MICROBIOLOGICAL NON-GMO APPROACHES FOR THE REDUCTION OF ETHANOL IN WINES

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WE STATE that the present study, entitled "**Microbiological non-GMO approaches for the reduction of ethanol in wines**", presented by **Xiaolin ZHU** for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university.

Doctoral Thesis Supervisors

Dr. Albert Mas | Dr. Gemma Beltran | Dr. Yurena Navarro

Tarragona, September 2021

致我的父母

致旭东

致我的女儿

错误经不起失败，但真理却不怕失败。

Wrong cannot afford defeat but right can.

Rabindranath Tagore

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Contents

OBJECTIVES AND OUTLINE

Before starting the PhD degree, I followed the studies to acquire the professional knowledge of wine systematically. The winemaking is a complex process determined by many factors. I felt that I was really interested in the application of wine microbiology during the alcoholic fermentation process. With this enthusiasm, I joined the Oenological Biotechnology Group of the Rovira i Virgili University. The group has experienced in technologies to study the microbial populations, being skilled in qualitive and quantitative analysis. It could also perform a rapid and sensitive detection of various fermentative products and had a complete and independent system to support the entire fermentation process, from yeast cultures to wine storage. This laid a foundation for me to complete my PhD study from 2017 to 2021.

As the increase in ethanol content in wine has attracted much attention, the pursuit of health and low-alcohol wine has urged researchers to find ways to reduce the ethanol content in wine. Changing the targeted metabolism of yeasts might be the simplest and most economical way to break away from ethanol yield. Genetic engineering methods can change the carbon metabolism pathway of yeasts. However, the public's attitude towards the application of GMO (genetically modified microorganisms) in foods makes that this goal should be achieved by non-GMO methods, such as strains selection and adaptive evolution. Metabolic differences between yeasts may bring the distinction in ethanol production. Therefore, the selection of yeasts species with low ethanol production has become a topic. Screening *Saccharomyces* or non-*Saccharomyces cerevisiae* species with lower ethanol yield and applying them to the alcoholic fermentation process can improve the ethanol reduction. Moreover, the inoculation strategy used with those non-*Saccharomyces* yeasts, such as coinoculation or sequential inoculation, can also have an effect on the final ethanol reduction. On the other hand, another non-GMO strategy used in the last decades to improve wine yeast has been the adaptive laboratory evolution (ALE), which increases genetic and phenotypic variation of yeast by exerting a selective pressure. This adaptation might result in changes in their metabolism profile, and

therefore, in ethanol production. This process requires cultivating the yeast species in a specific selective environment to develop the desired phenotype.

The hypothesis of this study was that **the application of non-GMO microbial technology can reduce the ethanol content in wine**. In order to demonstrate this hypothesis, our general objective was the selection and application of non-*Saccharomyces* yeast species to reduce ethanol content. Parallelly, different strategies of inoculation by selected non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* or ALE *S. cerevisiae* were evaluated (coinoculation or sequential inoculation), as well as different timing or proportions among them. This general goal can be divided into the following three specific objectives:

1. **To explore a rapid method to screen non-*Saccharomyces* yeast species with a low ethanol yield.**

Non-*Saccharomyces* yeasts are known to reduce ethanol yield during alcoholic fermentation. Due to the diversity of non-*Saccharomyces* yeast species, a rapid method for screening yeasts with a low ethanol yield is required.

The aim of this study was to propose a rapid method to screen yeasts with a low ethanol yield from 45 non-*Saccharomyces* yeast strains, belonging to 19 species. This method included two steps and was complete in 5 days to obtain the selected yeasts. The first stage of screening was done in a low concentration of sugar (YPD liquid medium) during 3 days; a second stage was performed in synthetic must during 2 days. The selection was based on the relationship of sugar consumption and ethanol production.

To verify the practicability of this rapid method, the selected yeasts were applied to a complete sequential fermentation with *S. cerevisiae* in laboratory conditions. Fermentation kinetics, yeast population dynamics and main fermentative by-products were detected to explore the characteristics of selected non-*Saccharomyces* yeasts to reduce ethanol.

The objective is stated in Chapter 1: A rapid method for selecting non-*Saccharomyces* strains with a low ethanol yield. The results are published in Microorganisms (2020) 8, 658.

2. To study the effect of a non-*Saccharomyces* multistarter on ethanol reduction and yeast population dynamics, using different inoculation ratios and strategies.

The use of non-*Saccharomyces* yeast strains in alcoholic fermentation to reduce ethanol is performed following different inoculation strategies, such as single and mixed fermentations. The growth and metabolism of non-*Saccharomyces* yeasts in the mixed fermentation can be affected by other yeasts, which may lead to improve the ethanol reduction and the concentration of main by-products. In addition, the traditional method of plate counting cannot achieve accurate results for a multistarter fermentation. The application of culture independent techniques, such as quantitative PCR, can accurately and effectively obtain the population dynamics for each strain. Exploring the population dynamics of non-*Saccharomyces* yeasts in mixed fermentations can provide a reference for the determination of the strain inoculation strategy in alcoholic fermentation to reduce ethanol content.

The aim of this study was to explore the effect of mixed inoculation of three selected non-*Saccharomyces* yeasts (from *M. pulcherrima*, *T.delbrueckii* and *Z.baili* species) and *S. cerevisiae* on ethanol reduction and population dynamics. As a single starter, all three strains could reduce ethanol content in wine. We hypothesized that a multistarter might improve the reduction of ethanol and the composition of metabolites. In order to achieve the appropriate inoculation strategy, the three strains were used as a multistarter in coinoculated or sequential fermentations with *S. cerevisiae* at different inoculum ratios. Yeast population was monitored by plating samples on WLN agar and by PMA-qPCR techniques, analyzing the evolution of different yeasts during the fermentation. The ethanol production, ethanol yield and the concentration of the main fermentative by-products fermentation characteristics were also determined at the end of the fermentation.

The objective is described in Chapter 2: Effect of a multistarter yeast inoculum on ethanol reduction and population dynamics in wine fermentation. The results are published in Foods (2020) 10, 623.

3. To evaluate the ethanol reduction by selected non-*Saccharomyces* yeast species and evolved *Saccharomyces cerevisiae*.

As mentioned above, the mixed fermentation of non-*Saccharomyces* yeasts and *S. cerevisiae* can reduce the ethanol content in wine. The application of evolved *S. cerevisiae* with a low ethanol production is also a research goal. Evolved *S. cerevisiae* strains can be isolated and obtained using adaptive evolution techniques (long-term and multi-generation cultivation of yeast under selective conditions) by analyzing their fermentative performances and ethanol productions. In a former study, a massive scale evolution was performed on different *S. cerevisiae* strains and different stress conditions related to winemaking process (Ghiacu et al., manuscript in preparation). Some of the evolved *S. cerevisiae* strains were screened for its ability to reduce ethanol.

In this study, two selected non-*Saccharomyces* yeast strains, from *M. pulcherrima* and *L. thermotolerans* species, and three selected ALE *S. cerevisiae* strains (evolved on iso-butanol or high sugar conditions), were used in simultaneous and sequential inoculated fermentations to evaluate their ability to reduce the alcohol content. Yeast population dynamics were monitored, and the concentration of main fermentative by-products was detected to characterize the correlation between fermentation strategies and ethanol reduction.

The objective is presented in Chapter 3: Evaluation of different inoculation strategies, using selected non-*Saccharomyces* and non-GMO enhanced *Saccharomyces cerevisiae* yeasts, on the reduction of the ethanol content in wines, manuscript in final stage of preparation.

INTRODUCTION

1. Yeast and alcoholic fermentation

1.1 Yeast

Yeast is a unicellular microorganism belonging to the class of fungi, ascomycetous genera. Yeast is a facultative anaerobe, and survive in the bark, leaves, flowers, and berries of grapes in the vineyard, therefore, it can be detected at any stage of the winemaking process. Different yeasts isolated from fermented grape juice that have been known belong to more than 40 species (Jolly et al., 2014). Indigenous yeasts found in grapes or grape juice before wine fermentation are broadly divided into two categories, the *Saccharomyces* species and non-*Saccharomyces* yeasts.

