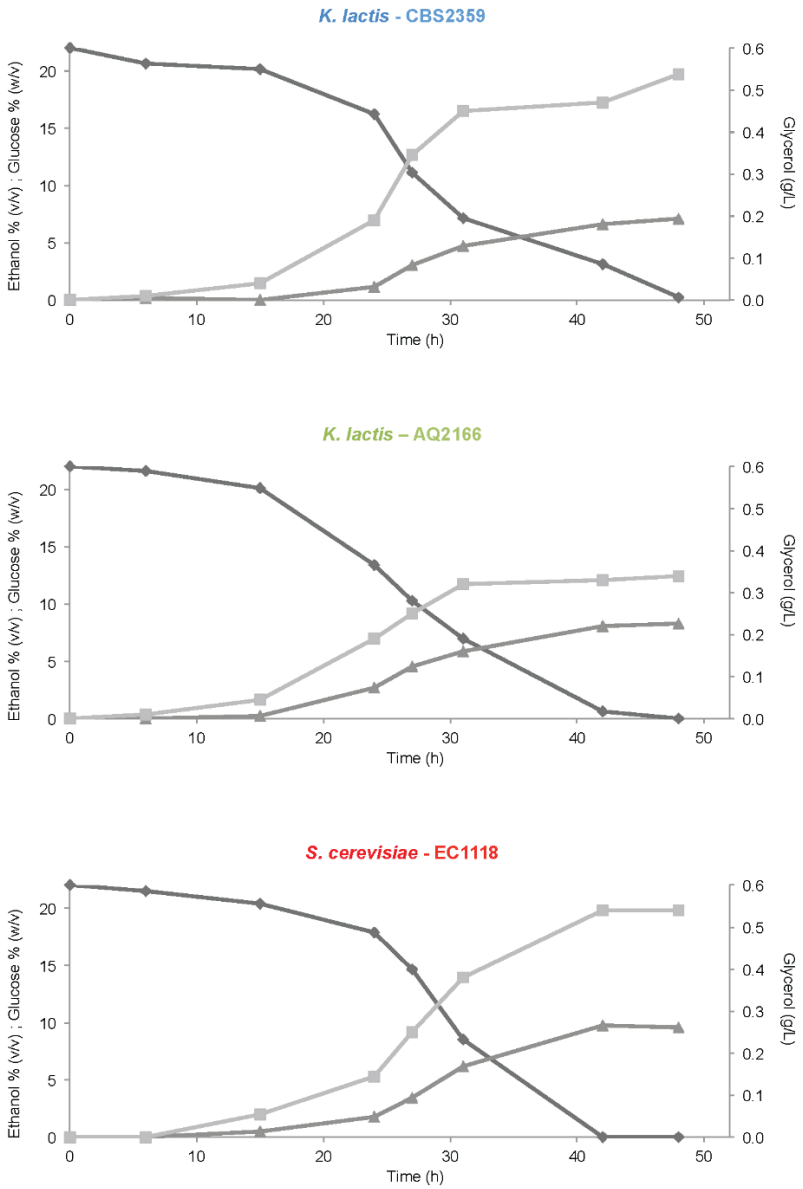

Figure 3.3. Evolution of main fermentation related metabolites (glucose: diamonds; ethanol: triangles; glycerol: squares) under aerated conditions.

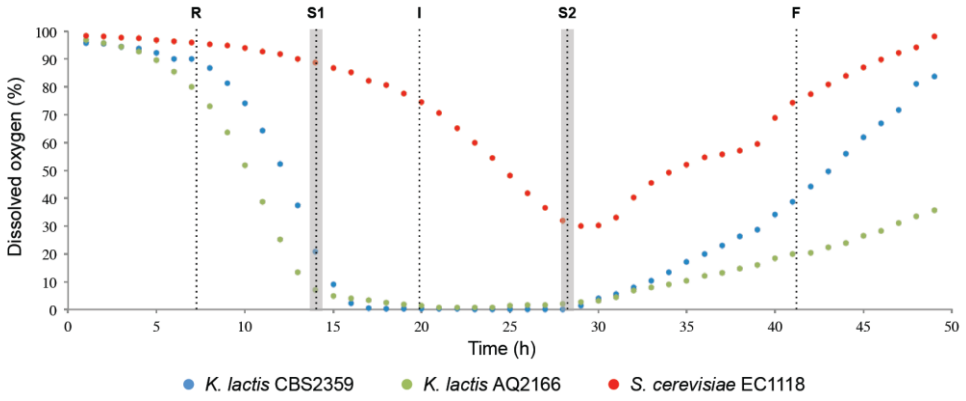

Figure 3.4. Evolution of dissolved oxygen levels under aerated conditions. RNAseq sampling time points are shadowed in grey. qPCR sampling time points are shown as dotted lines.

Oxygen demand decayed after about 30 hours of culture in all instances, probably due to the depletion of some essential nutrients, slow metabolic activity, and the entry of the cultures into stationary phase. These differences in dissolved oxygen profiles are in agreement with Respiratory Quotient (RQ) values for each strain (Figure 3.5). *K. lactis* strains showed steady RQ values around 1 (fully respiratory metabolism), until oxygen became a limiting factor. Once the increment in biomass results in an oxygen demand that cannot be fulfilled by the preset air flow (hypoxic conditions), an increase in RQ values is observed in the *K. lactis* strains.

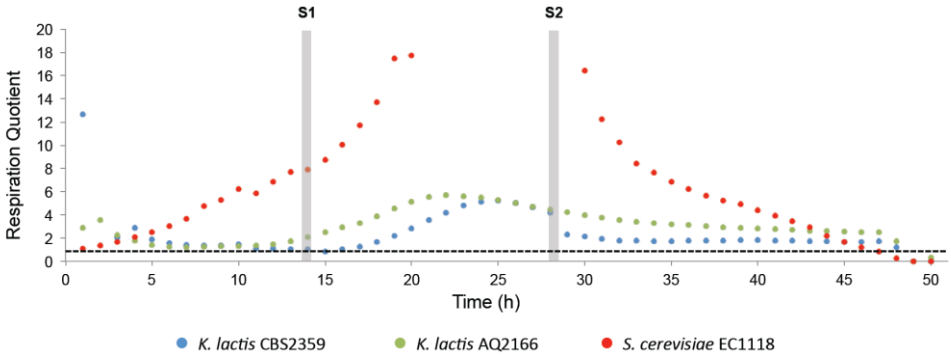

Figure 3.5. Evolution of respiration quotient under aerated conditions in natural must. RNAseq sampling time points are shadowed in grey. Discontinuous horizontal line shows RQ = 1 (Full Respiratory Metabolism).

S. cerevisiae, on the contrary, showed a quick increase in RQ values from the beginning of the experiment. Therefore, despite it consumes up to 30% of the oxygen available, RQ values indicate that *S. cerevisiae* is mainly fermenting. This behavior is in agreement with the well-known metabolic features of *S. cerevisiae*, an archetypical Crabtree-positive yeast. The Crabtree-negative nature of *K. lactis* allows these yeasts to keep RQ values always below those of *S. cerevisiae*, lowering the final ethanol production, as shown above. Despite these important differences in respiro-fermentative metabolism, all the yeast strains assayed showed similar sugar consumption kinetics (Figure 3.3).

The two *K. lactis* strains showed slight differences in oxygen consumption. The type strain showed a constant slope in dissolved oxygen decline, down to 0%, while AQ2166 slowed down oxygen consumption after reaching 10% DO, needing several hours to drop to 0%. This behavior correlates with the increase in RQ values, starting earlier for AQ2166 than for the *K. lactis* type

strain, and could indicate a higher sensitivity of AQ2166 to very low oxygen availability. Reaching 0% DO values is important in the context of wine aerobic fermentation, in order to protect wine color and aromatic features (Morales et al., 2015).

Sampling points for RNAseq and qPCR analyses were decided to focus on the two different stages in oxygen availability for *K. lactis*. Two sample points were defined for RNAseq analysis (Figure 3.4). Sample point S1 (12 hours) defined by no oxygen limitation, the RQ value was steady around 1 for *K. lactis* strains, indicating that glucose metabolism was fully respiratory. In contrast, sample point S2 (30 hours) was taken when hypoxic conditions (0% DO) had been running for several hours in *K. lactis* cultures. This point was also characterized by RQ values clearly above 1 (respiro-fermentative metabolism). In the case of *S. cerevisiae* S1 corresponds to the initial stages of oxygen consumption while S2 corresponds to the maximum oxygen uptake, both cases with RQ ratios higher than one, while there is a high oxygen availability.

Global analysis of the transcriptome

The main purpose of this work was to study the transcriptomic profile of *K. lactis*, in synthetic grape must with an aerated regime, under two very different metabolic states defined by the available dissolved oxygen. Neither *K. lactis* nor aerobic conditions are commonly used in wine fermentation, but they have been lately used to lower the ethanol content in wine (Morales et al., 2015; Chapter 2). Therefore, it is of great interest, to better understand how *K. lactis* responds to this new biotechnological application.

Samples for gene expression analysis were taken from S1 and S2 (see above), and analyzed by RNAseq as described in Methods. In a first attempt to obtain a global picture of the transcriptome data, a Principal Component Analysis (PCA) was ran (Figure 3.6).

PCA draw two very different scenarios, while samples from *K. lactis* cluster in two groups according to its time points, in *S. cerevisiae* samples from S1 and S2 cluster together. The results of the PCA analysis correlate well with the number of differentially expressed genes (DEG) for each species (S1 vs. S2). In *K. lactis* there is a high degree of divergence between sample points, with more than 12% of the transcriptome significantly modified (Figure 3.7), in contrast to the 2.5% modified for *S. cerevisiae*. Of the 623 genes showing modified expression in *K. lactis*, 337 were up-regulated, while 286 genes were down-regulated. However, only 68 up-regulated genes and 92 down-regulated genes were detected for *S. cerevisiae*. In addition, average fold-change values for genes differentially expressed in *K. lactis* was higher than for *S. cerevisiae*.

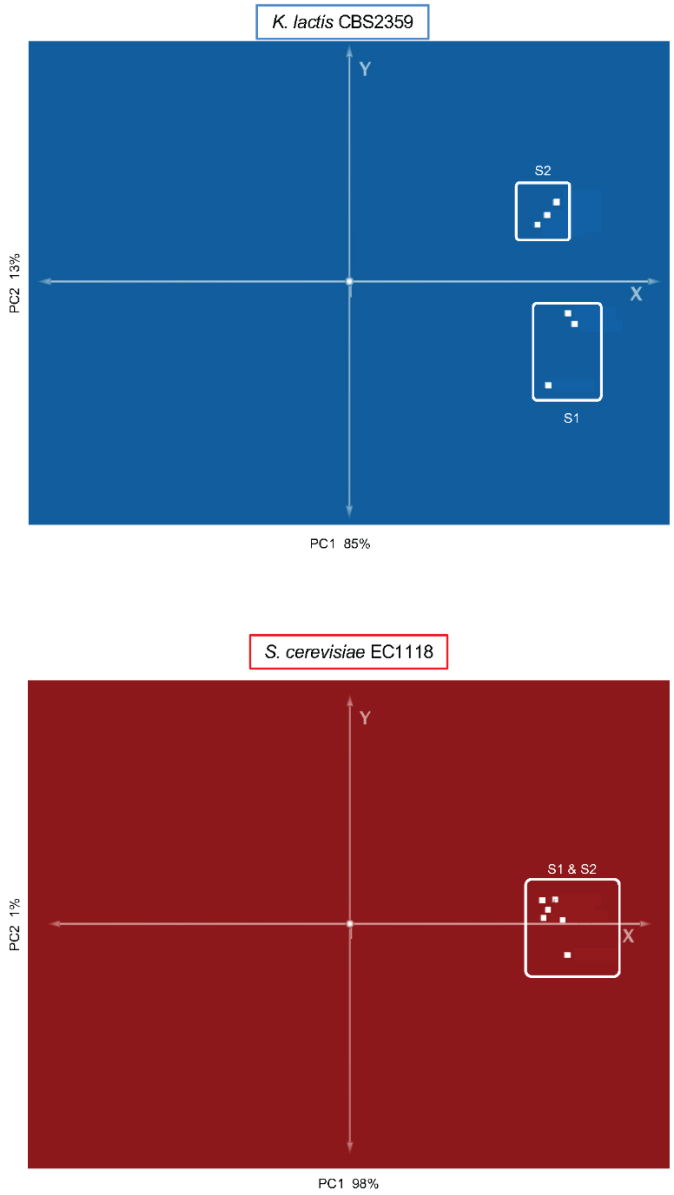

Figure 3.6. Principal Component Analysis for RNAseq data (TPM from biological triplicates) from *K. lactis* and *S. cerevisiae*.

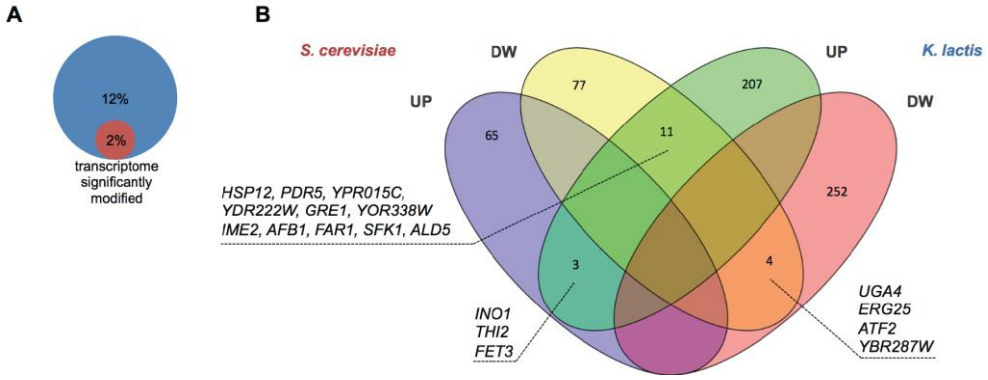

Figure 3.7. Analysis of the differentially expressed genes. (A) Percentage of transcriptome significantly modified for each species: Blue circle represents *K*. *lactis*; Red circle represents *S. cerevisiae*. (B) Venn diagram showing the number of differentially expressed genes for each species (S1 vs. S2).

These gene expression changes are in agreement with the DO profiles shown in Figure 3.4. *K. lactis* shows pure respiratory metabolism (RQ values around 1; Figure 3.5) in S1, with DO values above 30%, but the oxygen nutrient limitation (0% DO) in S2 re-shapes the transcriptome in response to the respiro-fermentative metabolism. The high impact of growth conditions in S2 on the transcriptome reprograming of *K. lactis* takes place despite oxygen is supplied at the same rate in both S1 and S2. In contrast to other studies with *K. lactis*, in this case the trigger for the observed transcriptomic changes is not a deliberated shift from aerobic to anoxic conditions (Blanco et al., 2007; David and Poyton, 2005) but a gradual modification from aerobic to hypoxic conditions due to increased biomass and metabolic activity of yeast cells. Under these conditions, *S. cerevisiae* shows respiro-fermentative metabolism in both sample points, with a clear preference for fermentation,

and DO values above 30% in both cases. Accordingly, changes in the expression pattern are much less pronounced than for *K. lactis*.

GO term enrichment among differentially expressed genes

The analysis of GO term enrichment is shown in Table 3.1. In order to ease the interpretation of results, the GO-Module tool (Yang et al., 2011) was used to prevent false positives and repeated terms. In the case of *K. lactis*, this analysis was performed by using the corresponding *S. cerevisiae* orthologs. Approximately four-fifths of the genes showing highly variable expression from *K. lactis* do have known orthologs in *S. cerevisiae* (around 500 genes).

Analysis of GO terms among *K. lactis* genes significantly up-regulated in S2 revealed an enrichment in oxidoreductase activity; iron ion transmembrane transporter activity; aldehyde dehydrogenase (NAD) activity, and catalytic activity (Table 3.1). Regarding genes in the iron ion transmembrane transporter activity category, *FET4* codes for a low-affinity iron (II) permease, also involved in the transport of copper and zinc. Fet3p and Ftr1p constitute the cell-surface high-affinity iron uptake system required for iron import when it is present at low concentrations. Fet3p is a multicopper ferroxidase that receives iron (II) ions from cell-surface iron reductases such as Fre3p and passes iron (III) ions to the iron permease Ftr1p. In *K. lactis* two different genes *KLLA0E14477g* and *KLLA0E05897g* show similarity to *FRE3* (also significantly up-regulated but not listed under GO:0005381) from *S. cerevisiae*. Both orthologs are overexpressed in *K. lactis* in S2. Also *ARN1* and *ARN2* are involved in iron transmembrane transport. They belong to a family of transporters for siderophore-iron chelates, responsible in *S.*

cerevisiae for the uptake of iron bound to different siderophores like ferrirubin, ferrirhodin or triacetylfusarinine C. Among genes up-regulated in *K. lactis*, four different genes, similar to *S. cerevisiae ARN1* (*KLLA0A10439g*, *KLLA0E14609g*) or *ARN2* (*KLLA0C00220g*, *KLLA0C19272g*) were found. In *S. cerevisiae*, the transcription of these genes is activated by Atf1p, which is expressed in response to low iron conditions.

The identification of iron as a major nutrient requirement for *K. lactis* under these culture conditions is reinforced by the finding of several other genes related to iron metabolism being overexpressed in S2 for *K. lactis* cultures. These include five orthologs of *FIT1*, *FIT2* and *FIT3* from *S. cerevisiae*. These genes code for GPI-anchored cell wall mannoproteins involved in the retention of siderophore-iron complexes. Yap5p is an iron-sensing transcription factor; while Cth1p is involved in iron homeostasis, as well as the putative protein Fmp23p. The iron dependence of the *K. lactis* life style is also illustrated by the number of copies found in the genome (Dujon et al., 2004; Sherman et al., 2004), and overexpressed in this set of experiments, also *ARN1-2* and *FRE3* orthologs, as mentioned above, not only of *FIT1-3* orthologs.

The overexpression of this set of genes in S2 indicates the importance of iron metabolism for *K. lactis* under aerated conditions. This is related to the high oxygen consumption levels at this time point, considering that the electron transport chain is the main intracellular sink of iron ions. In addition, in agreement to the relevance of iron metabolism for *K. lactis* is the observation that, at this sample point, bioreactors turned pink-red, as shown for *Metschnikowia pulcherrima* under similar growth conditions. Both species are known to produce pulcherrimin, an iron chelate molecule, with antimicrobial effect by limiting access of other microorganisms to iron

(Sipiczki, 2006). Indeed, the absorbance spectrum of the *K. lactis* supernatants showed the characteristic pulcherrimin peak at 385 nm.

According to the dissolved oxygen profiles, one major change in the environmental conditions between S1 and S2 for *K. lactis* cultures is the transition from oxygen sufficient to hypoxic conditions. Transition from aerobic to anoxic conditions has been shown to induce the production of reactive oxygen species (ROS) in *S. cerevisiae* (David and Poyton, 2005). ROS production might be a consequence of a sudden redox imbalance, when the excess NADH resulting from the activity of the TCA cycle can no longer be taken up by the electron transport chain in the absence of molecular oxygen (Murphy, 2009). Two enriched categories, oxidoreductase activity, and aldehyde dehydrogenase (NAD) activity, suggest this to be also the case for *K. lactis* cells under our experimental conditions. This happens despite oxygen is still available and being used in time point S2, and despite the gradual reduction in dissolved oxygen levels would be expected to allow for a smoother adaptation of yeast cells to oxygen depletion.

A high number of significantly enriched GO terms were found from the set of genes down-regulated in *K. lactis* (Table 3.1). As shown in Table 3.1, the Key Modules (Yang et al., 2011) are iron-sulfur cluster binding; ion transmembrane transporter activity; ubiquinol-cytochrome-c reductase activity; structural constituent of ribosome and monovalent inorganic cation transmembrane transporter activity. Down-regulation of iron-sulfur cluster binding in S2 is in agreement with the above observation of iron becoming a limiting nutrient for *K. lactis* under these culture conditions. Iron-sulfur proteins are considered as regulatory elements of iron metabolism. Yeast cells exhibit loss of iron-sulfur proteins in response to iron depletion (Shakoury-Elizeh et al., 2010).

The oxygen limiting conditions in *K. lactis* in S2 reduced the flux distribution towards respiration compared to S1. Therefore, the mitochondrial electron transport chain is expected to show lower relative activity in S2. Indeed, both the ion transmembrane transporter activity and ubiquinol-cytochrome-c reductase activity GO terms were significantly down-regulated (Table 3.1). These GO terms include, among others, genes coding for the cytochrome c oxidase (*COX12*, *COX13*, *COX4, COX5b*, *COX6*, *COX7*, *COX8*, and *COX9*), or other components of the electron transport chain (*ATP1, COR1, RIP1* and many QCR genes) as well as additional mitochondrial constituents (*AGC1, FSF1*). Also falling in this category are the down-regulated genes coding for permeases for amino acids and other nitrogen compounds (*GAP1*, *DIP5*, *DUR3*). This is probably related to the oxygen limitation observed in S2, since respiration is associated to higher biomass production rates. Also in agreement is the down-regulation of genes coding for structural components of the ribosomes, highlighted in the structural constituent of ribosome GO-term category (Table 3.1).

Table 3.1. GO term enrichment among differentially expressed genes for each species. GO terms were grouped in biomodules by GO-Module.

	Strain	GO ID	P-val.	Sign.*	GO terms	GO-Module ID
Up-regulated	*K. lactis*	GO:0016491	0	K	oxidoreductase activity	1
		GO:0004029	$6x10^{-3}$	K	aldehyde dehydrogenase (NAD) activity	2
		GO:0005381	$2x10^{-3}$	K	iron ion transmembrane transporter activity	3
	S. cerevisiae	GO:0000944	0	K	base pairing with rRNA	1
		GO:0030556	0	T	rRNA modification guide activity	1
		GO:0030559	0	K	rRNA pseudouridylation guide activity	2
Down-regulated	*K. lactis*	GO:0051536	$2x10^{-3}$	K	iron-sulfur cluster binding	1
		GO:0051539	$3x10^{-3}$	T	4 iron, 4 sulfur cluster binding	1
		GO:0015075	0	K	ion transmembrane transporter activity	2
		GO:0008324	0	T	cation transmembrane transporter activity	2
		GO:0022890	0	T	inorganic cation transmembrane transporter activity	2
		GO:0008121	0	K	ubiquinol-cytochrome-c reductase activity	3
		GO:0003735	0	K	structural constituent of ribosome	4
		GO:0015077	0	K	monovalent inorganic cation transmembrane transporter activity	5
		GO:0004129	0	T	cytochrome-c oxidase activity	5
		GO:0015078	0	T	hydrogen ion transmembrane transporter activity	5

*'K' refers to the key terms of GO biomodules, 'T' refers to the truly significant hierarchical descendents of the key terms.

Under these experimental conditions, for the industrial *S. cerevisiae* yeast strain, the GO terms that appeared to be significant from the up-regulated set of genes, were all of them associated with rRNA and rRNA pseudouridylation (Table 3.1). Almost half of the genes significantly over-expressed in S2 are small nucleolar RNAs (snoRNAs). These stable RNAs are found within small nucleolar ribonucleoprotein complexes (snoRNPs) and localize to the nucleoli of eukaryotic cells. The majority of the snoRNAs are involved in ribosomal RNA processing, including pseudouridylation, a frequent posttranscriptional modification of uridine in RNAs. Pseudouridine (Ψ), when incorporated into RNA, can modify its secondary structure by increasing base stacking, improving base pairing and rigidifying sugar-phosphate backbone. Therefore, it alters the chemical and physical properties of RNA molecules (Zhao and He, 2015).

Pseudouridylation can induce different stress factors (Wu et al., 2011; Ge and Yu, 2013; Schwartz et al., 2014; Karijolich et al., 2015) suggesting a regulatory role for Ψ. The replacement of multiple U sites with Ψ in synthetic RNA molecules results in an increased protein expression level (reviewed in Zhao and He, 2015). The high expression of genes involved in pseudouridylation observed in our work could be related to oxidative stress due to respiratory metabolism. Indeed, RNA post-transcriptional modifications have been previously shown to be important for recovery after an environmental stress (Biggar and Storey, 2015). In addition, Tronchoni et al. (2014) described the role of RNA maturation and transcription stability after cold shock in wine yeast strains. No significant enrichment was found for genes down-regulated in *S. cerevisiae* (Table 3.1).

Genes similarly regulated in both species point to nutrient requirements

Only seven differentially expressed genes (three up- and four down-regulated) were found to behave the same way for *K. lactis* and *S. cerevisiae* (Figure 3.7). This low similarity in their transcriptomic responses is explained by the evolutionary divergence in aerobic fermentation between both species. While, according to RQ values, *K. lactis* is exclusively respiring until the lack of oxygen forces it to initiate fermentation, *S. cerevisiae* under the Crabtree effect maintains the main metabolic flux towards fermentation. Despite these differences, several genes show a common regulation when comparing both time points.

The three genes up-regulated are *INO1*, *THI2* and *FET3*. *INO1* (Figure 3.7), coding for the first enzyme in the inositol biosynthesis pathway, is induced by inositol requirement (Culbertson and Henry, 1975). It has been previously shown that inositol might be one of the limiting nutrients in some yeast culture media (Hanscho et al., 2012; Novo et al., 2013). The synthetic must medium used in this work contained three times more inositol than standard synthetic grape must. However, the aeration of the media allows yeast to respire and probably, this increases biomass production and makes inositol to become a limiting compound (Quirós et al., 2014). Respiro-fermentative metabolism and higher biomass production take place in both species under aerated conditions, despite the Crabtree effect of *S. cerevisiae*. The other two commonly up-regulated genes seem also related to the demand of specific nutrients for biomass production. *THI2*, the transcriptional activator of thiamine biosynthetic genes, responds to thiaminediphosphate demand, and *FET3* (discussed above) is induced by low iron availability (Askwith et al., 1994). Among the four genes commonly down-regulated in S2 we found

ERG25 (Figure 3.7). Erg25p, a di-ferric protein, is essential for the synthesis of ergosterol, an oxygen-dependent process. Down-regulation of *ERG25* in S2, might be related to iron requirement, as discussed above for *K. lactis* electron transport chain genes and genes coding for other mitochondrial constituents.

Validation of RNAseq data by qPCR analysis

A selection of highly differentially expressed genes (log fold change above 1), representative of the main GO terms discussed above, were chosen to validate the RNAseq data and were analyzed across the fermentation to obtain a more detailed expression profile (Figures 3.8 and 3.9). The qPCR data for sample points S1 and S2 confirmed the RNAseq expression in all cases but two. In both cases, gene expression was very low and no solid conclusions can be drawn. For the remaining genes, results from the qPCR analysis largely confirmed the expression pattern observed for S1 and S2 in *K. lactis* and *S. cerevisiae*.

Besides S1 and S2 sample points, additional sample points were included: sample point R, used as a reference for relative quantification by qPCR; and sample point F, representative of an advanced stage of fermentation, with dissolved oxygen levels clearly above sample point S2; finally, given the important differences in metabolic state between S1 and S2 for *K. lactis*, sample point I (Intermediate time point) was included, in order to characterize this observed transition step (Figure 3.9). This sample point corresponds to approximately 30 minutes after the culture reached 0% DO.

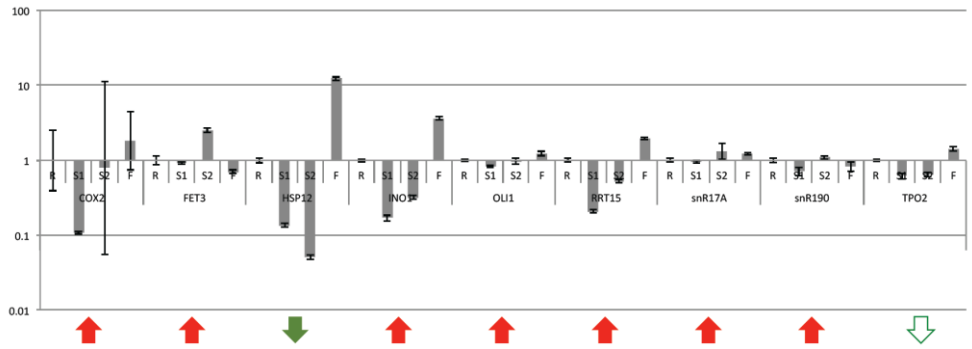

Figure 3.8. qPCR of *S. cerevisiae* selected genes. Sample points are described in Figure 3.4. Arrows represent the direction of gene expression between S2 and S1: Red arrows for genes showing higher expression in S2 compared to S1; Green arrows for genes showing lower expression in S2 compared to S1. Empty arrows represent a discrepancy between the RNAseq data and the qPCR data.

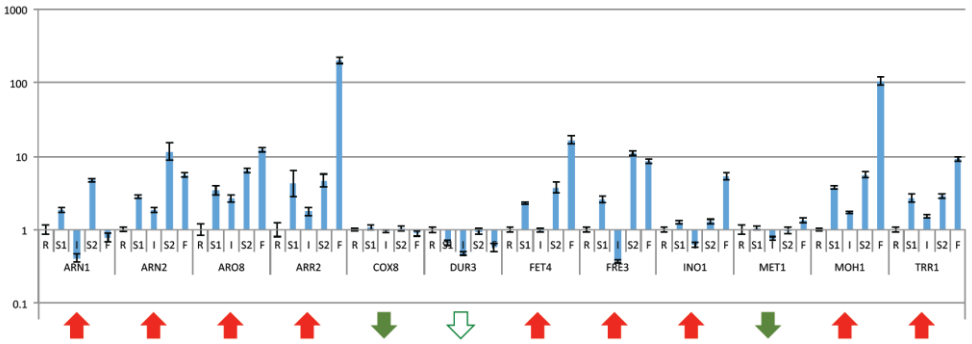

Figure 3.9. qPCR of *K. lactis* selected genes. Sample points are described in Figure 3.4. Arrows represent the direction of gene expression between S2 and S1: Red arrows for genes showing higher expression in S2 compared to S1; Green arrows for genes showing lower expression in S2 compared to S1. Empty arrows represent a discrepancy between the RNAseq data and the qPCR data.

The qPCR characterization of sample point I shows an interesting result, expression levels at this intermediate time point did not follow the trend between S1 and S2. On the contrary, the genes analyzed show a clear drop in expression at this sample point. Probably, this indicates a general reduction in gene transcription, in order to allow the cell to adapt its metabolism to the new growth conditions, with limiting oxygen availability.

As mentioned above, preliminary experiments showed a slightly different fermentation profile for *K. lactis* AQ2166, as compared to the *K. lactis* type strain. The main differential feature is a slower transition step for *K. lactis* AQ2166. For this reason, qPCR analysis was performed for this strain from sample point I, for the same genes than the type strain. As shown by the trend lines in Figure 3.10, the general behavior of both *K. lactis* strains is similar, concerning relative expression levels between sample points S2 and I, and between sample points F and S2. However, the general trend towards reduced expression levels during the transition step (sample point I), previously observed for the type strain, is less pronounced for *K. lactis* AQ2166 (with the noticeable exception of *ARR2*). This weaker response to the transition step is in agreement with the slower slope cultures of this strain reach 0% DO. This suggests *K. lactis* AQ2166 is able to perceive and anticipate oxygen limitation more efficiently than the type strain, and gradually adapt transcription levels, before oxygen availability becomes a limiting factor for growth.

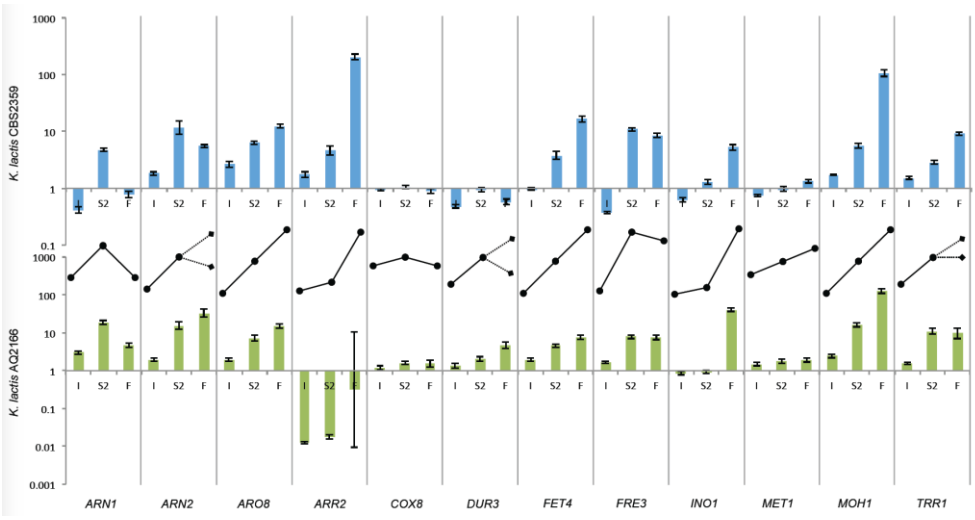

Figure 3.10. Comparative gene expression profile between *K. lactis* strains after reaching zero per cent of dissolved oxygen. Gene expression trends are shown by schematic lines. Continuous line: same gene expression trend; dotted lines: different expression trend.

Oxidative stress

A transitory oxidative stress response due to hypoxic conditions has been described for *S. cerevisiae* (Becerra et al., 2002) and *K. lactis* (Blanco et al., 2007). Despite some similarities, the hypoxic induced oxidative stress response seems to be different between both yeast species, probably due to the preference for respirative metabolism in *K. lactis*, in contrast to respiro-fermentative metabolism in *S. cerevisiae*. González-Siso and Cerdán (2012) proposed a set of gene types to be explored in order to characterize these transcriptional responses, including heme biosynthesis, ergosterol biosynthesis and supply, NAD(P)H consuming oxidative defense reactions, other oxidative defense reactions, NAD(P)-dehydrogenases from the inner

membrane of mitochondria, heme/respiration-related transcriptional factors, sterol-related transcriptional factors, peroxide-related transcriptional factors, life span-related proteins, and mitophagy-related proteins.

Given the hypoxic conditions identified in S2 for *K. lactis* cultures, we paid attention to changes in the expression levels of genes assigned by González-Siso and Cerdán (2012) to each of the above-mentioned gene types in both *K. lactis* and *S. cerevisiae*. Even though time point S2 cannot be compared between yeast species given the important metabolic differences, it was worth to study if oxidative stress response genes appeared significantly induced in *S. cerevisiae*. As shown in Table 3.2 and 3, the number of these genes differentially expressed between S1 and S2 is higher for *K. lactis* than for *S. cerevisiae*. This number of DEG is higher than observed by Blanco et al. (2007), probably due to differences in the experimental conditions and to the lower throughput techniques available at that time for *K. lactis*.

Table 3.2. Gene types responding to oxidative conditions due to hypoxia in *K. lactis*.

Gene	Orthologs	FC*	Category
KLLA0F15037g	*TRR1*	4.78	other oxidative defense reactions
KLLA0A00660g	*ATG32*	1.88	mitophagy-related proteins
KLLA0B07975g	*GRX8*	1.60	other oxidative defense reactions
KLLA0C17160g	*ATG1*	1.20	mitophagy-related proteins
KLLA0E14939g	*UTH1*	1.18	mitophagy-related proteins
KLLA0E20593g	*ATG8*	0.97	mitophagy-related proteins
KLLA0F20009g	*AHP1*	0.81	NAD(P)H consuming oxidative defense reactions
KLLA0D14333g	*DOT5*	0.79	NAD(P)H consuming oxidative defense reactions
KLLA0A00264g	*GTT1*	0.77	NAD(P)H consuming oxidative defense reactions
KLLA0B12133g	*ATG11*	0.61	mitophagy-related proteins
KLLA0E04181g	*BCY1*	0.53	life span-related proteins
KLLA0A09383g	*MTM1*	0.53	NAD(P)H consuming oxidative defense reactions
KLLA0E18547g	*MOT3*	0.52	heme/respiration-related transcriptional factors
KLLA0F26917g	*CCS1*	0.52	NAD(P)H consuming oxidative defense reactions
KLLA0F07557g	*GSH2*	0.46	NAD(P)H consuming oxidative defense reactions
KLLA0B03586g	*SCH9*	-0.42	life span-related proteins
KLLA0F22880g	*HAP1*	-0.57	heme/respiration-related transcriptional factors
KLLA0A05071g	*ERG4*	-0.59	ergosterol biosynthesis and supply
KLLA0E17733g	*GRX6*	-0.60	other oxidative defense reactions
KLLA0B11495g	*ROX1*	-0.68	heme/respiration-related transcriptional factors

KLLA0F15224g	*ERG1*	-0.74	ergosterol biosynthesis and supply
KLLA0F10285g	*MVD1*	-0.82	ergosterol biosynthesis and supply
KLLA0C15147g	*HEM3*	-0.83	heme biosynthesis
KLLA0D11242g	*ERG5*	-0.84	ergosterol biosynthesis and supply
KLLA0E03653g	*ERG11*	-0.92	ergosterol biosynthesis and supply
KLLA0C12265g	*ERG24*	-0.93	ergosterol biosynthesis and supply
KLLA0B09636g	*GRX5*	-0.95	other oxidative defense reactions
KLLA0E21891g	*NDE1*	-1.07	NAD(P)-dehydrogenases from the inner membrane of mitochondria
KLLA0B08085g	*ERG25*	-1.13	ergosterol biosynthesis and supply
KLLA0E20285g	*PRX1*	-1.17	NAD(P)H consuming oxidative defense reactions
KLLA0C06336g	*NDI1*	-1.19	NAD(P)-dehydrogenases from the inner membrane of mitochondria
KLLA0F06336g	*ERG2*	-1.28	ergosterol biosynthesis and supply
KLLA0D11660g	*CTA1*	-2.23	NAD(P)H consuming oxidative defense reactions

*FC, fold change

Differences between *K. lactis* and *S. cerevisiae* for the expression of this set of genes are in agreement with the general trend in gene expression discussed above, related to the more limited physiological changes observed for each species (Figure 3.4). Genes up-regulated in *K. lactis* under hypoxic conditions belong to the types “other oxidative defence reactions”, “mitophagy-related proteins”, and “NAD(P)H consuming oxidative defence

reactions", while genes down-regulated belong to "NAD(P)-dehydrogenases from the inner membrane of mitochondria and "ergosterol biosynthesis and supply", as well as two genes for "NAD(P)H consuming oxidative defence reactions" (Table 3.2). This expression pattern would confirm the adaptation responses already hypothesised by González-Siso and Cerdán (2012), that can be summarized as a reduction in mitochondrial activity through mitophagy, the down-regulation of oxygen consuming biosynthetic processes, like ergosterol or heme biosynthesis, and tuning of oxidative defence mechanisms (with more genes up-regulated than down-regulated). Down-regulation of genes typically related with ROS detoxification, like *CTA1* or *PRX1*, or mitochondrial NADH dehydrogenases, like *NDE1* and *NDI1*, seems to be paradoxical, but it is explained by a general reduction in mitochondrial synthesis in S2.

Table 3.3. Gene types responding to oxidative conditions due to hypoxia in *S. cerevisiae*.

Gene	FC*	Category
SOD1	0.92	NAD(P)H consuming oxidative defense reactions
NCP1	-0.46	ergosterol biosynthesis and supply
MGA2	-0.47	heme/respiration-related transcriptional factors
SUT2	-0.47	sterol-related transcriptional factors
DOT5	-0.48	NAD(P)H consuming oxidative defense reactions
ERG26	-0.49	ergosterol biosynthesis and supply
ROX1	-0.51	heme/respiration-related transcriptional factors
TPK2	-0.52	life span-related proteins
ERG7	-0.55	ergosterol biosynthesis and supply
HAP1	-0.59	heme/respiration-related transcriptional factors
ERG5	-0.61	ergosterol biosynthesis and supply
HOG1	-0.61	mitophagy-related proteins
GRX6	-0.63	other oxidative defense reactions
GTT1	-0.70	NAD(P)H consuming oxidative defense reactions
CTT1	-0.72	NAD(P)H consuming oxidative defense reactions
ECM22	-0.72	sterol-related transcriptional factors
NDE1	-0.76	NAD(P)-dehydrogenases from the inner membrane of mitochondria
ERG24	-0.90	ergosterol biosynthesis and supply
ERG1	-0.93	ergosterol biosynthesis and supply
SLT2	-1.10	mitophagy-related proteins
ERG4	-1.12	ergosterol biosynthesis and supply
HEM13	-1.17	heme biosynthesis
UPC2	-1.19	sterol-related transcriptional factors
ERG11	-1.24	ergosterol biosynthesis and supply
ERG3	-1.30	ergosterol biosynthesis and supply
ERG25	-1.43	ergosterol biosynthesis and supply

*FC, fold change

Oxidative stress by hypoxia has been associated to the sudden oxygen deficiency, resulting in leakage of electrons from the electron transport chain, mainly at the level of complex I (Murphy, 2009), leading to a drastic increase in the rate of formation of superoxide anion ($O_2^{\bullet -}$). This oxygen radical is readily converted to H_2O_2 by the activity of superoxide dismutases (encoded in *S. cerevisiae* by *SOD1* and *SOD2*). The expression of these genes is shown in Figure 3.11 as normalized RPKM values for *K. lactis* and *S. cerevisiae*. It shows that there is not a significant change in the expression of *SOD1* and *SOD2* between S1 and S2 in *K. lactis*. Interestingly, this is because this species already shows high levels of expression for these genes related to active respiratory metabolism. The final detoxification step is catalyzed by catalases, Ctt1p and Cta1p. Similar to *SOD* genes, expression levels of *CTA1* and *CTT1* are again higher for *K. lactis* than for *S. cerevisiae* (Figure 3.11).

An intriguing possibility to explain increased acetic acid production would be that the detoxification of $O_2^{\bullet -}$ produced by hypoxic conditions and reduction of H_2O_2 by catalases could be coupled to the oxidation of ethanol to acetaldehyde, as has been shown in human cells (Zimatkin et al., 2006). BioCyc (Caspi et al., 2016) data base collection for metabolic pathways predicts that the same reaction could be catalysed by Ctt1p or Cta1p in *S. cerevisiae* (Reaction: EC1.11.1.6) (Caspi et al., 2016). Further oxidation of acetaldehyde, catalysed by aldehyde dehydrogenases, would result in acetate production.

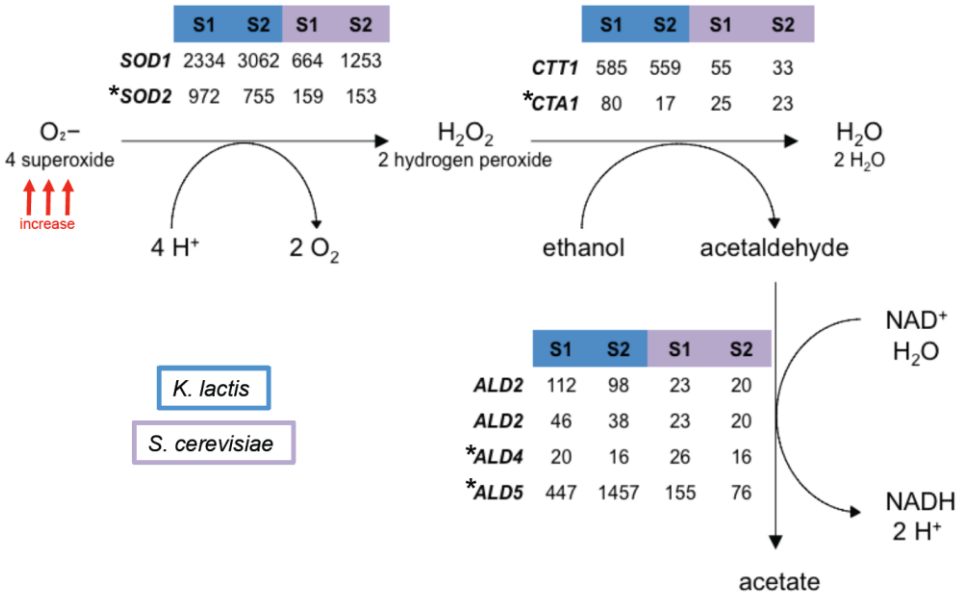

Figure 3.11. Gene expression of the enzymes possibly involved in acetic acid production linked to ROS detoxification. Expression values are shown as RPKM. S1: Sample time point 1; S2: Sample time point 2. *: mitochondrial enzymes.

In conclusion, gene expression analysis has confirmed the metabolic shift observed in DO and also the differences observed for *S. cerevisiae* and *K. lactis* under aerobic conditions. Two main drivers appear to be responsible in *K. lactis* for this transcriptomic remodeling. By one side, this species appears as a respiration and iron specialist, also indicated by the high copy number of genes required for iron uptake, or the high constitutive expression levels of genes involved in ROS detoxification. This species seems to activate two complementary mechanisms in order to cope with increasing iron requirements, activation of iron uptake mechanisms; and repression of

the biosynthesis of heme containing proteins. A second driver to downregulation of genes coding for heme containing proteins (e.g. *COX* genes) is oxygen starvation (hypoxia), which would limit the capacity of the electron transfer chain. Finally, oxidative stress caused by hypoxia might be counteracted by also repressing the synthesis of genes coding for this latter group of proteins, as well as mitochondrial activity, or oxygen consuming biosynthetic processes, like ergosterol or heme biosynthesis. The response of *K. lactis* during adaption to hypoxic conditions is also characterized by a transient general downregulation of the transcriptional activity.

The most striking feature of the transcriptional response of *S. cerevisiae* to continued growth under aerated conditions is the activation of genes involved in RNA pseudouridylation, which suggests this growth condition triggers a response based on RNA posttranscriptional modification, similar to what has been described for other stress conditions. The lack of a specific response involving genes related to respiration in this species correlates with low oxygen consumption throughout the experiment, in turn related to the Crabtree-positive character of *S. cerevisiae*.

The ability for continuous growth in grape must and to maintain a strong respiratory metabolism under hypoxic and respiratory conditions, is a remarkable feature of *K. lactis* strains, which contributes to the usefulness of this species for alcohol level reduction in wine. Our data (both transcriptomic and physiological) indicate that, *K. lactis* is actively and almost exclusively respiring sugars, until oxygen becomes a limiting factor. This suggest that, increasing oxygen availability during the first fermentation stages would help improve results in terms of sugar consumed by respiration and, consequently, alcohol level reduction. The only drawback to take into

account is the eventual impact of increased oxygenation on volatile acidity, at least for some *K. lactis* strains (like the type strain).

Metabolic diversity of *K. lactis* strains is revealed by different physiological and transcriptomic responses to progressive oxygen starvation. The yeast strain showing the smoother adaptation to hypoxic conditions is also the one producing the lowest acetate levels therefore being the strongest candidate to low ethanol production by respiration at initial stages of fermentation following this new methodology.

Chapter 4. Physiological studies of *Saccharomyces cerevisiae* mutant and industrial strains for lowering ethanol and acetic acid content

BACKGROUND

Rising sugar concentration of grape berries at harvest, due to global climate warming (Mira de Orduña, 2010), is a current matter of concern for winemakers, particularly in warm producing regions. A second factor contributing to increasing sugar levels in grapes is the current consumer preferences for well-structured, full body wines, which require an optimal phenolic maturity of grapes. Under standard winemaking conditions, excess sugar in grape must, combined with other changes in must composition, also related to global climate warming, results into fermentation troubles and, more significantly, into high alcohol content in the final wines. High ethanol content in wines can compromise product quality by exacerbating the perception of some mouthfeel features such as hotness and viscosity. Sweetness, acidity, aroma, flavour intensity, and texture properties can also be negatively impacted, albeit to a lesser extent (Gawel et al, 2007a; Gawel et al, 2007b; Guth and Sies, 2002). In addition, wines with a high alcoholic degree might be rejected by some consumers, as it will be perceived as a threat for health and road safety. International trade of such wines might also be hampered by significant increases in taxes, depending on the countries involved.

Currently, there is not a single approach that would completely solve this issue. Therefore, the wine industry is seeking for complementary solutions targeting different steps of the production cycle, including grapevine clonal selection, vineyard management, removal of excess sugar, adaptation of winemaking practices, using metabolic inhibitors, or partial ethanol removal (Teissedre, 2013). Concerning the fermentation step in winemaking, most of the efforts are currently focused on the use of non-*Saccharomyces* wine yeast species/strains showing lower ethanol yields than *Saccharomyces*

cerevisiae (Ciani et al. 2016; Loira et al., 2012). Some years ago, researchers from Instituto de Ciencias de la Vid y del Vino proposed taking advantage of the respiratory metabolism of yeasts as an approach for reducing ethanol yield (Gonzalez et al., 2013). Several yeast strains were screened for the ability to reduce ethanol yield under aerated conditions. Due to the Crabtree effect *S. cerevisiae* strains were not expected to show significant respiration levels, but some strains did show a noticeable respiration rate (Quirós et al. 2014). However, many of them, including all *S. cerevisiae* strains showed a significant increase in acetic acid production under aerobic conditions (Quirós et al. 2014; Chapter 2). This drawback cannot be solved by just selecting other yeast strains, since the proposed technology requires *S. cerevisiae* to be inoculated either simultaneously or subsequently to non-*Saccharomyces* starters, to ensure fermentation completion. In addition, albeit at low initial levels, *S. cerevisiae* will be almost invariably present in the natural microbiota of grape must. Therefore, volatile acidity due to the metabolic activity of *S. cerevisiae* would always remain a matter of concern for the fermentation of wine under aerated conditions, as shown in studies using a combination of *Metschnikowia pulcherrima* and *S. cerevisiae* (Morales et al., 2015).

In this chapter, two parallel strategies were used in order to tackle the problems related to the use of *S. cerevisiae* in winemaking under aerated conditions. On one hand, several computational modelling and other complementary approaches were used to identify yeast genes whose deletion would be predicted to result in reduced acetic acid yield under aerobic conditions. This was performed on a laboratory strain background, to take advantage of the availability of a yeast strains knock out collection in this background. Some of the predictions from computational simulation

could be experimentally confirmed, showing promise as targets for the genetic improvement of industrial yeast strains.

On the other hand, a *S. cerevisiae* wine yeast strains was constructed, aiming to reduce the Crabtree effect, as a way to improve ethanol content reduction by respiration. This metabolic trait makes *S. cerevisiae* preferentially consume sugars by fermentation, independently of oxygen availability, and seems to be regulated at various levels, from transcriptional repression of respiratory functions (Barnett and Entian, 2005), to kinetic features of enzymes involved in pyruvate metabolism in this species (Holzer, 1961; Pronk et al., 1996). Some mutations have been related to alleviated Crabtree effect in *S. cerevisiae*, including loss-of-function of *REG1* (Herwig and von Stockar, 2002) or *HXK2* (Diderich et al., 2001; Petit et al., 2000; Raamsdonk et al., 2001; Rossell et al., 2008); as well as reduced pyruvate kinase (Pyk1) levels (Pearce et al., 2001). *REG1* and *HXK2* are involved in carbon catabolite repression (CCR) in this species (Figure 4.1). Reg1 is the regulatory subunit of the Glc7-Reg1 protein phosphatase complex, targeting it to several CCR related substrates, including Snf1 or Mig1 (Novo et al., 2013). Hxk2 is a moonlighting protein. In addition to its hexokinase activity (it is the main cytoplasmic hexokinase during yeast growth on glucose), it participates in transcriptional repression in the nucleus, together with Mig1 (Novo et al., 2013). Nucleocytoplasmic localization of both Hxk2 and Mig1 depends on its phosphorylation state (Contreras et al., 2015). The impact of lowered Pyk1 activity on the Crabtree effect is probably related to the rate of pyruvate accumulation (Holzer, 1961; Pronk et al., 1996, Pearce et al., 2001) (Figure 4.2). Two additional target genes, not initially planned, were incorporated in this part of the work, based on experimental results obtained, *PDE2*, and *PDC1*. In this chapter I confirmed reduced ethanol yields in *S.*

cerevisiae during the fermentation of natural or synthetic grape must under aerobic conditions, despite the Crabtree effect, as recently described (Quirós et al. 2014, Chapter 2, Morales et al., 2015). However, these were not actually improved by the genetic modifications assayed. In contrast, we found an unexpected positive impact of some genetic modifications on volatile acidity (i.e. acetic acid), the main drawback of *S. cerevisiae* for this application (Quirós et al. 2014, Chapter 2, Morales et al., 2015).

METHODS

Yeast strains

S. cerevisiae wine yeast strain, EC1118 (Lallemand Inc., Ontario, Canada), two haploid laboratory strains CEN.PK113-7D (Nijkamp JF, et al., 2012) and S288C (Mortimer and Johnston,1986) were used in this work to evaluate their fermentation profile and verify the approximate effect of oxygen in acetic acid production. Yeast knock-out strains were obtained from Euroscarf (http://www.euroscarf.de). They were constructed in the BY4741 background and transformed with the plasmid pHLUM (Bürckstümmer et al., 2006) to render them prototroph and reduce the potential interference of auxotrophic markers. In addition, *S. cerevisiae* Zymaflore® FX10 (Laffort), a homozygous and homothallic commercial wine yeast strain was used as host strain for genetic modification of industrial yeasts. Homozygous Yeast knock-out strains (BY4743 background), used as the origin for the KANMX transformation cassettes, were obtained for Open Biosystems, Huntsville, USA.

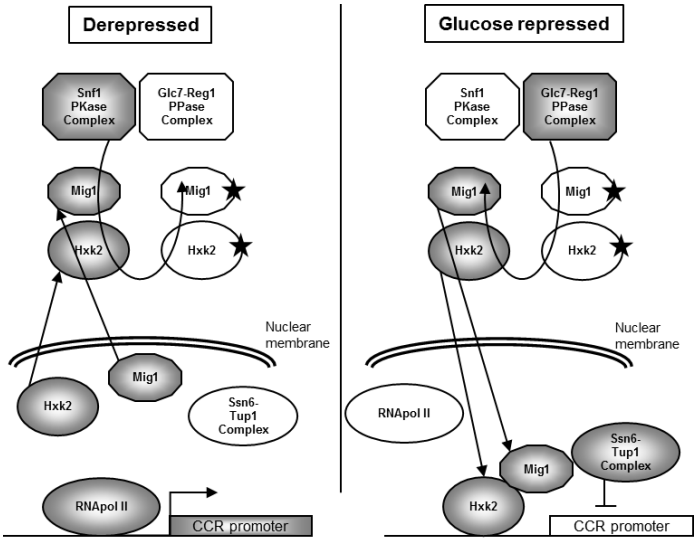

Figure 4.1. Simplified model showing the main role of Hxk2 and Reg1 in carbon catabolite repression (CCR). The different elements are shown in grey for the active state and empty for the inactive state. Stars on Mig1 and Hxk2 indicate a phosphorylated state. Several elements shown in the model have additional functions (either in CCR or not), and not all the factors involved in CCR are shown. Both Hxk2 and Reg1 must be active for efficient repression of many genes under CCR control. Model based on (Gancedo, 2008), with additional information from (Fernández-García et al., 2012).

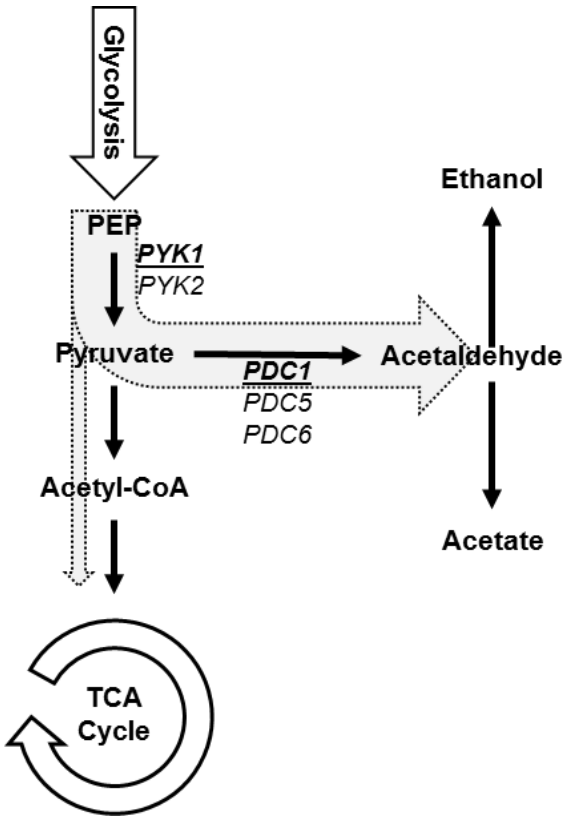

Figure 4.2. Schematic representation of the pyruvate node in *S. cerevisiae*. Background arrows indicate carbon flux distribution under excess sugar, aerobic conditions for Crabtree-positive yeasts (overflow metabolism at the pyruvate node level). Some of the genes deleted in this work (underlined) are shown; as well as genes coding for cognate isoenzymes. PEP phosphoenolpyruvate.

Construction of recombinant strains

The heterozygous mutants were constructed using the short flanking homology method (Güldener et al., 1996), by transforming FX10 using the lithium acetate procedure (Gietz, 2002) with a PCR fragment obtained by amplification of the KanMX4 cassette and flanking regions from the appropriate homozygous deletion strain in the BY4743 background. Selection of heterozygous FX10 transformants was performed in YPD solid media plates supplemented with 200 mg/L of geneticin (G418). Correct insertion of the KanMX4 cassette was verified by PCR using primers upstream and downstream of the deleted region combined with primers inside KanMX4. Primers used for the construction and verification of recombinant strains are shown in Annex 4.1. Amplification strategies are summarized in Figure 4.3. The homozygous mutants were constructed by sporulating the heterozygous mutants in a medium supplemented with geneticin. The geneticin resistance feature segregated 2:2 as expected. Since the original strain was homothallic, strains recovered from the segregation analysis plates were spontaneous autodiploids, homozygous for the corresponding gene deletion, as verified by PCR.

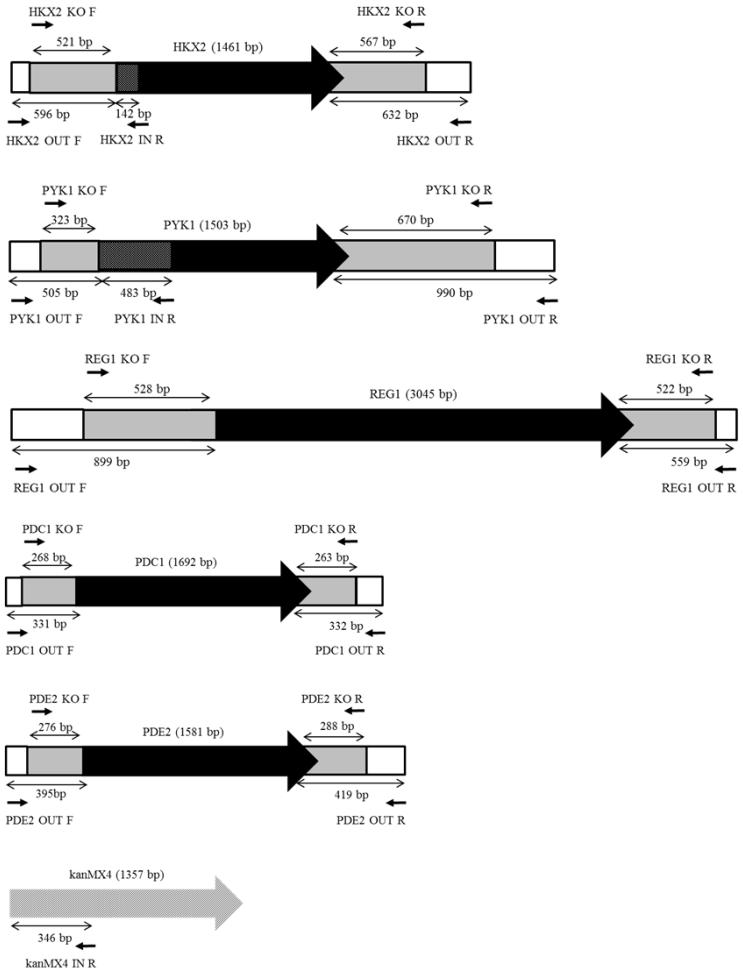

Figure 4.3. Summary of all PCR reactions performed in this work. In black, ORFs to be replaced in FX10. They were replaced by the kanMX4 cassette (shown in the bottom of the figure) by amplifying the whole region from the appropriate homozygous deletion strain in the BY4743 background, and using it to transform FX10. Primers with the "KO" label (see figure) were used for this purpose. Correct insertion was verified by using primers with the "OUT" label, in single and double FX10 deletion strains. In addition, in order to avoid ambiguities, additional confirmation PCR reactions were run by using one of the "OUT" primers from each pair and the "kanMX4 IN R" primer. In this case, additional control PCR reactions were run with the "IN" labelled primers (see figure). All PCR verification reactions were run in parallel with genomic DNA from the parent and the putative recombinant strain.

Dissection of yeast asci

Cells were incubated (25°C, 150 rpm) in sporulation medium (1% potassium acetate) after an overnight in pre-sporulation medium (YPG), in flasks allowing aeration at 250 rpm. When ascospores were visible by microscope at enough density, tetrads were dissected as follows: Ascus were digested with a zymolyase solution (2.5 mg/mL of zymolyase, 1 M sorbitol). A total of 500 µL of the solution with the sporulated cells was spun down and the cell pellets were resuspended in 500 µL of zymolyase solution (0.5 mg/mL) and incubated at room temperature for 10 min. Tetrad dissection was performed in a Singer micromanipulator (Singer Instruments) and grown in YPD plates.

Growth conditions

The strains were grown at 25 ºC, and maintained at 4 °C on YPD plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar), as well as in glycerol stocks at -80 °C. For *S. cerevisiae* S288C physiological characterization, batch cultures were performed in sterilized synthetic medium, based on Herwig synthetic medium (Herwig et al., 2001) containing 20 g/L glucose, 5 g/L (NH4)2SO4, 3 g/L KH2PO4, 0.5 g/L MgSO4 7H2O, 8 mL/L trace element solution, 8 mL/L vitamin solution, and 0.1 mL/L anti-foam (PPG P2000). The composition of both trace and vitamin solutions is described in Chapter 3. The pH of medium was adjusted to 3.5. Previously, parallel fermentations were performed in real wort and synthetic wort to ensure that the results were comparable.

Fermentation experiments in natural grape must contain approximately 200 g/L of sugar. Grape juice was stored at -20ºC. Before use, it was heated in

an autoclave until reaching 105 ºC for a few seconds, and then allowed to cool down.

For cultures in bioreactors, yeast inocula were grown in YPD broth for 48 h, at 25-28ºC (for other strains or FX10 based strains respectively) and 250 rpm and washed twice with water before use. The medium was inoculated to a final OD_{600} of 0.2. The experiments were performed in a DASGIP parallel fermentation platform (DASGIP AG, Jülich, Germany) equipped with four SR0400SS vessels. Bioreactors were filled with 250-300 mL of culture medium, for other strains or FX10 based strains respectively. Agitation was maintained at 250 rpm and the temperature kept 28 ºC with a recirculating chiller. Aerobic cultures were sparged with pure air at a gas flow of 1.0-2.5 L/h (for other strains or FX10 based strains respectively) controlled with gas flow controllers (MFC17, Aalborg). Anaerobic cultures were sparged with N_2 at the same regime. Gas flow calibration was regularly verified with a soap bubble flow meter.

Exhaust gas was cooled in a condenser and the instant concentrations of O_2 and CO_2 recorded with a GA4 gas analyser (DASGIP AG). For technical reasons gas exchanges were only determined for aerobic fermentation experiments. Oxygen consumption and CO_2 production were determined by taking into account the in and out gas flows, and their respective concentrations in air and in the off gas. Instant values were integrated over time. Respiration quotient (RQ) was calculated as que quotient between CO_2 production and oxygen consumption.

Determination of acetic acid production on shake flasks used 250 mL flasks (nominal volume), with 25 mL working volume of natural grape must. Samples for the determination of metabolite concentrations and OD_{600} were

withdrawn during the exponential phase at 64, 72, 88, 92, 116 and 120 hours of fermentation, for each of the biological replicates.

Determination of extracellular metabolites

The concentrations of glucose, glycerol, ethanol, and acetic acid were determined in duplicate by HPLC using a Surveyor Plus liquid chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively); and a HyperREZTM XP Carbohydrate H+ column (8µm particle size) and guard (Thermo Fisher Scientific). The column was maintained at 50 ºC and 1.5 mM H2SO4 was used as the mobile phase at a flow rate of 0.6 mL/min. Prior to injection in duplicate, the samples were filtered through 0.45 µm pore size nylon filters (Fisher Scientific, Madrid, Spain) and diluted 20-fold in MiliQ water.

Determination of dry weight

Nine millilitre samples of the culture were withdrawn from each fermenter and vacuum filtered in a Millipore discs (0,22 µm pore size). Samples were washed twice with deionized water. Discs were then dried for 24 hours on an oven at 65 ºC. Dry weight of the cells was determined as the difference between the weight of the dry discs before and after filtering the culture.

2-deoxy glucose sensitivity assay

Recombinant FX10 deletion strains were spotted at different dilutions (10^{-1} to 10^{-4}) on YP plates (10 g/L yeast extract and 20 g/L Bacto peptone) that contained 2% of galactose as carbon source, supplemented with 200 µg/mL of 2-deoxy glucose. Plates were incubated for 48 h at 28 °C.

Volatile compounds analysis

500 µL of sample (or water HPLC grade for control; Sigma-Aldrich, Spain) were placed in a 2 mL glass-vial with 1 mL of ammonium sulphate solution (45% w/v) and extracted with 250 µL of methyl acetate-ethanol solution (99.5:0.5, v/v) containing 50 ppm of internal standards (4-methyl 2-pentanol, 1-nonanol, and heptanoic acid). A 3 µL sample of upper, methyl acetate phase was injected with the SSL liner held at 180 ºC.

Gas chromatography-mass spectrometry was carried out in a Thermo TRACE GC Ultra device, equipped with a Thermo TriPlus autosampler with a fused-silica capillary column TG-WAXMS A (30 m long; 0.25 mm OD; 0.25 µm film thickness) coupled to a Thermo ISQ mass detector.

Chromatographic conditions were as follows: 5 min at 40 ºC, 3 ºC/min up to 200 ºC, 15 ºC/min up to 240 ºC, and 10 min at 240 ºC. Helium was used as carrier gas at a flow rate of 1 mL/min, operating in split mode (ratio 30). Detection was performed with the mass spectrometer operating in the Full Scan mode (dwell time 500 ms), with 70 eV ionization energy, and source and quadrupole temperatures of 250 ºC. Peaks were identified by comparison of retention times and ion spectra from real standards (Sigma-

Aldrich Química) and spectra from the NIST mass spectral library. For each compound, including internal standards, the sum of the areas of the peaks of up to five characteristic ions was obtained.

Statistical analysis

One-way analysis of variance was carried out on data from biological triplicates using Tukey's or the Dunnet unilateral tests (depending on the experiments, see below), with significance level set at 5%. Correlations between parameters were analysed by Pearson correlation analysis. All analyses were performed using SPSS Statistics v. 25 program (IBM, Armonk, NY).

Metabolic modelling

The iFF708 genomic scale model of *S. cerevisiae* employed in this study was used in its bioopt format. This model of *S. cerevisiae* contains 708 "Open Reading Frames" (ORFs) corresponding to approximately 12% of the ORFs identified in the genome of *S. cerevisiae*, according to the *Saccharomyces* Genome Database (SGD; http: //www.yeastgenome. org) until the date of its construction. This model also comprises 842 reactions distributed by subcellular compartments that include cytosol, mitochondria and extracellular space.

This study used FBA, MoMA and MiMBl algorithms for simulation of the fluxes at specific environmental conditions obtained from the aerobic

physiological characterization of *S. cerevisiae* S288C, to predict and investigate the deletion of different genes possibly related to the production of acetic acid in the presence of oxygen.

FBA simulations were performed using the GNU linear programming kit, MoMA calculations were performed using an object oriented quadratic programming package, while MiMBl solutions where determined using a software developed by (Brochado et al, 2012). The methods applied were made available by the group of Dr. K. Patil from EMBL

RESULTS

Suitability of simplified experimental conditions and laboratory strains to model *S. cerevisiae* production of acetic acid under aerobic conditions

Results prior to this investigation showed that *S. cerevisiae* greatly increases its acetic acid yield when the cultures are oxygenated. One of the main solutions found to try to understand this process was the prediction of the main yields and / or conversion rates of aerobic and anaerobic cultures of *S. cerevisiae*, as well as the prediction of genes/"target" reactions whose "knockout" represents an expression with an impact on the production of acetic acid under aerobic conditions.

The selection of the strains used in this work was critical. Although most strains are suitable for metabolite analysis, metabolic differences between

strains are common and should be considered, so this study was carried out using the findings based on batch cultures of strains selected in previous studies, which presented the best compromise between the consumption of sugar in synthetic wort and the yield of acetic acid. To verify the approximate effect of oxygen in the production of acetic acid, the physiological characterization of strains of *S. cerevisiae* was carried out. Initially the fermentation profile of several different strains (CEN.PK113-7D, EC1118, S288C) was evaluated. Batch cultures were carried out in a Dasgip bioreactor in both natural grape must and synthetic medium.

Strain S288C showed the expected behaviour, according to those previously observed for industrial strains. The main features considered are, faster fermentation kinetics under aerobic conditions, as indicated by CO_2 release profiles (Figure 4.4), and glucose consumption profiles (Figure 4.5); increased production of biomass (Figure 4.5) and acetic acid (Figure 4.5) under aerobiosis, and no noticeable effect of aeration on ethanol production. A higher production of glycerol and succinic acid under anaerobic conditions was also observed. Considering S288C is the best studied *S. cerevisiae* strain in terms of genome sequence and metabolic network it was concluded that those models can be used to analyse the problem addressed in this chapter, i.e. excess acetate production under aerobic conditions by *S. cerevisiae*.

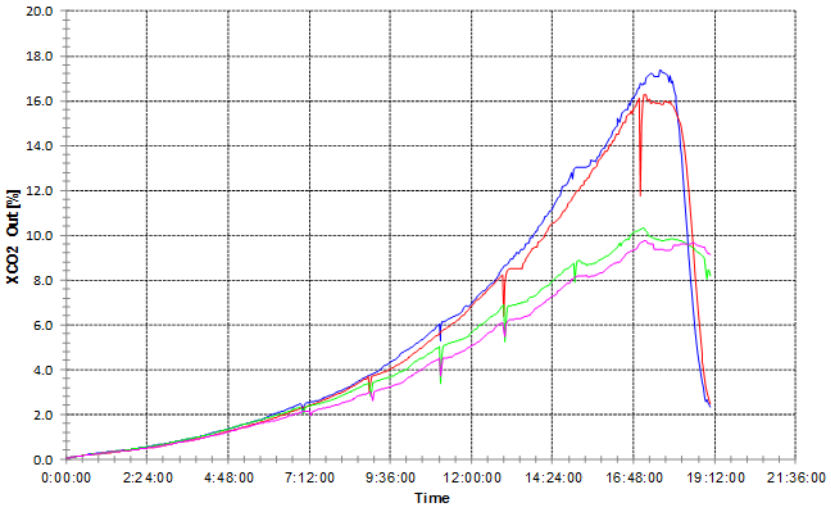

Figure 4.4. CO_2 profile of *S cerevisiae* S288C cultivated in duplicate in synthetic medium containing 2% glucose, under aerobic (blue and red lines) and anaerobic (green and pink lines) conditions. Sampling induced a sharp transient drop in the CO_2 profiles.

Metabolic modelling

It is often difficult to identify which genetic manipulations will lead to the desired phenotype. Thus, to understand the molecular mechanisms underlying the relationships between sugar content, alcohol production and acetate accumulation, mathematical models of metabolism were combined with experimental data.

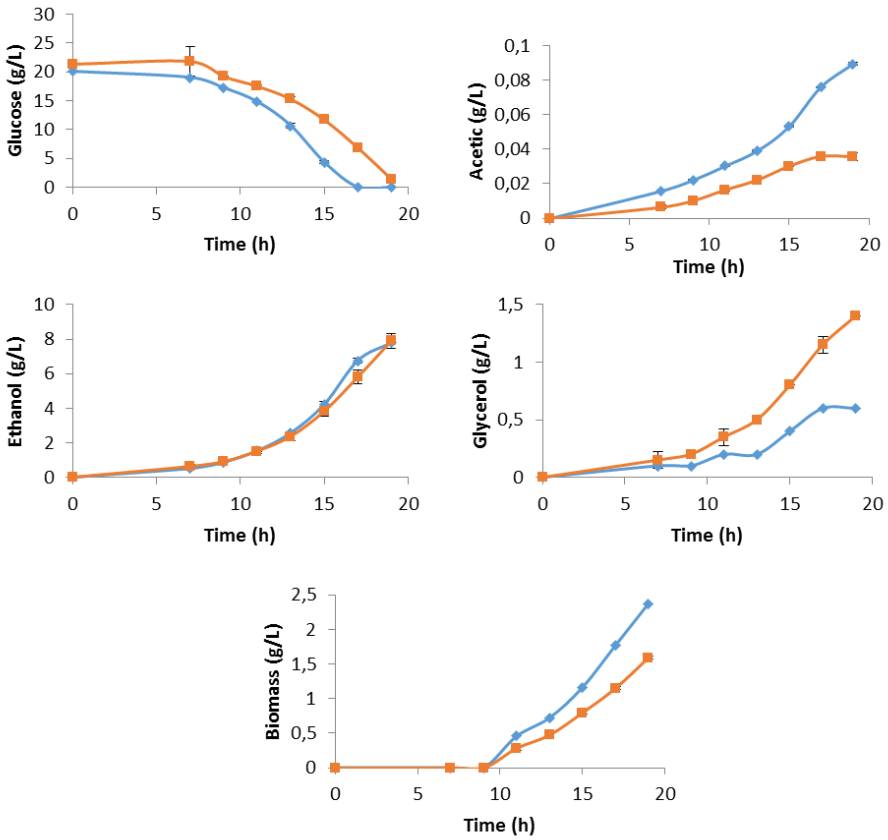

Figure 4.5. Concentration of biomass and main external metabolites under aerobic (blue lines) anaerobic (orange lines) batch fermentation conditions.

In particular, we used constraint-based models, which basically consist on the set of biochemical reactions happening in the cell, in this case in yeast. These models are typically built at genome-scale and also contain some thermodynamic information, which determines reaction reversibility. However, constraint-based models do not model enzyme regulation, neither transcriptional nor substrate-level regulation, which may lead to wrong predictions (Khodayari and Maranas, 2016). Still, when compared to kinetic models which include substrate-level regulation, these provide reasonable accuracy while being easier to build. Furthermore, kinetic models are generally small and hard to scale to the whole genome.

The prediction accuracy of genome-scale models can be improved by incorporating experimental data. For instance, by introducing metabolomics and thermodynamic data to determine reaction directionality we can remove futile cycles, and by including flux data we can impose constraints on the reaction fluxes, further constraining the model.

Common mathematical methods used to find which gene knockouts will lead to the reduction of acetic acid production/yield at maximum growth rate are FBA (Varma and Palsson, 1993), MoMa (Segre et al., 2002), and MiMBl (Brochado et al., 2012). FBA works by finding the reaction flux values that lead to reduced acetic acid production at maximum growth rate while satisfying the steady-state assumption (i.e. metabolite concentrations do not change), and the bounds imposed on the reactions fluxes. MoMa attempts to predict the short-term effect of a gene knockout by minimizing the Euclidean distance between the FBA solution for the strain before the knockout and after the knockout. The key difference between FBA and MoMa is that MoMa tries to predict the flux distribution in the cell before it has time to adjust to the gene knockout. While FBA predicts the flux

distribution that would be seen after the cell evolves and adapts to the gene knockout. MiMBl allows to predict genetic interactions in the metabolic network. It works as a variation of MoMA, where the objective function was reformulated to include metabolite turnovers instead of fluxes, taking into account that reactions that carry higher fluxes can have a stronger impact on the predicted flux distribution.

Since the methods are based on different assumptions, different methods provide different predictions. Therefore, in this study different optimization methods were used (FBA, MoMA and MiMBl) to predict acetic acid production while maximizing growth. Furthermore, in these problems there are more variables than equations, hence there are several solutions that satisfy all the constraints, i.e. the system is highly underdetermined. Therefore, we performed the predictions several times in order to be able to rely on the results.

Firstly, the model was pre-processed by removing duplicate reactions and isoenzymes. Secondly, the reference (wild-type) flux distribution was determined, in order to obtain the normalization factors that were used as input for MoMa and MiMBl. It is essential to use an accurate reference flux distribution in order to obtain biologically meaningful simulation results. The following step was to compare strains with single gene deletions to see which one produced more or less acetic acid in aerobic conditions, while maximizing growth. The growth of single gene deletion mutants in different environmental conditions was then simulated with MoMA and MIMBl, while using the optimal wild-type FBA flux distribution as reference. Only the essential genes were considered for these simulations. The conversion rates used as environmental conditions for the model are listed in Table 4.1. Original raw experimental data, as well as calculation details in order to

decide on these bounds are described in Annex 4.2. It is important to note that during the simulation process different outputs were generated, in order to see the model behaviour in different oxygen and glucose limitation conditions at different growth rates (changing the upper bound constrains between experimentally observed literature values, and unlimited production). This was performed in order to verify if the model was correctly predicting acetic acid production under aerobic conditions. From the observed results it is concluded that, for unlimited oxygen conditions, there was no production of acetic acid, whereas the production of ethanol was similar to the one determined experimentally. Therefore, it was essential to restrict the upper bound of oxygen to the experimental values. Annex 4.3 shows the final chosen output for the simulation of acetic acid production in aerobic conditions, while constraining the production of the experimentally measured metabolites, according to Table 4.1. Two of the methods, FBA and MoMA, produced solutions for 330 possible gene deletions, while MiMBl returned solutions for around 250 genes. In the case of FBA, the great majority of the results were considered unsuitable for acetic acid reduction because the predicted values for growth rate or extracellular acetate were out of the target range. For MoMa and MiMBl, the simulation produced several gene knock-outs with expected reduction in acetic acid production (Figure 4.6). There was no overlapping between FBA predictions, and those performed by the other two methods.

Table 4.1. Values of the different upper bounds used to perform simulations with FBA, MoMA, and MiMBl.

Replicate	Aerobic 1	Aerobic 2
Growth rate[1]	0.2233	0.2233
Glucose uptake[2]	10.369	10.509
Oxygen[2]	0.926	0.852
Ethanol[2]	15.607	15.360
Glycerol[2]	0.658	0.547
Acetate[2]	0.115	0.113
Succinate[2]	0.009	0.008
Carbon dioxide[2]	19.719	18.774

[1] h^{-1}
[2] $mmol.gDW.h^{-1}$

Genome-scale models still lack thermodynamic parameters which can generate futile cycles and unbalanced reactions. It is also true that the reversibility of all the reactions in the model used in the predictions is not yet 100% cured, since we would need information on internal metabolite concentrations to adjust the model. Considering these difficulties, I used three criteria in order to select candidate deletions for acetic acid reduction during the fermentation of grape juice in the presence of oxygen. First, from the list of single reaction knock-outs, some of the best solutions encountered, regarding growth rate and the value of acetic acid production, were selected taking into account results from both MoMA and MiMBl (Table 4.2); second, genes coding for transcription factors involved in central carbon metabolism; third, other genes that have been related to CCR in the literature (according to results from the parallel experiments described below). A summary of all the selected genes is shown in Table 4.3.

Table 4.2. Best candidate gene deletions according to the prediction method.