Saccharomyces yeasts

Saccharomyces yeasts are the main alcohol producers in fermentation. The species that have enological interest belonging to *Saccharomyces* genus (previously called *Saccharomyces sensu stricto*) have been revised several time in the last decades. Currently, based on the increasing number of sequenced strains, eight species are included in this genus containing *S. cerevisiae*, *S. uvarum*, *S. paradoxus*, *S. eubayanus*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, and *S. jurei* (Naumov et al., 2000; Borneman and Pretorius, 2015; Dujon and Louis, 2017). Two species of this genus are later classified as species hybrids: *S. bayanus* (*S. eubayanus x S. uvarum*) and *S. pastorianus* (*S. cerevisiae x S. eubayanus*) (Naumov et al., 2000; Gibson and Liti, 2014). The evolution of the *Saccharomcyes sensu stricto* group is shown in Figure 1 (cited from Dujon and Louis, 2017). According to the researches in these years, three new species *S. cariocanus*, *S. mikatae* and *S. kudriavzevii*, were discovered since 1990s. However, in recent years, the *S. cariocanus* species has disappeared while other three species were discovered (*S. arboricola*, *S. eubayanus* and *S. jurei*). Among the *Saccharomyces* species, *S. cerevisiae* is the most important species in wine fermentation.

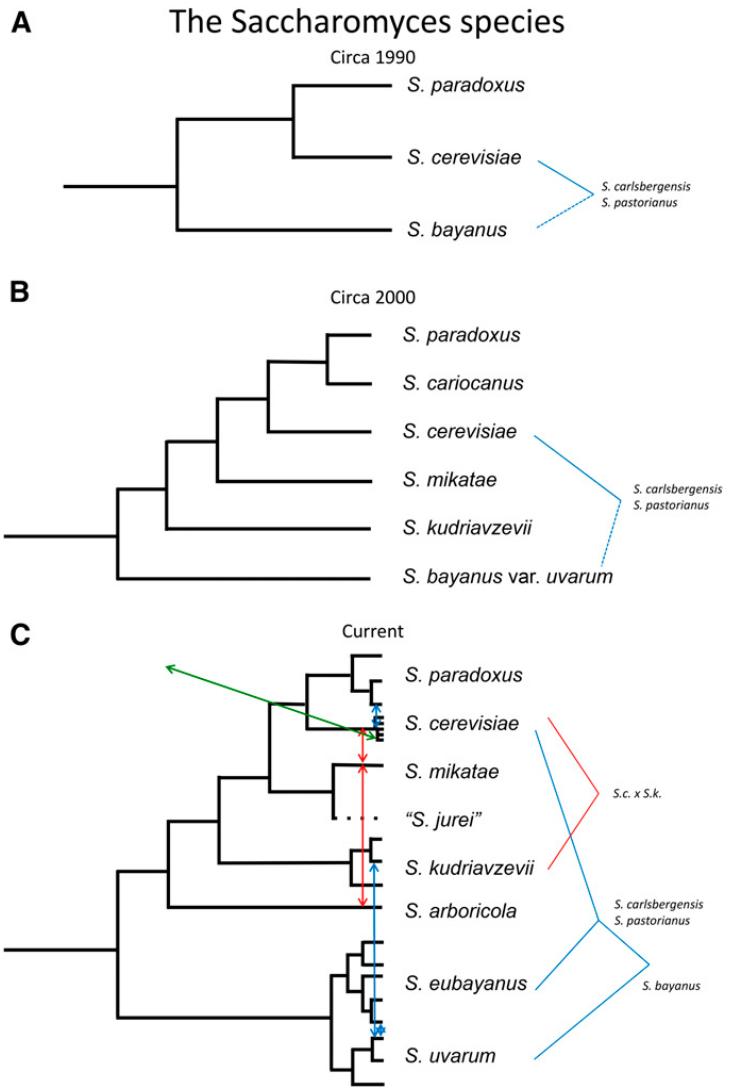

Figure 1. Evolution of the phylogeny of the *Saccharomyces* (formerly *S. sensu stricto*) group since the first genome sequence. (A) By the mid-1980s to mid-1990s, the use of DNA–DNA reassociation and the biological species definition led to the consolidation of the *Saccharomyces* yeasts into three species and one hybrid used in lager fermentation. This hybrid was between *S. cerevisiae* and something close to *S. bayanus*, but not *S. bayanus* itself. (B) By the late 1990s, the use of the biological species definition, along with electrophoretic karyotyping and presence/absence of specific repeated sequences, on isolates in various culture collections resulted in the discovery of three new species, *S. cariocanus*, *S. mikatae*, and *S. kudriavzevii*, and the refinement of *S. bayanus* var. *uvarum* as a species while *S. bayanus* itself appears to be a hybrid. (C) In recent years, whole genome sequencing along with genetic analysis has resulted in the current view of the group. One species (*S. cariocanus*) has disappeared based on phylogeny and three others have been discovered (*S. arboricola*; *S. eubayanus*, the other parent in the lager hybrids; and *S. jurei*). There are many examples of Horizontal Gene Transfer (HGT), red arrows, as well as introgressions, blue arrows. Perhaps the most interesting is the HGT of genes that provide useful traits in wine fermentation, green arrow, which distinguishes the wine yeast from wild European yeasts (Dujon and Louis, 2017).

Despite the abundant microbial diversity in winemaking, *S. cerevisiae* is the main yeast in the alcoholic fermentation process. The first stages of spontaneous fermentations are characterized by the interaction of multiple non-*Saccharomyces* yeasts, however, those species are soon replaced by indigenous *S. cerevisiae* strains who will be the main responsible of the alcoholic fermentation (Taylor et al., 2014; Morrison-Whittle et al., 2015; Varela et al., 2017a). The reason why *S. cerevisiae* can stand out in a multi-microbial environment and dominate the alcoholic fermentation is due to its high fermentation capacity and the ability to adapt to scarce oxygen and nutrients. Specifically, *S. cerevisiae* is tolerant to high concentration of ethanol and organic acids, has a low nitrogen requirement, and ferments sugars at low pH values, although it may bring less variety and flavour compounds to wines (Bisson, 1999; Albergaria and Arneborg, 2016). All characteristics described above make *S. cerevisiae* more advantageous when competing with other yeast species.

Non-*Saccharomyces* yeasts

Approximately twenty genera of non-*Saccharomyces* yeasts are relevant to winemaking, listed in Table 1 (Fleet, 1993; Kurtzman and Fell, 1998a). The non-*Saccharomyces* yeasts found in grape must are mainly belonging to *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia* and *Pichia* species. In addition, some studies stated that strains from *Dekkera*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* species can also be observed (Romano et al., 2003; Fleet, 2003 & 2008).

Non-*Saccharomyces* yeasts can also have a positive or negative impact on the quality of the wine, such as reducing ethanol production (Gobbi et al., 2014; Englezos et al., 2016; Martorell et al., 2019), affecting flavor substances which will enhance the complexity of the wine (Tronchoni et al., 2018; Dutraive et al., 2019; Gamero et al., 2020), or modulating the final wine acidity, by either reducing malic acid content (Thornton et al., 1996) or increasing the concentration of lactic acid (Kapsopoulou et

al., 2007), among others. Negatively, most of the non-*Saccharomyces* species have low fermentation power and low SO_2 resistance (Rementeria et al., 2003; Jolly et al., 2006).

Table 1. Non-*Saccharomyces* yeasts related to winemaking (Kurtzman and Fell, 1998a; Fleet, 1993).

Teleomorphic ascomycetous genera (Ascomycotina)	Anamorphic ascomycetous genera (Deuteromycotina)	Anamorphic heterobasidio-mycetous genera (Basidiomycotina)
Citeromyces, *Debaryomyces* *Dekkera*, *Hanseniaspora* *Issatchenkia*, *Kluyveromyces* *Lodderomyces*, *Metschnikowia* *Pichia*, *Saccharomycodes* *Schizosaccharomyces*, *Torulaspora* *Zygoascus*, *Zygosaccharomyces*	*Brettanomyces* *Candida* *Kloeckera*	*Cryptococcus* *Rhodotorula*

1.2 Alcoholic fermentation

Traditional fermentation is a spontaneous process characterized by a rich biodiversity, including bacteria, yeasts, and other fungi. As an important role in fermentation, yeast can metabolize glucose, fructose and other monosaccharides into carbon dioxide and ethanol under anaerobic conditions through the action of enzymes. This process is called alcoholic fermentation (Fleet and Heard, 1993; Fugelsang, 1997). In the alcoholic fermentation process, glucose and fructose are the main carbon sources. The metabolism of glucose by yeasts mainly includes two stages, glycolytic pathway, and anaerobic degradation of pyruvate. Specifically, the first stage is to metabolize glucose into pyruvate, and the second stage is to catalyze pyruvate to generate acetaldehyde and carbon dioxide through pyruvate decarboxylase, and then further reduce acetaldehyde to ethanol (reviewed by Varela et al., 2015). The metabolic pathways involved in ethanol production by *S. cerevisiae* is shown in Figure 2 (referring to Varela et al., 2015; Ciani et al., 2016; Hranilovic et al., 2018).

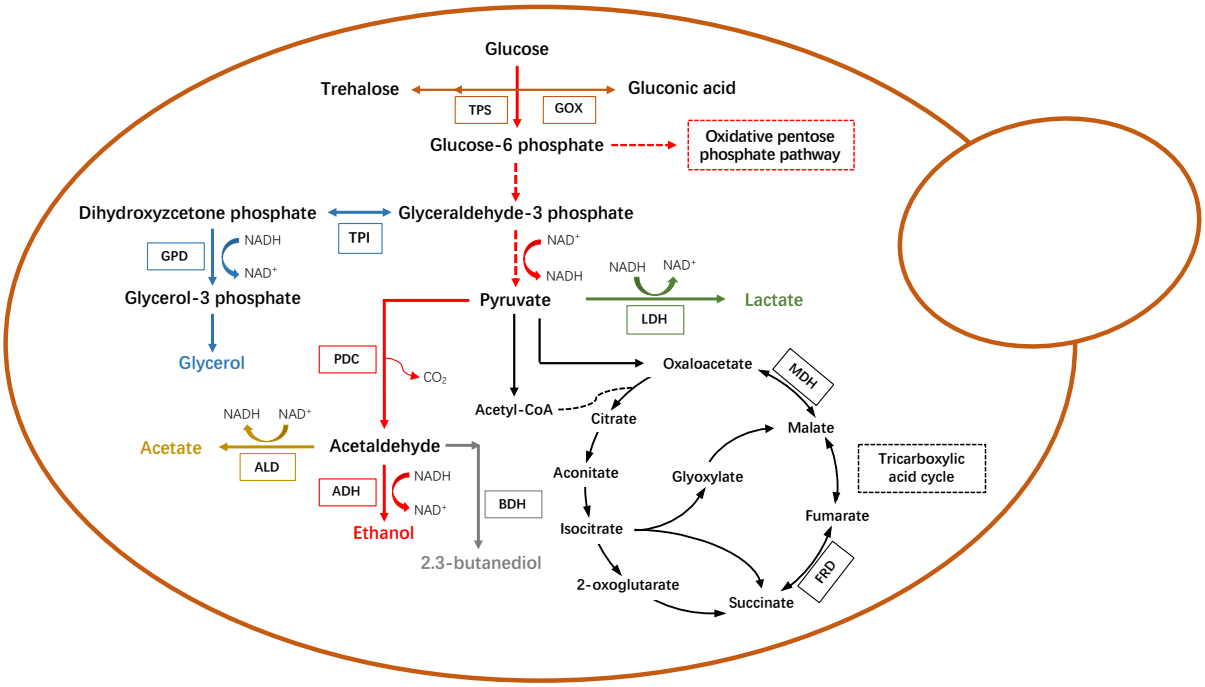

Figure 2. Metabolic pathways involved in ethanol production by *S. cerevisiae*. ADH, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; BDH, butanediol dehydrogenase; FRD, fumarate reductase; GOX, glucose oxidase; GPD, glycerol-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDC, pyruvate decarboxylase; TPI, triosephosphate isomerase; TPS, trehalose-6-phosphate synthase (referring to Varela et al., 2015; Ciani et al., 2016; Hranilovic et al., 2018).

The spontaneous fermentation is performed by the sequential action of different yeast strains which are naturally present on the grape berries or in the winery equipment (Ribereau-Gayon et al., 2000). Some yeast species are selected in reasons of ecological determinants and technological parameters during winemaking process (Ciani et al., 2010). The main purpose of using selected starter cultures is to standardize the quality of the wine, and to ensure the completion of the process, avoiding sluggish or stuck fermentations (Bisson, 1999). Spontaneous fermentation could be stagnant and accompanied by the production of unfavorable metabolites (Spano et al., 2010; Capozzi et al., 2011).

The use of yeast starters, mainly commercial *S. cerevisiae* strains in form of Active Dry Yeast (ADY), has been used for several decades in winemaking, as a way to control the fermentation process (Mas et al., 2016). However, with this inoculation

strategy, a single yeast strain is responsible of the fermentation, reducing the impact of other yeast species, as well as the overall complexity of the wine. More recently, non-*Saccharomyces* yeast species with different properties have been tested and used also as starters in winemaking (Cañas et al., 2011; Escribano-Viana et al., 2018; Gamero et al., 2020). Indeed, some non-*Saccharomyces* yeast species, such as *L. thermotolerans*, *M. pulcherrima* and *T. delbrueckii* (Padilla et al., 2016), have been commercialized as ADY in the past few years.

Those non-*Saccharomyces* yeast starters are usually used in mixed inoculum fermentations, together with *S. cerevisiae*, to ensure the completion of the fermentation. In this case, *S. cerevisiae* and non-*Saccharomyces* yeasts are present in the fermentation, being inoculated in two different ways: i) simultaneous inoculation, in which a mixture of non-*Saccharomyces* and *Saccharomyces* yeasts in equal or different ratios is inoculated to start fermentation; and ii) sequential inoculation, in which non-*Saccharomyces* yeasts are inoculation at the beginning of the fermentation, and *Saccharomyces* yeasts are inoculated after 24 – 72 h or when 20 – 50% sugar has been consumed (Contreras et al., 2014b; Canonico et al., 2016; Lleixà et al., 2016a; Varela et al., 2016; Hranilovic et al., 2020). In sequential fermentations, non-*Saccharomyces* yeasts consume part of the nutrients present in the media, affecting the nutrient availability for *S. cerevisiae*. A common practice to avoid this nutrient limitation and favor *S. cerevisiae* growth, is to use nutrient supplementation (Ciani et al., 2010; Lleixà et al., 2016a; Wang et al., 2016; Roca-Mesa et al., 2020). Since non-*Saccharomyces* yeasts have the potential risk of negatively affecting wine flavor and quality during winemaking process, the research on the oenological characteristic of strains to obtain "selected" yeasts has become a challenge.

1.3 Yeast physiology and growth

The environmental changes faced by yeast during alcoholic fermentation, such as low oxygen, pH, osmotic pressure, temperature, nutrient limitations, presence of SO_2 and other toxic substances, among others, may have negative effects on yeast growth,

and in the availability of nutrients (Heard and Fleet, 1988; Hansen et al., 2001; Jolly et al., 2003; Rementeria et al., 2003; Maturano et al., 2015) Therefore, some factors affecting the yeast growth will also affect fermentation performance and the final composition of the wine (Heard, 1999).

Ethanol

As mentioned above, non-*Saccharomyces* yeasts may be replaced by *S. cerevisiae* in the final stages of alcoholic fermentation. One of the reasons is that some non-*Saccharomyces* yeasts cannot tolerate high concentrations of ethanol. It is proved that some non-*Saccharomyces* yeasts belonging to genera *Kloeckera*/*Hanseniaspora* and *Candida* only survive at the early stage of fermentation due to their intolerance to ethanol (Padilla et al., 2016). However, other studies have confirmed that *H. guilliermondii* has a higher ethanol tolerance similar to *S. cerevisiae* (Pina et al., 2004). Similarly, results from Roca-Mesa et al. (2020) verified that *L. thermotolerans* and *T. delbrueckii* can tolerate high concentrations of ethanol, being able to complete the fermentation as single inoculum. In addition, the reduction of fermentation temperature enhances the tolerance of non-*Saccharomyces* yeasts to ethanol. For example, *C. stellata* and *H. uvarum* have higher ethanol tolerance at 10 °C than that at 25 °C (Gao and Fleet, 1988).

Sugar and nitrogen

The sugar and nitrogen content in grape must are important components that affect the growth of yeasts (Beltran et al., 2005; Lleixà et al., 2016a; Roca-Mesa et al., 2020). Specifically, the concentration of sugars (glucose and fructose) affects the osmotic pressure of the must, and due to the poor osmotic pressure tolerance of some non-*Saccharomyces* yeasts, must with high sugar concentration is not conductive to the survival of these non-*Saccharomyces* yeasts (Heard, 1999). In addition, it has been reported that high levels of sugar affect the population proportion of yeasts inoculated into fermentation (Lleixà et al., 2016a).

The shortage of nitrogen content in alcoholic fermentation is able to inhibit the growth of yeasts, and a nitrogen content of at least 140 mg/L is required for the yeasts to complete the fermentation (Bell and Henschcke, 2005; Martínez-Moreno et al. 2012). In sequential fermentations, non-*Saccharomyces* strains such as *S. bacillaris* and *T. delbrueckii*, consumed most of the nitrogen within 72 h, which limits the growth of the subsequent *S. cerevisiae* (Roca-Mesa et al., 2020). Lleixà et al. (2016a) concluded that the limited nitrogen content will extend the fermentation time and increase the proportion of non-*Saccharomyces* yeasts in the middle and end of fermentation. Therefore, exploring the appropriate nutrient composition ratio of must is assistant to obtain a stable fermentation, and to adjust the supplement of nutrients during fermentation process when yeasts need.