Gene name	MoMA[1]	MiMBI[1]
ADH3	✓	✓
ALD6	✓	✓
CIT1	✓	✓
CTP1	✓	✓
DIC1	✓	
GLT1	✓	
MDH1	✓	
MDH3	✓	✓
MIR1	✓	
NDI1	✓	✓
OAC1	✓	
PDA1	✓	✓
RIP1	✓	
THR1	✓	✓
THR4	✓	✓
TKL2[2]		

[1] ACxtO < 0.1 ∩ 0.18 < VGRO < 0.23
[2] ACxtO > 0.1 ∩ 0.18 < VGRO < 0.23 (used as negative control for acetic acid production).

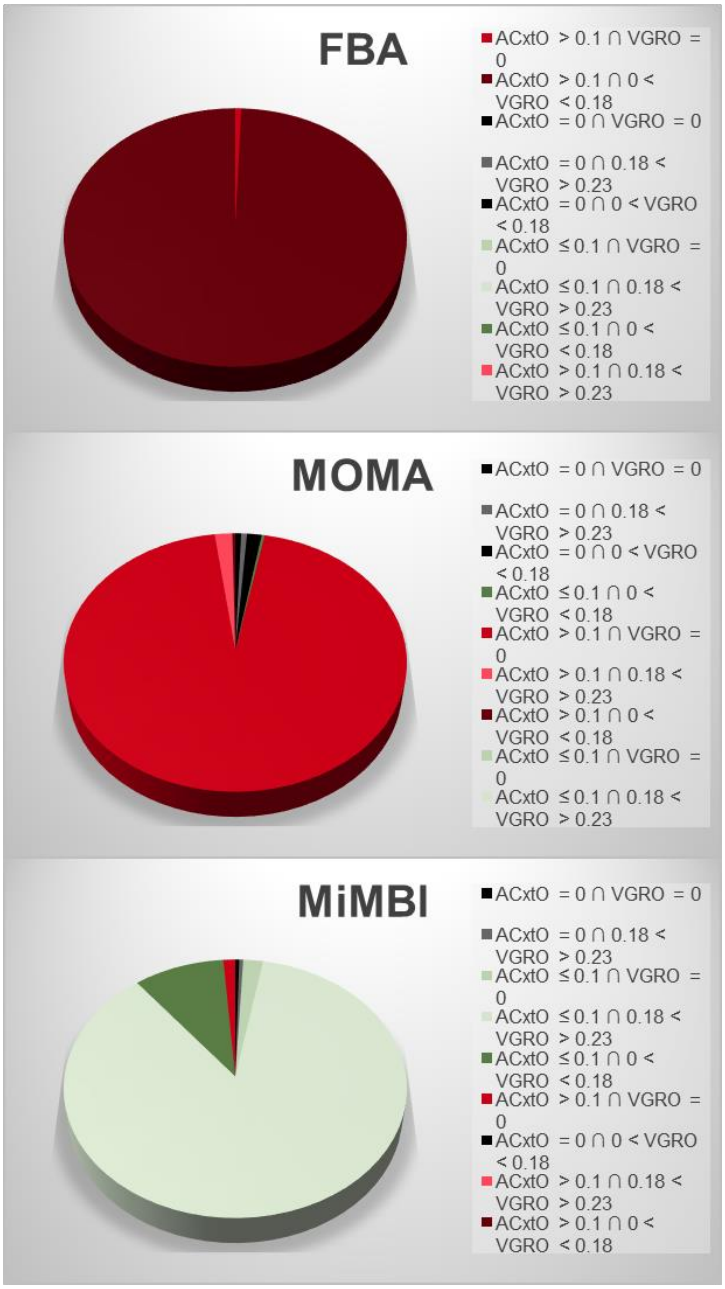

Figure 4.6. Summary of results of the different predictions perform with the different algorithms. VGRO, growth rate (h^{-1}); ACxtO, output for extracellular acetic acid (mmol.gDW.h^{-1}).

Table 4.3. Summary of all gene deletions retained as candidates to improve (reduce) acetic acid production under aerobic conditions according to the selection criteria.

Computational simulation	Regulatory genes	Other CCR related genes
ADH3	*GDH1*	*ADR1*
ALD6	*HAP4*	*CAT8*
CIT1	*HOR2*	*CYC1*
CTP1	*HXK2*	*CYC8*
DIC1	*MTH1*	*MIG1*
GLT1	*SNF1*	*MIG2*
MDH1	*STD1*	*REG1*
MDH3		*RGT1*
MIR1		*TUP1*
NDI1		
OAC1		
PDA1		
RIP1		
THR1		
THR4		
TKL22		

Experimental confirmation of aerobic acetic acid production in the BY4741 background

Production of acetic acid was evaluated for each of the strains in shake flasks, using natural grape must as culture medium. Acetic acid accumulation was monitored over time, and data were analysed in different ways. The main acetic production data are shown in Annex 4.4 for each of the mutants. It can be appreciated that some strains like *MDH3*, *ALD6*, and *RIP1* show much slower accumulation and lower final content of acetic acid than the control, while others do not differentiate from BY4741 pHLUM. It is also important to note that there were also strains that behave opposite to what was expected from simulation (see also Figure 4.7). According to this first overview, the 92 hours sample point was taken as the most informative one for statistical analysis. A statistical analysis was performed on acetic acid concentration at this timepoint (Annex 4.5). In parallel, acetic acid yield was compared for the same time point (Figure 4.7). According to all these data, the following gene deletions seem to be the most promising ones as targets for genetic improvement of industrial wine yeasts: *MDH3*, *ALD6*, and *RIP1*. *MDH3* encodes a peroxisomal malate dehydrogenase isozyme, involved in the glyoxylate cycle. *ALD6* codes for a cytosolic aldehyde dehydrogenase. This isozyme is activated by Mg^{2+} and utilizes NADP+ and has been described as a major contributor to acetate production by *S. cerevisiae* (Saint-Prix et al., 2004). *RIP1* codes for an ubiquinol-cytochrome-c reductase, a component of the mitochondrial cytochrome bc1 complex. It transfers electrons from ubiquinol to cytochrome c1 during respiration.

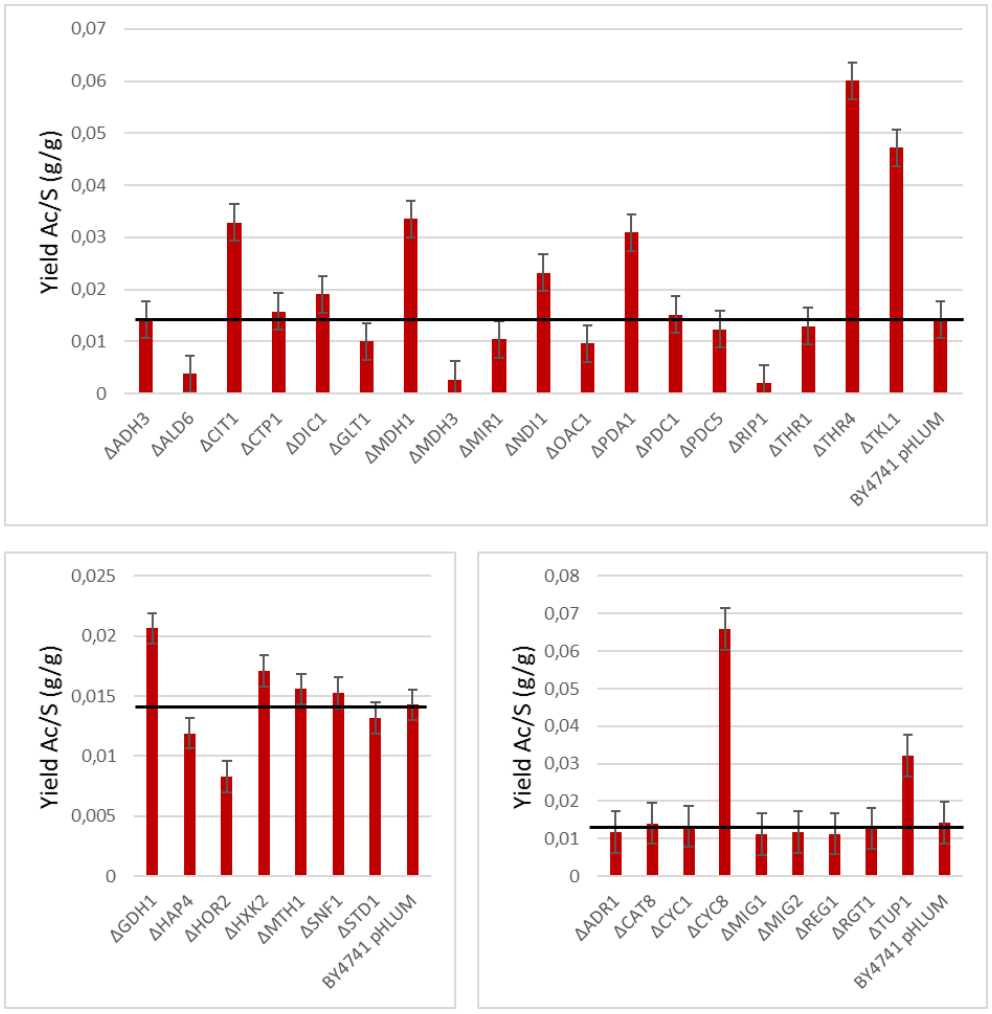

Figure 4.7. Acetate yield of all the laboratory yeast knock-out strain assayed. The horizontal line indicates the value for the control strain BY4741 pHLUM.

Selection of target genes for FX10 genetic manipulation

Given that the Crabtree effect is the major metabolic feature of *S. cerevisiae* restricting respiratory metabolism, three of these genes (*HXK2*, *REG1* and *PYK1*) were selected according to published information about the impact of gene deletions on respiro-fermentative metabolism. (Herwig and von Stockar, 2002) found that mutant yeast strains defective for either *HXK2* or *REG1* alleviated repression of respirative functions by external glucose. Several other authors have reported reduced formation of fermentation product as well as higher biomass yield by yeast strains carrying inactive alleles of *HXK2* (Diderich, et al., 2001; Petit et al. 2000; Raamsdonk et al., 2001; Rossel et al., 2008). On the other hand, Pearce et al. (Pearce et al., 2001) described recombinant yeast strains with reduced pyruvate kinase (Pyk1) levels, which showed increased flux trough the TCA pathway.

FX10 based recombinant strains defective for each one of these three genes were constructed as described in Methods. For *HXK2* or *REG1* both alleles were deleted (Table 4.4). However, *PYK1* being an essential gene, only one of the two alleles was deleted, in order to reduce gene dosage (Table 4.4). According to the functions previously described for these genes, we found that strains FREG1 and FHXK2 were defective for carbon catabolite repression, while FPYK1 was normally repressed (Figure 4.8). However, preliminary analysis of ethanol yields during the fermentation of natural grape must under moderate aeration, failed to identify a relevant impact of these gene deletions on respiro-fermentative metabolism. Interestingly, some of the recombinant strains showed reduced acetic acid production as compared to FX10.

Table 4.4. Yeast strains obtained in this Chapter in the FX10 background.

Strains	Genotype	Source
FX10	Homozygote industrial yeast derivative	Laffort
FHXK2	FX10 hxk2::kanmx4/hxk2::kanmx4	This study
FPYK1	FX10 PYK1/pyk1::kanmx4	This study
FREG1	FX10 reg1::kanmx4/reg1::kanmx4	This study
FPDE2	FX10 pde2::kanmx4/pde2::kanmx4	This study
FPDC1	FX10 pdc1::kanmx4/pdc1::kanmx4	This study

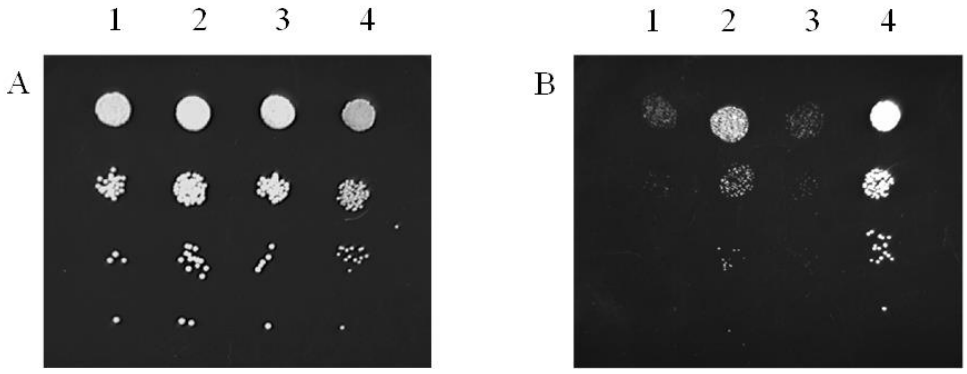

Figure 4.8. Growth of *S. cerevisiae* FX10 control (1), FHXK2 (2), FPYK1 (3), and FREG1 (4) on YPgalactose supplemented (B) or not (A) with 2-deoxy glucose.

We could draw three main observations from our preliminary analysis. First, despite the Crabtree effect, the wine yeast strain FX10 is able to respire a significant amount of sugar under aerobic conditions. Second, as previously shown (Quirós et al., 2014; Chapter 2; Morales et al., 2015; Contreras et al., 2015; Giovanelli et al., 1996; Papini et al., 2012), increased acetic acid production under aerobic conditions is a main limitation in order to use *S. cerevisiae* for alcohol level reduction. Finally, the main advantage observed for some of the recombinant strains tested above was, indeed, reduced acetic acid yields under aerobic conditions. For this reason, we decided to focus on the reduction of aerobic acetic acid yields as the main target for wine yeast improvement, to reach alcohol level reduction without the drawback of excess volatile acidity. Indeed, this was the main target of the parallel research line on this chapter. According to this new focus, we included an additional gene to be deleted in the industrial wine yeast background, *PDC1*. Pyruvate decarboxylase (Pdc1) is a key enzyme in alcoholic fermentation, catalysing the decarboxylation of pyruvate to acetaldehyde, as an intermediary step towards ethanol production (Barnett, 1976). Acetaldehyde can also be oxidized to acetic acid, and several authors have found decreased acetate production by Pdc1 defective yeast strains (Brochado et al., 2010). In addition, the work performed by other researchers in this group, using the BY4743 laboratory background, indicated that *PDE2* deletion might also result in reduced acetic acid yield under aerobic conditions. Hence, *PDE2* and *PDC1* deletions were introduced in the FX10 genetic background, in order to perform a comparative characterization (Table 4.4).

Main fermentation products for recombinant wine yeast strains

Characterization of these five recombinant strains was performed in natural grape must under aerobic and anaerobic conditions. Only one of the deletions resulted in a severe impairment of yeast growth, *PDE2*. Final biomass values for FPDE2 were about one-half those of the control strains (Table 4.5). Differences in biomass values were not supported by statistical analysis. The higher biomass production observed for all the strains under aerobic conditions (Table 4.5) is in agreement with a significant portion of sugar being consumed by respiration. Concerning residual sugar, data for FREG1 indicate *REG1* deletion is detrimental for yeast metabolism; despite no impact on cell numbers being observed. FREG1 is the only strain leaving some residual sugar after seven days of culture, under either aerobic or anaerobic conditions (Table 4.5). As expected, residual sugar was mainly constituted by fructose.

Comparison of ethanol yields on sugar between anaerobic and aerobic conditions (Table 4.6) confirms that, despite the Crabtree effect, even wild type strains of *S. cerevisiae* show a great deal of respiratory metabolism in grape must under aerobic conditions. FREG1 and FPDE2 show the extreme values (0.21 g/g and 0.27 g/g, respectively) of ethanol yield under aerobic conditions; while the ethanol yield for FREG1 is also the lowest (0.31 g/g) under anaerobic conditions. However, most of the recombinant strains showed ethanol yield values that were indistinguishable from the control FX10 strain, not only under anaerobic conditions (where no respiration can take place) but also under aerobic conditions.

Table 4.5. Main metabolites in fermentations run by the parent FX10 strain and recombinant derivatives.

	[1]Biomass (DO_{600} nm)		[2]Residual sugars (g/L)		[2]Ethanol (% vol/vol)		[2]Acetic acid (g/L)		[2]Glycerol (g/L)	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
FX10	13.93 ± 1.93^{b}	20.93± 1.53^{bc}	0.10 ± 0.17^{a}	0.03 ± 0.06^{a}	8.70 ± 0.24^{c}	5.63 ± 0.35^{b}	0.14 ± 0.03^{bc}	3.34 ± 0.48^{cd}	10.28 ± 0.24^{a}	5.80 ± 0.20^{a}
FHXK2	14.53 ± 1.52^{b}	22.70± 2.35^{bc}	0.10 ± 0.17^{a}	0.03 ± 0.06^{a}	8.71 ± 0.34^{c}	5.59 ± 1.01^{b}	0.16 ± 0.08^{c}	3.57 ± 0.38^{d}	11.00 ± 0.17 ^{ab}	5.98 ± 0.03^{a}
FPYK1	10.63 ± 1.67^{ab}	24.63 ± 1.38^{c}	0.17 ± 0.21^{a}	0.06 ± 0.06^{a}	8.51 ± 0.09^{c}	5.79 ± 0.21^{b}	0.11 ± 0.03^{abc}	4.57 ± 0.89^{d}	10.52 ± 0.03^{ab}	6.10 ± 0.20^{a}
FREG1	12.30 ± 0.70^{ab}	24.72± 2.02^{c}	22.50 ± 5.11^{b}	38.00 ± 2.17^{b}	6.70 ± 0.40^{a}	4.03 ± 0.34^{a}	0.15 ± 0.04^{c}	0.15 ± 0.02^{a}	10.73 ± 1.47 ^{ab}	17.57 ± 0.32^{b}
FPDC1	11.71 ± 2.78^{ab}	18.75± 2.19^{b}	ND	ND	8.19 ± 0.26^{bc}	5.64 ± 0.37^{b}	0.06 ± 0.04^{ab}	0.73 ± 0.47^{ab}	9.85 ± 0.06^{a}	5.55 ± 0.44^{a}
FPDE2	7.53 ± 2.05^{a}	13.13 ± 1.95^{a}	0.07 ± 0.12^{a}	ND	7.59 ±0.25^{b}	6.49 ± 0.71^{b}	0.04 ± 0.03^{a}	1.91 ± 0.76^{bc}	12.43 ± 0.91^{b}	6.43 ± 0.67^{a}

Values are shown as mean ± SD of three biological replicates. The glucose and fructose contents of the natural must in the fermentations were ranged in 190.4-195.8 g/L.
Different letters indicate statistically significant differences (HSD Tukey) for values in the same column.
[1]Analyses were performed after four days of fermentation.
[2]Analyses were performed after seven days of fermentation
ND, not detectable.

Table 4.6. Yields of ethanol, acetic acid and glycerol calculated for the parent FX10 strain and recombinant derivatives.

	$Y_{E/S}$ (g/g)		$Y_{A/S}$ (mg/g)		$Y_{G/S}$ (mg/g)	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
FX10	0.36 ± 0.01^{b}	0.23 ± 0.01^{ab}	0.76 ± 0.16^{bc}	17.56 ± 2.30^{cd}	54.04 ± 1.21^{a}	30.47 ± 1.04^{a}
FHXK2	0.36 ± 0.01^{b}	0.23 ± 0.01^{ab}	0.86 ± 0.04^{bc}	18.62 ± 2.22^{d}	57.27 ± 1.53^{ab}	31.15 ± 0.64^{a}
FPYK1	0.35 ± 0.01^{b}	0.24 ± 0.01^{ab}	0.59 ± 0.16^{abc}	23.84 ± 4.98^{d}	54.78 ± 2.16^{a}	31.76 ± 1.51^{a}
FREG1	0.31 ± 0.01^{a}	0.21 ± 0.01^{a}	0.94 ± 0.28^{c}	1.01 ± 0.17^{a}	63.74 ± 4.26^{bc}	115.29 ± 3.42^{b}
FPDC1	0.36 ± 0.02^{b}	0.24 ± 0.01^{ab}	0.35 ± 0.23^{ab}	3.91 ± 2.44^{ab}	54.27 ± 3.52^{a}	29.93 ± 1.89^{a}
FPDE2	0.34 ±0.02^{b}	0.27 ± 0.01^{b}	0.22 ± 0.20^{a}	10.04 ± 4.02^{bc}	71.21 ± 2.36^{c}	33.79 ± 3.50^{a}

Values are shown as mean ± SD of three biological replicates.
$Y_{E/S}$ ethanol yield on sugar, $Y_{A/S}$ acetic acid yield on sugar, $Y_{G/S}$ glycerol yield on sugar
Different letters indicate statistically significant differences (HSD Tukey) for values in the same column

Acetic acid production under anaerobic conditions by some of the strains assayed is suitable for the production of quality wines (Table 4.5), considering that, above 0.8 g/L, acetic acid may confer an undesirable acidic taste and unpleasant vinegar aroma to wine (Bely et al., 2003). FPDE2 showed the lowest acetic acid yield under these culture conditions (Table 4.6). On the other hand, the trend towards increased acetic acid production under aerobic conditions that was previously described (Quirós et al., 2014; Morales et al., 2015; Giovanelli et al., 1996) is confirmed for this set of yeast strains. Indeed, acetic acid production under aerobic conditions was unacceptably high for most of the strains, apart from FREG1 and FPDC1 (Table 4.5). Actually, FREG1 seems to be an exception to the general rule

of increasing acetic acid yield under aerobiosis. While the other yeast strains experience acetic acid yield increases ranging from 8 to 40 times under aerobic as compared to anaerobic conditions, acetic acid yield for FREG1 is similar under both culture conditions.

Quantitative differences in glycerol yields in anaerobiosis are relatively small (as compared for example with differences in acetic acid yields), even though FREG1 and FPDE2 show higher glycerol yields than the control strain, and these differences are statistically significant (Table 4.6). Aeration results in a reduction of about one half in glycerol yield for most of the strains. However, FREG1 shows the opposite behaviour, with twice the glycerol yield under aerobic conditions as compared to anaerobiosis (Table 4.6). The final glycerol content of aerobic FREG1 fermentation is indeed in the upper part of the normal window for quality wines (12-18 mg/L; Table 4.5).

Other metabolites in fermentations by FX10 derivatives

In view of the striking differences in acetic acid yields shown by the recombinant strains (Table 4.6), we wondered whether other metabolic by-products from the pyruvate node (acetaldehyde, acetoin and 2,3 butanediol) were also affected by the gene deletions tested. No statistically significant differences were found for the yeast strains concerning acetaldehyde production under anaerobic conditions (Table 4.7). The general trend for acetaldehyde levels was to higher values in aerobiosis, apart from FREG1. Deletion of *PDC1* results in a huge increase in acetaldehyde production under aerobic conditions (Table 4.7).

Table 4.7. Volatile metabolites produced by the parent FX10 strain and recombinant derivatives.

	Acetaldehyde		**Acetoin**		**2,3 butanediol**	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
FX10	0.094± 0.042[a]	0.463± 0.132[a]	0.006 ± 0.002[a]	35.017 ± 2.381[d]	0.518 ± 0.324[a]	6.287 ± 0.210[d]
FHXK2	0.144 ± 0.026[a]	0.553± 0.054[a]	0.007 ± 0.000[a]	48.212 ± 2.214[e]	0.482 ± 0.054[a]	7.380 ± 0.289[e]
FPYK1	0.145 ± 0.019[a]	0.409± 0.175[a]	0.009 ± 0.001[a]	26.628 ± 0.275[c]	0.599 ± 0.041[a]	7.100 ± 0.170[e]
FREG1	0.176 ± 0.068[a]	0.129 ± 0.029[a]	0.154 ± 0.021[b]	10.594 ± 2.920[a]	0.973 ± 0.119[a]	1.273 ± 0.178[a]
FPDC1	0.130 ± 0.034[a]	1.875 ± 0.478[b]	0.005 ± 0.001[a]	15.557 ± 1.273[b]	0.391 ± 0.069[a]	2.244 ± 0.173[b]
FPDE2	0.125 ± 0.039[a]	0.389 ± 0.055[a]	0.020 ± 0.005[a]	31.113 ± 1.961[cd]	1.943 ±0.549[b]	4.570 ± 0.500[c]

Values are shown as mean of relative abundance on the internal standard ± SD of at least two biological replicates, after seven days of fermentation.
Different letters indicate statistically significant differences (HSD Tukey) for values in the same column.

The relative similarity in acetaldehyde production shown by the yeast strains under anaerobic conditions does not translate on a similar uniformity concerning acetaldehyde derived products. Anaerobic cultures of FREG1 and FPDE2 show a clear increase in acetoin (25-fold or four-fold respectively) and 2,3 butanediol (two-fold increase and four-fold) levels, as compared to FX10 (Table 4.7). Following the trend seen for acetaldehyde, all the strains produced clearly increased amounts of acetoin or 2,3

butanediol under aerobic conditions. Again, the behaviour of strains deleted for *REG1* or *PDC1* falls clearly apart from other yeast strains. FREG1 and FPDC1 show the lowest values of both acetoin and 2,3 butanediol in aerobiosis (Table 4.7). In addition, the difference between aerobic and anaerobic conditions observed for FREG1, concerning the content of these two compounds is the smallest among all the strains analysed.

Finally, we performed a principal component analysis by considering ethanol, acetic acid, and glycerol, as well as acetaldehyde, acetoin, and 2,3 butanediol. The results confirm the important metabolic differences between aerobic and anaerobic cultures (Figure 4.9). Samples are clearly separated along the PC1 axis, depending on the oxygenation conditions, with anaerobic samples showing lower production of acetoin, 2,3 butanediol, and acetic acid; and high production of ethanol and glycerol, as already seen in Tables 4.6 and 4.7. However, two strains, FREG1 and FPDC1 show, under aerobic conditions, a behaviour that is reminiscent of anaerobic cultures. This is clear for acetic acid yield and 2,3 butanediol production in the case of FREG1, and also evident for FPDC1 concerning acetic acid yield, as well as acetoin and 2,3 butanediol production. Aerobic samples of FPDC1 are clearly separated from the rest of the samples along the PC2 axis, mostly due to the high acetaldehyde production of this strain in the presence of oxygen. The high production of glycerol by FREG1 under aerobic conditions explains the low position of these samples along the PC2 axis.

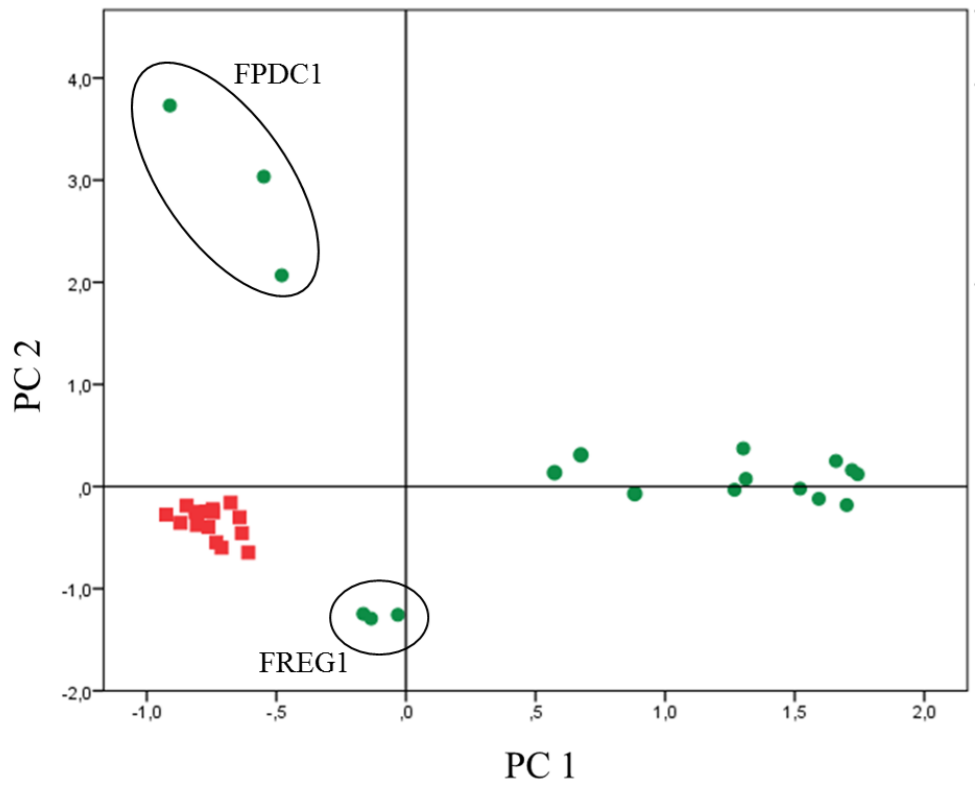

Figure 4.9. Principal Component Analysis based on the yields of main fermentation products and pyruvate derived metabolites. The PC1 and PC2 explained 64.89 and 81.97% respectively of the total yeast strains variance under aerobic (green dots) and anaerobic (red squares) conditions.

DISCUSSION

Computational modelling of yeast metabolism, combined with knowledge-based selection of additional genes, such as regulatory ones, allowed to identify a set of more than thirty candidate gene deletion yeast strains to test the impact of such deletions on acetic acid production under aerobic conditions. Only a subset of these gene deletions was confirmed to result in a relevant reduction of acetic acid yield during the fermentation of natural grape must under aerated conditions. Interestingly, none of the regulatory or CCR related genes were experimentally confirmed to impact acetic acid production under these conditions. All confirmed prediction derived from *in silico* simulation, highlighted the relevance of computational approaches to understand industrial yeast physiology. *REG1* deletion was not confirmed under these experimental conditions and genetic background (BY4741), despite it being later confirmed in bioreactor assays for the FX10 background. This points out to a delicate balance in oxygen availability, grape must composition, and genetic background to determine a boost in acetic acid production by *S. cerevisiae*. Also, the fact that several of the genes predicted by computational simulation resulted in higher instead of lower acetic acid production levels is indicative of the limitations of genome-scale models to correctly simulate metabolic features that were not part of the data used to build the models. However, computer simulation was indeed useful in order to predict some interesting genes as targets for genetic improvement. It would be worth, in the future, to develop wine yeast recombinant strains, deleted for some of these genes, in order to confirm it this can be extrapolated to industrial genetic backgrounds and winemaking conditions.

The result presented, also indicate that, despite the Crabtree effect, there is a big impact of oxygen availability on yeast metabolism for all the strains assayed in this work. Indeed, reduced ethanol yield in the fermentation of natural or synthetic grape must was already described for *S. cerevisiae* in previous articles from our research group (Quirós et al., 2014; Morales et al., 2015). This indicates a relevant portion of the sugar was metabolized by respiration under aerobic conditions, a conclusion that is also sustained by an important increase in biomass yield in aerobic cultures. For most of the yeast strains studied, production of other metabolites is also highly affected by culture under aerobic conditions, as compared to anaerobiosis, including acetic acid and glycerol, also in agreement with previous publications (Giovanelli et al., 1996) and results in Chapter 2; as well as other pyruvate derived metabolites, like acetaldehyde, acetoin or 2,3 butanediol. This is indicative of the importance of redox balance and the pyruvate metabolic branch point in order to determine acetic acid production by *S. cerevisiae*.

A clear alleviation of the Crabtree effect was previously described for loss-of-function mutations in several of the genes studied in this work, including *HXK2*, *PYK1* and *REG1*(Herwig and von Stockar, 2002; Pearce et al., 2001). Surprisingly, we found little or no impact of these gene deletions on ethanol or biomass yield in the FX10 genetic background and under our experimental conditions. Claims on the relief of the Crabtree effect for these gene deletions were mostly based on chemostat cultures under carbon limited conditions. In contrast, natural grape must is quite restrictive concerning yeast assimilable nitrogen, while carbon sources (glucose and fructose) are in great excess. Our results illustrate the relevance of the cultivation mode to assess yeast metabolic features. In this way, genetic modification resulting in an important change in the critical dilution rate under chemostat growing

conditions might appear as almost irrelevant for cultures in batch, especially for growth in high sugar content media. Several authors postulate that the Crabtree effect in *S. cerevisiae* is mostly a manifestation of an overflow metabolism at the level of pyruvate (Holzer, 1961; Pronk et al., 1996). Indeed, a total relief of the Crabtree effect in this species has only been attained by an almost complete impairment of glucose intake by the cells (Henricsson et al., 2005). According to this model, some gene deletions affecting the glycolytic rate (i.e. the rate of production of pyruvate), or the capacities of enzymes involved in further pyruvate metabolism, might have a clear impact on the critical dilution rate (Herwig and von Stockar, 2002; Pearce et al., 2001). However, the extreme overflow we can expect for batch cultures with around 200 g/L initial sugar content might be almost insensitive to the same gene modifications. This is exactly what we observed for some of the gene modifications initially selected in this work.

Despite the low impact of the assayed gene modifications on ethanol yields under aerobic conditions, one important observation, from a practical point of view, is a clear reduction in ethanol levels under aerobic compared to standard fermentation conditions. We must remember that the final goal of this research line was alcohol level reduction in wine. One of the problems associated with aeration during wine fermentation is increased acetic acid production, as shown in Table 4.5 and as already observed in previous works (Quirós et al., 2014; Chapter 2; Morales et al., 2015). Our preliminary results showed that, despite not being intended for that purpose, some gene modifications seemed to result in clearly reduced volatile acidity. Therefore, I incorporated some additional genes in the study, paying attention to these results.

According to results in other genetic backgrounds (Herwig and von Stockar, 2002; Raamsdonk et al, 2001; Pearce et al., 2001), deletion of *PYK1* (hemizygous) or *HXK2* in FX10 results in glucose derepression. Other authors have described low ethanol yield in aerobic batch cultures of strains deleted for *HXK2* or showing reduced levels of pyruvate kinase activity (Diderich et al., 2001; Pearce et al., 2001). However, the behaviour of FHXK2, FPYK1 and FX10 strains in this work was almost identical, only minor (although statistically significant) differences in metabolic footprint were found for acetoin and 2,3 butanediol production, and only for aerobic cultures. The low impact of these gene deletions during the fermentation of natural grape must is thus in contrast with the results by Diderich et al. (2001) and Pierce et al. (2001). There are at least two non-exclusive explanations to this discrepancy. One is based in media composition. Initial glucose content in the batch cultures by the later authors ranged from 10 g/L to 20 g/L, while natural grape juice used in this chapter contained about 200 g/L (equimolar amounts of glucose and fructose). In addition, yeast assimilable nitrogen in grape must is limiting, so that most of the sugar is metabolized under nitrogen limitation. This is in contrast with synthetic media for which nitrogen sources were in excess. A second explanation in the case of *PYK1* deletion is FPYK1 was hemizygous for that deletion (*PYK1* is an essential yeast gene). The maximal reduction in pyruvate kinase activity we would expect from this construction is 50%. In contrast, Pierce et al. (2001) used a construction resulting in reduction of pyruvate kinase levels down to 20-25% of normal values. The high similarity of FX10, FHXK2 and FPYK1 under our experimental conditions, either aerobic or anaerobic, despite the different behaviour shown under chemostat conditions (Herwig and von Stockar, 2002; Pearce et al., 2001) is illustrative of the lack of predictive power of

standard Crabtree assays for certain industrially relevant conditions, as discussed above.

Strain FPDE2 shows the lowest biomass production under both aerobic and anaerobic growth conditions, despite being to reach complete fermentation of grape must with a kinetics similar to FX10 (Figure 4.9). Since the cyclic AMP phosphodiesterase encoded by *PDE2* is involved in reducing cAMP levels, and despite it can be partially substituted by Pde1p (Park et al, 2005), we would expect cell functions regulated by PKA to be overexpressed or overrepressed in FPDE2 (Rødkær and Færgeman, 2014). Two opposite effects on biomass production would be expected. On one hand, increased PKA activity would involve activation of glycolysis, growth and proliferation. On the other hand, stress response would be reduced, resulting in low tolerance to the harsh conditions in grape must, and notably osmotic stress. Our results indicate the later effect would be dominant and result in the low biomass of FPDE2 cultures under both aerobic and anaerobic conditions. The expected positive impact of the deletion of *PDE2* on glycolysis rate might be responsible for the fact that FPDE2 appears in the upper part of the distribution of ethanol yield under aerobic conditions, being the strain showing the minor reduction in ethanol yield from anaerobic to aerobic fermentation conditions. In addition to ethanol, this gene deletion has a limited impact on the yields of acetic acid, glycerol and 2,3 butanediol in anaerobiosis; as well as on acetic acid yield in aerobic fermentations. In practical terms, the reduction of acetic acid yield under aerobic conditions by FPDE2 might have an advantage over FX10 in order to attain alcohol level reduction by aeration of the fermenting must, by limiting the problem of excess volatile acidity. However, other mutant strains assayed in this work seem to be more interesting in this respect (see below).

PDC1 codes for the main pyruvate decarboxylase isozyme in *S. cerevisiae*. Deletion of this gene in the FX10 background results in little impact under anaerobic conditions, but it has relevant consequences for aerobic fermentation. Under these conditions, the ethanol yield of FDC1 is similar to the control strain, but it shows a clear reduction in acetic acid yield, as well as acetoin and 2,3 butanediol. In contrast, a relevant increase in acetaldehyde production is observed. Although the main activity of Pdc1 is the conversion of pyruvate to acetaldehyde, acetoin has been described as one main side product of the reaction. Acetoin can in turn be transformed to 2,3 butanediol. It has been shown that Pdc5, the other major pyruvate decarboxylase isozyme, is able to warrant about 70% of the pyruvate decarboxylase activity required by the cell in Δ*pdc1* strains (Wang et al., 2015). However, our results suggest differences in acetoin production between Pdc1 and Pdc5, resulting in acetaldehyde accumulation at the expense of acetoin and 2,3butanediol. Perhaps the most interesting feature of FPDC1 concerning its application in wine making is the low acetic acid yield in aerobic fermentation, resulting in volatile acidity values around the 0.8 g/L threshold.

Deletion of *REG1* is probably the most pleiotropic gene modification among those assayed in this work. This was to be expected, given the upstream position of Reg1 in the glucose sensing signal transduction pathway. Concerning anaerobic conditions, FREG1 is almost the only strain showing statistically significant differences with the control strain for ethanol yield or acetoin production. However, the most interesting impact of this gene modification is shown for aerobic cultures. On one hand, it shows the lower ethanol yield values among the yeast strains used in this work. More interesting is the fact that this strain shows also the lower values for acetic

acid yield under aerobic conditions, as well as for acetaldehyde, acetoin or 2,3 butanediol. In contrast to all the other yeast strains, almost no difference in acetic acid yield was observed for this strain between aerobic and anaerobic fermentations, and those values are similar to anaerobic cultures of FX10. Also relevant is the increase in the aerobic glycerol yield for this strain as compared to anaerobic growth (about two-times). This was indeed opposite to the other strains, showing a two-fold decrease in glycerol yield for the same growth conditions. This result seems to be in contrast with reports showing an increase in the production of acetic acid for genetic modifications aiming to glycerol overproduction (Remize et al., 1999). However, these reports are based on growth under standard fermentation conditions while glycerol overproduction by FREG1 without an increase in acetic acid production takes place under aerobic fermentation conditions. Obviously, the redox compensation mechanisms involved in linking acetic acid and glycerol metabolism under anaerobic conditions are not operating the same way in the presence of oxygen. An interesting feature of *REG1* loss-of-function yeast mutants, is they can be easily obtained and selected by random mutagenesis (Neigeborn and Carlson, 1987; Zimmermann and Scheel, 1977). This opens the way for obtaining non-GMO wine yeast strains similar to FREG1, which would be readily available for winemakers, avoiding the limitations associated to recombinant wine yeasts (Cebollero et al., 2007). The hurdle imposed by the recessive character of *REG1* defective mutants can be overcome by sporulating yeast strains before or after random mutagenesis. The use of homozygous wine yeast strains, like FX10, would be advisable for this approach.

Global discussion

The biotechnological problem addressed in this thesis is alcohol level reduction in grape wine. Increasing alcohol content in quality wines has been a trend during the last thirty or forty years and is mainly related to global climate warming; although other drivers might also play a role, like consumers (or wine prescriptors) demand for full bodied and strongly aromatic wines. Whereas climate change results in an imbalance during grape ripening, by boosting sugar accumulation to a greater extent than phenolic or aromatic maturity, these current trends in wine consumption require fully mature grapes. Together these two drivers lead to harvest grapes with increasing sugar content, and this results in a parallel increase in the alcohol level of the wines produced.