1.4 Microbial interactions

Microbial interactions can also affect yeast growth, involving either interaction between yeasts or between yeast and bacteria (Fleet, 2003). Specifically, the competition mechanism triggered by the utilization of nutrients such as amino acids and vitamins by yeasts may be detrimental to other species. Some metabolites or proteins released in the media by different microorganisms, such as ethanol or some glycoproteins can inhibit or destroy other species (Fleet et al., 2003; Albergaria et al., 2010). In addition, some fermentative by-products derived from yeast metabolism, such as acetic acid, medium-chain fatty acids, acetaldehyde, and the synergistic effect triggered by the combination of these substances are also important roles in the inhibition mechanism; on the contrary, the cellular material released by yeast autolysis during metabolism can promote the growth of other yeasts (Ludovico et al., 2001; Fleet, 2003).

During the interaction, the growth of non-*Saccharomyces* yeasts is limited by *S. cerevisiae*. For example, some toxic substances produced by *S. cerevisiae* are suspected to be responsible for the death of *H. guilliermondii* (Pérez-Nevado et al., 2006). In addition, high concentrations of *S. cerevisiae* as a starter culture can inhibit the growth

of non-*Saccharomyces* yeast through cell-to-cell mechanism (Nissen et al., 2003; Wang et al., 2016). Some studies have found that killer strains of *S. cerevisiae* can be isolated from fermented grape juice, and become the dominate when fermentation is complete (Musmanno et al., 1999; Guriérrez et al., 2001; Fleet et al., 2003). On the contrary, Killer strains can also be isolated in wines made by non-*Saccharomyces* strains, such as *Candida*, *Hanseniaspora*, *Pichia* and *T. delbrueckii*, which have a killer effect on *S. cerevisiae* (Fleet and Heard, 1993; Ramirez et al., 2015). The interaction between killer strains may determine the evolution of species. Therefore, through studying the killer interaction, as well as inoculation amount and inoculated time of *S. cerevisiae* and non-*Saccharomyces* strains, the contribution of non-*Saccharomyces* yeasts to alcohol fermentation can be controlled, highlighting its advantages.

1.5 Main by-products and effect on wine

During alcoholic fermentation, the metabolism of *Saccharomyces* and non-*Saccharomyces* yeasts produce, a part of ethanol, different by-products that affect to the quality and flavor of the wine. Yeast strains have abilities to produce different volatile compounds through metabolism during alcoholic fermentation (Figure 3) (Pretorius et al., 2012).

Those compounds, include glycerol, esters, higher alcohols, organic acids, fatty acids, aldehydes, and sulfides, are part of the secondary aroma of wine. The content of these metabolites is affected by the inoculation of *Saccharomyces* and non-*Saccharomyces* yeasts, thereby affecting the sensory characteristics and quality of wine. As mentioned above, non-*Saccharomyces* yeasts have shown potential in enhancing wine aroma, however, their negative effects cannot be ignored. Table 2 describes the oenological characteristic and negative effects of some non-*Saccharomyces* species on wine (reviewed by Ciani and Comitini, 2011; Capozzi et al., 2015).

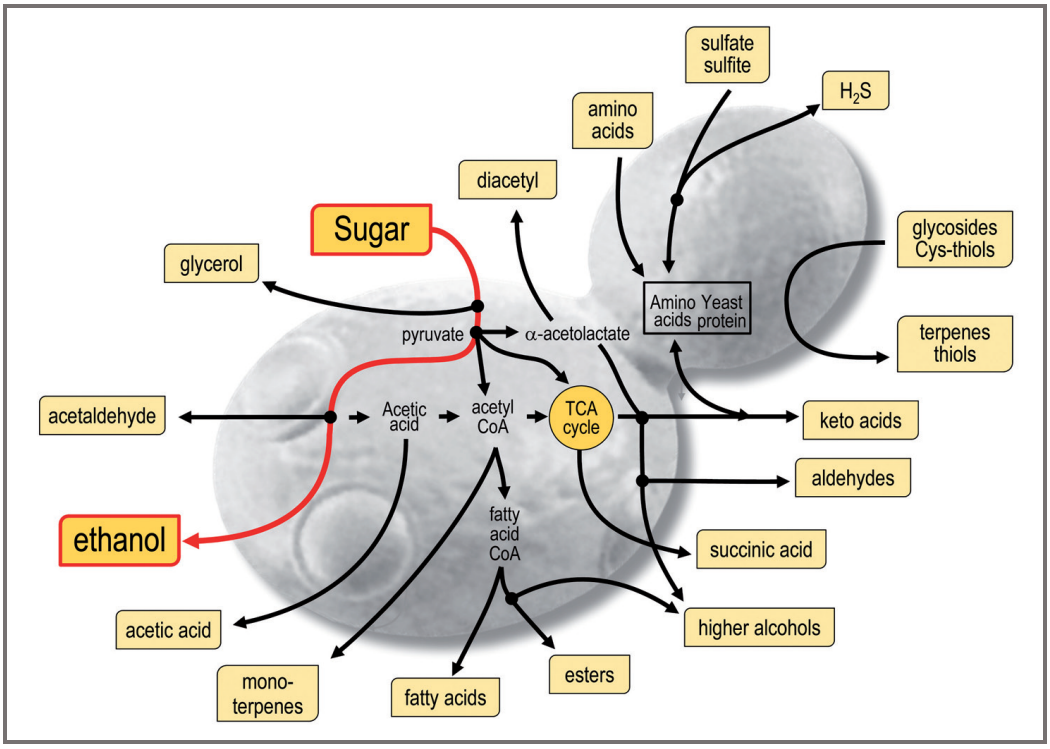

Figure 3. Commercial yeast strains possess different abilities to form and modulate compounds that impact on wine sensory properties. These compounds are produced as a result of yeast metabolic processes (Pretorius et al., 2012).

In addition to studying the alcoholic fermentation characteristics of different yeast genera, several studies have summarized the specificity of fermentation by-products produced by different non-*Saccharomyces* yeast species. For example, yeasts from *M. pulcherrima* species can increase the concentration of higher alcohols, while may reduce acetaldehyde and volatile phenols content; fermentation by *T. delbrueckii* and *Rhodotorula* species achieved a higher concentration of esters in alcoholic fermentation process; the content of higher alcohols and lactic acid are increased in fermentation by *L. thermotolerans* (reviewed by Padilla et al., 2016). The production of several metabolites, such as glycerol and acetic acid can affect wine flavour and aroma (Goold et al., 2017). Glycerol can provide mellowness and sweetness and make a positive contribution to wine quality. It is usually present at a concentration of 5 – 9 g/L (Noble and Bursick, 1984). Excessive accumulation of acetic acid (greater than 0.8 g/L) is undesirable for sensory quality of wine (Fleet and Heard, 1993).

Considering the positive and negative effects of the enumerated different non-*Saccharomyces* yeasts in alcoholic fermentation process, mixed starters to be applied in fermentation should be designed (Ciani and Comitini, 2011). The fermentation of mixed inoculation in different grape juices should be verified since different nutrient compositions of grape juice. More importantly, due to the expansion of fermentation volume and the limitation of oxygen, the application of mixed fermentation on an industrial or semi-industrial scale also needs to be clarified (Beltran et al., 2008; Viana et al., 2009). Therefore, a better management of mixed starter cultures can modify alcoholic fermentation reasonably.

Table 2. Principal oenological characteristic and risk of non-*Saccharomyces* yeast of wines (reviewed by Ciani and Comitini, 2011; Capozzi et al., 2015).

Yeast genera	Oenological characteristics	Negative affect	Effects produced by mixed fermentation with *S. cerevisiae*, compared with pure *S. cerevisiae* culture
Torulaspora	Low concentration of acetic acid	Slower fermentation rate; Production of sulphur compounds	Low concentration of acetic acid (*delbrueckii*)
Debaryomyces	High level of *β*-glucosidase activity	No describe	Increase in terpenols content (*variji*)
Starmerella	Fructophilic yeast combined consumption of reduced sugars; High glycerol producer; High succinic acid producer; High acetaldehyde producer; High acetoin producer; Low ethanol yield	No describe	High glycerol producer; High succinic acid producer; Low ethanol yield (*bombicola*)
Metschnikowia	High concentration of esters; Increase wine flavor and aroma; Antimicrobial activity (*pulcherrimin*)	Delays in fermentation due to antimicrobial activity	High concentration of esters; Increase wine flavor and aroma; Increase glycerol content (*pulcherrimin*)
Hanseniaspora	Increased amounts of 2-phenyl-ethyl acetate, higher alcohols, acetate, ethyl esters and medium-chain fatty acids; Reduced level ocratoxine A; High acetic acid producer	Negative compounds (volatile acidity, sulphur compounds, etc.); Biogenic amine production; Production of acetoin; Sluggish or stuck fermentation	Slight increase in ethyl acetate production (strong reduction in comparison with pure culture)(*uvarum*); Increase in 2-phenyl ethyl acetate (*osmophila*)
Candida	High glycerol producer (up to 14 gl-1), low acetic acid concentration; Increased concentrations of terpinol; Decreased concentrations of aldehydes and acetate esters	Production of sulphur compounds; Slower kinetics rate (low ethanol concentration)	No describe
Kluyveromyces	Enhancement of aroma and flavor; Increased concentrations of lactic acid, glycerol and 2-phenylethanol; Low acetaldehyde producer	Higher “spicy” and “acidity” attributes	Reduction in final acetaldehyde formation; Increase in titratable acidity (*thermotolerans*)

Yeast genera	Oenological characteristics	Negative affect	Effects produced by mixed fermentation with *S. cerevisiae*, compared with pure *S. cerevisiae* culture
Issatchenkia	Increase free monoterpenes and non-isoprenoids; Reduction of malic acid content	Production of biogenic amines	No describe
Pichia	Increased concentrations of volatile compounds (acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl octanoate, isoamyl acetate, 3-mercaptohexyl acetate, 2,3-butanediol and glycerol); Increased concentration of polysaccharides	Antimicrobial activity against S. cerevisiae	Increase in isoamyl acetate production (*anomala*); Increase in thiols content (*kluyveri*)
Zygosaccharomyces	Low concentration of acetic acid, H_2S, SO_2, malic acid degradation; High fermentative power; Increased concentration of polysaccharides	High amount of acetic acid	Low concentration of succinic acid (*bailii*)
Schizosaccharomyces	Degradation of malic and gluconic acid	Increased concentration of acetaldehyde, propanol and 2,3-butandiol; Low concentration of esters	Reduction in titratable acidity

2. Monitoring of the alcoholic fermentation

To monitor the alcoholic fermentation process, some physical and microbiological indicators are usually measured during the fermentation process. On the one hand, physical indicators mainly include the measurement of weight loss, must density, temperature, and production of carbon dioxide (CO_2). On the other hand, the yeast population dynamics is analyzed to reflect the fermentation process.

2.1 Physicochemical indicators

During the activity of microorganisms, nutrients are constantly being consumed in the fermentation process, such as sugars, nitrogen, oligoelements, vitamins and mineral salts. The consumption of sugars can be directly and easily monitored by either the decrease of the density of the media, or by the decrease of the weight. Gravimetric determination is the easiest way to monitor fermentation kinetics, which can be used to determine the termination of fermentation through detecting the daily weight of fermented must until a constant value (Tristezza et al., 2016). A similar method is density detection, which usually uses a densitometer to measure the daily density of the suspension after centrifuging fermenting must until a stable value is detected (Lleixà et al., 2016b). The main basis for density monitoring to judge the alcoholic fermentation process is the consumption of sugars. Studies have concluded that in a simulated wine solution containing ethanol, glycerol and sugar, sugar is the substance that can most influence the density value, followed by ethanol (Nurgel et al., 2005). Therefore, researchers track the fermentation process by directly measuring the residual sugar content daily until a stable value (Varela et al., 2016 & 2017b; Lin et al., 2020). The amount of carbon dioxide (CO_2) released is also considered as an indicator, which is due to the release of CO_2 caused by yeast metabolism (Maturano et al., 2019), and directly related to the production of ethanol (Zhang et al., 2019). Also, the respiratory quotient (RQ), which is related to glucose consumption, can be used to investigate the level of respiro-fermentative metabolism of different yeasts (Quirós

et al., 2014; Morales et al., 2015). The application of above indicators to map fermentation kinetics is usually accompanied by the measurement of temperature, because of the change of temperature affect the growth rate of microorganisms and thus affect the consumption of nutrients (Tronchoni et al., 2018).

2.2 Population dynamics and its monitorization

The monitoring of fermentation is often accompanied by the population dynamics of yeasts which follows the yeasts population in the early, middle and end stages of the fermentation process. The spontaneous fermentations are characterized by a diversity of microorganisms, being different yeast species are present at different stages of fermentation. Yeasts from *Kloeckera*, *Hanseniaspora*, *Metschnikowia*, *Candida* and *Pichia* genera tend to dominate the early stages of fermentation (Barnett, 2000). At the beginning of fermentation, the number of non-*Saccharomyces* yeasts in grape juice is approximately 10^4 to 10^5 cells/mL, growing to a final population of 10^6 to 10^8 cells/mL during fermentation. It has been reported that the initial population of *S. cerevisiae* is much lower than that of non-*Saccharomyces* yeasts, being almost not recovered in healthy grapes or grape must (Jemec et al., 2001; Beltran et al, 2002). However, due to the excellent adaptation to the fermentative environment, such as low pH, high sugar content and resistance to alcohol, *S. cerevisiae* dominates the end of fermentation despite the small population at the beginning. *Candida* species (lately renamed as *Starmerella*) are present at the beginning of fermentation, but they can be also recovered in the last stages of fermentation (Llauradó et al, 2002; David et al., 2014). In addition, the population dynamics of strains from *Hanseniaspora* species is prolonged in low temperature environments (Andorrà et al., 2010b; Maturano et al., 2015).

The traditional counting methods of yeasts are mainly based on their macroscopic, microscopic, physiological and biochemical characteristics (Barnett, 1992; Yarrow, 1998), so microscope observation, optical density at 600 nm (OD600) and culture-based techniques are frequently used. Overall, microscope observation and OD600 are

used to monitor the total yeast population (Ribéreau-Gayon et al., 2006). As culture-based techniques, the plating the samples on general, differential or selective media, such as YPD (Yeast Extract Peptone Dextrose) agar, WLN (Wallerstein Laboratory Nutrient) agar and lysine agar medium, is often used.

The application of molecular identification techniques has been widely used in wine microbiology, being more time efficient and reliable (Andorrà et al., 2010a & b; Albertin et al., 2014; Wang et al., 2015b; Navarro et al., 2020). Compared with traditional physiological identification methods, the results from molecular identification are more consistent because changes may occur in the physiological states of yeasts during different adaptation processes as they occur during fermentation (Mitrakul, 1999).

2.2.1 Culture-dependent techniques

Due to a complex microbial system in spontaneous fermentation, the qualitative and quantitative progression of wine microorganisms have been thoroughly studied. The traditional yeast viability analysis is the counting of colony forming units (CFU/mL) after plating the samples in the appropriate medium. This method is a culture-dependent technique. For example, WLN agar and lysine agar media can be used for monitoring the viable yeast population during the alcoholic fermentation. The WLN agar is a differential medium, which can distinguish different yeast species based on their color and colony morphology (Medina et al., 1997; Pallmann et al., 2001; Li et al., 2018) (Figure 4), while the lysine agar is a selective medium for non-*Saccharomyces* yeasts, as those species can grow using lysine as a single nitrogen source, but not *Saccharomyces* yeasts (Aa Kühle and Jespersen, 1998).

However, some yeast derived from different genera or species may present similar colony morphologies on plates, and would not be differentiated and properly identified in mixed fermentations or cultures. Therefore, DNA-based technologies should be combined to properly identify yeast species.