Researchers at Instituto de Ciencias de la Vid y del Vino have been working on biotechnological strategies to address this problem, in order to reduce ethanol yield during the fermentation of grape juice by yeasts. One of the main strategies proposed was taking advantage of the respiratory metabolism of yeasts, by diverting carbon coming from sugars to CO_2, instead of ethanol. This was mostly based on the use of non-*Saccharomyces* yeast strains, since it was expected that the Crabtree effect would limit respiration of sugars by *S. cerevisiae*. One important observation was that, under aerobic conditions, *S. cerevisiae* significantly increases the production of acetic acid, which would result in wine spoilage. Considering that this yeast species will always be present in grape must or the cellar environment, this problem is not restricted to its use under aerobic conditions. The use of selected non-*Saccharomyces* strains for sugar respiration will require grape must oxygenation, and available oxygen might result in excess acetic acid production by *S. cerevisiae* (and some other yeast species).

Taking into account the previous work, this thesis includes work performed with both *S. cerevisiae* and non-*Saccharomyces* wine yeast strains; and took both ethanol and acetic acid yields as metabolic targets for improvement (i.e. reduction). This required research on the processes and pathways involved in the regulation of the respiro-fermentative metabolism of several yeast species, as well as on the factors related with the increment of acetic acid yield observed in aerated cultures of *S. cerevisiae*.

Considering the development of aerobic fermentation processes based on respiration by non-*Saccharomyces* yeasts, and according to results shown in Chapter 2, it is worth noting that several parameters among those that can be easily controlled during winemaking, can have a huge impact on the metabolism of both *S. cerevisiae* and alternative yeast species. Anyway, according to its Crabtree status, *S. cerevisiae* tends to produce high amounts of ethanol compared to the other species assayed in this thesis. In addition, as previously described, this species produces high amounts of acetic acid in all aerated conditions, while *C. sake* or *M. pulcherrima* are much less problematic for this trait across most of the conditions tested. Metabolic features of some yeast species appear as strongly dependent on oxygen supply, as shown for *C. sake* in Chapter 2, or *K. lactis* in Chapter 3. Curiously, in some instances, yeast assimilable nitrogen or temperature show a stronger impact on ethanol and acetic acid yields than does oxygen supply itself. In practical terms, this must be managed with care, especially for mixed inoculation fermentations, since different combinations of environmental factors might have opposite effects, also depending on the yeast strain used. Transcriptomic analysis of *K. lactis* cultures, described in Chapter 3, has been key to highlight medium constituents, usually not taken into account in the oenological context, that might become relevant for the new wine

fermentation procedures required for alcohol reduction by respiration. This is the case of oxygen itself (also observed in Chapter 2); but it is also true for iron. Since this species was selected for transcriptome analysis in part based on its similarity to *M. pulcherrima* under aerated wine fermentation conditions, it would be interesting to explore whether the dependence on iron availability observed for *K. lactis* metabolism is reproduced for other strongly respiring wine yeast species.

As mentioned above, the problem of high acetate production by *S. cerevisiae* will always be present when fermentation batches are aerated, due to the ubiquity of this species in the winemaking environment. The use of computational biology and genome-scale models to understand the metabolic features of *S. cerevisiae* wine yeasts, and its interaction with environmental factors, is a key contribution of this thesis. This kind of approach has been very useful for the improvement of other industrial processes involving this yeast species, like the production of bioethanol or recombinant proteins. However, there are big differences between the laboratory strains and standard growth conditions used to build these models, and the actual genome constitution of wine yeasts and environmental conditions found in winemaking. This might explain the relatively low prediction power of this approach in this thesis. Some of the most promising gene deletions analysed in Chapter 4 (CCR related) where not anticipated by metabolic modelling, while many of those actually predicted where not confirmed. Anyway, several of the predictions were confirmed in natural grape must for the laboratory background. This shows that metabolic modelling was useful despite the above-mentioned limitations. Those gene modifications are worth to be tried in a wine relevant genetic background in the near future. Interestingly, in Chapter 4, devoted to

metabolic modelling, there was also room for serendipity. Looking for Crabtree alleviated yeast strains it was found that some genetic modifications resulted in lower acetate yields under aerobic conditions. This work also confirmed one important previous observation, i.e. significant alcohol level reduction through respiration by *S. cerevisiae* in grape must, despite the Crabtree effect. In contrast, it was found that mutations described in the literature as affecting the Crabtree effect or CCR under standard laboratory conditions and genetic backgrounds (e.g. gene deletions involving *HXK2*, *REG1*, or *PYK1*), did not result in further reduction in ethanol yield under aerated winemaking conditions (as in the FX10 background).

In summary, results in this thesis provide new knowledge to understand the metabolism of wine yeasts, both *S. cerevisiae* and some non-*Saccharomyces* species, under aerobic winemaking conditions. This will contribute to the future development on yeast strains and production procedures allowing to counteract one of the main negative consequences of global warming on wine quality. In addition, it will help understanding the metabolism of non-*Saccharomyces* wine yeast strains. The use of new yeast species as starter cultures is becoming a trend in winemaking apart from the use suggested in this thesis for alcohol level reduction (e.g. improved aroma, body, or acidity). Finally, some of the findings in Chapter 4 might help refine our understanding of aerobic metabolism of the model species *S. cerevisiae*.

In practical terms, results from Chapter 2 and 3 might be useful to design process conditions for alcohol level reduction with strains of *C. sake*, *M. pulcherrima*, and eventually *K. lactis*. On the other side, according to results from Chapter 4, one way to obtain non-recombinant wine yeast strains with low acetate yield under aerobic conditions would be selecting for CCR mutant derivatives from an industrial wine yeast strain, either by random

mutagenesis or by adaptive laboratory evolution (e.g. using non-preferred carbon sources in the presence of 2-deoxy glucose). In addition, results on *MDH3*, *ALD6*, and *RIP1* must be confirmed in an industrial genetic background. In case results were confirmed, it would be interesting to develop strategies to obtain similar mutants by non-GMO approaches.

Coming back to the general scheme shown in Figure 1.1, an optimized process taking advantage of the results of this thesis would start as an aerated process, co-inoculated with a balanced combination of at least two yeast strains. One would be a non-*Saccharomyces* strain, used under optimal conditions, according to results from Chapter 2 (or characterized in a similar way). The second one would be a *S. cerevisiae* wine yeast strain. This strain would ideally dominate over the native yeast microbiota, so avoiding acetate production by indigenous yeasts; and show itself low acetic acid yield under aerated conditions. Such *S. cerevisiae* strain would have been selected for low aerobic acetate yield or, most probably, genetically improved by non-GMO techniques. After the aeration period, the process would continue under anaerobic conditions, and the fermentation would be taken to completion by the same or by a second *S. cerevisiae* yeast strain.

Conclusions

1. Temperature and nitrogen sources affect differently to distinct yeast strains/species concerning the yield of ethanol, acetate and other metabolites.
2. Some *Metschnikowia pulcherrima* and *Candida sake* strains seem to be good candidates for developing fermentation processes focused on low alcohol and acetate yields.
3. For some yeast strains, inside the range of aerobic conditions tested, there are factors that are more determinant for alcohol yield than oxygen abundance.
4. Transcriptional changes in *Kluyveromyces lactis* throughout aerobic fermentation can be explained in relation to changes in oxygen and iron availability.
5. Many of the predictions obtained by mathematical modelling of *Saccharomyces cerevisiae* metabolism, concerning reduction in acetate yields, could not be confirmed experimentaly.
6. MoMA simulations of gene deletions allowed the identification of the best candidates for acetate yield reduction.
7. Deletion of *MDH3*, *ALD6*, and *RIP1*, generated good results for aerobic acetate reduction in the BY4741 genetic background.
8. Deletion of *REG1* and *PDC1* in the FX10 background produced good results for the reduction of acetic acid production under aerated conditions.

Conclusiones

1. La temperatura y la disponibilidad de fuentes de nitrógeno afectan de manera diferente a distintas cepas/especies de levaduras en cuanto a rendimiento en etanol, acetato, y otros metabolitos.
2. Algunas cepas de *Metschnikowia pulcherrima* y *Candida sake* parecen buenos candidatos para desarrollar procesos de fermentación con bajo rendimiento en alcohol y acetato.
3. Para algunas cepas de levadura, dentro del intervalo de condiciones aeróbicas que hemos estudiado, la abundancia de oxígeno no es el factor más determinante del rendimiento alcohólico.
4. Los cambios transcripcionales que experimenta *Kluyveromyces lactis* a lo largo de una fermentación aireada se pueden explicar fundamentalmente en función de los cambios en la disponibilidad de oxígeno y hierro.
5. Muchas de las predicciones sobre reducción del rendimiento en acetato, basadas en el modelado matemático del metabolismo de *Saccharomyces cerevisiae*, no se pudieron confirmar experimentalmente.
6. La simulación de deleciones con MoMA permitió la identificación de los mejores genes candidatos para la reducción del rendimiento en acetato.
7. La deleción de los genes *MDH3*, *ALD6*, y *RIP1* ha dado buenos resultados de reducción de acetato en condiciones de aireación en el fondo BY4741.
8. La deleción de los genes *REG1* y *PDC1* ha dado lugar a buenos resultados de reducción de acetato en condiciones de aireación en el fondo industrial FX10.

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List of abbreviations

To avoid an unnecessarily long list of abbreviations, only abbreviations used in more than one chapter are included in the list. The meaning of other abbreviations is explained in the corresponding chapter. Units of the International System of Units (SI), chemical symbols, gene names, and gene product names, are not included in this list.

bp: Base pairs

CCR: Carbon catabolite repression

DO: Dissolved oxygen

FBA: Flux balance analysis

Gbp: 10^9 base pairs

GMO: Genetically modified organisms

HPLC: High performance liquid chromatography

LAB: Lactic acid bacteria

Mbp: 10^6 base pairs

MoMA: Minimization of metabolic adjustment

PCA: Principal component analysis

PCR: Polymerase chain reaction

Annexes

Annex 1.1

Summary of the main positive and negative characteristics of non-*Saccharomyces* yeasts with oenological interest (adapted from Pretorius et al., 2000).

Genera	Oenological properties	Negative effect
Aureobasidium *A. pullulans*	Pectinolytic enzymes producer; Hydrolyses grape glycosides with β-D-glucosidase, α-L-arabinofuranosidase and α-L-rhamnosidase activities.	
Candida *Candida spp.* *C. stellata* *C. pyralidae*	High glycerol producer; Increased concentrations of terpenol; Decreased concentrations of acetic acid, aldehydes and acetate esters; *C. pyralidae* produces two killer toxins (CpKT1 and CpKT2) that are able to control the development of spoilage yeast *B. bruxellensis*. Without inhibit *S. cerevisiae* or the LAB strains.	May produce sulphur compounds, acetaldehyde, volatile acids and esters; Present lower kinetics rate (low ethanol concentration); Wine exposed to air develops film layers.
Cryptococcus *Cryptococcus spp.* *C. saitoi*	Pectinolytic enzymes producer.	
Debaryomyces *D. hansenii (anamorph: C. famata)* *D. vanrijiae* *D. vanriji* *D. pseudopolymorphus*	Increased concentration of terpenols (citronellol, nerol, and geraniol) in co-fermentations of grape juice with *D. pseudopolymorphus* and *S. cerevisiae;* Increased concentration of geraniol, esters and fatty acids in sequential fermentation of grape juice	May spoil fermented food

	with *D. vanriji* and *S. cerevisiae.*	
Hanseniaspora *H. osmophila* *H. uvarum (anamorph: Kloeckera apiculata)* *H. vineae (anamorph: Kloeckera africana)* *H. anomala*	Increased concentration of 2-phenyl-ethyl acetate, higher alcohols, acetate, ethyl esters and medium-chain fatty acids; Reduced level ochratoxin A; Extracellular enzymes producer.	Produces volatile acidity, sulphur compounds, biogenic amines, acetoin, acetic acid and its esters and killer toxins; Promotes sluggish or stuck fermentation;
Lachancea *L. thermotolerans*	Produces low levels of volatile acidity and undesirable flavour compounds; Produces lactic acid, limiting the concentration of malic acid.	
Kazachstania *K. aerobia* *K. gamospora*	Increased concentration of acetate, ethyl esters and phenethyl propionate; Increased concentration of ethyl acetate in co-inoculated fermentation of grape juice with K. aerobia and S. cerevisiae.	
Kluyveromyces *K. lactis (anamorph: Candida spherica)*	Enhanced aroma and flavour; Increased concentrations of lactic acid, glycerol and 2-phenylethanol; Pectinase producer.	Wines present higher “spicy” and “acidity” attributes.
Kregervanrija *K. fluxuum (anamorph: Candida vini)*		
Metchnikowia *M. pulcherrima (anamorph: Candida pulcherrima)* *M. fructicola*	Increased concentration of esters; Increases wine flavour and aroma (terpenes and thiols); High producer of β-glucosidase; Pectinase producer;	Promotes delays in fermentation due to antimicrobial activity; Grows as a film layer; Produces high levels of acetaldehyde.

	Presents antimicrobial activity (pulcherrimin); M. fructicola dominates the must during cold soak in order to offer a natural protection against spoilage microorganisms. The use of this yeast allows winemaker to reduce the SO2 at crushing.	
Meyerozyma *M. guilliermondii (anamorph: Candida guilliermondii)* *Millerozyma* *M. farinose (synonym: Pichia farinose)*	Presents glycosidase activity, producing β-D-glucosidase and α- L-rhamnosidase	
Pichia *Pichia spp.* *P. fermentans* *P. kluyveri* *P. kudriavzevii (anamorph: C. krusei synonym: Issatchenkia orientalis)* *P. manshurica (synonym: P. membranaefaciens)*	Increased concentrations of volatile compounds (acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl octanoate, 2,3-butanediol and glycerol) and polysaccharides; Increased amount of free monoterpenes and non-isoprenoids; Reduced malic acid content; Enhances varietal aromas, and thiols aromas. Sequential inoculation with *P. kluyveri* and *S. cerevisiae* increased the 'peach/apricot' character; Co-fermentation of grape juice with *P. kluyveri* and *S. cerevisiae* leaded to higher levels of 3-mercaptohexyl acetate; Co-inoculation of *P. kudriavzevii* with *S. cerevisiae* enhanced the catalysis of malic acid in grape juice fermentation;	Produces biogenic amines, and high levels of acetaldehyde; Production of chalky film. Co-fermentation of grape juice with P. kluyveri and S. cerevisiae produced many off odor compounds

	P. membranifaciens increased esters production in Muscat wine.	
Priceomyces *P. carsonii (synonym: Pichia vini)*		
Rhodotorula *R. mucilaginosa* *R. dairenensis*	Pectinolytic enzymes producer; Presents cellulose activity at low temperatures and low pH.	
Saccharamycodes *S. ludwigii*		Increased concentrations of acetaldehyde; Highly tolerant to ethanol and resistant to SO_2 and sorbate; Flocculent masses settle as chunks and form a sliminess;
Schizosaccharomyces *S. pombe*	Degradation of malic and gluconic acid Improve fermentation behavior of yeast starter cultures Improve aroma complexity High production of pyruvic acid which contributes to the formation of vinylphenolic pyranoanthocyanins. Allows maloalcoholic deacidification	Increased concentration of acetaldehyde, propanol and 2,3-butandiol; Low concentration of esters; Deacidification; Re-fermentation of bottled wine.
Schwanniomyces *S. vanrijiae (synonym: D. vanrijiae)*		
Starmerella *S. bacillaris* *S. bombicola*	*S. bacillaris* can survive until the end of the alcoholic fermentation, tolerating high concentrations of ethanol; High glycerol producer; Presents reduced ethanol yield and acid (in combination with *S. cerevisiae*);	

	Increases aroma complexity.	
Torulaspora *T. delbrueckii (anamorph: Candida colliculosa)*	High purity fermentation; Lower production of glycerol, acetaldehyde, acetic acid, ethyl acetate, and volatile phenols; Increased production of higher alcohols, terpenes, and extracellular enzymes of oenological interest; Depending on the strain provides aromatic and mouthfeel complexity, and fresh fruit characteristics.	Presents slower fermentation rate; Sulphur compounds producer
Wickerhamomyces *W. anomalus (anamorph: Candida pelliculosa)*	Tolerant toward environmental stress factors like low pH, and high osmolarity; Presents metabolic versatility; Exoenzymes producer; Sequential inoculation of *W. anomalus* and *S. cerevisiae* showed increased concentration of acetate- and ethyl- esters.	Excessive production of acetic acid and ethyl acetate; Promotes wine spoilage.
Williopsis *W. saturnus*	Increased levels of terpenols (linalool, citronellol, and α-terpineol); Terpenoid esters (citronellyl and neryl acetate) producer; Co-inoculation with *W. saturnus* and *S. cerevisiae* shows higher concentrations of acetic acid, ethyl acetate and isoamyl acetate.	
Zygotorulaspora *Z. florentina (synonym: Zygosaccharomycodes florentinus)*	Mixed fermentations of *Z. florentina* with *S. cerevisiae* increased the production of polysaccharides, glycerol and esters; and a reduction of volatile acidity.	

Annex 1.2

Main bacterial species found by NGS analysis of vineyard and wine making samples (adapted from Morgan et al., 2017; and Pereira et al., 2000)

Acinetobacter	***Candidatus***	***Komagataeibacter***	***Methylobacterium***	***Sphingomonas***
A. baumannii	*C. accumulibacter*	*K. europaeus*	*M. adhaesivum*	*S. aerolata*
A. calcoaceticus	*C. blochmannia floridanus*	*K. hansenii*	*M. dankookense*	*S. aquatilis*
A. guillouiae	*C. blochmannia pennsylvanicus*	*K. intermedius*	*M. extorquens*	*S. echinoides*
A. johnsonii	*C. carsonella ruddii*	*K. maltaceti*	*M. fujisawaense*	*S. endophytica*
A. junii	*C. desulforudis audaxviator*	*K. medellinensis*	*M. longum*	*S. insulae*
A. lwoffii	*C. liberibacter*	*K. oboediens*	*M. mesophilicum*	*S. melonis*
A. rhizosphaerae	*C. pelagibacter ubique*	*K. rhaeticus*	*M. populi*	*S. mucosissima*
	C. phytoplasma	*K. saccharivorans*	*M. radiotolerans*	*S. phyllosphere*
	C. sulcia muelleri	*K. sucrofermentans*	*M. rhodesianum*	*S. pseudosanguinis*
	C. vesicomyosocius okutanii	*K. xylinus*		*S. wittichii*
				S. yunnanensis
Chryseobacterium	***Halomonas***	***Ralstonia***	***Wolbachia***	***Streptomyces***
Chryseobacterium spp.	*H. desiderata*	*R. solanacearum*	*W. endosymbionts*	*Streptomyces spp.*
	H. elongate			
	H. phoceae			
	H. rifensis			

Annexes 2.1. and 2.2.

See Excel files on the digital version of this PhD.

Annex 2.1. This file includes 9 tabs corresponding to the 9 conditions shown in Table 2.1. Each of them presents the analytic data for all the parameters measured from the fermentation experiments carried out with each one of the four yeast strains tested, as well as a graphic representation of them.

Annex 2.2. Raw data and calculations for ethanol yield, acetic acid yield, RQ, and efficacy for each combination of yeast strain and condition in 72 hours time point.

Annex 3.1

Primers used in Chapter 3.

Strain	Gene	Forward Primer	Tm	Reverse Primer
S. cerevisiae	*RRT15*	AGATTTCTGTTCTCCATGAGTCC	*60.32*	ACAATAAATTTCGCCAGGAGAG
	INO1	AACCAAACTACTTCGGCTCC	60.45	GCGTTATTGATGTCCCAACCA
	COX2	TCAAAGAGAAGGTGTCTTCTATGG	60.30	ATTGGCATATTTGCATGACCTG
	snR190	GCCCTGATGATAATGGTGTCTC	61.06	AAATCCCTTGTCGTCATGGTC
	OLI1	TGGTTTATTAGGAGCAGGTATTGG	60.67	AATAGCCATAGGGAATACTAGGTC
	snR17a	GCGATGATCTTGACCCATCC	61.09	TTGTCAGACTGCCATTTGTACC
	FET3	CCCAGAATACCCAATGAGAAGAG	60.70	TCCACGAGAACAAGACCCA
	TAF10	TACTCCTCCTATCATTCCCGA	59.44	CGTTTCACTCGTACATCTGCT
	PDA1	TCACCAATACAAGAACGAGGAC	60.53	GATTAGAGGCACCATCACCA
	HSP12	CAAGGGTGTCTTCCAAGGTG	61.02	GCGGCTCCCATGTAATCTC
	TPO2	CCGTACCGCTACCAATTCC	60.69	CGTTCTTCAGTTTCTTCCTCTTCC
K. lactis	*KLLA0A 04906g*	GCTCTATAAACTTACCAATCTCGG	*59.28*	ACTTTGGATGCTTGTGAGGG
	KLLA0E 05875g	GGGAAGTACATAGTTTGCGAC	59.20	ACTGCTGGTTGCTCTTCTC
	KLLA0D 16412g	GTCATAACAGAATCTCCATCCAC	59.12	TAGCCAACAAAGAATCCTCACAG
	KLLA0F 15037g	CGAGCCCTAATGAGATAGCAG	60.28	CGTTCCTTCAACACTAATAGCAC
	KLLA0C 00220g	CTTACAATACCCATTCGCTCCT	60.40	CTTCCAAACACATCAGACAATCC
	KLLA0E 14477g	GACCAGAGCACAATCGCA	60.50	TAGACCAAGTAACCAATCCTCCA
	KLLA0E 00243g	AGAACCAAGCAGAGATTTGAACAG	61.81	ACTCTTCCCAAGCATAGTATCCT

KLLA0E 14499g	TCATCGGTATTCTTGTCTTCACTG	60.90	TTAACCACCAATTATCACTCCACC
KLLA0E 14609g	GTATGCAACCAACTCTTACCGT	61.12	CCAAACACATCCGACAATCTC
KLLA0F 12001g	GATACCGTCATCACCTCTTACAG	60.50	CACACCAGTTCTTCTACCCA
KLLA0D 17204g	CTCCAGTAGACAACGAAGCA	59.87	CATCCTCTATCTTAGTCTTGCCT
KLLA0B 03311g	CATCCCAGTTAAGATTCACAACAG	60.12	AAACCTAGAGCAAAGAAACCGA
KLLA0F 16170g	TACTGAGACCTTCATCGCCA	60.82	AAGTTCTTCTTGTGCCCTTTCC
KLLA0C 17468g	GCATTCACAGTTTACGCCA	59.21	ACCACCAGCATTACCCTC

Annex 4.1

Primers used in Chapter 4.

Primer	Sequence (5' → 3')
HXK2 KO F	TTCTGAACCTCCTCGCACAT
HXK2 KO R	AGCGTAGTGAGGTGGAGACC
HXK2 OUT F	CTCCAGAGCTCCACATTGGT
HXK2 OUT R	TGTGATTTGCGGTGTTCATC
HXK2 IN R	AGTGCTTGGTAACGGCTTGT
PYK1 KO F	GCTTGTGATGTCTTCCAAGTGA
PYK1 KO R	CCACTTCAGTTTTCTTCCCATCT
PYK1 OUT F	CCATCGACAGATTGGGAGAT
PYK1 OUT R	CGGCCTTCTTTGTTGCTACT
PYK1 IN R	CTTGTCGTCAACGACTTCCA
REG1 KO F	GGATATTGAAGGAAGGAATCAGC
REG1 KO R	TTGACATTGGCCAGATACCTC
REG1 OUT F	GCGGATCCATCTTTGAATGT
REG1 OUT R	TGCAGTCCCTGGCTTTTATT
PDC1 KO F	CCTTGGTTCCACTAATTCATCGG
PDC1 KO R	TGCTATCGTTCAACACCACCT
PDC1 OUT F	TATTGTCCGCTGCCCCTTTT
PDC1 OUT R	TCAGGGTTTTGGAAACCACAC
PDE2 KO F	ACTCGGGAAATATGTATCACTAT
PDE2 KO R	TGGCTTAGAGAGAATATACTTGC
PDE2 OUT F	AAGGGTCCTGCGTCCTTTTC
PDE2 OUT R	TGCATATACCAACACAGGGAACA
kanMX4 IN R	CTGCAGCGAGGAGCCGTAAT

Annexes 4.2. and 4.3.

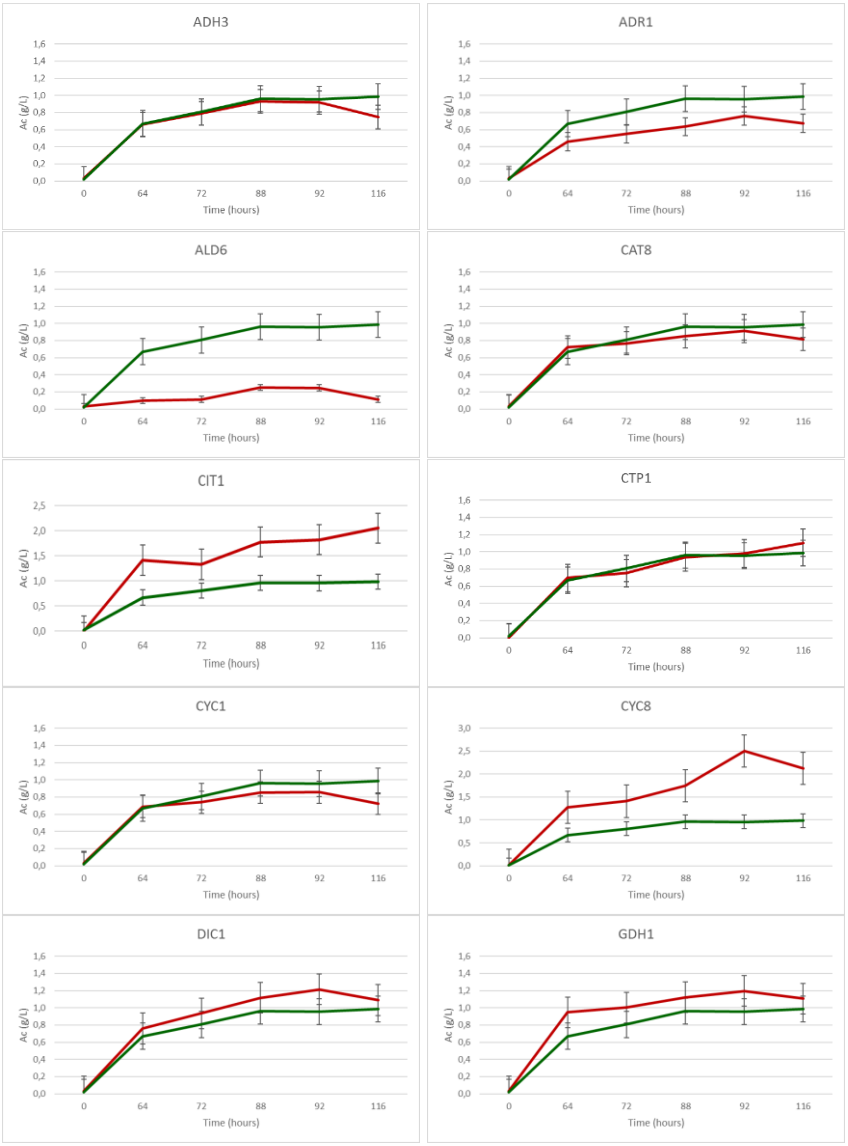
See Excel files on the digital version of this PhD.

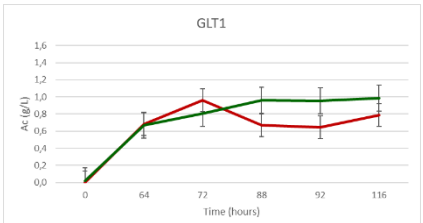
Annex 4.2. Calculations performed to determine conversion rates mmol/gDW.h^{-1} between 11 and 19 hours of culture for all the measured metabolites under aerobic or anaerobic conditions (two replicates each). The final tab shows a summary of the conversion rates used to perform the different steps of computations simulations. Data and calculations from anaerobic cultures, as well as published growth rates (VGRO) were used for comparison purposes.

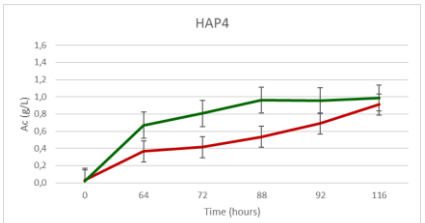
Annex 4.3. Results of knock-out for the solutions that involved a single deletion. Growth rate (VGRO), and acetate fluxes are shown for each solution. Results for gene deletions selected for experimental testing are compiled in the second tab.

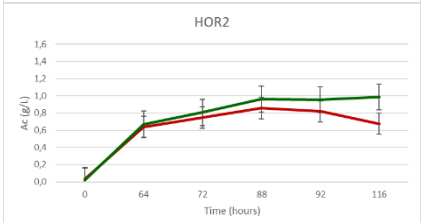
Annex 4.4.

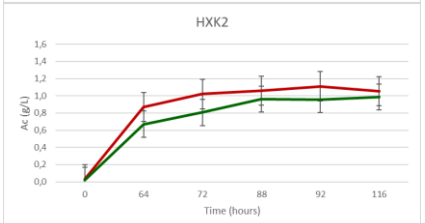
Acetate production by the different yeast knock-out strains (red lines) in natural grape must, compared to BY4741 pHLUM (green lines).

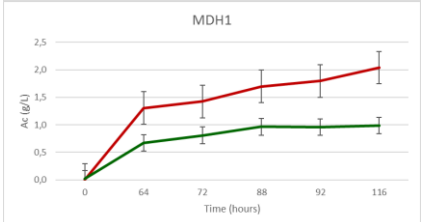


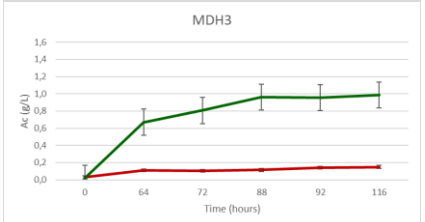

GLT1
Ac (g/L)
Time (hours)

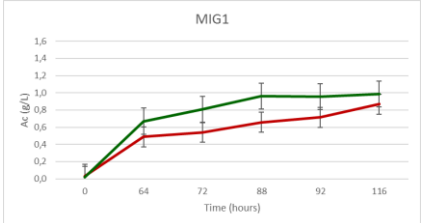

HAP4
Ac (g/L)
Time (hours)

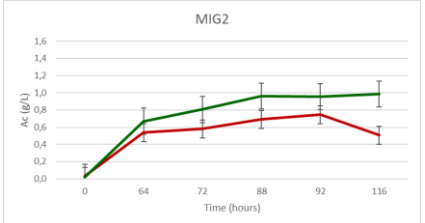

HOR2
Ac (g/L)
Time (hours)


HXK2
Ac (g/L)
Time (hours)


MDH1
Ac (g/L)
Time (hours)


MDH3
Ac (g/L)
Time (hours)


MIG1
Ac (g/L)
Time (hours)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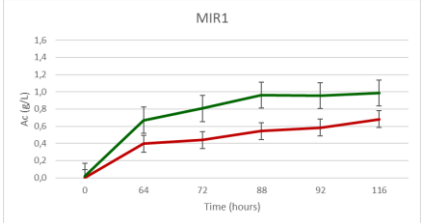
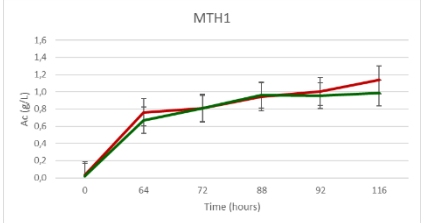
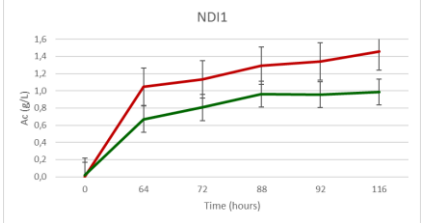
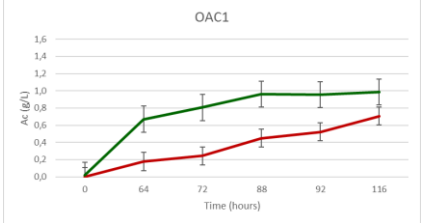
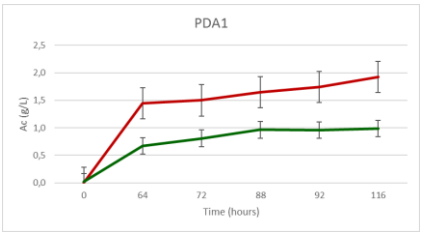
Ac (g/L)
Time (hours)

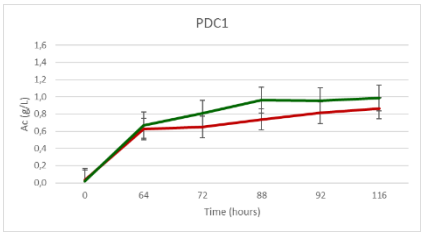

MIR1
Ac (g/L)
Time (hours)


MTH1
Ac (g/L)
Time (hours)


NDI1
Ac (g/L)
Time (hours)


OAC1
Ac (g/L)
Time (hours)


PDA1
Ac (g/L)
Time (hours)
0
64
72
88
92
116


PDC1
Ac (g/L)
Time (hours)
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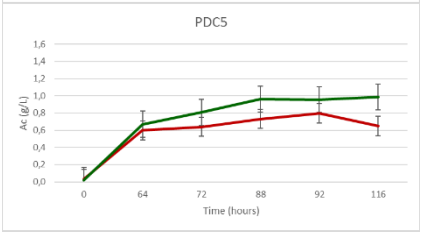
64
72
88
92
116


PDC5
Ac (g/L)
Time (hours)
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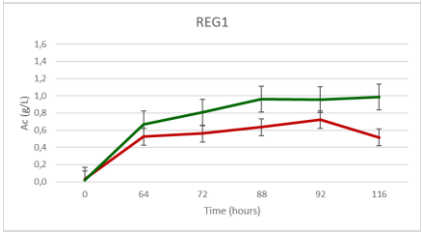
64
72
88
92
116


REG1
Ac (g/L)
Time (hours)
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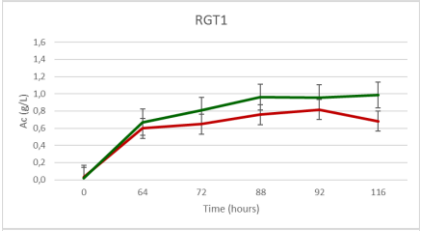
64
72
88
92
116


RGT1
Ac (g/L)
Time (hours)
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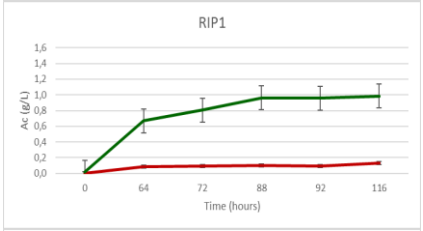
72
88
92
116


RIP1
Ac (g/L)
Time (hours)
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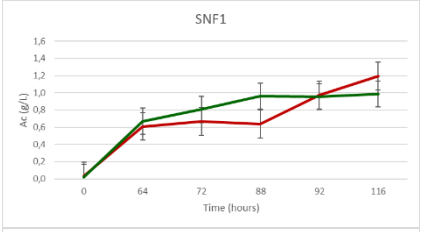
72
88
92
116


SNF1
Ac (g/L)
Time (hours)
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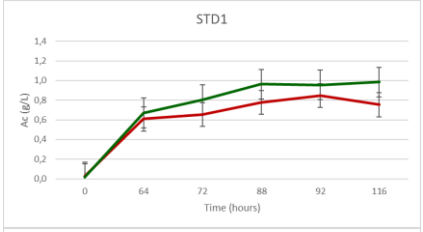
72
88
92
116


STD1
Ac (g/L)
Time (hours)
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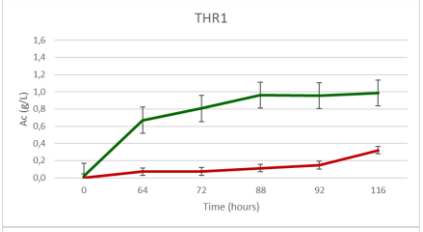
72
88
92
116


THR1
Ac (g/L)
Time (hours)
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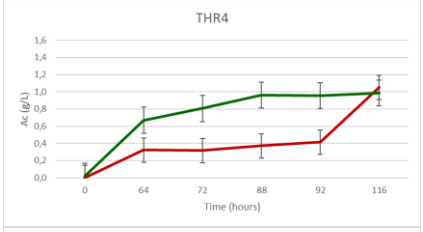
64
72
88
92
116


THR4
Ac (g/L)
Time (hours)
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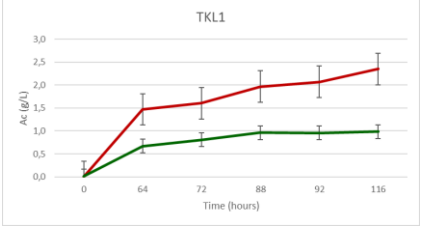
72
88
92
116


TKL1
Ac (g/L)
Time (hours)
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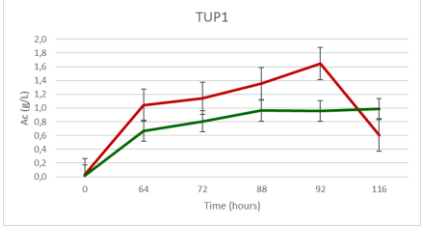
64
72
88
92
116


TUP1
Ac (g/L)
Time (hours)
0
64
72
88
92
116

Annex 4.5.

Acetic acid produced (g/L) in the 92 hours time point for all the laboratory strains analysed in Chapter 4.

Genotype	Mean ± Standard deviation	Sig.	+
ΔADH3	0.92±0.04	0.904	
ΔADR1	0.76±0.04	0.353	
ΔALD6	0.25±0.18	0	*
ΔCAT8	0.91±0.10	0.893	
ΔCIT1	1.80±0.18	1	
ΔCTP1	0.98±0.08	0.978	
ΔCYC1	0.86±0.22	0.741	
ΔCYC8	2.51±0.21	1	
ΔDIC1	1.22±0.18	1	
ΔGDH1	1.20±0.29	1	
ΔGLT1	0.61±0.37	0.029	*
ΔHAP4	0.69±0.32	0.14	
ΔHOR2	0.82±0.09	0.597	
ΔHXK2	1.11±0.11	1	
ΔMDH1	1.80±0.05	1	
ΔMDH3	0.14±0.03	0	*
ΔMIG1	0.71±0.05	0.201	
ΔMIG2	0.74±0.14	0.299	
ΔMIR1	0.58±0.02	0.018	*
ΔMTH1	1.01±0.15	0.989	
ΔNDI1	1.34±0.05	1	
ΔOAC1	0.52±0.04	0.004	*
ΔPDA1	1.74±0.07	1	
ΔPDC1	0.81±0.05	0.58	
ΔPDC5	0.80±0.12	0.502	
ΔREG1	0.72±0.08	0.227	
ΔRGT1	0.82±0.04	0.592	
ΔRIP1	0.09±0.04	0	*
ΔSNF1	0.97±0.20	0.972	

ΔSTD1	0.85±0.12	0.704	
ΔTHR1	0.15±0.08	0	*
ΔTHR4	0.41±0.07	0	*
ΔTKL1	2.07±0.06	1	
ΔTUP1	1.65±0.03	1	
BY7471 pHLUM (control)	0.97±0.16		

* Signification below 0.05 for T de Dunnett's test - H0: Phenotype < control

The present work originated, until the present date, the following scientific publications:

Published articles

The following published articles contain parts of the work described in this PhD thesis:

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Environmental factors influencing the efficacy of different yeast strains for alcohol level reduction in wine by respiration

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ABSTRACT

We have recently shown that ethanol yields in winemaking can be reduced by taking advantage of the respiratory metabolism of some non-*Saccharomyces* yeast species. Using an orthogonal design we have now addressed the impact of three environmental factors (temperature, nitrogen source, and oxygen supply level) on the aerobic metabolism in synthetic must of *Saccharomyces cerevisiae*, *Metschnikowia pulcherrima*, *Kluyveromyces lactis*, and *Candida sake*. An integrative parameter, Efficacy (efficacy for alcohol level reduction) was designed to simplify comparisons between strains or growth conditions. It integrates sugar consumption, ethanol yield, and acetic acid production data. We found a high relative impact of nitrogen source availability and temperature, as compared to aeration conditions, for several fermentation parameters, including ethanol yield. However, increasing oxygen supply showed a positive impact in terms of alcohol reduction and Efficacy for all the strains tested. The best results across assays were obtained for *C. sake* CBS 5093, with high sugar consumption rates, associated to low ethanol yields, and very low acetic acid production. Processes involving this yeast strain would benefit from high aeration levels and low nitrogen source availability; while fermentation temperatures would have little impact on its Efficacy for alcohol level reduction.