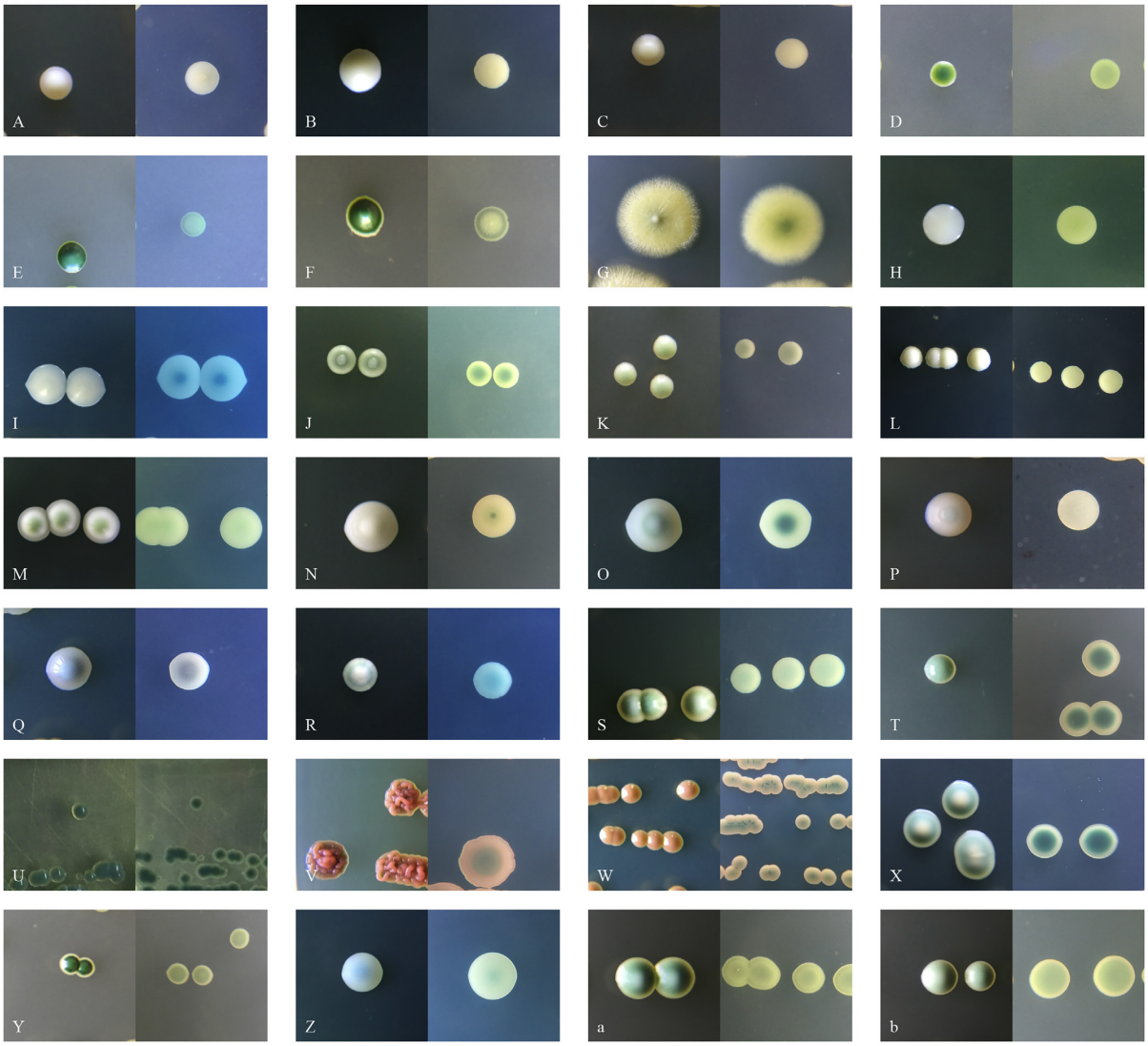

Figure 4. The photographs of twenty-eight colony morphotypes on the WLN agar (both front and back sides). A-*Zygosaccharomyces parabailii*, B-*Zygosaccharomyces bailii*, C-*Saccharomyces cerevisiae*, D-*Hanseniaspora uvarum*, E-*Hanseniaspora uvarum*, F-*Hanseniaspora osmophila*, G-*Aureobasidium pullulans*, H-*Candida zeylanoides*, I-*Candida railenensis*, J-*Candida argentea*, K, L, M-*Torulaspora delbrueckii*, N, O-*Debaryomyces hansenii*, P-*Metschnikowia pulcherrima*, Q-*Metschnikowia sinensis*, R, S-*Zygotorulaspora florentina*, T-*Cryptococcus flavescens*, U-*Cryptococcus magnus*, V-*Sporidiobolus pararoseus*, W-*Rhodotorula babjevae*, X-*Curvibasidium pallidicorallinum*, Y-*Starmerella bacillaris*, Z-*Candida kofuensis*, a-*Saccharomyces uvarum* and b-*Saccharomyces sp.* (Li et al., 2018).

The DNA-based technologies used for species identification are usually based on the polymerase chain reaction (PCR) of ribosomal DNA (rDNA) sequence. The structure of rDNA contains encoding units (18S, 5S, 5.8S, and 26S rDNA), internal transcriber spacers (ITS) and external transcriber spacers (ETS) (Fernández-Espinar et

al., 2006). The yeast species can be identified using rDNA sequence analysis including 5.8S ITS1/ITS4 (by using the amplification and restriction analysis of this region) or 26S D1/D2 (by sequencing this region) (Kurtzman and Robnett, 1998b; Hierro et al., 2006; Nisiotou et al., 2007; Bovo et al., 2009; Li et al., 2018). Esteve-Zarzoso et al. (1999) used PCR-RFLP (Restriction Fragment Length Polymorphism) of 5.8S-ITS rDNA to identify 132 yeast species from 25 different genera, which was confirmed as a rapid and easy method for species identification. In addition, Terminal Restriction Fragment Length Polymorphism (T-RFLP) can also be used to species identification of yeasts (Efriwati et al., 2013).

2.2.2 Culture-independent techniques

The application of culture-independent techniques can be used to analyze accurately the yeast population dynamics during alcoholic fermentation. Although plate counting has been the most used methodology to monitor the yeast growth, it presents some disadvantages. It is a time consuming technique, since plates have to be incubated for 2 – 3 days. In addition, as described in 2000 by Millet and Lonvaud-Funel, some cells could not produce colonies on the plate but could be counted by the direct epifluorescence technique (DEFT), they stated these cells in a state of viable but not-culturable (VBNC). Thus, in order to better determine the yeast viability during fermentation, the culture-independent techniques are required for the identification of yeast species.

Culture-independent techniques that are used in most studies include quantitative PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), massive sequencing and techniques combined with fluorescence microscopy or flow cytometry (Hierro et al, 2006 & 2007; Díaz et al. 2010; Andorrà et al., 2011; Wang et al., 2014, 2015a & b; Bachmann et al., 2015; Portillo et al., 2016; Navarro et al., 2020). The qPCR technology will be further discussed since it will be applied in the present thesis.

2.2.3 qPCR analysis

Quantitative PCR (qPCR), also named real time qPCR (RT-qPCR) (Figure 5) (VanGuilder et al., 2008), is a sensitive and rapid method to detect and quantify the population of each yeast species in a specific sample. qPCR monitors the amplification of a targeted DNA molecule during the PCR, in real time, not at its end. This technique uses specific primers for each yeast species, and DNA-binding fluorescent chemicals to measure the total amount of DNA product present after each PCR cycle. In the exponential increase of the PCR amplification, the threshold cycle (Ct) value obtained has a linear correlation with the initial DNA concentration of the sample, thus it serves as the basis for quantification. It is a method for quantitative analysis of specific DNA sequences in the samples, which corresponds to the quantification of a specific microorganism in the samples.

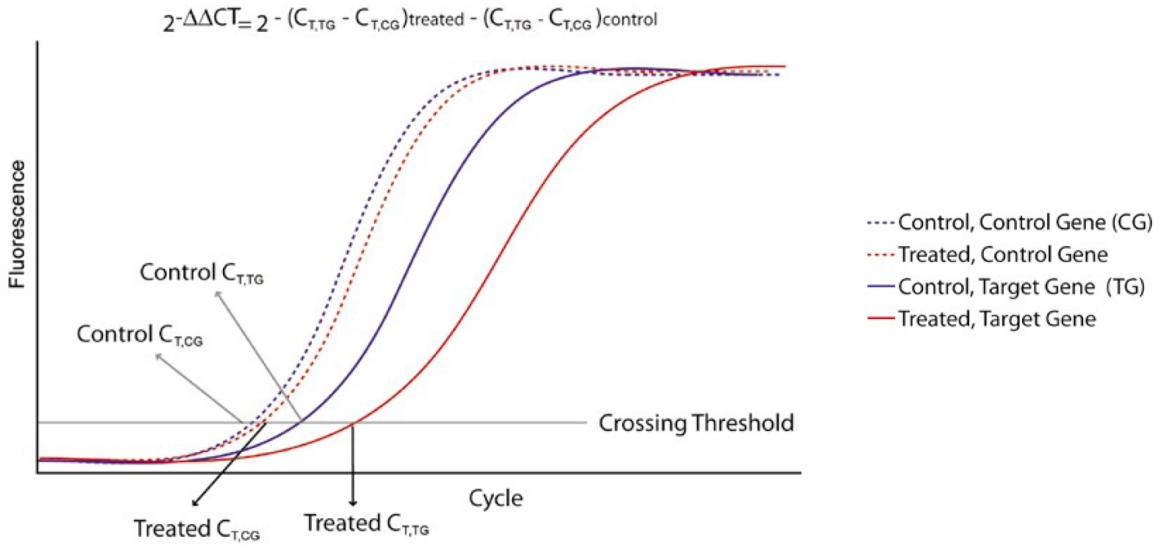

Figure 5. Quantitation by Real-Time qPCR. Mathematical basis of the 2-ΔΔCT method. The 2-ΔΔCT method enables relative quantitation (treated sample is X fold of control sample) through measurements of crossing thresholds (CT) (VanGuilder et al., 2008).