1. Introduction

The steady increase in alcohol levels in wine is one of the main challenges faced by the enological industry in recent decades. The problem is related in part to global warming, which results in faster grape ripening, as well as an imbalance between sugar accumulation and the phenolic maturity of berries (Jones, White, Cooper, & Storchmann, 2005; Mira de Orduña, 2010). This trend to increased sugar content in grape must is also driven by current consumer preferences for well-structured and full-bodied wines, which require late harvest in order to warrant proper aromatic and phenolic maturity. However, high sugar content leads to elevated ethanol production during must fermentation, and contribute to stuck or sluggish alcoholic or malolactic fermentation, due to alcohol toxicity on microbial cells. On the commercial side, excess ethanol might impair wine sensory quality; discourage consumers, due to health and road safety considerations; or become a hurdle in the global market, due to regulations and taxes associated to the alcohol content of beverages. The goal of reducing alcohol content of wines is being addressed by researchers involved in all the stages of wine production, from vine clone selection to partial dealcoholisation of the finished wine (Teissedre, 2013).

Development of *Saccharomyces cerevisiae* yeast strains showing reduced alcohol yield during the fermentation of grape must has been a recurrent topic in wine biotechnology during the last thirty years. It was initially explored by genetic engineering approaches (Heux, Sablayrolles, Cachon, & Dequin, 2006; Michnick, Roustan, Remize, Barre, & Dequin, 1997; Rossouw, Heyns, Setati, Bosch, & Bauer, 2013; Varela et al., 2012); and more recently by evolutionary engineering (Cadière, Ortiz-Julien, Camarasa, & Dequin, 2011; Tilloy, Ortiz-Julien, & Dequin, 2014). However, fine-tuning of *S. cerevisiae* metabolism to low ethanol yield is still a tough work.

Despite *S. cerevisiae* is the main yeast species responsible of transforming grape must into wine, many other yeast species participate in the initial stages of the process (Ciani, Comitini, Mannazzu, & Domizio, 2010; Cordero-Bueso, Esteve-Zarzoso, Cabellos, Gil-Díaz, & Arroyo, 2013; Fleet, 2003; Guadalupe-Medina et al., 2013; Rojas, Gil, Piñaga, & Manzanares, 2003; Sadoudi et al., 2012). Our research group recently proposed using the respiratory

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metabolism of some of these non-*Saccharomyces* yeasts as a tool for reducing the alcohol content of wine (Gonzalez, Quirós, & Morales, 2013). The proposed procedure involves the use of a non-*Saccharomyces* yeast strains and controlled oxygenation during the first two to four days of fermentation. The final steps of the fermentation would be carried out under standard conditions and driven by *S. cerevisiae* (Morales, Rojas, Quirós, & Gonzalez, 2015). Several yeast strains, including *Metschnikowia pulcherrima*, *Kluyveromyces lactis*, and *Candida sake* isolates were found to be good candidates to develop fermentation procedures aiming at reducing alcohol content in wine by respiration (Quirós, Rojas, Gonzalez, & Morales, 2014). Results of previous work also indicated that, besides the ability to respire sugars under aerated winemaking conditions, production of volatile acidity under such conditions had to be taken into account. Differences of up to one order of magnitude in acetic acid yield were found among the different yeast strains studied (Quirós et al., 2014).

However, our current knowledge of the metabolic features of these alternative yeast species is limited, including the two main parameters to be considered for an effective alcohol content reduction in wine, alcohol and acetic acid yields on sugar. In order to help optimization of alcohol level reduction with these yeast species we have addressed the impact of three different and easily controllable environmental factors (fermentation temperature, nitrogen source availability, and oxygen supply) on sugar consumption, as well as ethanol, acetate and glycerol production, in a model wine fermentation system, for four yeast strains belonging to different species and selected according to previous results (Quirós et al., 2014).

2. Materials and methods

2.1. Yeast strains

A commercial *S. cerevisiae* wine yeast strain, EC1118 (Lallemand Inc., Ontario, Canada), and three non-*Saccharomyces* strains, *M. pulcherrima* CECT 12898 (labeled as IFI1459 in previous works), *C. sake* CBS 5093, and *K. lactis* CECT 10669, all of them selected from a previous study (Quirós et al., 2014), were used in this work. The strains were grown at 25 °C, and maintained at 4 °C on YPD plates (2% glucose, 2% peptone, 1% yeast extract, and 2% agar), as well as in glycerol stocks at −80 °C.

2.2. Batch cultures

Batch cultures were performed in a defined medium containing 200 g/L glucose, 6 g/L citric acid, 1.7 g/L YNB without amino acids and ammonium sulfate (Difco™, Becton Dickinson, New Jersey, USA), 0.018 g/L myo-inositol and ammonium chloride. The amounts of ammonium chloride used to get different YAN levels (Yeast Assimilable Nitrogen in mg N/L) were: 0.573 g/L for 150 YAN, 0.764 g/L for 200 YAN, and 0.955 g/L for 250 YAN. The pH of medium was adjusted to 3.5 with NaOH. Yeast inocula were grown in YPD broth for 48 h, at 25 °C and 250 rpm and washed twice with water before use. The medium was inoculated to 0.2 final OD_{600}. Experiments were performed in a DASGIP parallel fermentation platform (DASGIP AG, Jülich, Germany) equipped with four SR0400SS vessels. Bioreactors were filled with 200 mL of culture medium and 200 μL (aprox.) of antifoam 204 (Sigma–Aldrich) were added. Agitation was maintained at 250 rpm and the temperature kept at 15, 20 or 25 °C with a recirculating chiller. The pH of the medium was kept at 3.5 by the automated addition of 2 N NaOH. The cultures were sparged with either pure air or mixtures of air, O_2 and N_2, to get 10%, 21% (pure air) and 50% oxygen content in the in gas, at a gas flow of 1.0 L/h. In order to standardize ethanol stripping, we chose stablishing the different aeration regimes by keeping the gas flow constant and varying oxygen content with gas mixtures. Gas flow was controlled with gas flow controllers (MFC17, Aalborg), whose calibration was regularly verified with a soap bubble flow meter. Samples for determination of metabolite concentrations and OD_{600} were withdrawn twice a day during 7 days. Exhaust gas was cooled in a condenser and the instant concentrations of O_2 and CO_2 recorded with a GA4 gas analyzer (DASGIP AG). For technical reasons gas exchanges were only determined for fermentation experiments sparged with pure air, but not for gas mixtures.

2.3. Extracellular metabolites and calculations

The concentrations of glucose, glycerol, ethanol, and acetic acid were determined by HPLC using a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively); and a HyperREZ™ XP Carbohydrate H+ 8 μm column and guard (Thermo Fisher Scientific). The column was maintained at 50 °C and 1.5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} was used as the mobile phase. Prior to injection in duplicate, samples were filtered through 0.45 μm pore size nylon filters (Fisher Scientific, Madrid, Spain) and diluted 20-fold.

Two calculations were performed with analytical data from the 72 h samples. Alcohol level reduction was calculated as the difference between the expected increase in ethanol content, according to sugar consumed up to this time point (17 g/L of sugar consumed for an increase of 1% alcohol by volume; ABV), and the actually measured ethanol content. Even though this alcohol level reduction value is useful for comparison between conditions and strains, it is probably overestimated, since part of the ethanol produced will have been lost by stripping, and must be taken with caution outside this context. In addition, an integrative parameter, Efficacy (efficacy for alcohol level reduction) was designed to simplify comparisons between strains or growth conditions. It was calculated as follows: $\text{Efficacy} = AR \times 2 \times (0.5 - AA)$. Were AR is the alcohol level reduction (expressed as % ABV), and AA is acetic acid content (g/L), all values referred to 72 h. The 0.5 g/L value was chosen as a maximum tolerable content on acetic acid at this time point. Higher values would result in excess volatile acidity by the end of the process. The 2× factor allows for a better comparison with the alcohol level reduction value (Efficacy equals alcohol level reduction when no acetic acid is produced).

Oxygen consumption and CO_2 production were determined by taking into account the in and out gas flows, and their respective concentrations in air and in the off gas. Instant values were integrated over time. Respiration quotient (RQ) was calculated as que quotient between CO_2 production and oxygen consumption.

2.4. Experimental design and statistical analysis

An orthogonal design was used to get the best combination of factors with the minimal number of experiments. Orthogonal design and boxplot figures were calculated and drawn with the IBM SPSS Statistics v19 software. Table 1 shows the values for each variable in the design obtained. Results were analyzed by means of the IBM SPSS Conjoint v19 software. Importance values obtained in this analysis (0–100) provide a measure of how important each factor was to determine the overall result for each parameter and strain.

3. Results and discussion

Three non-*Saccharomyces* yeast strains, belonging to three

Table 1
Summary of the nine combinations of the three parameters evaluated resulting from an orthogonal design, performed with IBM SPSS conjoint program.

Condition	Aeration (%O_2)	YAN (mg N/L)	Temperature (°C)
1	21	150	20
2	10	150	25
3	50	250	20
4	21	250	25
5	50	150	15
6	21	200	15
7	50	200	25
8	10	250	15
9	10	200	20

different yeast species were selected according to results from a previous work (Quirós et al., 2014) in order to explore the potential of different yeast species to contribute to ethanol content reduction by respiration during the transformation of grape must into wine. *S. cerevisiae* EC1118 was also included in the study for comparison. We addressed the impact on yeast metabolism of three easily manageable factors, fermentation temperature, nitrogen nutrient availability (YAN), and aeration regime. Nine combinations of these three factors at three different levels were assayed for each strain, as stablished in an orthogonal design (Table 1), and results were further analyzed by IBM SPSS Conjoint program. Fermentation experiments were run for six days, and samples were withdrawn twice a day (Supplementary file 1). Data from the 72 h sample were chosen for conjoint analysis. This choice was based on three considerations: i) according to previous results (Morales et al., 2015), three days was considered as a suitable oxygenation interval in order to reach the desired ethanol content reduction (indeed the average alcohol level reduction by 72 h was 2.1% ABV); ii) in most fermentation experiments sugar consumption was sufficient after 72 h in order to calculate metabolic yields with acceptable precision; iii) none of the fermentation experiments was stuck at this time point. Values for the main metabolic parameters at this time point are summarized in Supplementary file 2, and Fig. 1.

Boxplot for sugar consumption, considering all nine fermentation conditions, shows different median values but overlapping distributions and similar boundaries for all four yeast strains (Fig. 1A). The only evident extreme value was obtained for *S. cerevisiae* under condition 4, showing the highest sugar consumption after 72 h. This result would be in agreement with the adaptation of *S. cerevisiae* to growth in high sugar content substrates. Indeed, *S. cerevisiae* shows the highest consumption values among the four strains for six out of the nine growth conditions studied (Supplementary file 2). Also noticeably, condition 4, oxygenated with pure air (i.e. 21% O_2), and involving the highest YAN and temperature values (250 mg N/L and 25 °C), results in the highest sugar consumption for all the strains used in this study (ex aequo with condition 9 in the case of *K. lactis*).

The most striking differences between the four strains were found for ethanol yield values (Fig. 1B). The distribution for *S. cerevisiae* is narrower, and values, including median, clearly higher than for any other strain. Actually, *S. cerevisiae* showed the highest ethanol yield in all but one of the growth conditions assayed (Supplementary file 2). This trend towards high ethanol production under most culture conditions is in agreement with the well described metabolic features and the evolutionary history of this species (Piškur, Rozpedowska, Polakova, Merico, & Compagno, 2006), including the Crabtree effect (Pronk, Steensma, & van Dijken, 1996), which favors fermentative over respiratory metabolism despite oxygen availability. Another confirmation of the specific adaptation of *S. cerevisiae* to fermentative metabolism is that it was unable to grow under hyperoxygenated conditions (sparging with 100% O_2), in contrast to all the other yeast strains tested (data not shown). Low tolerance to oxygen might be due to high sensitivity of specific metabolic pathways or cell components to oxidative stress in this species, or to higher intracellular stationary levels of molecular oxygen due to low oxygen consumption by respiration. This would be in agreement with the higher dissolved oxygen levels generally appreciated in *S. cerevisiae* cultures, as compared to the other strains (Supplementary file 1).

RQ was calculated for all the experiments sparged with pure air. The results are in line with ethanol yields, with RQ values for *S. cerevisiae* ranging from 4.2 to 11.9, while the range of RQ values observed for all the other strains fell between 1.3 and 3.5 (Supplementary file 2). These values indicate the portion of carbon source metabolized by respiration was 3%–9% for *S. cerevisiae*, and 12%–53% for the other strains. These data confirm respiratory metabolism as one major determinant of ethanol yields under these fermentation conditions. The RQ values obtained for *S. cerevisiae* are in agreement with our previous results (Quirós et al., 2014), as well as other authors, depending on the strain and growth conditions, RQ values ranging from 2.8 to ∞ have been described for *S. cerevisiae* under aerated glucose rich conditions (Aceituno et al., 2012; de Deken, 1966; Franzen, 2003). It is worth noting that oxygen consumed after 72 h in these cultures ranged from 1.9 g/L to 19.4 g/L (Supplementary file 2), far apart from the microgram or milligram range used in other enological applications. Any trials aiming to scale up the process of alcohol level reduction in wine by respiration should take into account the relatively strong aeration conditions required.

Concerning acetic acid yield, the highest median value and overall distribution were observed for *S. cerevisiae* (Fig. 1C). This species was the highest acetic acid producer in eight out of the nine growth conditions tested (Supplementary file 2), with all acetic acid production values above the overall median of the experiment (considering all strains and growth conditions). This is in agreement with some of our previous results with this *S. cerevisiae* strain (Morales et al., 2015); and confirm volatile acidity production as the major drawback of using *S. cerevisiae* under aerated winemaking conditions. In contrast, the low median and distribution of values observed for this parameter in *C. sake* (Fig. 1C) suggest this species might be an interesting option for alcohol level reduction. Glycerol yield showed similar distributions for most strains apart for *M. pulcherrima*, which showed both the highest median and extreme values (Fig. 1D). This strain showed also the highest glycerol yields for all the growth conditions tested (Supplementary file 2). It would be a very interesting option for wine styles where high glycerol content is perceived as a positive quality trait.

Results of the conjoint analysis are summarized in Table 2. Glucose consumption is favored both by increasing nitrogen availability (YAN) and increasing fermentation temperature, with quite similar relative impact for all the strains in the study. However, a clear effect of increasing oxygen supply on glucose consumption was only revealed for *C. sake*, being the most important factor governing glucose consumption for this strain.

Surprisingly, YAN shows the highest relative impact on ethanol yield for all the strains apart *K. lactis*. In all four cases the correlation of YAN and ethanol yield was direct (Table 2). This suggests that nitrogen source availability stimulates sugar uptake, resulting in a higher rate of fermentation over respiration. This might be due to limitations in the oxygen transfer rate or to overflow metabolism, as described for Crabtree effect in *S. cerevisiae* (Pronk et al., 1996). On the other side, increasing oxygen supply shows a negative impact on ethanol yield, also compatible with the expected impact of oxygen availability on respiratory metabolism, for all strains but *C. sake*. It seems that the stimulation of sugar uptake induced by oxygen in this later species might go beyond its respiratory

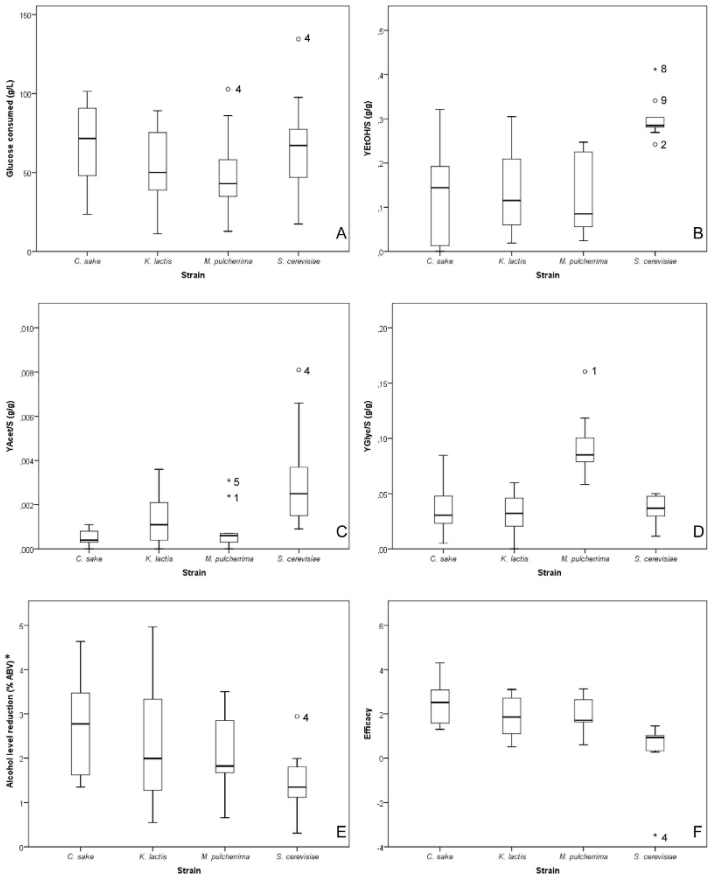

Fig. 1. Boxplots of the mean sugar consumption (A), ethanol yield on glucose (B), acetic acid yield on glucose (C), glycerol yield on glucose (D), alcohol level reduction (E), and Efficacy (F), by the strains analyzed in all conditions tested. Numbers indicate growth conditions for outliers. *Alcohol level reduction values cannot be taken as absolute, they can be overestimated by ethanol stripping, but they are useful for comparative purposes.

capacity, resulting in glycolytic overflow and increased ethanol yields. On the other side, *K. lactis* is the only yeast strain for which oxygen supply appears as the most relevant factor affecting ethanol yield (Table 2). According to this analysis, in order to reduce ethanol yield by respiration with *S. cerevisiae*, *M. pulcherrima*, or *C. sake*, either YAN or fermentation temperature would be better targets for process optimization than oxygen supply (always considering the range of values used in this work).

As a rule, we found a direct correlation between oxygen supply and acetic acid production (Table 2). This is, indeed, the most important factor influencing volatile acidity production by *C. sake*. However, considering this strain showed very low acetic acid yields under all growth conditions tested (Fig. 1C), the technological relevance of acetic acid production would be negligible for this species. On the other side, both YAN and fermentation temperature showed clearly different effects for each of the strains (Table 2). YAN is negatively correlated with acetic acid production for all strains but *C. sake*; while the correlation of acetic acid production

Table 2
Importance values, calculated by Conjoint analysis, of the effect of the three different environmental parameters (O_2 supplied – O_2; Nitrogen availability – YAN; Temperature – TEMP) on different fermentation parameters for different yeast species.

		S. cerevisiae[a]		*M. pulcherrima*[a]		*K. lactis*[a]		*C. sake*[a]	
Consumed glucose	O_2	2	+	5	+	11	–	44	+
	YAN	45	+	45	+	49	+	29	+
	TEMP	53	+	50	+	40	+	27	+
Ethanol yield	O_2	26	–	31	–	51	–	19	+
	YAN	44	+	52	+	34	+	62	+
	TEMP	30	–	17	+	15	+	19	+
Acetic acid yield	O_2	37	+	33	+	38	+	70	+
	YAN	15	–	38	–	36	–	24	+
	TEMP	48	+	29	–	26	–	6	–
Glycerol yield	O_2	79	–	22	–	62	–	75	–
	YAN	2	+	71	–	3	–	12	–
	TEMP	19	+	7	+	35	+	13	–
Alcohol reduction	O_2	12	+	21	+	7	+	47	+
	YAN	27	+	30	+	47	+	35	–
	TEMP	61	+	49	+	46	+	18	+
Efficacy	O_2	19	+	18	+	13	+	44	+
	YAN	46	–	32	+	45	+	41	–
	TEMP	35	–	50	+	42	+	15	+

[a] Symbols indicate direct (+) or inverse (–) correlation. Importance values can vary from 0 to 100.

with temperature is negative for all of them but *S. cerevisiae*. The opposite impact of YAN and temperature on acetic acid production, depending on the yeast strain, should be taken into account for the development of fermentation processes based on mixed cultures. In contrast to acetic acid, a negative correlation was found for glycerol yield with oxygen supply for all the strains assayed (Table 2). Oxygen supply is the most important factor affecting glycerol yield for all strains but *M. pulcherrima*. In contrast, YAN showed the strongest impact on glycerol yield for this strain (with a negative correlation).

In the context of developing procedures for alcohol level reduction in wine by sugar respiration it would be difficult to take decisions based on a single parameter, like sugar consumption, ethanol production, or acetate production. For this reason we decided to integrate the most relevant information for this purpose in a single parameter, Efficacy (see Materials and methods). This parameter takes into account sugar consumption, as well as ethanol and acetate production. *C. sake* showed the best distribution of Efficacy values among the strains tested in this study (Fig. 1F). In turn, *S. cerevisiae* showed the lowest Efficacy values in all but one of the growth conditions analyzed (Fig. 1F, and Supplementary file 2). The conjoint analysis showed a positive correlation of Efficacy with oxygen supply, for all the strains tested (Table 2). However, the relative impact is different for each strain, with *C. sake* sowing the strongest dependence of Efficacy on oxygen supply, while *K. lactis* showed very limited impact. Concerning Efficacy, YAN is a relevant variable to take into account for all yeast strains tested (Table 2). However, the direction of the correlation depends on the strain; it is positive for *M. pulcherrima* and *K lactis*, and negative for the other two strains. Similarly, Efficacy is affected by fermentation temperature in opposite directions depending on the strain, negatively for *S. cerevisiae* and positively for the other yeasts (Table 2). The lowest impact of temperature on Efficacy was observed for *C. sake*.

In summary, we have analyzed the impact of three easily manageable environmental factors on the production of the main fermentation metabolites by three non-*Saccharomyces* yeast strains during the fermentation of synthetic grape must (and compared results with *S. cerevisiae*). The different levels for each environmental factor were hence chosen in a reasonable range for process optimization, considering alcohol level reduction trough sugar respiration by yeast cells (Gonzalez et al., 2013). We must however consider that conclusions of this analysis cannot be extrapolated beyond the range of values assayed. For example, it is obvious that respiration would not take place in fully anaerobic cultures. Similarly, as mentioned above, 100% oxygen seems to be toxic for *S. cerevisiae*. This means including a 0% or 100% oxygenation level would change the result of the analysis, but it would be less relevant for practical purposes. Surprisingly, apart from glycerol production, oxygen supply is not the main driver of the differences observed for most parameters and strains. The different impact of the variations in the three environmental factors on the metabolism of each yeast strain is also appreciated by taking Efficacy as an integrative parameter. Both the relevance of each factor, and the direction they affect Efficacy, are different for each strain.

Concerning future process optimization, the main conclusions of this work would be that increasing oxygen supply (up to the upper levels assayed in this work) would positively contribute to alcohol level reduction and Efficacy of the process (despite the negative impact on acetic acid yields). In addition, *C. sake* CBS 5093 appears as the most promising strain among those tested. Using *C. sake* for alcohol level reduction by respiration would avoid most of the problems associated to volatile acidity (Fig. 1C), while alcohol level reduction and Efficacy would be high. According to Table 2, optimal conditions for this strain would probably involve initial YAN close to 150 mg N/L, 25 °C, and high oxygenation levels.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2015.09.046.

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Non-conventional Yeast Species for Lowering Ethanol Content of Wines

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Rising sugar content in grape must, and the concomitant increase in alcohol levels in wine, are some of the main challenges affecting the winemaking industry nowadays. Among the several alternative solutions currently under study, the use of non-conventional yeasts during fermentation holds good promise for contributing to relieve this problem. Non-*Saccharomyces* wine yeast species comprise a high number or species, so encompassing a wider physiological diversity than *Saccharomyces cerevisiae*. Indeed, the current oenological interest of these microorganisms was initially triggered by their potential positive contribution to the sensorial complexity of quality wines, through the production of aroma and other sensory-active compounds. This diversity also involves ethanol yield on sugar, one of the most invariant metabolic traits of *S. cerevisiae*. This review gathers recent research on non-*Saccharomyces* yeasts, aiming to produce wines with lower alcohol content than those from pure *Saccharomyces* starters. Critical aspects discussed include the selection of suitable yeast strains (considering there is a noticeable intra-species diversity for ethanol yield, as shown for other fermentation traits), identification of key environmental parameters influencing ethanol yields (including the use of controlled oxygenation conditions), and managing mixed fermentations, by either the sequential or simultaneous inoculation of *S. cerevisiae* and non-*Saccharomyces* starter cultures. The feasibility, at the industrial level, of using non-*Saccharomyces* yeasts for reducing alcohol levels in wine will require an improved understanding of the metabolism of these alternative yeast species, as well as of the interactions between different yeast starters during the fermentation of grape must.

Keywords: non-*Saccharomyces* yeasts, low alcohol wine, ethanol yield, yeast respiration, mixed starters

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INDUSTRIAL AND SOCIAL INTEREST IN REDUCING ALCOHOL LEVELS IN WINE

The ethanol content in wine increased considerably over the past 20 years due to two main factors: the impact of climate change upon the global production of grapes, and the current quest for new wine styles, often requiring increased grape maturity (Jones et al., 2005; Grant, 2010; MacAvoy, 2010; Alston et al., 2011; Gonzalez et al., 2013). Late harvests are indeed required to meet present consumer's preferences toward well-structured, full body wines, and optimal phenolic maturity of grapes. This practice results in a noticeable increase in the sugar content of the berries (Mira de Orduña, 2010) with consequent higher alcohol levels in wine. On the other hand, global

climate change has deeply influenced the vine phenology and the grape composition, resulting in grapes with lower acidity, altered phenolic maturation and tannin content, and increasing sugar concentration (Jones et al., 2005). These changes further contribute to rising alcohol content in wines, in addition to modifying other wine sensory attributes as well as wine microbiology (Mira de Orduña, 2010). Alston et al. (2015) reported that the ethanol content in New World wines was higher than in European wines (13.65 vs. 13.01% v/v). The ethanol contents found in North American, Argentinean, Australian, and Chilean wines were 13.88, 13.79, 13.75, and 13.71% v/v, respectively. In Europe, Spain accounted the highest values (13.43% v/v). The high ethanol content in wine can lead to stuck and sluggish fermentations (Coulter et al., 2008) and to unbalanced wines that are unpleasant for consumers. Indeed, several studies reported that high ethanol concentration increase hotness and bitterness perceptions, while it decreases acidity sensations and masks the perception of some important aroma compounds such as higher alcohols, esters and monoterpenes (Escudero et al., 2007; Robinson et al., 2009; Fischer, 2010; Frost et al., 2015). This trend brings about some troubles for the wine industry, as well as social and public safety problems related to alcohol consumption (Grant, 2010; MacAvoy, 2010). In order to overcome these issues, the market focus is directed to wines with a moderate alcohol content. In addition, lowering ethanol content has an economic interest due to the high taxes imposed in some countries (Gil et al., 2013).

Pickering (2000) and Saliba et al. (2013) reported that wines with reduced ethanol content have been classified as dealcoholized or alcohol free (<0.5% v/v), low alcohol (0.5–1.2% v/v), reduced alcohol (1.2 to 5.5–6.5% v/v) and lower alcohol wine (5.5–10.5% v/v), even if these classifications, which are loosely based on labeling and legislative requirements, vary between different countries (Pickering, 2000). However, most winemakers are interested in developing practices aiming to reduce the alcohol concentration in wine by just 1–3% v/v, in order to compensate the impact of global warming and to obtain better-balanced wines (Meillon et al., 2010a,b; Gambuti et al., 2011). The winemaking industry is addressing this challenge by targeting almost all the different steps of the production cycle (Teissedre, 2013), starting from grapevine clonal selection, vineyard management, pre-fermentation and winemaking practices, microbiological approaches and post-fermentation and processing technologies (García-Martín et al., 2010; Gil et al., 2013; Poni, 2014; Varela et al., 2015).

In this regard, the viticultural practices to reduce ethanol content in wine act to manage grapes sugar content through different approaches such as reducing leaf area (defoliation or topping of shoots; Martinez de Toda et al., 2013; Poni, 2014), pre-harvest irrigation to cause a significant delay of ripening (Mendez-Costabel, 2007), application of growth regulators to postpone ripening (Symons et al., 2006) and manage harvest date (Bindon et al., 2013). At pre-fermentative stage the reduction of sugar concentration in must could be achieved by dilution of grape must with water (depending of country regulation) or using nanofiltration technologies (Harbertson et al., 2009; García-Martín et al., 2010). Another pre-fermentative strategy to remove sugar from must could be the addition of glucose oxidase enzyme (Pickering, 2000). The ethanol reduction in wine could be also achieved at post-fermentation stage. In this regard, it is possible to mention the blending of low-high alcohol wines or physical removal of alcohol from wine with membrane-based system, vacuum distillation and supercritical CO_2 extraction (Gambuti et al., 2011; Kontoudakis et al., 2011; Schmidtke et al., 2012).

S. cerevisiae IS NOT THE BEST YEAST SPECIES FOR REDUCING ALCOHOL LEVELS IN WINE

Development and application of yeast strains showing below normal alcohol production has been a recurrent objective for wine biotechnology for more than 20 years, starting even before increasing ethanol content in wines was widely perceived as a problem. Low alcohol production by yeasts might be related with two distinct metabolic features, alcohol tolerance, or ethanol yield on sugar. Traditional scientific literature on wine yeast often use the term fermentative power, to refer to the amount of alcohol produced by different yeast strains from natural or synthetic grape must (Lopes et al., 2006). Due to the assay conditions, this parameter is mainly related to alcohol tolerance, and tells little about the usefulness of yeast strains for alcohol level reduction. Indeed, oenological use of yeast strains having low fermentative power would result in either stuck fermentation or the starter being quickly replaced by native yeasts.

To attain a relevant alcohol level reduction in wine (fermented to dryness), the appropriate yeast metabolic trait to take into account is alcohol yield on sugar. Ethanol yield on sugar is formally expressed as grams of ethanol produced per gram of glucose or fructose consumed (g/g). The rule of thumb says consumption of 17 g/L of sugar will result in an increase of 1% v/v in alcohol content. Not surprisingly, being *Saccharomyces cerevisiae* the main yeast species responsible of alcoholic fermentation during winemaking, it has almost invariably been the species of choice for all research efforts aiming to reduce ethanol yields. However, evolution has shaped this species to quickly and efficiently produce ethanol from sugars under most environmental conditions, following the make-accumulate-consume life strategy (Piskur et al., 2006). Although, some natural variability can be found among wild isolates of this species, the distribution of ethanol yield values is rather narrow (around the values mentioned above).

Researchers have designed several alternative genetic engineering approaches in order to partially redirect *S. cerevisiae* normal carbon flux, starting with the overexpression of *GPD1* or *GPD2*, coding for isozymes of glycerol 3-phosphate dehydrogenase. The choice of *GPD* genes was additionally driven by glycerol contribution to sweetness, smoothness and wine body. Other strategies aiming to reducing alcohol yields also involve genetic manipulation of the central carbon and energy metabolism of *S. cerevisiae*. Target genes include for example *PDC2*, coding for pyruvate decarboxylase; *ADH1*, coding for alcohol dehydrogenase; or *TPI1*, coding for triose phosphate isomerase. An excellent review by Kutyna et al. (2010) gathers

additional genetic engineering strategies in order to reduce alcohol yield during wine fermentation. However, a recent experimental evaluation of genetic modifications to develop low ethanol yield wine yeast strains concluded that overexpression of *GPD1* was the most efficient strategy to lower alcohol yield (Varela et al., 2012). Also in agreement with early studies in this field (Remize et al., 1999; Cambon et al., 2006) they found overexpression of *GPD1* resulted in the overproduction of some metabolites negatively affecting wine quality. In order to avoid these drawbacks additional genetic modifications were required (Cambon et al., 2006; Ehsani et al., 2009). Metabolic pathways mentioned in this paragraph are summarized in **Figure 1**.

Limitations of the genetic engineering approach are twofold. First, commercial use of genetically engineered wine yeast strains does not seem to be feasible in the short term (Gonzalez et al., 2013). In order to circumvent this problem, some researchers are now using adaptive laboratory evolution (Cadière et al., 2011; Kutyna et al., 2012). Second, the increase in concentration required to reach a relevant impact on wine final alcohol content (2–3% v/v), would certainly compromise wine quality for most alternative metabolites. This holds true even for glycerol, one of the preferred targets for researchers in this field. Reduction of 2% v/v ethanol by diverting carbon flux toward glycerol production would result in more than 30 g/L extra glycerol (about five times the usual values). Almost any other chemical compound would also become unacceptable in wine at such elevated concentrations. Carbon dioxide is perhaps the only metabolite that would cause no trouble when overproduced by yeast during wine fermentation, in part because it is readily released to the atmosphere. The two main metabolic pathways for CO_2 production are respiration and fermentation. Concerning alcohol reduction, the advantage of respiration is that no ethanol is produced, since all six carbon atoms from each molecule of sugar end up as CO_2. Some researchers have suggested partial respiration of sugars from grape must as a way to decrease ethanol yield during winemaking (Gonzalez et al., 2013 and references therein). A possible way to reach this goal is shown in **Figure 2**.

There are, however, two restrictions to make yeast cells respire sugars under standard winemaking conditions, oxygen requirement and the Crabtree effect. Respiratory metabolism has a huge oxygen demand, but it is known to participate in many other chemical reactions that can be detrimental to wine quality. Proper management of dissolved oxygen during wine fermentation will be required in order to meet respiration requirements while preserving other wine compounds from excessive oxidation (see below). On the other side, *S. cerevisiae* is the archetype Crabtree-positive yeast species. This metabolic feature strongly favors fermentative over respiratory metabolism, despite oxygen availability (Pronk et al., 1996), and have played a key role in the adaptation of this species to sugar rich environments (Piskur et al., 2006). In *S. cerevisiae* "aerobic fermentation" involves usually above 98% of the sugars consumed in the presence of oxygen (de Deken, 1966). Only under conditions of very low sugar availability (which is not obviously the case for grape must), is respiration the main energetic metabolic pathway in this species (Pronk et al., 1996). The possibility of reducing ethanol yields by promoting respiration of sugars by *S. cerevisiae* or other yeast species was initially suggested by Smith (1995), and the idea has been independently recovered and developed to different levels in recent years (Erten and Campbell, 2001; Contreras et al., 2015b; Morales et al., 2015; see below).

SUGAR METABOLISM OF NS YEASTS

Common ethanol yields on sugar after complete grape juice fermentation are 90–95% of theoretical, with the remaining 5–10% being explained by biomass biosynthesis, ethanol stripping, and alternative metabolic pathways (Konig et al., 2009). This mainly reflects anaerobic carbon flux distribution in *S. cerevisiae*. However, NS wine yeasts usually differ from *S. cerevisiae* in metabolic flux distribution during fermentation and, consequently, in ethanol production, biomass synthesis, and by-product formation (Ciani et al., 2000; Magyar and Toth, 2011; Milanovic et al., 2012; Tofalo et al., 2012). Under anaerobic conditions, the diversion of alcoholic fermentation and the abundant formation of secondary compounds may in part explain the low ethanol yield of some of these NS yeast species/strains. Indeed, some of these species are strongly characterized by species-specific patterns of fermentation by-products, which allows the differentiation of the majority of these yeast strains according to the species (Domizio et al., 2011).

The production of ethanol and the other main fermentation compounds are metabolically linked. In *S. cerevisiae* glycerol production is highly correlated with the production of acetic acid (Ciani and Rosini, 1995). Indeed, as mentioned above, genetic engineering of *S. cerevisiae* for glycerol overproduction often results a large production of acetic acid (Remize et al., 1999, 2000; Eglinton et al., 2002). The evaluation of the relation between fermentation by products and ethanol production among several NS wine yeasts revealed both direct and inverse correlations between acetic acid and ethanol production, for *Saccharomycodes ludwigii* and *Kloeckera apiculata*, respectively (Ciani and Maccarelli, 1998). In contrast, *Torulaspora delbrueckii*, *Candida stellata*, and *Hanseniaspora uvarum* did not show any correlation between the two fermentation products. Ethanol is positively correlated with glycerol and ethyl acetate in *C. stellata* and *K. apiculata* respectively, while an inverse correlation between ethanol and succinic acid production was shown for *T. delbrueckii*.

The most striking metabolic trait of *S. cerevisiae* is perhaps the Crabtree effect. This feature makes *S. cerevisiae* preferentially consume sugars by fermentation in almost any growth condition, apart from carbon limited chemostat operated at low dilution rates. This trait has been often related to glucose triggered transcriptional repression of genes involved in respiratory functions (Barnett and Entian, 2005). However, current understanding of the Crabtree effect points to overflow metabolism at the level of the pyruvate node, as the main mechanism contributing to the observed distribution of carbon

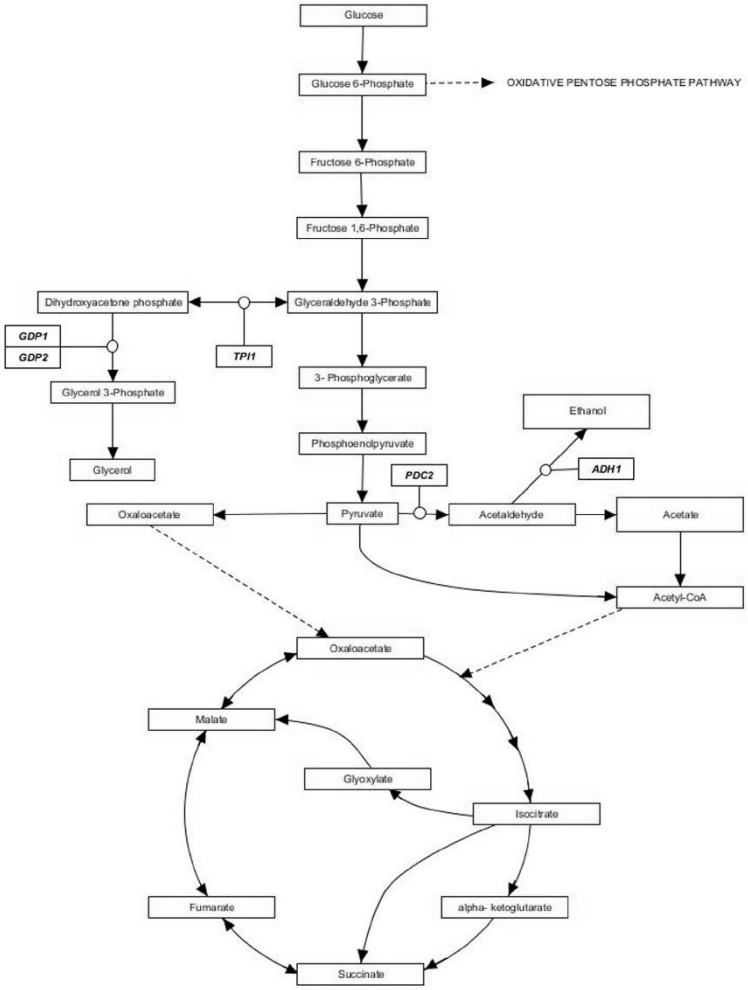

FIGURE 1 | Metabolic pathways involved in ethanol production by *Saccharomyces cerevisiae*. Genes targeted by genetic engineering strategies mentioned in the text are indicated in black boxes.

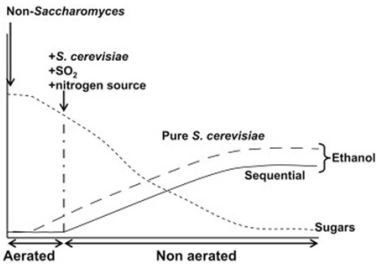

FIGURE 2 | Idealized representation of the expected evolution of ethanol production during grape must fermentation in a sequential inoculation with a Crabtree-negative non-*Saccharomyces* yeast strain, followed by *S. cerevisiae* at the moment indicated (continuous line). Aeration would be restricted to the first stages of alcoholic fermentation, as indicated. The expected evolution of ethanol production for a pure *S. cerevisiae* starter in the same conditions is indicated by a dashed line. For simplicity, sugar consumption has been assumed to follow a similar pattern in both situations. Reproduced from Gonzalez et al. (2013) with permission of the copyright owner.

flux toward ethanol production (Holzer, 1961; Pronk et al., 1996). In addition, Aceituno et al. (2012) found cytoplasm-to-mitochondria NADH transport to be a limiting factor to get a fully aerobic metabolism in the presence of oxygen. Independent of the mechanism, the critical factor determining the respiro-fermentative balance in *S. cerevisiae* seems to be the rate of sugar consumption. Indeed, mutations slowing down the glycolytic rate result in a noticeable relief of the Crabtree effect (Otterstedt et al., 2004; Jansen et al., 2005).

Several classifications of yeast species, according to the way they regulate respiro-fermentative metabolism have been proposed (Gancedo and Serrano, 1989; Alexander and Jeffries, 1990). In general, they are categorized as either Crabtree-positive or Crabtree-negative, or as obligate respiratory. Assessment of the Crabtree status is generally based on studies under carbon limited chemostat conditions (Pronk et al., 1996). So, despite most yeast species found in the oenological environment have shown fermentative capacity (Kurtzman et al., 2011), most of them have never been evaluated for Crabtree status. Furthermore, according to recent studies (Quirós et al., 2014; Contreras et al., 2015b) the classification based on standard Crabtree assays has little prediction power on the behavior of yeasts under growth conditions more closely mimicking those found in wine fermentation. In addition, important differences can be found among yeast strains belonging to the same species.

Analysis of the respiro-fermentative behavior of yeast strains under controlled aeration conditions in high sugar containing media has usually confirmed *S. cerevisiae* as one of the most fermentation-prone yeast species. However, strains from some other species have shown even higher ethanol yield or RQ values than control *S. cerevisiae* yeast strains under such assay conditions (Quirós et al., 2014; Contreras et al., 2015b). Interestingly, respiratory behavior of yeast strains seems to be strongly affected by other environmental factors, not only sugar abundance or oxygen availability (Rodrigues et al., 2016). The extent to which these environmental factors affect yeast respiro-fermentative metabolism, and secondary by-products like glycerol or acetic acid, is species or strain-specific. Further research is required in order to understand the metabolic diversity of NS yeast species and the relevance of this diversity for oenological applications, including reducing ethanol content of wines.

SELECTION OF NON-*Saccharomyces* WINE YEAST TO REDUCE THE ETHANOL CONTENT

During wine production, the non-*Saccharomyces* (NS) yeasts contribute to the fermentation process, either directly or through their effect on both growth kinetics and metabolic activity of *S. cerevisiae* (Ciani and Comitini, 2015). These NS yeasts are capable of anaerobic or aerobic growth and may persist during the fermentation, competing with *Saccharomyces* for nutrients, producing secondary compounds or modifying the *S. cerevisiae* metabolism (Milanovic et al., 2012; Sadoudi et al., 2012; Barbosa et al., 2015).

NS wine yeasts have been shown to modulate wine fermentation and to enhance sensorial complexity and aroma profile of wines (Fleet, 2008; Ciani et al., 2010). In addition, some of these NS species/strains are able to combat spoilage yeasts (Comitini et al., 2011; Oro et al., 2014; Alonso et al., 2015). Thus, over the last years, the role of NS yeasts in winemaking, previously neglected or demonized, has been re-evaluated, and their use has been proposed in controlled mixed fermentation with the aim to improve wine complexity, aroma profile and control of spoilage microorganisms (Rojas et al., 2001; Swiegers et al., 2005; Domizio et al., 2007; Renouf et al., 2007; Anfang et al., 2009; Comitini et al., 2011; Jolly et al., 2014). In this context, the metabolic traits of NS wine yeasts could also be profitably used to reduce the alcohol content in wine. This application would benefit from a better understanding of the metabolic pathways diverting carbon flux from ethanol production in NS yeasts, as well as the biological variability of these yeast species in terms of ethanol yield. One of these alternative pathways would be sugar respiration under suitable fermentation conditions, especially for Crabtree-negative yeast species, as discussed in other sections of this review. In summary, our current knowledge suggests several promising approaches for the use of NS wine yeast to limit ethanol production. However, taking into account the current interest on NS wine yeasts is mostly related to their impact on wine sensory quality (Lambrechts and Pretorius, 2000; Romano et al., 2003; Ciani et al., 2010; Belda et al., 2015, 2016; Wang et al., 2015; Hu et al., 2016; Masneuf-Pomarede et al., 2016; Medina et al., 2016), a positive contribution to wine aromatic complexity would certainly be a plus in yeast strain selection for this purpose.

Screening Based on Low Ethanol Yield under Anaerobic Fermentation Conditions

Over the recent years, there was a rising interest to investigate on the wine yeast variability in ethanol yield as a potential tool for the reduction of alcohol content in wine. Variability between different yeasts genera and species could be exploited at industrial level to produce wines better fitting consumer preferences. Reduction in ethanol yield is strictly dependent on the microbial strategies that divert sugar-carbon away from ethanol production.

As mentioned above, *S. cerevisiae*, shows high fermentation performance with high ethanol yield and fermentation efficiency, exhibiting a low intraspecies variability for these characters. In contrast, NS wine yeasts show, as a trend, lower ethanol production and lower ethanol resistance. Overall these features are considered to be a major factor of the dominance of *S. cerevisiae* over NS species during wine fermentation. Generally, the species belonging to *Hanseniaspora, Candida, Pichia, Kluyveromyces, Metschnikowia, Torulaspora,* and *Issatchenkia* genera, widely or occasionally found in grape juice, are not tolerant to ethanol concentrations above 5–7% v/v. Their decline and death as the fermentation progresses can be mostly explained by their low alcohol tolerance, even though recent studies indicate that the interactions with *S. cerevisiae* might be more complex (Arneborg et al., 2005; Branco et al., 2014; Ciani and Comitini, 2015). On the other hand, NS wine yeasts exhibit a broad spectrum of fermentation by-products, low fermentation purity (volatile acidity g/L ÷ ethanol % v/v) and, often, low ethanol yield (Muller-Thurgau, 1896; Ribéreau-Gayon and Peynaud, 1960; Romano et al., 1992). A systematic investigation on fermentation by-products formed by a wide collection of NS wine yeasts, belonging to five different species, was carried out by Ciani and Maccarelli (1998). In that work, "apiculate" yeast species showed a high production of acetaldehyde, ethyl acetate and acetoin; *C. stellata* exhibited high production of glycerol and succinic acid, while *T. delbrueckii* was shown to be a lower producer of secondary products of fermentation. In the various NS species tested, the ethanol production is differently related with the fermentation by-products. NS wine yeasts are generally low-ethanol producing yeasts. However, this feature does not necessarily mean that they exhibit also low ethanol yield.

In this context, only recent studies addressed the interspecies and/or intraspecies variability in ethanol yield among NS wine yeasts (Magyar and Toth, 2011; Contreras et al., 2014, 2015b; Gobbi et al., 2014). In a comparative evaluation of some oenological properties in several wine strains, Magyar and Toth (2011) found a very low ethanol yield for four *Candida zemplinina* strains. Gobbi et al. (2014), investigating on several NS wine yeast species, showed that strains belonging to the species of *H. uvarum, Zygosaccharomyces sapae, Zygosaccharomyces bailii,* and *Zygosaccharomyces bisporus* exhibited significant low ethanol yield and fermentation efficiency in comparison with *S. cerevisiae* under anaerobic conditions and using different grape juices. For *H. uvarum*, these data confirm the low ethanol yield previously described (Ciani et al., 2006), in contrast to species belonging to the *Zygosaccharomyces* genus. Moreover, they found that ethanol yield, like other fermentation features, is a species-related trait. However, as indicated previously for other fermentation parameters (Ciani and Maccarelli, 1998; Comitini et al., 2011; Domizio et al., 2011), a pronounced intraspecies variability was also evident. In another recent work a screening on 50 different NS strains belonging to 24 different genera for their ethanol yield was carried out (Contreras et al., 2014). This led to the identification of four NS yeast strains (two strains of *Metschnikowia pulcherrima* and one strain each of *Schizosaccharomyces malidevorans* and *C. stellata*) that showed low ethanol yield. In a different study, under semi-aerobic condition, nine out of 48 NS strains showed ethanol yields lower than the *S. cerevisiae* control strain. Three of them (*T. delbrueckii* AWRI1152, *Pichia kudriavzevii* AWRI1220, and *Z. bailii* AWRI1578) gave promising results in the subsequent aerobic sequential trials, with *S. cerevisiae* AWRI1631 (Contreras et al., 2015b; see below for further discussion on this work).

Some *Saccharomyces* species, other than *S. cerevisiae*, have also shown potential for ethanol reduction. This is the case for *Saccharomyces uvarum*, a cryophilic species that has been described as a low ethanol and high glycerol producer (Giudici et al., 1995). Fermentation kinetics in must at 13°C is better for *S. uvarum* than for *S. cerevisiae*, but some strains get stuck at 8% v/v alcohol when run at 24°C (Kishimoto et al., 1994; Masneuf-Pomarede et al., 2010). In a sequential inoculation of *S. uvarum* (AWRI 2846) and *S. cerevisiae*, Contreras et al. (2015a) found an ethanol reduction of 0.8% v/v, and an increase of glycerol of 6.4 g/L. The decrease in ethanol production was not fully explained by the increase in glycerol, in terms of carbon mass balance.

Respiration Based Screening

As mentioned above, development of respiration based methods to reduce alcohol content in wine requires the use of NS yeast strains showing no or weak Crabtree effect. However, this metabolic feature, which is indeed rather common across the yeast phylogeny (de Deken, 1966), is not sufficient to warrant the utility of a given yeast species/strain for such purpose. Suitable yeast strains must be able to develop in grape must, a relatively harsh growth medium due to osmotic stress, low pH, and the presence of natural or added inhibitors of microbial growth. In addition sugar consumption kinetics should be relatively fast, in order to be compatible with industrial procedures; as well as being able to dominate fermentation processes, in competition with the microbiota naturally present in grape must. Finally, they must not generate secondary metabolic products that would result in wine spoilage, in either aerobic or anaerobic conditions.

Initial trials to follow the sugar respiration strategy analyzed the behavior of three to four yeast strains in synthetic or natural grape juice under aerobic or microaerobic conditions (Smith, 1995; Barwald and Fischer, 1996; Erten and Campbell, 2001). More recent studies use a higher number of yeast strains (around 60) and milder aeration regimes, than previous studies (Quirós et al., 2014; Contreras et al., 2015b). Quirós et al. (2014) chose respiratory quotient (RQ) as an indicator of the respiration capabilities of each yeast strain. RQ can be calculated as the

ratio of CO_2 produced to O_2 consumed. When hexoses are used as substrate RQ can range from 1 (full respiration) to ∞ (full fermentation). The relationship between RQ and the percentage of sugar consumed by respiration (%SR) can be expressed as follows: %SR = 100/(3RQ-2). They calculated RQ values, in synthetic medium containing 200 g/L sugar, pH 3.5, and high biomass content (OD600 = 20), under strongly aerated conditions, and identified strains from several yeast species with RQ values close to 1 under these specific growth conditions. The advantage of RQ over direct calculation of ethanol yields is it is not affected by ethanol stripping. One alternative which is especially valid for mild aeration regimes is comparing ethanol yields with *S. cerevisiae*, in order to identify low yield candidates, and setting control experiments with pure nitrogen gas at the same flow rate, in order to compare ethanol yields between anaerobic and aerobic (or micro-aerobic) conditions (Contreras et al., 2015b; Morales et al., 2015). We must, however, stress that Contreras et al. (2015b) considered their aeration conditions to be not strong enough to trigger respiratory metabolism.

However, low respiratory quotient or low ethanol yields are not enough to ensure the usefulness of yeast strains for the purposes discussed in this review. Indeed, strains showing a strong preference for respiratory metabolism would be completely useless if the amount of sugar they metabolized were negligible (in a reasonable fermentation time). Hence, authors took into account sugar consumption after 3 or 4 days on synthetic grape must in order to identify interesting strains (Quirós et al., 2014; Contreras et al., 2015b).

The other main aspect to be taken into consideration for a proper yeast strain selection in this context is volatile acidity. There are already several reports showing an important increase in acetic acid yield for *S. cerevisiae* under aerated conditions, as compared to anaerobic growth (Giovanelli et al., 1996; Papini et al., 2012; Quirós et al., 2014; Contreras et al., 2015b; Rodrigues et al., 2016). Strains from other yeast species have also been found to produce high amounts of acetic acid under oxygenation (Quirós et al., 2014; Contreras et al., 2015b); and some of them also under standard fermentation conditions (Ciani and Picciotti, 1995; Viana et al., 2008).

MANAGING MIXED FERMENTATIONS

Apart from reducing ethanol yields, the main driver for the current development of NS commercial starters is related to the increasing consumer demand for wines showing improved sensorial properties and distinctive flavor (Pretorius and Hoj, 2005; Belda et al., 2015, 2016; Masneuf-Pomarede et al., 2016; Medina et al., 2016), in contrast to the limited complexity attributed to wines fermented with *S. cerevisiae* starter strains (Heard, 1999; Rojas et al., 2003; Romano et al., 2003; Ciani et al., 2006, 2010; Jolly et al., 2006). However, NS wine yeasts often show low fermentation power. For this reason *S. cerevisiae* starters have to be used to ensure consumption of all sugars from grape must, and to bring the fermentation process to completion. In addition, the interactions between *Saccharomyces* and NS yeasts can be exploited to modulate the content of ethanol in wine (Ciani and Comitini, 2015; Wang et al., 2015). Temperature, sulphite content, sugar concentration, nitrogen composition, oxygen and pH, which influence glycerol and ethanol biosynthesis, must also be modulated and controlled.

Mixed starters can be used by either simultaneous or sequential inoculation. This later modality allows to take advantage of the metabolism of the first inoculated NS yeast without the influence of the *Saccharomyces* starter culture. In this way, the reduction in ethanol content will depend on the metabolic characteristics of the NS strain used, and on the actual opportunity it will have to stamp its metabolic footprint before *S. cerevisiae* takes over. Some important control parameters should be taken in account for this purpose: the inoculation concentration and the timing between the first and second inoculation, nutrient consumption and sulphite content. High inoculation level of NS yeast improves the competitiveness toward *S. cerevisiae* and other wild yeasts; while the interval between the first and the second inoculation affects the duration of this metabolic activity, which will quickly decline upon inoculation of *S. cerevisiae*. However, attention must also be paid to the consumption of nitrogen sources and vitamins from grape must by NS yeasts during the first stage of sequential inoculation fermentation (Kemsawasd et al., 2015). This consumption often requires to be compensated by suitable yeast nutrients in order to prevent stuck fermentations after inoculation of *S. cerevisiae* (Medina et al., 2012; Lage et al., 2014). Special attention is required in oxygenated fermentations, since a strong nutrient depletion is expected due to high biomass production by NS yeasts under these growth conditions. Concerning sulphite concentration, it must be adjusted during the first stage to the actual tolerance of the NS yeast strain used, since it will usually fall below the standard values for *S. cerevisiae* strains. Eventually, they might be raised to ordinary winemaking concentration after the second inoculation. Interestingly, controlled fermentation by sequential inoculation has been proposed as a way to reduce sulphite contents in the final wine.

The sequential inoculation strategy, using NS/*S. cerevisiae* has been employed in several studies. Many of them use *Starmerella bombicola* (formerly *C. stellata*) as the NS counterpart to *S. cerevisiae*. In these investigations a high production of glycerol and succinic acid and interactions involving some by-products (acetaldehyde, acetoin) with a consequent reduction of final ethanol amount were found (Ciani and Ferraro, 1996, 1998; Ferraro et al., 2000). The reduction in ethanol content in these assays varied from 0.64% v/v at pilot scale in natural grape juice to 1.60% v/v at laboratory scale using synthetic grape juice. Sequential fermentation trials using *Lachancea thermotolerans* (formerly *Kluyveromyces thermotolerans*) were carried out under industry condition using a high inoculation level (10^7 cell/ml) with a delay of the second inoculum (*S. cerevisiae* strain) of 2 days resulting in an ethanol reduction of 0.7% v/v (Gobbi et al., 2013). A sequential inoculation of *M. pulcherrima* AWRI1149 followed by a *S. cerevisiae* wine strain gave rise to a wine with an ethanol concentration lower than that achieved with *S. cerevisiae* (0.9 and 1.6% v/v in Chardonnay and Shiraz wines, respectively; Contreras et al., 2014). Di Maio et al. (2012) showed that *C. zemplinina* may be used in mixed fermentation with *S. cerevisiae* to reduce the

ethanol content in wine (0.32% v/v) and to increase the glycerol content More recently, the use of sequential fermentation with immobilized non-*Saccharomyces* wine yeast, was proposed to reduce the ethanol content in wine using *S. bombicola*, *M. pulcherrima*, *H. uvarum*, and *Hanseniaspora osmphila* selected strains, in Verdicchio grape juice. Sequential fermentation of 72-h showed an ethanol reduction of 1.64% (v/v) for *S. bombicola*, 1.46% (v/v) for *M. Pulcherrima*, 1.21% (v/v) for *H. uvarum*, and 1.00% (v/v) for *H. osmophila*. The wines obtained did not exhibit any negative fermentation products, but rather an increase of some desirable compounds (Canonico et al., 2016). In **Table 1** are summarized the anaerobic sequential fermentations of some NS yeasts as compared to control *S. cerevisiae* proposed to reduce the ethanol content in wine.

As a general trend, using NS/*S. cerevisiae* pairs in mixed fermentation did not result in the overproduction of undesirable by-products, in contrast to some *S. cerevisiae* genetically engineered strains, which can dramatically accumulate acetic acid or other metabolites with a negative impact on wine sensorial quality (Michnick et al., 1997; Remize et al., 1999). Indeed, apart from the reduction of ethanol content in wine, positive interactions in fermentation by-products have been shown during sequential fermentation. In NS/*S. cerevisiae* mixed cultures, the interactions due to the wide inter-generic metabolic diversity should be higher. These interactions were investigated in *S. cerevisiae* and *S. bombicola* (Sipiczki et al., 2005) mixed fermentation. In this co-culture complementary consumption of glucose and fructose was observed (Ciani and Ferraro, 1998). Using sequential, continuous fermentation and immobilized yeast cells, preliminary evidence has highlighted the exchange of acetaldehyde between these two yeast species. The excess of acetaldehyde production by *S. bombicola*, due to the low activity of alcohol dehydrogenase (Ciani et al., 2000), was quickly metabolized by *S. cerevisiae*, which is a more active alcoholic fermentation species (Ciani and Ferraro, 1998). In this context, an acetaldehyde flux between *S. cerevisiae* and *Saccharomyces bayanus* has also been reported (Cheraiti et al., 2005). These interactions in acetaldehyde reduction, were also detected in mixed fermentations using *S. cerevisiae*, *T. delbrueckii* (Ciani et al., 2006; Bely et al., 2008; Belda et al., 2015) and *L. thermotolerans* (Ciani et al., 2006). Another compound involved in interactions between two yeast species in mixed fermentation is acetoin; this is largely accumulated by *S. bombicola* in pure culture, and completely metabolized by *S. cerevisiae* in mixed fermentation (Ciani and Ferraro, 1998). More recently, the *influence Hanseniaspora guilliermondii* on genomic expression of *S. cerevisiae* in mixed culture wine fermentation was investigated (Barbosa et al., 2015)

TABLE 1 | Reduction of ethanol content in anaerobic sequential fermentations of some NS yeasts as compared to control *Saccharomyces cerevisiae*.

Sequential fermentation	Grape juice	Ethanol reduction % (v/v)	Reference
S. bombicola/S. cerevisiae	White	0.64	Ferraro et al., 2000
S. bombicola/S. cerevisiae	Synthetic	1.60	Ciani and Ferraro, 1998
S. bombicola/S. cerevisiae	White	1.64	Canonico et al., 2016
H. uvarum/S. cerevisiae	White	1.21	Canonico et al., 2016
H. osmophila/S. cerevisiae	White	1.00	Canonico et al., 2016
M. pulcherrima/S. cerevisiae	White	0.90	Contreras et al., 2014
M. pulcherrima/S. cerevisiae	Red	1.60	Contreras et al., 2014
M. pulcherrima/S. cerevisiae	Red	0.90	Contreras et al., 2015a
M. pulcherrima/S. cerevisiae	White	1.46	Canonico et al., 2016
L. thermotolerans/S. cerevisiae	Red	0.70	Gobbi et al., 2013
C. zemplinina/S. cerevisiae	Red	0.32	Di Maio et al., 2012

On the other hand, oxygenated fermentation, as proposed above to stimulate yeast respiration, introduce a new challenge for managing mixed fermentations. Oxygen supply has a positive impact in several microbial and chemical processes during winemaking. It activates *S. cerevisiae* metabolism, in part because it is required for the biosynthesis of plasma membrane sterols, so aeration practices are often used in order to ensure good initial fermentation kinetics or to help recover sluggish fermentation (Alexandre and Charpentier, 1998; Valero et al., 2001; Fornairon-Bonnefond et al., 2002). Oxygen is also used in hyper-oxygenation treatments, in order to get rid of compounds highly sensitive to oxidation that would contribute to browning of white wines if oxidized in later stages of the winemaking process. In turn, macro- and micro-oxygenation of wines are used, alone or in combination with other oenological practices, in order to improve and stabilize wine color during the aging of red wines, or to avoid the "reduced" character sometimes associated to aging on yeast lees (Fornairon-Bonnefond et al., 2003).

Nevertheless, oxygen supply amounts required to ensure efficient yeast respiration are far beyond requirements for even the most demanding oxygenation practices, among those described above. There is a risk that the strong oxygenation levels required for yeast respiration would promote, as a side effect, the oxidation of key components for the sensory quality of wines, namely phenolics and aroma compounds. However, oxygen affinity of fermenting yeast cells has been determined to be about 1000 times higher than wine polyphenols (Salmon, 2006). Accordingly, the target to avoid oxidative damage to wine phenolics would be coupling air supply to oxygen consumption by yeast cells. Being able to keep dissolved oxygen values around 0% would be a good indicative of success for this objective. This goal was shown to be feasible by using controlled aeration conditions and an appropriate *M. pulcherrima* strain (Morales et al., 2015).

An additional major issue of strong aeration of wine during the fermentation step is acetic acid production. Several authors have described a boost in acetic acid production by *S. cerevisiae* when fermenting under aerobic or micro-aerobic conditions (Giovanelli et al., 1996; Papini et al., 2012; Quirós et al., 2014; Contreras et al., 2015b; Rodrigues et al., 2016). Other yeast species have also been shown to negatively impact volatile acidity under aerated growth conditions in synthetic grape must (Quirós et al., 2014). Rodrigues et al. (2016) analyzed volatile acidity across several growth conditions for four different yeast strains. They found a clear correlation between oxygen supply and acetic acid production. The good news is that some yeast species produce very little volatile acidity even under oxygenated

conditions (Quirós et al., 2014; Rodrigues et al., 2016). It is possible to manage oxygen supply in fermentation trials driven by simultaneous inoculation of *S. cerevisiae* and a NS strain (Morales et al., 2015). However, the strict control of the process required under such growth conditions suggests that a better control of volatile acidity would be achieved by inoculating *S. cerevisiae* only after oxygen supply has been arrested (i.e., by sequential inoculation). In addition, proper control of yeast metabolism in aerated fermentations would benefit from the development of dedicated devices, able to monitor oxygen consumption and to adapt air supply to yeast requirements, so avoiding both excess oxidation and excess acetic acid production.

CONCLUSION

Research on yeast based strategies in order to reduce ethanol content of wines started about 20 years ago (Michnick et al., 1997). The growing evidence about global climate warming and its impact on sugar content of grapes at harvest has contributed to an ever increasing interest on this topic. The biotechnological strategies initially explored were based on genetic engineering of *S. cerevisiae*, a rational choice considering the preponderant role of this species in both spontaneous and inoculated fermentation. However, the use of these recombinant strategies soon faced hurdles coming from both yeast metabolism and the regulatory framework for genetically modified organisms. The fact that further genetic modification was required in order to overcome initial problems did not help much.

In this context, the intense research activity around NS wine yeasts, our increasing awareness about the metabolic diversity of yeasts, and the arrival to the market of NS starters, opened new opportunities to exploit yeast metabolism with the aim of reducing ethanol content of wines. Current knowledge indicate that, similar to other metabolic traits, ethanol yield on sugar is not only species-specific, but often strain-specific. Some NS yeast species can show ethanol yields similar or higher than *S. cerevisiae*, but many of them show reduced ethanol yields. It has also been shown that oxygenation during wine fermentation can help further reduce ethanol yields by these, often Crabtree negative, NS yeast species.

Since most NS wine yeasts are sensitive to ethanol concentrations above 6–8%, in order to keep the microbiological control of the fermentation process, and to avoid stuck or sluggish fermentation (and wine spoilage), the use of *S. cerevisiae* starters, in either sequential or simultaneous inoculation, will still be required. The introduction of mixed starter inoculation to routine winemaking practices also demands for a better control of the fermentation parameters, adapted to each specific combination of yeast starters. Some parameters to take into account are sulphite concentration, temperature, pH adjustments, inoculation levels (and timing, for sequential inoculation), yeast nutrition, and eventually oxygenation levels and timing, among other parameters.

In order to perform knowledge based decisions in this field, further research will be required. Some of the topics that need to be addressed are common to other oenological applications of NS wine yeasts, while other are more specific for alcohol level reduction. Environmental factors influencing ethanol yield by different wine yeast species warrant a special attention, both for respiration-based and anaerobic fermentation strategies. As a general rule, research projects on NS wine yeasts should always pay attention to the production of unwanted metabolites, including acetic acid, which has been identified as a serious drawback, especially for respiration-based strategies or certain yeast strains. Reliable assessment of the impact of new yeast strains/species, and new oenological practices on quality related features of wines would require pilot scale experiments, use of natural grape must, and rigorous sensory analysis. One complex but very relevant aspect that is indeed already attracting attention by wine biotechnologists is physiological and ecological interactions between cells from the starter cultures, among them and with the natural microbiota. The few articles already published on this topic are just opening the window to a world of interactions, including competitions, metabolite exchanges, and production of narrow and wide spectrum antimicrobials. All these phenomena have a potential to impact alcohol level and overall quality of wines. Yeast inter and intraspecific diversity must always be taken into account both in the design of experiments and to draw general conclusions. Finally, the interaction of starter cultures with natural microbiota is a very relevant but complex topic, which might eventually benefit from the increasing availability of high throughput technologies, including metagenomic analysis.

AUTHOR CONTRIBUTIONS

MC, RG, and PM conceived the idea and outline of the review. All authors contributed to writing specific sections and approved the final version of the manuscript.

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Microbial Cell Factories

RESEARCH Open Access

Identification of target genes to control acetate yield during aerobic fermentation with *Saccharomyces cerevisiae*

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Abstract

Background: Aerobic fermentation of grape must, leading to respiro-fermentative metabolism of sugars, has been proposed as way of reducing alcohol content in wines. Two factors limit the usefulness of *Saccharomyces cerevisiae* for this application, the Crabtree effect, and excess volatile acidity under aerobic conditions. This work aimed to explore the impact on ethanol acetate production of different *S. cerevisiae* strains deleted for genes previously related with the Crabtree phenotype.

Results: Recombinant strains were constructed on a wine industrial genetic background, FX10. All yeast strains, including FX10, showed respiro-fermentative metabolism in natural grape must under aerobic conditions, as well as a concomitant reduction in ethanol yield. This indicates that the Crabtree effect is not a major constrain for reaching relevant respiration levels in grape must. Indeed, only minor differences in ethanol yield were observed between the original and some of the recombinant strains. In contrast, some yeast strains showed a relevant reduction of acetic acid production. This was identified as a positive feature for the feasibility of alcohol level reduction by respiration. Reduced acetic acid production was confirmed by a thorough analysis of these and some additional deletion strains (involving genes *HXK2*, *PYK1*, *REG1*, *PDE2* and *PDC1*). Some recombinant yeasts showed altered production of glycerol and pyruvate derived metabolites.

Conclusions: *REG1* and *PDC1* deletion strains showed a strong reduction of acetic acid yield in aerobic fermentations. Since *REG1* defective strains may be obtained by non-GMO approaches, these gene modifications show good promise to help reducing ethanol content in wines.

Keywords: Aerobic fermentation, Crabtree effect, Volatile acidity, Alcohol level reduction, Carbon catabolite derepression, Wine

Background

Rising sugar concentration of grape berries at harvest, as a consequence of global climate change [1], has become a matter of concern for winemakers, particularly those from viticultural regions located in warm countries. A second factor contributing to increasing sugar levels in grape must is the current consumer preferences for well-structured, full body wines, which require an optimal phenolic maturity of grapes. Under standard winemaking conditions, excess sugar in grape must, together with other changes in must composition, also related to global climate warming, translates into fermentation troubles and, more significantly, into high alcohol content in the final wines. High ethanol content in wines can compromise product quality by exacerbating the perception of some mouth feel features such as hotness and viscosity. Sweetness, acidity, aroma, flavor intensity, and textural

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properties can also be negatively impacted, to a lesser extent [2–4]. In addition, wines with a high alcoholic degree might be rejected by some consumers on the ground of health or road safety considerations. International trade of such wines might also be hampered by significant increases in taxes, depending on the countries involved.

Currently, there is not a single approach that would completely solve this issue. Therefore, the wine industry is seeking for complementary solutions targeting different steps of the production cycle, including grapevine clonal selection, vineyard management, removal of excess sugar, adaptation of winemaking practices, using metabolic inhibitors, or partial ethanol removal [5]. Concerning the fermentation step in winemaking, most of the efforts are currently focused on the use of non-*Saccharomyces* wine yeast species/strains showing lower ethanol yields than *Saccharomyces cerevisiae* [6, 7]. Some years ago, our research group proposed taking advantage of the respiratory metabolism of yeasts as an approach for reducing ethanol yield [8]. Several yeast strains were shown to be able to reduce ethanol yields during aerated fermentation, as compared to standard conditions [9]; but many of them, including *S. cerevisiae* strains, were discarded for this application, due to a significant increase in acetic acid production under aerobic conditions [9, 10]. Nevertheless, the proposed technology requires *S. cerevisiae* to be inoculated either simultaneously or subsequently to non-*Saccharomyces* starters, in order to ensure fermentation completion. In addition, *S. cerevisiae* will be almost invariably present in the natural microbiota of grape must. Therefore, volatile acidity due to the metabolic activity of *S. cerevisiae* would always remain a matter of concern for the fermentation of wine under aerated conditions, as shown in studies using a combination of *Metschnikowia pulcherrima* and *S. cerevisiae* [11].

This work started with the aim of constructing *S. cerevisiae* wine yeast strains showing an alleviated Crabtree effect, as a way to improve ethanol content reduction by respiration. This metabolic trait makes *S. cerevisiae* preferentially consume sugars by fermentation, independently of oxygen availability, and seems to be regulated at various levels, from transcriptional repression of respiratory functions [12], to kinetic features of enzymes involved in pyruvate metabolism in this species [13, 14]. Some mutations have been related to alleviated Crabtree effect in *S. cerevisiae*, including loss-of-function of *REG1* [15] or *HXK2* [16–19]; as well as reduced pyruvate kinase (Pyk1) levels [20]. *REG1* and *HXK2* are involved in carbon catabolite repression (CCR) in this species (Fig. 1). Reg1 is the regulatory subunit of the Glc7-Reg1 protein phosphatase complex, targeting it to several CCR related substrates, including Snf1 or Mig1 [21]. Hxk2 is a moonlighting protein. In addition to its hexokinase activity (it is the main cytoplasmic hexokinase during yeast growth on glucose), it participates in transcriptional repression in the nucleus, together with Mig1 [21]. Nucleocytoplasmic localization of both Hxk2 and Mig1 depends on its phosphorylation state [22]. The impact of lowered Pyk1 activity on the Crabtree effect is probably related to the rate of pyruvate accumulation [13, 14, 20] (Additional file 1: Figure S1).

In this work we found reduced ethanol yields in *S. cerevisiae* during the fermentation of natural or synthetic grape must under aerobic conditions, despite the Crabtree effect. This confirmed recent results from parallel research lines [9–11]. In contrast, we found an unexpected positive impact of some genetic modifications on volatile acidity (i.e. acetic acid), the main drawback of *S. cerevisiae* for this application [9–11].

Results

Selection of target genes for alcohol level reduction by respiration

We analyzed the metabolic impact of the deletion of five target genes in a *S. cerevisiae* industrial wine yeast background (Table 1). The aim was the identification of yeast genetic modification strategies that would improve alcohol level reduction during wine fermentation under aerobic conditions. Given that the Crabtree effect is the major metabolic feature of *S. cerevisiae* restricting respiratory metabolism, three of these genes were selected according to published information about the impact of gene deletions on respiro-fermentative metabolism. Herwig and von Stockar [15] found that mutant yeast strains defective for either *HXK2* or *REG1* alleviated repression of respirative functions by external glucose. Several other authors have reported reduced formation of fermentation products, as well as higher biomass yield, by yeast strains carrying inactive alleles of *HXK2* [16–19]. On the other side, Pearce et al. [20] described recombinant yeast strains with reduced pyruvate kinase (Pyk1) levels, which showed increased relative flux trough the TCA pathway.

FX10 based recombinant strains defective for each one of these three genes were constructed as described in Methods. For *HXK2* or *REG1* both alleles were deleted (Table 1). However, *PYK1* being an essential gene in *S. cerevisiae*, only one of the two alleles was deleted, in order to reduce gene dosage (Table 1). According to the functions previously described for these genes, we found that strains FREG1 and FHXK2 were defective for carbon catabolite repression, while FPYK1 was normally repressed, as expected (Fig. 2). However, preliminary analysis of ethanol yields during the fermentation of natural grape must under moderate aeration, failed

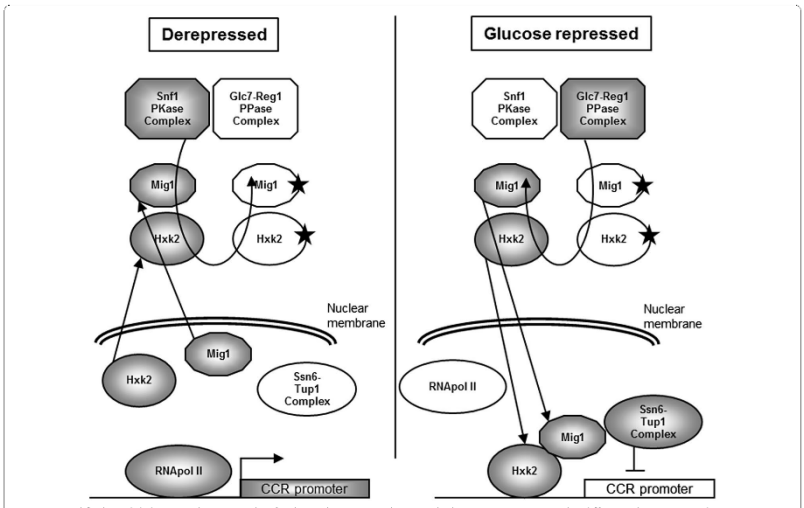

Fig. 1 Simplified model showing the main role of Hxk2 and Reg1 in carbon catabolite repression (CCR). The different elements are shown in grey for the active state and empty for the inactive state. Stars on Mig1 and Hxk2 indicate a phosphorylated state. Several elements shown in the model have additional functions (either in CCR or not), and not all the factors involved in CCR are shown. Both Hxk2 and Reg1 must be active for efficient repression of many genes under CCR control Model based on [21], with additional information from [22]

Table 1 Yeast strains used for this study

Strains	Genotype	Source
FX10	Homozygote industrial yeast derivative	Laffort
FHXK2	FX10 hxk2::kanMX4/hxk2::kanMX4	This study
FPYK1	FX10 PYK1/pyk1::kanMX4	This study
FREG1	FX10 reg1::kanMX4/reg1::kanMX4	This study
FPDE2	FX10 pde2::kanMX4/pde2::kanMX4	This study
FPDC1	FX10 pdc1::kanMX4/pdc1::kanMX4	This study

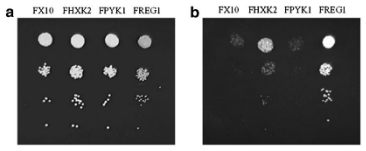

Fig. 2 Growth of S. cerevisiae FX10 and different recombinant derivatives on YPgalactose. **a** Without 2-deoxy glucose, **b** supplemented with 2-deoxy glucose

to identify a relevant impact of these gene deletions on respiro-fermentative metabolism. Interestingly, some of the recombinant strains showed reduced acetic acid production as compared to FX10 (data not shown).

In order to identify additional potential targets for genetic improvement of acrobic metabolism, with the aim of reducing alcohol levels, we performed a competition experiment of the homozygous deletion collection. The continuous cultivation described by Herwig and von Stockar [15] was modified by running in synthetic medium ($D = 0.18\ h^{-1}$), with 10 g/L glucose and full acration for 12 generations. Results of the competition experiment were analyzed as described [23]. Five gene deletions, showing improved growth according to this study, were tested for the ethanol and acetate yields during the fermentation of grape must under aerobic conditions. While none of the strains showed reduced ethanol yield, the strain deleted for *PDE2* showed a clear reduction in acetic acid yield.

We could draw three main observations from our preliminary analysis. First, despite the Crabtree effect, the wine yeast strain FX10 was able to respire a significant

amount of sugar under aerobic conditions. Second, as previously shown [9–11, 24–26], increased acetic acid production under aerobic conditions is a chief limitation in order to use *S. cerevisiae* for alcohol level reduction. Finally, the main advantage observed for some of the recombinant strains tested above was, indeed, reduced acetic acid yields under aerobic conditions. For this reason, we decided to focus on the reduction of aerobic acetic acid yields as the main target for wine yeast improvement. This would allow alcohol level reduction without the drawback of excess volatile acidity. According to this new focus, we included an additional gene in the study, *PDC1* (Additional file 1: Figure S1) Pyruvate decarboxylase (encoded by *PDC1*) is a key enzyme in alcoholic fermentation, catalyzing the decarboxylation of pyruvate to acetaldehyde, as an intermediary step towards ethanol production [27]. Acetaldehyde can also be oxidized to acetic acid, and several authors have found decreased acetate production by Pdc1 defective yeast strains [28]. Hence, *PDE2* and *PDC1* deletions were introduced in the FX10 genetic background, in order to perform a comparative characterization (Table 1).