The key parameters in the quantification process are related to the minimum concentration of the target which can be described as the limit of the detection (LoD, the lowest amount of measurand in a sample that can be probability detected) and the limit of quantification (LoQ, the lowest amount of measurand in a sample that can be quantitatively determined with acceptable accuracy under stated experimental

conditions) (Bustin et al., 2009; Forootan et al., 2017). Studies concluded that the LoD and LoQ of one yeast do not change even in the presence of interference from living cells of other species (Andorrà et al., 2010a; Wang et al., 2015a).

A wide detection range can be achieved by qPCR analysis, ranging from 10 to 10^8 cells/mL (Rawsthorne and Phister, 2006); however, different yeast species can have different LoD values. For example, some yeast species reached the minimum LoD of approximately 10 cells/mL such as *Hanseniaspora spp.*, *Saccharomyces*, *I. orientalis*, *T. delbrueki* and *Z. bailii*; others such as *C. zemplinina* and *L. thermotolerans* have the minimum LoD of 10^2 cells/mL; whereas that of *M. pulcherrima* is 10^3 cells/mL (Rawsthorne and Phister, 2006; Zott et al., 2010; Navarro et al., 2020). Although qPCR technique can detect extremely low concentrations of yeast populations, the determination of the LoQ of different strains in qPCR analysis is worth exploring, which provides the basis for the treatment of cells when monitoring the population of yeast during fermentation.

During alcoholic fermentation, some wine related yeasts were identified and enumerated using qPCR technique, such as *A. pullulans* (Wang et al., 2020), *B. bruxellensis* (Vendrame et al., 2014; Wang et al., 2020), *C. californica* (Wang et al., 2020), *C. stellata* (Hierro et al., 2006; García et al., 2017a), *C. zemplinina* (Hierro et al., 2007; Zott et al., 2010; Andorrà et al., 2010a & 2012), *C. pallidicorallinum* (Wang et al., 2020), *D. bruxellensis* (Phister and Mills, 2003; Hierro et al., 2006; Andorrà et al., 2010a), *H. uvarum* (Hierro et al., 2006 & 2007; Andorrà et al., 2010a & 2012; Wang et al., 2015a; Padilla et al., 2017), *Hanseniaspora spp.* (Andorrà et al., 2010b; Zott et al., 2010), *I. orientalis* (Zott et al., 2010), *L. Kononenkoae* (Hierro et al., 2007), *L. thermotolerans* (García et al., 2017a; Navarro et al., 2020), *M. caribbica* (Wang et al., 2020), *M. chrysoperlae* (Wang et al., 2020), *M. guilliermondii* (Wang et al., 2020), *M. Pulcherrima* (Zott et al., 2010; García et al., 2017a; Padilla et al., 2017; Navarro et al., 2020; Wang et al., 2020), *T. delbrueckii* (Zott et al., 2010; García et al., 2017a; Padilla et al., 2017; Navarro et al., 2020), *S. bacillaris* (Wang et al., 2015a; Padilla et al., 2017), *Saccharomyces spp.* (Andorrà et al., 2010b; Zott et al., 2010), *S. cerevisiae* (Andorrà et al., 2010a & 2012; Wang et al., 2015a;

García et al., 2017a; Padilla et al., 2017; Navarro et al., 2020; Wang et al., 2020), *S. pombe* (García et al., 2017a) and *Z. Bailii* (Rawsthorne and Phister, 2006; Andorrà et al., 2010a).

2.2.4 Detection of viable but not-culturable yeasts (VBNC)

The interaction between yeasts causes cell death and accumulation during alcoholic fermentation. Compared with culture-dependent techniques, qPCR technology has high sensitivity, however, the results are not so accurate since the basis for monitoring is the DNA, which is rather stable with time, even when cells are dead. Thus, it detects the total cell population. The dynamics of yeast population is often characterized by the population of living cells during fermentation, but the presence of dead cells can interfere the enumeration. To solve this problem, some modifications have been applied and incorporated into qPCR. For example, reverse-transcription PCR (RT-PCR) can be considered a better technique to quantify viable yeasts from rRNA (Hierro et al., 2006; Wang et al., 2015a). However, some concerns have been raised on the stability of this molecule and its use for the determination of total viable cells (Sunyer-Figueras et al, 2018). Similarly, viability-qPCR is also regarded as an effective strategy. This has an extra pretreatment procedure before qPCR analysis using a DNA-binding dye, such as ethidium monoazide (EMA) or propidium monoazide (PMA). These dyes bind to DNA of dead cells which cannot be amplified during the subsequent qPCR analysis, thereby, detecting only the population of living cells (Nocker et al., 2006; Andorrà et al, 2010a; Elizaquível et al., 2014). This working principle is shown in Figure 6 (Emerson et al., 2017). Although these two dyes behave similarly when embedded in cells, they have different permeability when crossing the cell membrane. Fittipaldi et al. (2012) concluded that PMA is more effective and selective than EMA in discrimination of living and dead cells. The effect of PMAxx treatment in living and dead cells is shown in Figure 7 (Navarro et al., 2020).

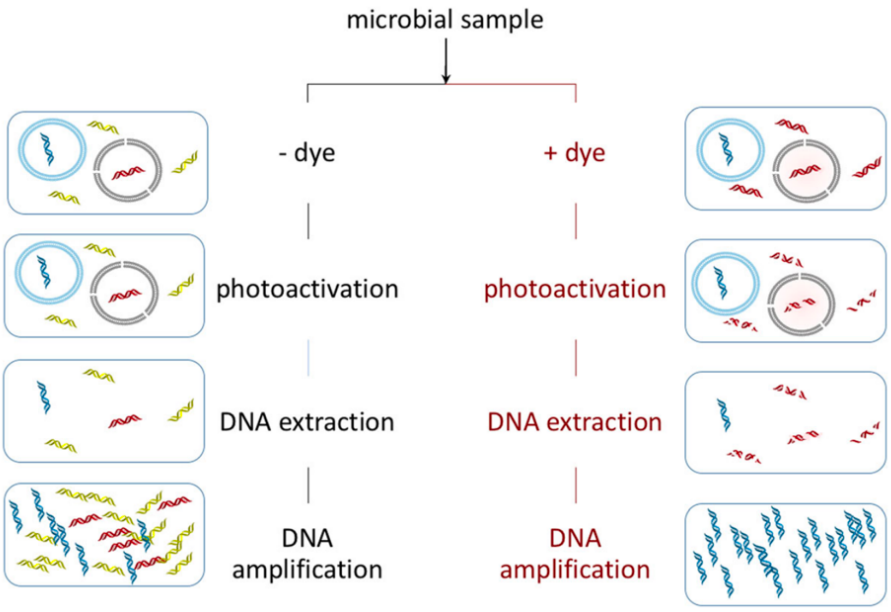

Figure 6. Viability PCR workflow (e.g., using EMA, PMA, or similar dyes). The initial sample is divided in two. One sample (left side) remains untreated, leaving total DNA—including extracellular DNA (yellow) and DNA in living (blue DNA, blue membrane) and dead (red DNA, black membrane) cells—relatively intact and available for downstream applications. The other sample (right side) is stained with a viability dye that binds to free DNA and to DNA in cells with compromised membranes. Upon photoactivation in the treated sample, bound DNA is degraded, such that it is no longer a suitable template for amplification. After amplification, a comparison of treated versus untreated samples can reveal relative proportions and/or types of living and dead microorganisms (Emerson et al., 2017).