Main fermentation products

Characterization of the five recombinant strains mentioned above was performed in natural white grape must under aerobic conditions (as well as under anaerobic conditions for comparison purposes). Only one of the gene deletions assayed resulted in a severe impairment of yeast growth, *PDE2*. Final biomass values for FPDE2 were about one-half those of the control strains, for anaerobic or aerobic conditions respectively (Table 2). The higher biomass production observed for all the strains under aerobic conditions (Table 2) is in agreement with a significant portion of sugar being consumed by respiratory metabolism. Concerning residual sugar, data for FREG1 indicate *REG1* deletion is detrimental for yeast metabolism; despite no impact on cell numbers was observed. FREG1 was the only strain leaving some residual sugar after seven days of culture, under either aerobic (38 g/L) or anaerobic (22 g/L) conditions (Table 2). This was in agreement with results from chemostat cultures mentioned above. Residual sugar was almost exclusively constituted by fructose (Additional file 2: Figure S2). Indeed, FREG1was the only strain showing an altered preference for the two monosaccharides present in grape must (Additional file 2: Figure S2).

Comparison of ethanol yields on sugar between anaerobic and aerobic conditions (Table 3) confirmed that, despite the Crabtree effect, even wild type strains of *S. cerevisiae* showed a great deal of respiratory metabolism in grape must under aerobic conditions. FREG1 and FPDE2 show the extreme values (0.21 and 0.27 g/g, respectively) of ethanol yield under aerobic conditions; while the ethanol yield for FREG1 was also the lowest one (0.31 g/g) under anaerobic conditions. However, most of the recombinant strains showed ethanol yield values that were indistinguishable from the control FX10 strain, not only under anaerobic conditions (where no respiration can take place) but also under aerobic conditions.

Acetic acid production under anaerobic conditions by some of the strains assayed is suitable for the production of quality wines (Table 2), considering that, above 0.8 g/L, acetic acid may confer an undesirable acidic taste and unpleasant vinegar aroma to wine [29]. FPDE2 showed the lowest acetic acid yield under anaerobic conditions (Table 3). On the other side, the trend towards increased acetic acid production under aerobic conditions that was previously described [9, 11, 25] was confirmed for this set of yeast strains. Indeed, acetic acid production under aerobic conditions was unacceptably high for most of the strains, apart from FREG1 and FPDC1 (Table 2). Actually, FREG1 seems to be an exception to the general rule of increasing acetic acid yield under aerobiosis. While the other yeast strains experienced an increase in acetic acid yield, ranging from 8 to 40 times under aerobic as compared to anaerobic conditions, acetic acid yield for FREG1 was similar under both culture conditions.

Quantitative differences in glycerol yields in anaerobiosis were relatively small (as compared for example with differences in acetic acid yields), even though FREG1 and FPDE2 showed higher (statistically significant) glycerol yields than the control strain (Table 3). Aeration resulted in a reduction of about one half in glycerol yield for most of the strains. However, FREG1 showed the opposite behavior, with twice as much glycerol yield under aerobic conditions as compared to anaerobiosis (Table 3). The final glycerol content of aerobic FREG1 fermentation is indeed in the upper part of the normal range accepted for quality wines (12–18 mg/L; Table 2).

Other pyruvate derived metabolites

In view of the striking differences in acetic acid yields shown by the recombinant strains (Table 3), we wondered whether other metabolic by-products from the pyruvate node (acetaldehyde, acetoin and 2,3 butanediol) were also affected by the gene deletions tested. No statistically significant differences were found for the yeast strains concerning acetaldehyde production under anaerobic conditions (Table 4). The general trend for acetaldehyde levels was towards higher values in aerobiosis, with the exception of FREG1. Deletion of *PDC1* results in a huge increase in acetaldehyde production under aerobic conditions (Table 4).

Table 2 Main metabolites in fermentations run by the parent FX10 strain and recombinant derivatives

	[1]Biomass (DO_{600} nm)		[2]Residual sugars (g/L)		[2]Ethanol (% vol/vol)		[2]Acetic acid (g/L)		[2]Glycerol (g/L)	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
FX10	13.93 ± 1.93^{b}	20.93 ± 1.53^{bc}	0.10 ± 0.17^{a}	0.03 ± 0.06^{a}	8.70 ± 0.24^{c}	5.63 ± 0.35^{b}	0.14 ± 0.03^{bc}	3.34 ± 0.48^{cd}	10.28 ± 0.24^{a}	5.80 ± 0.20^{a}
FHXK2	14.53 ± 1.52^{b}	22.70 ± 2.35^{bc}	0.10 ± 0.17^{a}	0.03 ± 0.06^{a}	8.71 ± 0.34^{c}	5.59 ± 1.01^{b}	0.16 ± 0.08^{c}	3.57 ± 0.38^{d}	11.00 ± 0.17^{ab}	5.98 ± 0.03^{a}
FPYK1	10.63 ± 1.67^{ab}	24.63 ± 1.38^{c}	0.17 ± 0.21^{a}	0.06 ± 0.06^{a}	8.51 ± 0.09^{c}	5.79 ± 0.21^{b}	0.11 ± 0.03^{abc}	4.57 ± 0.89^{d}	10.52 ± 0.03^{ab}	6.10 ± 0.20^{a}
FREG1	12.30 ± 0.70^{ab}	24.72 ± 2.02^{c}	22.50 ± 5.11^{b}	38.00 ± 2.17^{b}	6.70 ± 0.40^{a}	4.03 ± 0.34^{a}	0.15 ± 0.04^{c}	0.15 ± 0.02^{a}	10.73 ± 1.47^{ab}	17.57 ± 0.32^{b}
FPDC1	11.71 ± 2.78^{ab}	18.75 ± 2.19^{b}	ND	ND	8.19 ± 0.26^{bc}	5.64 ± 0.37^{b}	0.06 ± 0.04^{ab}	0.73 ± 0.47^{ab}	9.85 ± 0.06^{a}	5.55 ± 0.44^{a}
FPDE2	7.53 ± 2.05^{a}	13.13 ± 1.95^{a}	0.07 ± 0.12^{a}	ND	7.59 ± 0.25^{b}	6.49 ± 0.71^{b}	0.04 ± 0.03^{a}	1.91 ± 0.76^{bc}	12.43 ± 0.91^{b}	6.43 ± 0.67^{a}

Values are shown as mean ± SD of three biological replicates. The glucose and fructose contents of the natural must in the fermentations were ranged in 190.4–195.8 g/L. Different letters indicate statistically significant differences (HSD Tukey) for values in the same column

ND not detectable

[1] Analyses were performed after 4 days of fermentation

[2] Analyses were performed after 7 days of fermentation

Table 3 Yields of ethanol, acetic acid and glycerol calculated for the parent FX10 strain and recombinant derivatives

	$Y_{E/S}$ (g/g)		$Y_{A/S}$ (mg/g)		$Y_{G/S}$ (mg/g)	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
FX10	0.36 ± 0.01^{b}	0.23 ± 0.01^{ab}	0.76 ± 0.16^{bc}	17.56 ± 2.30^{cd}	54.04 ± 1.21^{a}	30.47 ± 1.04^{a}
FHXK2	0.36 ± 0.01^{b}	0.23 ± 0.01^{ab}	0.86 ± 0.04^{bc}	18.62 ± 2.22^{d}	57.27 ± 1.53^{ab}	31.15 ± 0.64^{a}
FPYK1	0.35 ± 0.01^{b}	0.24 ± 0.01^{ab}	0.59 ± 0.16^{abc}	23.84 ± 4.98^{d}	54.78 ± 2.16^{a}	31.76 ± 1.51^{a}
FREG1	0.31 ± 0.01^{a}	0.21 ± 0.01^{a}	0.94 ± 0.28^{c}	1.01 ± 0.17^{a}	63.74 ± 4.26^{bc}	115.29 ± 3.42^{b}
FPDC1	0.36 ± 0.02^{b}	0.24 ± 0.01^{ab}	0.35 ± 0.23^{ab}	3.91 ± 2.44^{ab}	54.27 ± 3.52^{a}	29.93 ± 1.89^{a}
FPDE2	0.34 ± 0.02^{b}	0.27 ± 0.01^{b}	0.22 ± 0.20^{a}	10.04 ± 4.02^{bc}	71.21 ± 2.36^{c}	33.79 ± 3.50^{a}

Values are shown as mean ± SD of three biological replicates. $Y_{E/S}$ ethanol yield on sugar, $Y_{A/S}$ acetic acid yield on sugar, $Y_{G/S}$ glycerol yield on sugar. Different letters indicate statistically significant differences (HSD Tukey) for values in the same column

Table 4 Volatile metabolites produced by the parent FX10 strain and recombinant derivatives

	Acetaldehyde		Acetoin		2,3 butanediol	
	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
FX10	0.094 ± 0.042^{a}	0.463 ± 0.132^{a}	0.006 ± 0.002^{a}	35.017 ± 2.381^{d}	0.518 ± 0.324^{a}	6.287 ± 0.210^{d}
FHXK2	0.144 ± 0.026^{a}	0.553 ± 0.054^{a}	0.007 ± 0.000^{a}	48.212 ± 2.214^{e}	0.482 ± 0.054^{a}	7.380 ± 0.289^{e}
FPYK1	0.145 ± 0.019^{a}	0.409 ± 0.175^{a}	0.009 ± 0.001^{a}	26.628 ± 0.275^{c}	0.599 ± 0.041^{a}	7.100 ± 0.170^{e}
FREG1	0.176 ± 0.068^{a}	0.129 ± 0.029^{a}	0.154 ± 0.021^{b}	10.594 ± 2.920^{a}	0.973 ± 0.119^{a}	1.273 ± 0.178^{a}
FPDC1	0.130 ± 0.034^{a}	1.875 ± 0.478^{b}	0.005 ± 0.001^{a}	15.557 ± 1.273^{b}	0.391 ± 0.069^{a}	2.244 ± 0.173^{b}
FPDE2	0.125 ± 0.039^{a}	0.389 ± 0.055^{a}	0.020 ± 0.005^{a}	31.113 ± 1.961^{cd}	1.943 ± 0.549^{b}	4.570 ± 0.500^{c}

Values are shown as mean of relative abundance on the internal standard ± SD of at least two biological replicates, after 7 days of fermentation. Different letters indicate statistically significant differences (HSD Tukey) for values in the same column

The relative similarity in acetaldehyde production shown by the yeast strains under anaerobic conditions does not translate into a similar uniformity concerning acetaldehyde derived products. Anaerobic cultures of FREG1 and FPDE2 showed a clear increase in acetoin (25-fold or four-fold respectively) and 2,3 butanediol (two-fold increase and four-fold) levels, as compared to FX10 (Table 4). Following the trend seen for acetaldehyde, all the strains produced clearly increased amounts of acetoin or 2,3 butanediol under aerobic conditions. Again, the behavior of strains deleted for *REG1* or *PDC1* was clearly different from other yeast strains. FREG1 and FPDC1 showed the lowest values of both acetoin and 2,3 butanediol in aerobiosis (Table 4). In addition, the difference between aerobic and anaerobic conditions observed for FREG1, concerning the content of these two compounds was the smallest among all the strains analyzed.

Finally, we performed a principal component analysis by taking into account ethanol, acetic acid, and glycerol, as well as acetaldehyde, acetoin, and 2,3 butanediol. The results confirm the important metabolic differences between aerobic and anaerobic cultures (Fig. 3). Samples were clearly separated along the PC1 axis, depending on the oxygenation conditions, with anaerobic samples showing lower production of acetoin, 2,3 butanediol, and acetic acid; and high production of ethanol and glycerol, as already seen in Tables 3 and 4. However, two strains, FREG1 and FPDC1 showed, under aerobic conditions,

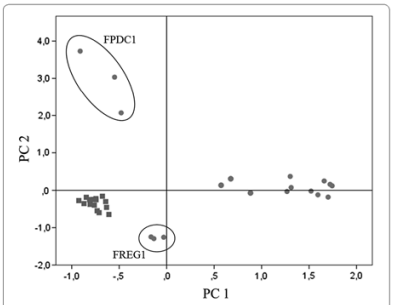

Fig. 3 Principal component analysis based on the yields of main fermentation products and pyruvate derived metabolites. The PC1 and PC2 explained 64.89 and 81.97 % respectively of the total yeast strains variance under aerobic (*green dots*) and anaerobic (*red squares*) conditions

a behavior reminiscent of anaerobic cultures. This was clear for acetic acid yield and 2,3 butanediol production in the case of FREG1, and also evident for FPDC1 concerning acetic acid yield, as well as acetoin and 2,3 butanediol production. Aerobic samples of FPDC1 were clearly separated from the rest of the samples along the PC2 axis, mostly due to the high acetaldehyde production of this strain in the presence of oxygen. The high production of glycerol by FREG1 under aerobic conditions explains the low position of these samples along the PC2 axis.

Discussion

Despite the Crabtree effect, there was a huge impact of oxygen availability on yeast metabolism for all the strains assayed in this work. Indeed, reduced ethanol yield in the aerobic fermentation of natural or synthetic grape must was already described for *S. cerevisiae* in our previous work [9, 11]. This indicates a relevant portion of the sugar was metabolized by respiration under aerobic conditions, a conclusion that is also sustained by an important increase in biomass yield in aerobic cultures. For most of the yeast strains studied, production of other metabolites was also highly affected by culture under aerobic conditions, as compared to anaerobiosis, including acetic acid and glycerol, also in agreement with previous publications [10, 25]; as well as other pyruvate derived metabolites, like acetaldehyde, acetoin or 2,3 butanediol.

A clear alleviation of the Crabtree effect was previously described for loss-of-function mutations in several of the genes studied in this work, including *HXK2*, *PYK1* and *REG1* [15, 20]. Surprisingly, we found little or no impact of these gene deletions on ethanol or biomass yield in the FX10 genetic background under our experimental conditions. Claims on the relief of the Crabtree effect for these gene deletions are mostly based on chemostat cultures under carbon limited conditions. In contrast, natural grape must contains limiting amounts of yeast assimilable nitrogen, while carbon sources (glucose and fructose) are in great excess. Our results illustrate the impact of the cultivation mode in order to assess yeast metabolic features. In this way, genetic modification resulting in an important change in the critical dilution rate under chemostat growing conditions might appear as almost irrelevant for cultures in batch, especially for growth in high sugar content media. Several authors postulate that the Crabtree effect in *S. cerevisiae* is mostly a manifestation of an overflow metabolism at the level of pyruvate [13, 14]. Indeed, a total relief of the Crabtree effect in this species has only been attained by an almost complete impairment of glucose intake by the cells [30]. According to this model, some gene deletions affecting the glycolytic rate (i.e. the rate of production of pyruvate), or the capacities of enzymes involved in further pyruvate metabolism, might have a clear impact on the critical dilution rate [15, 20]. However, the extreme overflow we can expect for batch cultures with around 200 g/L initial sugar content might be almost insensitive to the same gene modifications. This is exactly what we observed for some of the gene modifications initially selected in this work.

Despite the low impact of the assayed gene modifications on ethanol yields under aerobic conditions, a clear reduction in ethanol levels, as compared to anaerobic conditions, was observed for all strains. One of the problems associated with aeration during wine fermentation is increased acetic acid production, as shown in Table 2 and as already observed in previous works [9–11]. Our preliminary results showed that, despite not being intended for that purpose, some gene modifications seemed to result in clearly reduced volatile acidity. The practical implications of this finding prompted us to include some additional genes in the study.

According to results in other genetic backgrounds [15, 18, 20], deletion of *PYK1* (hemizygous) or *HXK2* in FX10 results in glucose derepression. Other authors have described low ethanol yield in aerobic batch cultures of strains deleted for *HXK2*, or showing reduced levels of pyruvate kinase activity [16, 20]. However, the behavior of FHXK2, FPYK1 and FX10 strains in this work was almost identical, only minor (although statistically significant) differences in metabolic footprint were found for acetoin and 2,3 butanediol production, and only for aerobic cultures. The low impact of these gene deletions during the fermentation of natural grape must is thus in contrast with the results by Diderich et al. [16] and Pierce et al. [20]. There are at least two non-exclusive explanations to this discrepancy. One is based in media composition. Initial glucose content in the batch cultures by the later authors ranged from 10 to 20 g/L, while our natural grape must contain about 200 g/L (equimolar amounts of glucose and fructose). In addition, yeast assimilable nitrogen level in grape must is low, so that most of the sugar is metabolized under nitrogen limitation. This is in contrast with synthetic media for which nitrogen sources were in excess. A second explanation in the case of *PYK1* deletion is FPYK1 was hemizygous for that deletion (*PYK1* is an essential yeast gene). The maximal reduction in pyruvate kinase activity we would expect from this construction is 50 %. In contrast, Pierce et al. [20] used a construction resulting in a reduction of pyruvate kinase levels down to 20–25 % of normal values. The high similarity of FX10, FHXK2 and FPYK1 under our experimental conditions, either aerobic or anaerobic, despite the different behavior shown under chemostat conditions [15, 20] is illustrative of the lack of predictive power of standard Crabtree assays for certain industrially relevant conditions, as discussed above.

Strain FPDE2 shows the lowest biomass production under both aerobic and anaerobic growth conditions, despite it is able to reach complete fermentation of grape must with a kinetics similar to FX10 (Fig. 4). Since the cyclic AMP phosphodiesterase encoded by *PDE2* is involved in reducing cAMP levels, and despite it can be partially substituted by Pde1p [31], we would expect cell functions regulated by PKA to be altered FPDE2 [32]. Two opposite effects on biomass production would be expected. By one side, increased PKA activity would involve activation of glycolysis, growth, and proliferation. By the other side, stress response would be reduced, resulting in low tolerance to the harsh conditions in grape must, and notably osmotic stress. Our results indicate the later effect would be dominant and result in the low biomass of FPDE2 cultures under both aerobic and anaerobic conditions. The expected positive impact of the deletion of *PDE2* on glycolysis rate might exacerbate overflow metabolism and be responsible for the fact that FPDE2 appears in the upper part of the distribution of ethanol yield under aerobic conditions, being the strain showing the minor reduction in ethanol yield from anaerobic to aerobic fermentation conditions. In addition to ethanol, this gene deletion has a limited impact on the yields of acetic acid, glycerol and 2,3 butanediol in anaerobiosis; as well as on acetic acid yield in aerobic fermentations. In practical terms, the reduction of acetic acid yield under aerobic conditions by FPDE2 might have an advantage over FX10, in order to attain alcohol level reduction by aeration of the fermenting must. However, other mutant strains assayed in this work seem to be more interesting for the development of industrial strains (see below).

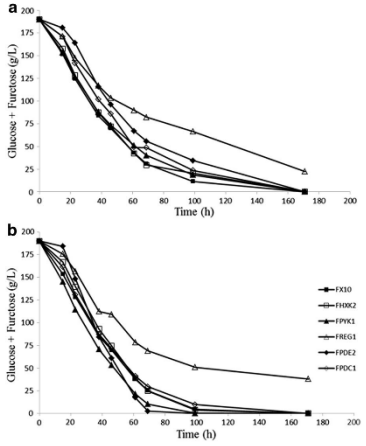

Fig. 4 Sugar consumption kinetics during anaerobic (**a**) and aerobic (**b**) fermentations of the strains indicated. Results are the average of biological triplicates Please, refer to Additional file 2: Figure S2 for SD (removed in this graph for clarity)

PDC1 codes for the main pyruvate decarboxylase isozyme in *S. cerevisiae*. Deletion of this gene in the FX10 background resulted in little impact under anaerobic conditions, but it had relevant consequences for aerobic fermentation. Under these conditions, the ethanol yield of FDC1 was similar to the control strain, but it showed a clear reduction in acetic acid yield, as well as acetoin and 2,3 butanediol. In contrast, a relevant increase in acetaldehyde production was observed. Although the main activity of Pdc1 is the conversion of pyruvate to acetaldehyde, acetoin has been described as one main side product of the reaction. Acetoin can in turn be transformed to 2,3 butanediol. It has been shown that Pdc5, the other major pyruvate decarboxylase isozyme, is able to warrant about 70 % of the pyruvate decarboxylase activity required by the cell in *Δpdc1* strains [33]. However, our results suggest differences in acetoin production between Pdc1 and Pdc5, resulting in acetaldehyde accumulation at the expense of acetoin and 2,3 butanediol. Perhaps the most interesting feature of FPDC1 concerning its application in wine making is the low acetic acid yield in aerobic fermentation, resulting in volatile acidity values around the 0.8 g/L threshold.

Deletion of *REG1* is probably the most pleiotropic gene modification among those assayed in this work. This was to be expected, given the upstream position of Reg1 in the glucose sensing signal transduction pathway (Fig. 1). In addition, the substrate of the GLC7-Reg1 protein phosphatase complex is Snf1, and this protein kinase is involved in the regulation of many cellular functions. Concerning anaerobic conditions, FREG1 was almost the only strain showing statistically significant differences with the control strain for ethanol yield or acetoin production. However, the most interesting impact of this gene modification was observed for aerobic cultures. By one side, it showed the lower ethanol yield values among the yeast strains used in this work. More interesting is the fact that this strain also showed the lower values for acetic acid yield under aerobic conditions, as well as for acetaldehyde, acetoin or 2,3 butanediol. In contrast to all the other yeast strains, almost no difference in acetic acid yield was observed for this strain between aerobic and anaerobic fermentations, and those values were

similar to anaerobic cultures of FX10. Also relevant is the increase in the aerobic glycerol yield for this strain as compared to anaerobic growth (about two-times). This was indeed opposite to the other strains, showing a two-fold decrease in glycerol yield for the same growth conditions. This result seems to be in contrast with reports showing an increase in the production of acetic acid for genetic modifications aiming to glycerol overproduction [34]. However, these reports are based on growth under standard fermentation conditions while glycerol overproduction by FREG1 without an increase in acetic acid production takes place under aerobic fermentation conditions. Obviously, the redox compensation mechanisms involved in linking acetic acid and glycerol metabolism under anaerobic conditions are not operating the same way in the presence of oxygen. An interesting feature of *REG1* loss-of-function yeast mutants, is they can be easily obtained and selected by random mutagenesis [35, 36]. This opens the way for obtaining non-GMO wine yeast strains similar to FREG1, which would be readily available for winemakers, avoiding the limitations associated to recombinant wine yeasts [37]. The hurdle imposed by the recessive character of *REG1* defective mutants can be overcome by sporulating yeast strains before or after random mutagenesis. The use of homozygous wine yeast strains, like FX10, is advisable for this approach.

Conclusions

We report the metabolic characterization of five gene deletions in a commercial *S. cerevisiae* yeast background during anaerobic and aerobic fermentation of natural grape must. Oxygenation of grape must is sufficient to warrant a relevant reduction in final ethanol content. The impact on ethanol yields of the gene deletions assayed in this work was negligible, both under aerobic and anaerobic fermentation conditions. However, some of these deletions did contribute to solve the main drawback of aerobic fermentation concerning winemaking, excess acetic acid production (resulting in high volatile acidity). The most promising results from this point of view were shown by the carbon catabolite derepressed strain FREG1, deleted for *REG1*. Similar strains would be easily obtained by classical genetic techniques. Such strains, in combination with a wild type strain, would be useful for the commercial production of wines with reduced ethanol content.

Methods

Strains and growth conditions

Saccharomyces cerevisiae Zymaflore® FX10 (Laffort), a homozygous and homothallic commercial wine yeast strain was used as host strain for genetic modification. The heterozygous mutants were constructed using the short flanking homology method [38], by transforming FX10 using the lithium acetate procedure [39] with a PCR fragment obtained by amplification of the kanMX4 cassette and flanking regions from the appropriate homozygous deletion strain in the BY4743 background (Open Biosystems, Huntsville, USA). Selection of heterozygous mutants was performed in YPD solid media plates supplemented with 200 mg/L of geneticin (G418). Correct insertion of the kanMX4 cassette was verified by PCR using primers upstream and downstream of the deleted region combined with primers inside kanMX4. Primers used for the construction and verification of recombinant strains are shown in Additional file 3: Table S1. Amplification strategies are summarized in Additional file 4: Figure S3. The homozygous mutants were constructed by sporulating the heterozygous mutants in a medium supplemented with geneticin. The geneticin resistance feature segregated 2:2 as expected. Since the original strain was homothallic, strains recovered from the segregation analysis plates were spontaneous autodiploids, homozygous for the corresponding gene deletion, as verified by PCR (see above).

Yeast strains were grown at 28 °C and maintained at 4 °C on yeast peptone dextrose (YPD) plates (2 % glucose, 2 % peptone, 1 % yeast extract, and 2 % agar), as well as in glycerol stocks at −80 °C. Fermentation experiments were performed in natural grape must (ca. 200 g/L sugars).

Fermentation assays

Batch fermentation were carried out in triplicate in bioreactors equipped with refrigerated gas condensers (Dasgip, Eppendorf, Germany). Bioreactors were filled with 250 mL of natural must and sparged at a gas flow rate of 2.5 L/h with either air or nitrogen. Gas flow was controlled with MFC17 mass flow controller (Aalborg Instruments and Controls, Inc., Orangeburg, NY), whose calibration was regularly verified with automatic flowmeters. Temperature was set to 28 °C, stirring to 200 rpm and inoculation to approximately 0.2 initial optical density at 600 nm (OD_{600}). pH was continuously adjusted to 3.5 during fermentation progress by the automatic addition of 2 M NaOH.

2-Deoxy glucose sensitivity assay

Yeast strains were spotted at different dilutions (10^{-1} to 10^{-4}) on YP plates (10 g/L yeast extract and 20 g/L Bacto peptone) that contained 2 % of galactose as carbon source, supplemented with 200 µg/mL of 2-deoxy glucose. Plates were incubated for 48 h at 28 °C.

Quantification of main fermentation-related metabolites

Production or consumption of glucose, fructose, glycerol, acetic acid and ethanol, were determined by HPLC in duplicate, using a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively). A Hyper REZ XP carbohydrate H+ 8 µm column and guard (Thermo Fisher Scientific) were used and maintained at 50 °C. Elution was performed with 1.5 mM H_2SO_4 as mobile phase, at a flow rate of 0.6 mL/min. Prior to injection, samples were filtered through 0.22-µm-pore-size nylon filters and diluted 10-fold.

Analysis of volatile compounds

500 µL of sample were placed in a 2 mL glass-vial with 1 mL of ammonium sulphate solution (45 % w/v) and extracted with 250 µL of methyl acetate-ethanol solution (99.5:0.5, v/v) containing 50 ppm of internal standards (4-methyl 2-pentanol, 1-nonanol, and heptanoic acid). A 3 µL sample of the upper, methyl acetate phase, was injected with the SSL liner held at 180 °C.

Gas chromatography–mass spectrometry was carried out in a Thermo TRACE GC Ultra apparatus equipped with a Thermo TriPlus autosampler with a fused-silica capillary column TG-WAXMS A (30 m long; 0.25 mm OD; 0.25 µm film thickness) coupled to a Thermo ISQ mass detector.

Chromatographic conditions were as follows: 5 min at 40 °C, 3 °C/min up to 200 °C, 15 °C/min up to 240 °C, and 10 min at 240 °C. Helium was used as carrier gas at a flow rate of 1 mL/min, operating in split mode (ratio 30). Detection was performed with the mass spectrometer operating in the Full Scan mode (dwell time 500 ms), with 70 eV ionization energy, and source and quadrupole temperatures of 250 °C. Peaks were identified by comparison of retention times and ion spectra from real standards (Sigma-Aldrich Química) and spectra from the NIST mass spectral library. For each compound, including internal standards, the sum of the areas of the peaks of up to five characteristic ions was obtained.

Statistical analysis

One way analysis of variance was carried out on the main fermentation metabolites found on day 7. Average of biological triplicates was compared using Tukey's test, with significance level set at 5 %. All analyses were performed using SPSS Statistics v. 20 program (IBM, Armonk, NY).

Additional files

Additional file 1: Figure S1. Schematic representation of the pyruvate node in *S. cerevisiae*. Background arrows indicate carbon flux distribution under excess sugar, aerobic conditions for Crabtree-positive yeasts (overflow metabolism at the pyruvate node level). Some of the genes deleted in this work (underlined) are shown; as well as genes coding for cognate isoenzymes. *PEP* phosphoenolpyruvate.

Additional file 2: Figure S2. Kinetics of glucose and fructose consumption under aerobic (A) or anaerobic (B) conditions. Results are the average of biological triplicates. Error bars correspond to ±SD from three biological replicates.

Additional file 3: Table S1. Primers used in this study.

Additional file 4: Figure S3. Summary of all PCR reactions performed in this work. In black, ORFs to be replaced in FX10. They were replaced by the kanMX4 cassette (shown in the bottom of the figure) by amplifying the whole region from the appropriate homozygous deletion strain in the BY4743 background, and using it to transform FX10. Primers with the "KO" label (see figure) were used for this purpose. Correct insertion was verified by using primers with the "OUT" label, in single and double FX10 deletion strains. In addition, in order to avoid ambiguities, additional confirmation PCR reactions were run by using one of the "OUT" primers from each pair and the "kanMX4 IN R" primer. In this case, additional control PCR reactions were run with the "IN" labelled primers (see figure). All PCR verification reactions were run in parallel with genomic DNA from the parent and the putative recombinant strain.

Authors' contributions

PM, ZS and RG conceived the study. ZS and JAC (equally) as well as JT, PM, AJR and MQ performed different parts of the experimental section like fermentation experiments, strain construction, or instrumental analysis. JAC and RG wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Recombinant strains described in this work are made available upon request to the corresponding author. Receivers must adhere to applicable regulations concerning the use of GMOs. Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Hypoxia and iron requirements are the main drivers in transcriptional adaptation of *Kluyveromyces lactis* during wine aerobic fermentation

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ABSTRACT

The respiratory metabolism of yeast species alternative to *Saccharomyces cerevisiae* has been explored in recent years as a tool to reduce ethanol content in grape wine. The efficacy of this strategy has been previously proven for mixed cultures of non-*Saccharomyces* and *S. cerevisiae* strains. In this work, we perform a transcriptomic analysis of the Crabtree-negative yeast *Kluyveromyces lactis* under tightly controlled growth conditions in order to better understand physiology of non-*Saccharomyces* yeasts during the fermentation of grape must under aerated conditions. Transcriptional changes in *K. lactis* are mainly driven by oxygen limitation, iron requirement, and oxidative stress. Oxidative stress appears as a consequence of the hypoxic conditions achieved by *K. lactis* once oxygen supply is no longer sufficient to sustain fully respiratory metabolism. This species copes with low oxygen and iron availability by repressing iron consuming pathways and activating iron transport mechanisms. Most of the physiological and transcriptomic features of *K. lactis* in aerobic wine fermentation are not shared with the Crabtree-positive yeast *S. cerevisiae*.

1. Introduction

Despite *Saccharomyces cerevisiae* is the main yeast species responsible for wine fermentation, other yeasts, including *Hanseniaspora/Kloeckera*, *Pichia*, *Candida* or *Metschnikowia* strains, develop during the first stages of grape must fermentation (Fleet and Heard, 1993). The metabolic footprint of these alternative yeast species is now recognized as a relevant contribution to wine aromatic complexity and sensory quality (Jolly et al., 2014). However, an uncontrolled fermentation process driven by non-*Saccharomyces* yeasts and bacteria would often result in wine spoilage. Historically, the use of *S. cerevisiae* starter cultures constituted an inflection point in the microbiological control of wine fermentation (Carrascosa et al., 2011), but it has been blamed for wine aromatic standardization and lack of complexity. In order to recover some of the positive contribution of "wild yeasts" to wine quality, while keeping a reasonable control of the fermentation process, wine microbiologists have been suggesting the use of non-*Saccharomyces* yeasts in mixed starter fermentations with *S. cerevisiae*, either by simultaneous or sequential inoculation (Jolly et al., 2014). Indeed, several non-*Saccharomyces* yeast starters are currently available in the market and their use by winemakers is growing. Most of these new starters are selected according to their impact on wine aromatic profile, glycerol or mannoprotein content, volatile acidity, or colour stability (Gonzalez et al., 2016).

Crabtree-negative non-*Saccharomyces* yeasts have been suggested in the context of climate change as an instrument to reduce ethanol content of wines (Gonzalez et al., 2013). The proposal for alcohol level reduction with these alternative yeast species is based on their capacity to respire sugars from grape must under aerated fermentation conditions (Quirós et al., 2014). Under oxygen sufficient conditions, Crabtree-negative yeasts consume all sugar by respiration, while most of the carbon flux goes to ethanol production in the case of Crabtree positive species, like *S. cerevisiae*. Our research group has shown the usefulness of several non-*Saccharomyces* yeasts, including *Kluyveromyces lactis*, for this purpose (Morales et al., 2015; Rodrigues et al., 2016). These works identified the overproduction of acetic acid, above commercially accepted levels, as a critical point for the interest of this approach. The increase in acetic acid production occurred either under aerobic conditions or after growing in aerobic conditions and then switching to anaerobic conditions. A tight control of the dissolved oxygen levels reduced the production of acetic acid. In addition to oxygen supply, other environmental factors, namely temperature and nitrogen source availability have also been shown to impact the output of aerated fermentation experiments with different non-*Saccharomyces* yeasts (Rodrigues et al., 2016).

Reducing ethanol content of wines by the strategies mentioned above involves the use of yeast species usually poorly characterized as well as growth conditions (aerated wine fermentation) that are fairly unexplored from a biotechnological viewpoint. In order to make

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advances in the development of fermentation procedures based on respiratory metabolism of non-*Saccharomyces* yeasts, it was judged interesting to know the transcriptional changes experienced by yeast cells under these previously unexplored growth conditions. *K. lactis* was chosen because of its good properties for alcohol level reduction by respiration (Rodrigues et al., 2016). In addition, the genome of this species is well sequenced and annotated (Dujon et al., 2004; Sherman et al., 2004). The aim of this work was hence using *K. lactis* as a model system to study changes, at the transcription level, relevant for our understanding of yeast physiology under aerated fermentation conditions, as those employed for alcohol level reduction in wine.

2. Methods

2.1. Strains and media

Three yeast strains were used in this work, *S. cerevisiae* EC1118, a widely used industrial wine yeast strain; *K. lactis* type strain, CBS2359; and *K. lactis* AQ2166, a natural oak isolate from Hungary. In order to mimic industrial wine fermentations, we used a synthetic grape must recipe based on the Herwig complex synthetic media (Herwig et al., 2001) with some modifications as follows (per liter): glucose 200 g; $(NH_4)_2SO_4$ 5 g, KH_2PO_4 3 g, $MgSO_4$ $7H_2O$ 0.5 g, trace element solution 2.67 mL, vitamin solution 2.67 mL, anti-foam (PPG P2000) 0.1 mL. The vitamin solution, stored in refrigerator and sterilized by filtration, contained per liter: biotine 0.05 g, Ca-D(+)panthothenate 1 g, nicotinic acid 1 g, myo-inositol 25 g, thiamine hydrochloride 1 g, pyridoxal hydrochloride 1 g, para-amino benzoic acid 0.2 g. The autoclaved trace element solution contained per liter: EDTA 15 g, $ZnSO_4 \cdot 7H_2O$ 45 g, $MnCl_2 \cdot 4H_2O$ 1 g, $CoCl_2 \cdot 6H_2O$ 0.3 g, $CuSO_4 \cdot 5H_2O$ 0.3 g, $Na_2MoO_4 \cdot 2H_2O$ 0.4 g, $CaCl_2 \cdot 2H_2O$ 4.5 g, $FeSO_4 \cdot 7H_2O$ 3 g, H_3BO_3 1 g, KI 0.1 g. Fermentations in real grape must were previously performed to ensure that our results were comparable to synthetic grape must (Supplementary Fig. S1).

2.2. Synthetic must fermentations under controlled aerobic conditions and RQ determination

Fermentation experiments were performed in triplicate in small bioreactors MiniBio (Applikon Biotechnology B.V., Delft, The Netherlands) coupled to BlueInOne Cell gas analyzer units (BlueSens, Germany). This setup allows to monitor different parameters: temperature, pH and dissolved oxygen (DO) in the media, as well as O_2 and CO_2 from the output gas.

Seed cultures were grown in YPD broth for 48 h, at 25 °C and 250 rpm. Bioreactors were filled in with 150 mL of synthetic grape must. Temperature was set to 25 °C, stirring to 1000 rpm, pH to 3.5, and inoculation to approximately 0.2 initial optical density at 600 nm (OD_{600}). The cultures were sparged with air at 25 mL/min (10 gas volumes/culture volume/h (vvh)). Gas flow was controlled with MFC17 mass flow controllers (Aalborg Instruments and Controls, Inc., Orangeburg, NY), whose calibration was regularly verified with an electronic flowmeter (Agilent Technologies, Santa Clara, CA).

CO_2 and O_2 readings from the gas analyzer were recorded every minute and used to calculate RQ in three steps. First the contribution of gas exchanges to changes in gas volume was taken into account to calculate actual amount (per minute) of CO_2 and O_2 coming out of the bioreactor. Then, CO_2 release and O_2 consumption rates were calculated as the difference between in and out values (per minute). Finally, RQ was estimated as the ratio between CO_2 production and O_2 consumption rates for each time point.

2.3. Analytical methods

Production and consumption of the main metabolites, glucose, fructose, glycerol, and ethanol, were determined in duplicate using a Surveyor Plus Liquid Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively) on a 300 × 7.7 mm HyperREZ™ XP Carbohydrate H+ (8 μm particle size) column and guard (Thermo Fisher Scientific). The column was maintained at 50 °C, and 1.5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Prior to injection in duplicate, the samples were filtered through 0.22 μm pore size nylon filters (Micron Analitica, Madrid, Spain) and diluted 10-fold in MilliQ water.

2.4. RNA sequencing and data analysis

Total RNA from biological triplicates was extracted using RNeasy® mini kit (QIAGEN) and subjected to DNAase treatment using the Ambion DNA-free™ kit according to the manufacturers' instructions. Concentration, purity and integrity of RNA samples were determined by spectrophotometric analysis considering the absorbance ratio at 260/280 nm and at 230/260 nm. Library preparation and sequencing of RNA was performed at Institute of Biomedicine & Biotechnology of Cantabria (Santander, Spain). After poly-A filtering, libraries were generated for the different time points and conditions. From these libraries, 50-bp single-end sequence reads were produced with Illumina HiSeq 2000. All raw RNAseq data have been deposited in NCBI under Sequence Read Archive SRP064945 (BioProject PRJNA298965) accession number.

Alignment of reads to the S288c *S. cerevisiae* yeast reference genome assembly or to CBS2359 *K. lactis* reference genome assembly was carried out using TopHat2 v.2.0.13 (Kim et al., 2013). Only uniquely mapped single copy, ≤1 polymorphism per 25 bp reads with quality ≥20 were kept for further analysis. The htseq-count tool (v.0.5.4p5) from HTSeq (Anders et al., 2015) was used to estimate unambiguous read count per genome assembly annotated transcript. Normalization following the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010), as well as a time-points DEGs searches (adjusted Benjamini–Hochberg $P \leq 0.05$ and ≥2-fold change) were performed in edgeR v.2.2.6 (Robinson et al., 2010). Finally, fragments per kb of exon per million fragments mapped (RPKM) was calculated using Cuffdiff v.2.2.1 (Trapnell et al., 2013) and low-expressed transcripts were filtered out when RPKM was <1 in both samples.

2.5. Real-time quantitative PCR

RNA was prepared as described above, and quantification was run in triplicate. All the reactions were run in a LightCycler® 480 Real-Time PCR System. The gene expression levels are shown as the changes in the concentration of the studied gene as compared to the control sample and were normalized with the concentration of the housekeeping *ACT1* gene and *PCA1* gene, with similar results (Teste et al., 2009) (only *ACT1* is shown in the figures) using the ΔΔCt method. Primers used in this study are listed in Supplementary Table S1.

2.6. Statistical analysis

Principal Component Analysis of the normalized RNAseq data transcripts per million (TPM) was done using MeV software (4.8v10.2). The remaining statistical analyses were done using STATA-SE. Venn diagram was drawn by using Venny 2.1 on-line tool software (Oliveros, J.C. (2007–2015) Venny. An interactive tool for comparing lists with Venn's diagrams: http://bioinfogp.cnb.csic.es/tools/venny/index.html). GO term analysis was performed using YeastMine (Balakrishnan et al., 2012). The *p-values* were corrected for multiple testing by the Bonferroni test for functional associations and GO analyses. The statistical level of significance was set at $p \leq 0.05$. Then, GO terms were grouped in biomodules by GO-Module (Yang et al., 2011) to prioritize Gene Ontology terms.

3. Results and discussion

3.1. Fermentation profile under aerated conditions

Fermentation assays of synthetic grape must were performed with two different *K. lactis* yeast strains, the type strain CBS 2359, with complete annotated genome sequence available (Dujon et al., 2004; Sherman et al., 2004) and *K. lactis* AQ2166. This second *K. lactis* strain was selected as a more suitable yeast strain for winemaking applications, especially considering acetic acid production under aerated conditions (Fig. 1). An industrial *S. cerevisiae* wine yeast strain, EC1118, was also included in the experiments in order to illustrate the impact of aerated fermentation methodology in an industrial wine yeast background (Novo et al., 2009). The experimental conditions, including aeration regime, were chosen according to previous results (Morales et al., 2015; Rodrigues et al., 2016). A validation of the aeration regime and synthetic grape must composition was done by comparing dissolved oxygen (DO) profiles in fermentations carried out in natural grape must. Similar DO profiles were obtained in natural or synthetic grape must (Supplementary Fig. S1).

Under these experimental conditions total sugar consumption was achieved in a relatively short time for all the strains assayed, 50 h for both *K. lactis* strains, and slightly faster for the industrial *S. cerevisiae*, 42 h (Fig. 2). Ethanol production under these aerated conditions was low as compared to regular (anaerobic) fermentation conditions; and lower for both strains of *K. lactis* (7 to 8.30% v/v ethanol) than for *S. cerevisiae* (10% v/v ethanol). This is in agreement with previous results by us and other authors, under both standard laboratory conditions (Quirós et al., 2014; Contreras et al., 2015) and in natural grape must (Morales et al., 2015). Also, as shown previously (Morales et al., 2015), oxygenation results in increased acetic acid production for *S. cerevisiae* and the type strain of *K. lactis* CBS2359, from 30 h. Although acetic acid is the primary volatile acid in wine, its production over detectable levels remembers vinegar and is considered as wine spoilage (Carrascosa et al., 2011). However, the second *K. lactis* strain showed very low acetic acid production (Fig. 1).

Aeration regime, as mentioned above was based on previous results, with the main goal of inducing a quick oxygen consumption by *K. lactis* strains, that will protect grape must components against oxidation. Accordingly, the two *K. lactis* strains showed a high consumption rate after the initial 5 h, reaching DO values below 10% after 15 h. The decline in DO was slightly faster for the *K. lactis* strain AQ2166. On the other hand, *S. cerevisiae*'s oxygen consumption was noticeable after around 12 h with a much slower decay, and never fell below 30% dissolved oxygen (Fig. 3). Oxygen demand decayed after about 30 h of culture in all instances, probably due to the depletion of some essential nutrients, slow metabolic activity, and the entry of the cultures into stationary phase. These differences in dissolved oxygen profiles are in agreement with Respiratory Quotient (RQ) values for each strain (Fig. S2). *K. lactis* strains showed steady RQ values around 1 (fully respiratory metabolism), until oxygen became a limiting factor. Once the increment in biomass results in an oxygen demand that cannot be fulfilled by the preset air flow (hypoxic conditions), an increase in RQ values is observed in the *K. lactis* strains.

S. cerevisiae, on the contrary, showed a quick increase in RQ values from the beginning of the experiment. Therefore, despite it consumes up to 30% of the oxygen available, RQ values indicate that *S. cerevisiae* is mainly fermenting. This behavior is in agreement with the well-known metabolic features of *S. cerevisiae*, an archetypical Crabtree-positive yeast. The Crabtree-negative nature of *K. lactis* allows these yeasts to keep RQ values always below those of *S. cerevisiae*, lowering the final ethanol production, as shown above. Despite these important differences in respiro-fermentative metabolism, all the yeast strains assayed showed similar sugar consumption kinetics (Fig. 2).

The two *K. lactis* strains showed slight differences in oxygen consumption. The type strain showed a constant slope in dissolved oxygen decline, down to 0%, while AQ2166 slowed down oxygen consumption after reaching 10% DO, needing several hours to drop to 0%. This behavior correlates with the increase in RQ values, starting earlier for AQ2166 than for the *K. lactis* type strain, and could indicate a higher sensitivity of AQ2166 to very low oxygen availability. Reaching 0% DO values is important in the context of wine aerobic fermentation, in order to protect wine colour and aromatic features (Morales et al., 2015).

Sampling points for RNAseq and qPCR analyses were decided focusing on the two different stages in oxygen availability for *K. lactis*. Two sample points were defined for RNAseq analysis (Fig. 3). Sample point S1 (12 h) defined by no oxygen limitation, the RQ value was steady around 1 for *K. lactis* strains, indicating that glucose metabolism was fully respiratory. In contrast, sample point S2 (30 h) was taken when hypoxic conditions (0% DO) had been running for several hours in *K. lactis* cultures. This point was also characterized by RQ values clearly above 1 (respiro-fermentative metabolism). In the case of *S. cerevisiae* S1 corresponds to the initial stages of oxygen consumption while S2 corresponds to the maximum oxygen uptake, both cases with RQ ratios higher than one, while there is a high oxygen availability.

3.2. Global analysis of the transcriptome

The main purpose of this work was to study the transcriptomic profile of *K. lactis*, in synthetic grape must with an aerated regime, under two very different metabolic states defined by the available dissolved oxygen. Neither *K. lactis* nor aerobic conditions are commonly used in wine fermentation, but they have been lately used to lower the ethanol content in wine (Morales et al., 2015; Rodrigues et al., 2016). Therefore,

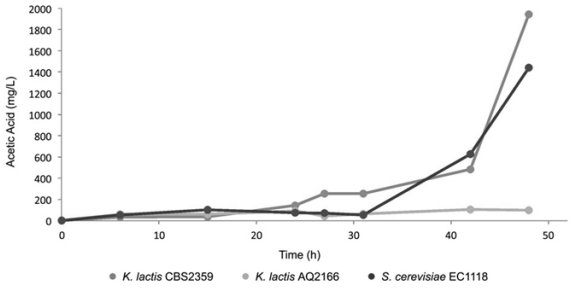

Fig. 1. Acetic acid production under aerated conditions.

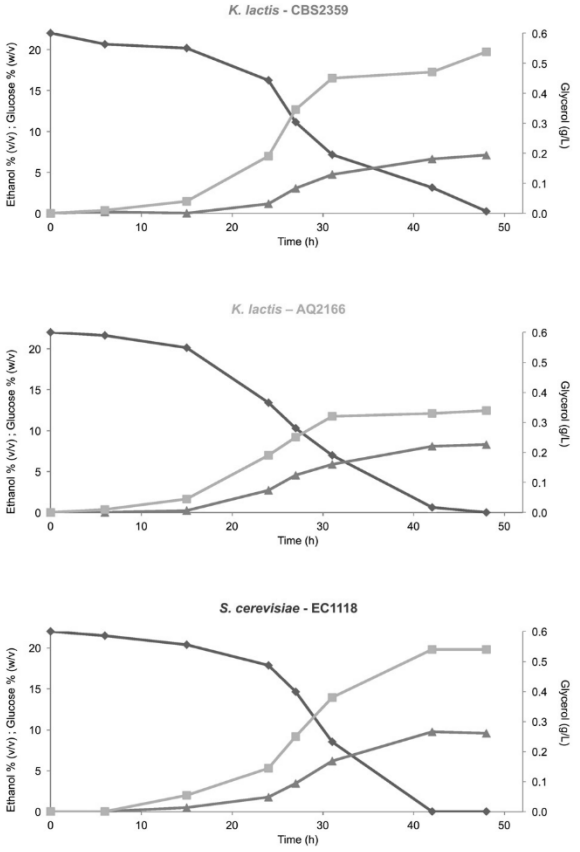

Fig. 2. Evolution of main fermentation related metabolites (glucose: diamonds; ethanol: triangles; glycerol: squares) under aerated conditions.

it is of great interest, to better understand how *K. lactis* responds to this new biotechnological application.

Samples for gene expression analysis were taken from S1 and S2 (see above), and analyzed by RNAseq as described in Materials and Methods. In a first attempt to obtain a global picture of the transcriptome data, a Principal Component Analysis (PCA) was ran (Supplementary Fig. S3). PCA draw two very different scenarios, while samples from *K. lactis* cluster in two groups according to its time points, in *S. cerevisiae* samples from S1 and S2 cluster together. The results of the PCA analysis correlate well with the number of differentially expressed genes (DEG) for each species (S1 vs. S2). In *K. lactis* there is a high degree of divergence between sample points, with >12% of the transcriptome significantly modified (Fig. 4), in contrast to the 2.5% modified for *S. cerevisiae*. Of the 623 genes showing modified expression in *K. lactis*, 337 were up-regulated, while 286 genes were down-regulated. However, only 68 up-regulated genes and 92 down-regulated genes were detected for *S. cerevisiae*. In addition, average fold-change values for genes differentially expressed in *K. lactis* was higher than for *S. cerevisiae*.

These gene expression changes are in agreement with the DO profiles shown in Fig. 3. *K. lactis* shows pure respiratory metabolism (RQ values around 1; Supplementary Fig. 2S) in S1, with DO values above 30%, but the oxygen nutrient limitation (0% DO) in S2 re-shapes the transcriptome in response to the respiro-fermentative metabolism. The high impact of growth conditions in S2 on the transcriptome reprograming of *K. lactis* takes place despite oxygen is supplied at the same rate in both S1 and S2. In contrast to other studies with *K. lactis*, in this case the trigger for the observed transcriptomic changes is not

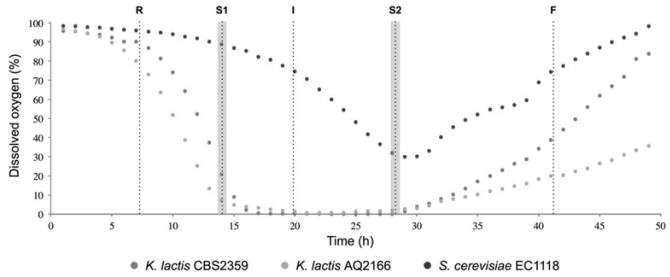

Fig. 3. Evolution of dissolved oxygen levels under aerated conditions. RNAseq sampling time points are shadowed in grey. qPCR sampling time points are shown as dotted lines.

a deliberated shift from aerobic to anoxic conditions (Blanco et al., 2007; David and Poyton, 2005) but a gradual modification from aerobic to hypoxic conditions due to increased biomass and metabolic activity of yeast cells. Under this conditions, *S. cerevisiae* shows respiro-fermentative metabolism in both sample points, with a clear preference for fermentation, and DO values above 30% in both cases. Accordingly, changes in the expression pattern are much less pronounced than for *K. lactis*.

3.3. GO term enrichment among differentially expressed genes

The analysis of GO term enrichment is shown in Table 1. In order to ease the interpretation of results, the GO-Module tool (Yang et al., 2011) was used to prevent false positives and repeated terms. In the case of *K. lactis*, this analysis was performed by using the corresponding *S. cerevisiae* orthologs. Approximately four-fifths of the genes showing highly variable expression from *K. lactis* do have known orthologs in *S. cerevisiae* (around 500 genes).

Analysis of GO terms among *K. lactis* genes significantly up-regulated in S2 revealed an enrichment in oxidoreductase activity; iron ion transmembrane transporter activity; aldehyde dehydrogenase (NAD) activity, and catalytic activity (Table 1). Regarding genes in the iron ion transmembrane transporter activity category, *FET4* codes for a low-affinity iron (II) permease, also involved in the transport of copper and zinc. Fet3p and Ftr1p constitute the cell-surface high-affinity iron uptake system required for iron import when it is present at low concentrations. Fet3p is a multicopper ferroxidase that receives iron (II) ions from cell-surface iron reductases such as Fre3p and passes iron (III) ions to the iron permease Ftr1p. In *K. lactis* two different genes *KLLA0E14477g* and *KLLA0E05897g* show similarity to *FRE3* (also significantly up-regulated but not listed under GO:0005381) from *S. cerevisiae*. Both orthologs are overexpressed in *K. lactis* in S2. Also *ARN1* and *ARN2* are involved in iron transmembrane transport. They belong to a family of transporters for siderophore-iron chelates, responsible in *S. cerevisiae* for the uptake of iron bound to different siderophores like ferrirubin, ferrirhodin or triacetylfusarinine C. Among genes up-regulated in *K. lactis*, four different genes, similar to *S. cerevisiae ARN1* (*KLLA0A10439g*, *KLLA0E14609g*) or *ARN2* (*KLLA0C00220g*, *KLLA0C19272g*) were found. In *S. cerevisiae*, the transcription of these genes is activated by Atf1p, which is expressed in response to low iron conditions.

The identification of iron as a major nutrient requirement for *K. lactis* under these culture conditions is reinforced by the finding of several other genes related to iron metabolism being overexpressed in S2 for *K. lactis* cultures. These include five orthologs of *FIT1*, *FIT2* and *FIT3* from *S. cerevisiae*. These genes code for GPI-anchored cell wall mannoproteins involved in the retention of siderophore-iron complexes. Yap5p is an iron-sensing transcription factor; while Cth1p is involved in iron homeostasis, as well as the putative protein Fmp23p. The iron dependence of the *K. lactis* life style is also illustrated by the number of copies found in the genome (Dujon et al., 2004; Sherman et al., 2004), and overexpressed in this set of experiments, also *ARN1-2* and *FRE3* orthologs, as mentioned above, not only of *FIT1-3* orthologs.

The overexpression of this set of genes in S2 indicates the importance of iron metabolism for *K. lactis* under aerated conditions. This is

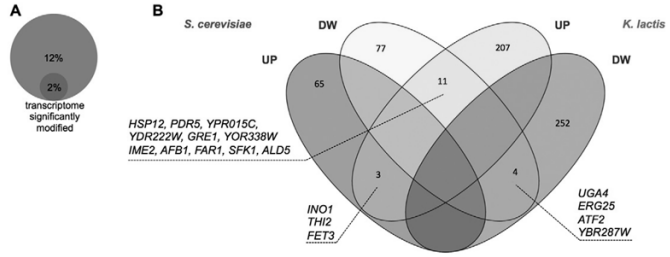

Fig. 4. Analysis of the differentially expressed genes. (A) Percentage of transcriptome significantly modified for each species: Blue circle represents *K. lactis*; Red circle represents *S. cerevisiae*. (B) Venn diagram showing the number of differentially expressed genes for each species (S1 vs. S2).

Table 1
GO term enrichment among differentially expressed genes for each species. GO terms were grouped in biomodules by GO-Module.

Expression	Strain	GO IDs	*P*-value	Significance[a]	GO terms	GO-Module IDs
Up-regulated	*K. lactis*	GO:0016491	0.000	K	Oxidoreductase activity	1
		GO:0004029	0.006	K	Aldehyde dehydrogenase (NAD) activity	2
		GO:0005381	0.002	K	Iron ion transmembrane transporter activity	3
	S. cerevisiae	GO:0000944	0.000	K	Base pairing with rRNA	1
		GO:0030556	0.000	T	rRNA modification guide activity	1
		GO:0030559	0.000	K	rRNA pseudouridylation guide activity	2
Down-regulated	*K. lactis*	GO:0051536	0.002	K	Iron-sulfur cluster binding	1
		GO:0051539	0.003	T	4 iron, 4 sulfur cluster binding	1
		GO:0015075	0.000	K	Ion transmembrane transporter activity	2
		GO:0008324	0.000	T	Cation transmembrane transporter activity	2
		GO:0022890	0.000	T	Inorganic cation transmembrane transporter activity	2
		GO:0008121	0.000	K	Ubiquinol-cytochrome-c reductase activity	3
		GO:0003735	0.000	K	Structural constituent of ribosome	4
		GO:0015077	0.000	K	Monovalent inorganic cation transmembrane transporter activity	5
		GO:0004129	0.000	T	Cytochrome-c oxidase activity	5
		GO:0015078	0.000	T	Hydrogen ion transmembrane transporter activity	5

[a] 'K' refers to the key terms of GO biomodules, 'T' refers to the truly significant hierarchical descendents of the key terms.

related to the high oxygen consumption levels at this time point, considering that the electron transport chain is the main intracellular sink of iron ions. Also in agreement to the relevance of iron metabolism for *K. lactis* is the observation that, at this sample point, bioreactors turned pink-red, as shown for *Metschnikowia pulcherrima* under similar growth conditions. Both species are known to produce pulcherrimin, an iron chelate molecule, with antimicrobial effect by limiting access of other microorganisms to iron (Sipiczki, 2006). Indeed, the absorbance spectrum of the *K. lactis* supernatants showed the characteristic pulcherrimin peak at 385 nm.

According to the dissolved oxygen profiles, one major change in the environmental conditions between S1 and S2 for *K. lactis* cultures is the transition from oxygen sufficient to hypoxic conditions. Transition from aerobic to anoxic conditions has been shown to induce the production of reactive oxygen species (ROS) in *S. cerevisiae* (David and Poyton, 2005). ROS production might be a consequence of a sudden redox imbalance, when the excess NADH resulting from the activity of the tricarboxylic acid (TCA) cycle can no longer be taken up by the electron transport chain in the absence of molecular oxygen (Murphy, 2009). Two enriched categories, oxidoreductase activity, and aldehyde dehydrogenase (NAD) activity, suggest this to be also the case for *K. lactis* cells under our experimental conditions. This happens despite oxygen is still available and being used in time point S2, and despite the gradual reduction in dissolved oxygen levels would be expected to allow for a smoother adaptation of yeast cells to oxygen depletion.

A high number of significantly enriched GO terms were found from the set of genes down-regulated in *K. lactis* (Table 1). As shown in Table 1, the Key Modules (Yang et al., 2011) are iron-sulfur cluster binding; ion transmembrane transporter activity; ubiquinol-cytochrome-c reductase activity; structural constituent of ribosome and monovalent inorganic cation transmembrane transporter activity. Down-regulation of iron-sulfur cluster binding in S2 is in agreement with the above observation of iron becoming a limiting nutrient for *K. lactis* under these culture conditions. Iron-sulfur proteins are considered as regulatory elements of iron metabolism. Yeast cells exhibit loss of iron-sulfur proteins in response to iron depletion (Shakoury-Elizeh et al., 2010).

The oxygen limiting conditions in *K. lactis* in S2 reduced the flux distribution towards respiration compared to S1. Therefore, the mitochondrial electron transport chain is expected to show lower relative activity in S2. Indeed, both the ion transmembrane transporter activity and ubiquinol-cytochrome-c reductase activity GO terms were significantly down-regulated (Table 1). These GO terms include, among others, genes coding for the cytochrome *c* oxidase (*COX12*, *COX13*, *COX4*, *COX5b*, *COX6*, *COX7*, *COX8*, and *COX9*), or other components of the electron transport chain (*ATP1*, *COR1*, *RIP1* and many QCR genes) as well as additional mitochondrial constituents (*AGC1*, *FSF1*). Also falling in this category are the down-regulated genes coding for permeases for amino acids and other nitrogen compounds (*GAP1*, *DIP5*, *DUR3*). This is probably related to the oxygen limitation observed in S2, since respiration is associated to higher biomass production rates. Also in agreement is the down-regulation of genes coding for structural components of the ribosomes, highlighted in the structural constituent of ribosome GO-term category (Table 1).

Under this experimental conditions, for the industrial *S. cerevisiae* yeast strain, the GO terms that appeared to be significant from the up-regulated set of genes, were all of them associated with rRNA and rRNA pseudouridylation (Table 1). Almost half of the genes significantly over-expressed in S2 are small nucleolar RNAs (snoRNAs). These stable RNAs are found within small nucleolar ribonucleoprotein complexes (snoRNPs) and localize to the nucleoli of eukaryotic cells. The majority of the snoRNAs are involved in ribosomal RNA processing, including pseudouridylation, a frequent posttranscriptional modification of uridine in RNAs. Pseudouridine (Ψ), when incorporated into RNA, can modify its secondary structure by increasing base stacking, improving base pairing and rigidifying sugar-phosphate backbone. As a consequence, it alters the chemical and physical properties of RNA molecules (Zhao and He, 2015).

Pseudouridylation can induce different stress factors (Wu et al., 2011; Ge and Yu, 2013; Schwartz et al., 2014; Karijolich et al., 2015) suggesting a regulatory role for Ψ. The replacement of multiple U sites with Ψ in synthetic RNA molecules results in an increased protein expression level (reviewed in Zhao and He, 2015). The high expression of genes involved in pseudouridylation observed in our work could be related to oxidative stress due to respiratory metabolism. Indeed, RNA post-transcriptional modifications have been previously shown to be important for recovery after an environmental stress (Biggar and Storey, 2015). In addition, Tronchoni et al. (2014) described the role of RNA maturation and transcription stability after cold shock in wine yeast strains. No significant enrichment was found for genes down-regulated in *S. cerevisiae* (Table 1).

3.4. Genes similarly regulated in both species point to nutrient requirements

Only seven differentially expressed genes (three up- and four down-regulated) were found to behave the same way for *K. lactis* and *S. cerevisiae* (Fig. 4). This low similarity in their transcriptomic responses is explained by the evolutionary divergence in aerobic fermentation between both species. While, according to RQ values, *K. lactis* is exclusively respiring until the lack of oxygen forces it to initiate fermentation, *S. cerevisiae* under the Crabtree effect maintains the main metabolic flux towards fermentation. Despite these differences, several genes show a common regulation when comparing both time points.

The three genes up-regulated are *INO1*, *THI2* and *FET3*. *INO1* (Fig. 4), coding for the first enzyme in the inositol biosynthesis pathway, is

induced by inositol requirement (Culbertson and Henry, 1975). It has been previously shown that inositol might be one of the limiting nutrients in some yeast culture media (Hanscho et al., 2012; Novo et al., 2013). The synthetic must medium used in this work contained three times more inositol than standard synthetic grape must. However, the aeration of the media allows yeast to respire and probably, this increases biomass production and makes inositol to become a limiting compound (Quirós et al., 2014). Respiro-fermentative metabolism and higher biomass production take place in both species under aerated conditions, despite the Crabtree effect of *S. cerevisiae*. The other two commonly up-regulated genes seem also related to the demand of specific nutrients for biomass production. *THI2*, the transcriptional activator of thiamine biosynthetic genes, responds to thiamine diphosphate demand, and *FET3* (discussed above) is induced by low iron availability (Askwith et al., 1994). Among the four genes commonly down-regulated in S2 we found *ERG25* (Fig. 4). Erg25p, a di-ferric protein, is essential for the synthesis of ergosterol, an oxygen-dependent process. Down-regulation of *ERG25* in S2, might be related to iron requirement, as discussed above for *K. lactis* electron transport chain genes and genes coding for other mitochondrial constituents.

3.5. Validation of RNAseq data by qPCR analysis

A selection of highly differentially expressed genes (log fold change above 1), representative of the main GO terms discussed above, were chosen to validate the RNAseq data and were analyzed across the fermentation to obtain a more detailed expression profile (Supplementary Figs. S4 and S5). The qPCR data for sample points S1 and S2 confirmed the RNAseq expression in all cases but two. In both cases gene expression was very low and no solid conclusions can be drawn. For the remaining genes, results from the qPCR analysis largely confirmed the expression pattern observed for S1 and S2 in *K. lactis* and *S. cerevisiae*.

Besides S1 and S2 sample points, additional sample points were included: sample point R, used as a reference for relative quantification by qPCR; and sample point F, representative of an advanced stage of fermentation, with dissolved oxygen levels clearly above sample point S2; finally, given the important differences in metabolic state between S1 and S2 for *K. lactis*, sample point I (Intermediate time point) was included, in order to characterize this observed transition step (Supplementary Fig. S5). This sample point corresponds to approximately 30 min after the culture reached 0% DO.

The qPCR characterization of sample point I shows an interesting result, expression levels at this Intermediate time point did not follow the trend between S1 and S2. On the contrary, the genes analyzed show a clear drop in expression at this sample point. Probably, this indicates a general reduction in gene transcription, in order to allow the cell to adapt its metabolism to the new growth conditions, with limiting oxygen availability.

As mentioned above, preliminary experiments showed a slightly different fermentation profile for *K. lactis* AQ2166, as compared to the *K. lactis* type strain. The main differential feature is a slower transition step for *K. lactis* AQ2166. For this reason, qPCR analysis was performed for this strain from sample point I, for the same genes than the type strain. As shown by the trend lines in Fig. 5, the general behavior of both *K. lactis* strains is similar, concerning relative expression levels between sample points S2 and I, and between sample points F and S2. However, the general trend towards reduced expression levels during the transition step (sample point I), previously observed for the type strain, is less pronounced for *K. lactis* AQ2166 (with the noticeable exception of *ARR2*). This weaker response to the transition step is in agreement with the slower slope cultures of this strain reach 0% DO. This suggests *K. lactis* AQ2166 is able to perceive and anticipate oxygen limitation more efficiently than the type strain, and gradually adapt transcription levels, before oxygen availability becomes a limiting factor for growth.

3.6. Oxidative stress

A transitory oxidative stress response due to hypoxic conditions has been described for *S. cerevisiae* (Becerra et al., 2002) and *K. lactis* (Blanco et al., 2007). Despite some similarities, the hypoxic induced oxidative stress response seems to be different between both yeast species, probably due to the preference for respirative metabolism in *K. lactis*, in contrast to respiro-fermentative metabolism in *S. cerevisiae*. González-Siso and Cerdán (2012) proposed a set of gene types to be explored in order to characterize these transcriptional responses, including heme biosynthesis, ergosterol biosynthesis and supply, NAD(P)H consuming oxidative defense reactions, other oxidative defense reactions, NAD(P)-dehydrogenases from the inner membrane of mitochondria, heme/respiration-related transcriptional factors, sterol-related transcriptional factors, peroxide-related transcriptional factors, life span-related proteins, and mitophagy-related proteins.

Given the hypoxic conditions identified in S2 for *K. lactis* cultures, we paid attention to changes in the expression levels of genes assigned by González-Siso and Cerdán (2012) to each of the above mentioned gene types in both *K. lactis* and *S. cerevisiae*. Even though time point S2 cannot be compared between yeast species given the important metabolic differences, it was worth to study if oxidative stress response genes appeared significantly induced in *S. cerevisiae*. As shown in Tables 2 and 3, the number of these genes differentially expressed between S1 and S2 is higher for *K. lactis* than for *S. cerevisiae*. This number of DEG is higher than observed by Blanco et al. (2007), probably due to differences in the experimental conditions and to the lower throughput techniques available at that time for *K. lactis*.

Differences between *K. lactis* and *S. cerevisiae* for the expression of this set of genes are in agreement with the general trend in gene expression discussed above, related to the more limited physiological changes observed for each species (Fig. 3). Genes up-regulated in *K. lactis* under hypoxic conditions belong to the types "other oxidative defense reactions", "mitophagy-related proteins", and "NAD(P)H consuming oxidative defense reactions", while genes down-regulated belong to "NAD(P)-dehydrogenases from the inner membrane of mitochondria and "ergosterol biosynthesis and supply", as well as two genes for "NAD(P)H consuming oxidative defense reactions" (Table 2). This expression pattern would confirm the adaptation responses already hypothesised by González-Siso and Cerdán (2012), that can be summarized as a reduction in mitochondrial activity through mitophagy, the down-regulation of oxygen consuming biosynthetic processes, like ergosterol or heme biosynthesis, and tuning of oxidative defense mechanisms (with more genes up-regulated than down-regulated). Down-regulation of genes typically related with ROS detoxification, like *CTA1* or *PRX1*, or mitochondrial NADH dehydrogenases, like *NDE1* and *NDI1*, seems to be paradoxical, but it is explained by a general reduction in mitochondrial synthesis in S2.

Oxidative stress by hypoxia has been associated to the sudden oxygen deficiency, resulting in leakage of electrons from the electron transport chain, mainly at the level of complex I (Murphy, 2009), leading to a drastic increase in the rate of formation of superoxide anion ($O_2^{\bullet -}$). This oxygen radical is readily converted to H_2O_2 by the activity of superoxide dismutases (encoded in *S. cerevisiae* by *SOD1* and *SOD2*). The expression of these genes is shown in Fig. 6 as normalized RPKM values for *K. lactis* and *S. cerevisiae*. It shows that there is not a significant change in the expression of *SOD1* and *SOD2* between S1 and S2 in *K. lactis*. Interestingly, this is because this species already shows high levels of expression for these genes related to active respiratory metabolism. The final detoxification step is catalysed by catalases, Ctt1p and Cta1p. Similar to *SOD* genes, expression levels of *CTA1* and *CTT1* are again higher for *K. lactis* than for *S. cerevisiae* (Fig. 6). An intriguing possibility to explain increased acetic acid production would be that the detoxification of $O_2^{\bullet -}$ produced by hypoxic conditions and reduction of H_2O_2 by catalases could be coupled to the oxidation of ethanol to acetaldehyde, as has been shown in human cells (Zimatkin et al., 2006). BioCyc (Caspi

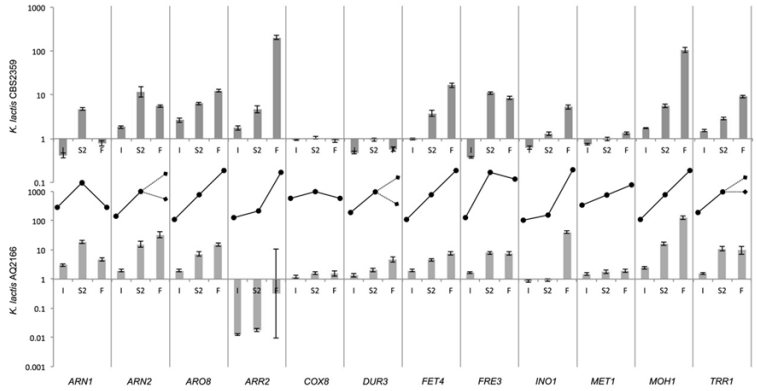

Fig. 5. Comparative gene expression profile between *K. lactis* strains after reaching 0% of dissolved oxygen. Gene expression trends are shown by schematic lines. Continuous line: same gene expression trend; dotted lines: different expression trend.

et al., 2016) data base collection for metabolic pathways predicts that the same reaction could be catalysed by Ctt1p or Cta1p in *S. cerevisiae* (Reaction: EC1.11.1.6) (Caspi et al., 2016). Further oxidation of acetaldehyde, catalysed by aldehyde dehydrogenases, would result in acetate production.

In conclusion, gene expression analysis has confirmed the metabolic shift observed in DO and also the differences observed for *S. cerevisiae* and *K. lactis* under aerobic conditions. Two main drivers appear to be responsible in *K. lactis* for this transcriptomic remodeling. By one side, this species appears as a respiration and iron specialist, also indicated by the high copy number of genes required for iron uptake, or the high constitutive expression levels of genes involved in ROS detoxification. This species seems to activate two complementary mechanisms in order to cope with increasing iron requirements, activation of iron uptake

Table 2
Gene types responding to oxidative conditions due to hypoxia in *K. lactis*.

Gene	Orthologs	FC[a]	Category
KLLA0F15037g	*TRR1*	4.78	Other oxidative defense reactions
KLLA0A00660g	*ATG32*	1.88	Mitophagy-related proteins
KLLA0B07975g	*GRX8*	1.60	Other oxidative defense reactions
KLLA0C17160g	*ATG1*	1.20	Mitophagy-related proteins
KLLA0E14939g	*UTH1*	1.18	Mitophagy-related proteins
KLLA0E20593g	*ATG8*	0.97	Mitophagy-related proteins
KLLA0F20009g	*AHP1*	0.81	NAD(P)H consuming oxidative defense reactions
KLLA0D14333g	*DOT5*	0.79	NAD(P)H consuming oxidative defense reactions
KLLA0A00264g	*GTT1*	0.77	NAD(P)H consuming oxidative defense reactions
KLLA0B12133g	*ATG11*	0.61	Mitophagy-related proteins
KLLA0E04181g	*BCY1*	0.53	Life span-related proteins
KLLA0A09383g	*MTM1*	0.53	NAD(P)H consuming oxidative defense reactions
KLLA0E18547g	*MOT3*	0.52	Heme/respiration-related transcriptional factors
KLLA0F26917g	*CCS1*	0.52	NAD(P)H consuming oxidative defense reactions
KLLA0F07557g	*GSH2*	0.46	NAD(P)H consuming oxidative defense reactions
KLLA0B03586g	*SCH9*	−0.42	Life span-related proteins
KLLA0F22880g	*HAP1*	−0.57	Heme/respiration-related transcriptional factors
KLLA0A05071g	*ERG4*	−0.59	Ergosterol biosynthesis and supply
KLLA0E17733g	*GRX6*	−0.60	Other oxidative defense reactions
KLLA0B11495g	*ROX1*	−0.68	Heme/respiration-related transcriptional factors
KLLA0F15224g	*ERG1*	−0.74	Ergosterol biosynthesis and supply
KLLA0F10285g	*MVD1*	−0.82	Ergosterol biosynthesis and supply
KLLA0C15147g	*HEM3*	−0.83	Heme biosynthesis
KLLA0D11242g	*ERG5*	−0.84	Ergosterol biosynthesis and supply
KLLA0E03653g	*ERG11*	−0.92	Ergosterol biosynthesis and supply
KLLA0C12265g	*ERG24*	−0.93	Ergosterol biosynthesis and supply
KLLA0B09636g	*GRX5*	−0.95	Other oxidative defense reactions
KLLA0E21891g	*NDE1*	−1.07	NAD(P)-dehydrogenases from the inner membrane of mitochondria
KLLA0B08085g	*ERG25*	−1.13	Ergosterol biosynthesis and supply
KLLA0E20285g	*PRX1*	−1.17	NAD(P)H consuming oxidative defense reactions
KLLA0C06336g	*NDI1*	−1.19	NAD(P)-dehydrogenases from the inner membrane of mitochondria
KLLA0F06336g	*ERG2*	−1.28	Ergosterol biosynthesis and supply
KLLA0D11660g	*CTA1*	−2.23	NAD(P)H consuming oxidative defense reactions

[a] FC, fold change.

Table 3
Gene types responding to oxidative conditions due to hypoxia in *S. cerevisiae*.

Gene	FC[a]	Category
SOD1	0.92	NAD(P)H consuming oxidative defense reactions
NCP1	−0.46	Ergosterol biosynthesis and supply
MGA2	−0.47	Heme/respiration-related transcriptional factors
SUT2	−0.47	Sterol-related transcriptional factors
DOT5	−0.48	NAD(P)H consuming oxidative defense reactions
ERG26	−0.49	Ergosterol biosynthesis and supply
ROX1	−0.51	Heme/respiration-related transcriptional factors
TPK2	−0.52	Life span-related proteins
ERG7	−0.55	Ergosterol biosynthesis and supply
HAP1	−0.59	Heme/respiration-related transcriptional factors
ERG5	−0.61	Ergosterol biosynthesis and supply
HOG1	−0.61	Mitophagy-related proteins
GRX6	−0.63	Other oxidative defense reactions
GTT1	−0.70	NAD(P)H consuming oxidative defense reactions
CTT1	−0.72	NAD(P)H consuming oxidative defense reactions
ECM22	−0.72	Sterol-related transcriptional factors
NDE1	−0.76	NAD(P)-dehydrogenases from the inner membrane of mitochondria
ERG24	−0.90	Ergosterol biosynthesis and supply
ERG1	−0.93	Ergosterol biosynthesis and supply
SLT2	−1.10	Mitophagy-related proteins
ERG4	−1.12	Ergosterol biosynthesis and supply
HEM13	−1.17	Heme biosynthesis
UPC2	−1.19	Sterol-related transcriptional factors
ERG11	−1.24	Ergosterol biosynthesis and supply
ERG3	−1.30	Ergosterol biosynthesis and supply
ERG25	−1.43	Ergosterol biosynthesis and supply

[a] FC, fold change.

mechanisms; and repression of the biosynthesis of heme containing proteins. A second driver to downregulation of genes coding for heme containing proteins (e.g. *COX* genes) is oxygen starvation (hypoxia), which would limit the capacity of the electron transfer chain. Finally, oxidative stress caused by hypoxia might be counteracted by also repressing the synthesis of genes coding for this latter group of proteins, as well as mitochondrial activity, or oxygen consuming biosynthetic processes, like ergosterol or heme biosynthesis. The response of *K. lactis* during adaption to hypoxic conditions is also characterized by a transient general downregulation of the transcriptional activity.

The most striking feature of the transcriptional response of *S. cerevisiae* to continued growth under aerated conditions is the activation of genes involved in RNA pseudouridylation, which suggests this growth condition triggers a response based on RNA posttranscriptional modification, similar to what has been described for other stress conditions. The lack of a specific response involving genes related to respiration in this species correlates with low oxygen consumption throughout the experiment, in turn related to the Crabtree-positive character of *S. cerevisiae*.

The ability for continuous growth in grape must and to maintain a strong respiratory metabolism under hypoxic and respiratory conditions, is a remarkable feature of *K. lactis* strains, which contributes to the usefulness of this species for alcohol level reduction in wine. Our data (both transcriptomic and physiological) indicate that, *K. lactis* is actively and almost exclusively respiring sugars, until oxygen becomes a limiting factor. This suggest that, increasing oxygen availability during the first fermentation stages would help improve results in terms of sugar consumed by respiration and, consequently, alcohol level reduction. The only drawback to take into account is the eventual impact of increased oxygenation on volatile acidity, at least for some *K. lactis* strains (like the type strain).

Metabolic diversity of *K. lactis* strains is revealed by different physiological and transcriptomic responses to progressive oxygen starvation. The yeast strain showing the smoother adaptation to hypoxic conditions is also the one producing the lowest acetate levels therefore being the strongest candidate to low ethanol production by respiration at initial stages of fermentation following this new methodology.

Abbreviations

DO: dissolved oxygen
RQ: Respiratory Quotient
PCA: Principal Component Analysis
PC: Principal Component
DEG: differentially expressed genes
S1: RNAseq Sample Point 1
S2: RNAseq Sample Point 2
ROS: reactive oxygen species
TCA: Tricarboxylic Acid Cycle
snoRNAs: small nucleolar RNAs
snoRNPs: small nucleolar ribonucleoprotein complexes
Ψ: Pseudouridine
R: qPCR Reference time point
I: qPCR Intermediate time point

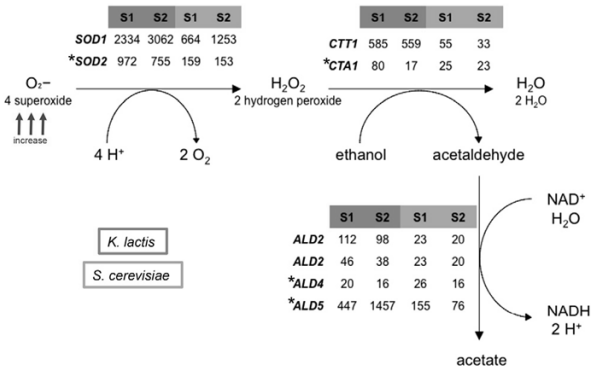

Fig. 6. Gene expression of the enzymes possibly involved in acetic acid production linked to ROS detoxification. Expression values are shown as RPKM. S1: Sample time point 1; S2: Sample time point 2. *: mitochondrial enzymes.

F: qPCR Final time point
$O_2^{\bullet-}$: superoxide anionTPM: transcripts per million
RPKM: reads per kilobase per million mapped reads
GO: Gene Ontology
FDR: False Discovery Rate
LogFC: log fold change
OD_{600}: optical density measured at a wavelength of 600 nm

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2017.01.014.

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Availability of data and material

The data set supporting the results of this article is available in the NCBI repository under Sequence Read Archive SRP064945 (BioProject PRJNA298965} accession number (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement). The data set supporting the results of this article is included in the article (and its Additional files}

Competing interests

The authors declare that they have no competing interests.

Author's contributions

RG, PM and JT, conceived the study; JT, AJR and JAC conducted the experiments; JT analyzed the data, JT and RG wrote the manuscript. All the authors read and approved the final manuscript.

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