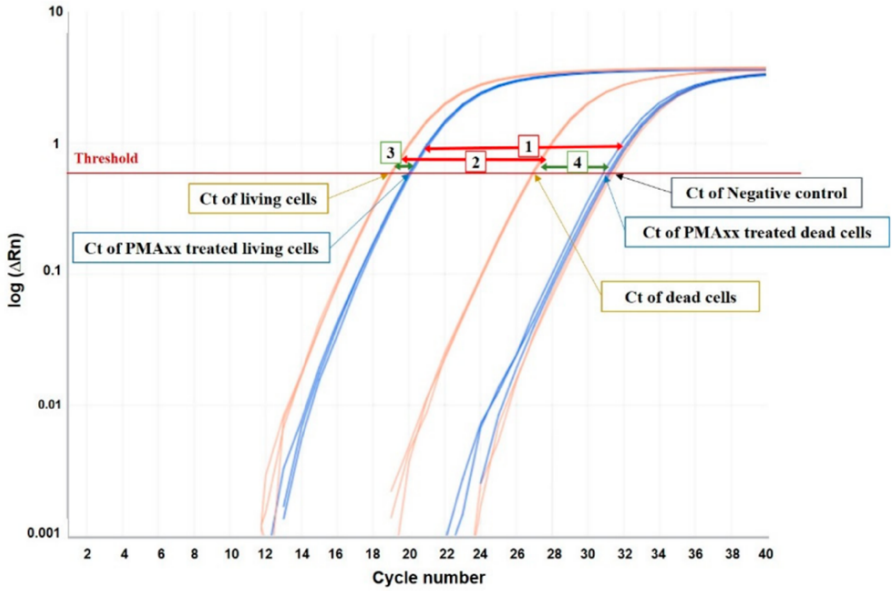

Figure 7. Schematic representation of the effect of PMAxx treatment in living and dead cells. Red arrows represent the cycle threshold (Ct) reduction obtained by subtracting the mean Ct values of living cells from those of dead cells, (1) with or (2) without PMAxx treatment. Green arrows represent the Ct reduction obtained by subtracting the mean Ct values obtained from PMAxx-qPCR of (3) living or (4) dead cells from the mean Ct values obtained from non-dyed qPCR (Navarro et al., 2020).

Viable but not-culturable yeasts are not able to grow in the culture medium, however, they still affect the characteristics of alcoholic fermentation. A recent research revealed that *M. pulcherrima* and *L. thermotolerans* yeasts are in the VBNC state at the end of fermentation, which could not be observed on WLN agar but well counted by PMA-qPCR analysis (Navarro et al., 2020). In general, the techniques for the detection of VBNC yeasts compensate the main drawback of traditional techniques of viable cell counting, and can estimate the survival of some non-*Saccharomyces* yeasts at the end of fermentation.

3. Ethanol and ethanol reduction

3.1 Changes of ethanol content in wine

In recent years, global warming and consumer requirements have increased the ethanol content in wine. Specifically, the content of ethanol in wine was increased by 2 – 3% (v/v) in the last two decades (Jones et al., 2011; Godden et al., 2015), which was attributed to climate change characterized by increasing temperature. The climate change results in faster grape ripening and lower total acidity, as well as the increase of sugar accumulation in grapes with an insufficiently phenolic maturity (Jones et al., 2005; Koufos er al., 2014; Godden et al., 2015). This tendency is common in most wine producing regions. In addition, consumer's demand for full-bodied and deeply colored red wines drove the evolution of winemaking styles to encourage the harvest of full-ripe grapes containing a high phenol concentration that could be extracted. Fully ripe grapes are usually rich in high concentration of glucosides and give wines higher alcohol content.

The changes in grape berry composition caused by the maturity affect the wine flavor and quality. The increased temperature during the ripening of the berries will cause faster pulp maturation and increase the content of the must soluble solids. Another related trend is the reduction of total acidity which has a negative effect on the sensory profile and microbial stability of wine, and higher pH value can reduce the antibacterial effect of sulfites.

3.2 The effects of ethanol on wine

Ethanol is the most important volatile compound in wine, it affects both the sensory perception of aromatic compounds and the detection of aromatic complexity (Goldner et al., 2009). In addition, ethanol can influence wine viscosity and body, and also our perceptions of sourness, sweetness, aroma and flavor, enhancing our perception of hotness and bitterness while diminishing the astringency of tannins (Fishcer and Noble, 1994; Pickering et al., 1998a; Fontoin et al., 2008; Meillon et al.,

2009). A high alcohol content in wine has negative effects on human health and, presently, is not appreciated by a wide part of consumers that prefer drinking lighter and responsibly (Salamon, 2006), thus it discourages wine consumption. Higher alcohol build-up during fermentation increases the risk of stuck or sluggish fermentation because the growth of microorganisms is limited with the increasing ethanol content during fermentation (Bisson, 1999). This fact can result in severe economic losses in the wine industry, which could be worsen by the increase in taxes during the export process due to high alcohol content.

3.3 Approaches to reduce ethanol content

Despite the increasing ethanol content in wine, a great number of consumers from several countries, especially from Europe, demand low ethanol beverages (9 – 13%, v/v) as a result of health and social concerns (Masson et al., 2008; Labanda et al., 2009; Saliba et al., 2013). Combined with the above description, the higher alcohol content in wine stimulates great attention of wine researchers to develop different approaches to reduce ethanol content in wine.

3.3.1 Viticultural interventions

The ethanol content in wine is mainly determined by the concentration of sugar accumulation in grapes. The sugar concentration gradually increases as the grape ripens, therefore, the intervention in the vineyard can reduce the accumulation of sugars, thereby reducing the content of ethanol in wine. The interventions to reduce the sugar accumulation through vineyard management is shown in table 3. The strategies are mainly including increase yield, remove leaves, planting environment, change irrigation, choose rootstocks and harvest time. In general, the choice of these strategies can bring some negative effects, mainly due to substandard maturity of grape berries, which affects the yield and quality of the wine.

Table 3. Vineyard management to reduce ethanol content in wine (summarized from Qzturk et al., 2014; Varela et al., 2015).

Methods	Implement	Influences	References
Increasing yield	Enhancing the bud load, lowering cluster thinning and choosing a vigorous rootstock	The potential detrimental effects on grape and wine quality	Novello and Palma, 2013; Kliewer and Dokoozlian, 2005
Leaf area management	Basal leaf removal	Increase phenolic development; better synchronization of sugar; delay in maturity; reduced soluble solids content; reduced anthocyanins content	Korkutal and Bahar, 2013; Di Profio et al., 2011; Stoll et al., 2010; Poni et al.,2013 & 2014; Filippetti et al., 2015
Vineyard site	Slopes shaded, reduce sun exposures and the environmental temperature	Grape yield and quality	Salamon, 2006; Smart and Robinson, 1991; Novello and Palma,2013
Soil composition	Acidity and mineral combinations: reduce magnesium and increase nitrogen	Delay ripening, loss of aromatic quality, increase dehydration, increase grape production and decrease fruit quality	Salamon, 2006; Bottcher et al., 2011
Irrigation strategy	Reduce shoot vigor, get small berries and clusters	Reduce grape maturity level	Clingeleffer, 2007
Rootstocks	Clonal selection for managing the vigor of the plant and scion	Enhance color and phenolics, and reduce the content of soluble solid	Bordenave et al., 2013; Novello and Palma, 2013
Harvest time	Early grape harvest or sequencing harvest	Organoleptic defects: herbaceous character and high acidity levels, lower pH and higher titratable acidity; Less fruity attributes, viscosity and hotness	Novello and Palma, 2013; Longo et al., 2018; Bindon et al., 2013 & 2014

3.3.2 Pre-fermentative applications

The pre-fermentation treatment is mainly dedicated to the use of various techniques to reduce the sugar content and change the composition of grape juice (Salamon, 2006; Schmidtke and Blackman, 2012; Bauer et al., 2013). However, the method like dilution of the must is illegal according to the wine regulations in most wine-producing countries. (Salamon, 2006).

A variety of membrane filtration techniques are applied to the wine production at different stages for different purposes. For example, the ultrafiltration and nanofiltration are used to clarify wine or removal sugars in must (Varsari et al., 2003;

Rektor et al., 2007; Labanda et al., 2009; Mihnea et al., 2012). As membrane treatments, based on the fractionalization of the sample into permeate and retentate streams, nanofiltration membranes have been used to remove sugar from must before alcoholic fermentation (Saha et al., 2013). According to the results from Martin et al. (2010), an ethanol reduction of 2% (v/v) was achieved by two steps of nanofiltration, however, the produced wine lost color and aromas.

In addition, the glucose oxidase (GOX) enzyme treatment is another popular method that can be used into the must to reduce ethanol content in wine. This kind of method is based on the glucose conversion, specifically, the oxidation of β-D-glucose is catalyzed to gluconic acid and hydrogen peroxide by the GOX instead of ethanol (Pickering et al., 1998b; Bankar et al., 2009). As a previous research concluded, via the treatment of GOX, the ethanol content could be reduced by 1 – 1.3% (v/v) in aerated synthetic grape must (Biyela et al., 2009). However, the concentration of carbonyl compounds content increased with GOX treatment, and those can be combined with sulfur dioxide leading to a higher demand of SO_2 (Pickering et al., 2001). In addition, the possible reduction of the microbial ability, the loss of aroma and the increase of processing costs should be considered when using this enzyme treatment method (Pickering et al., 1999a & b).

3.3.3 Fermentative interventions

Different techniques have been applied during fermentation process to reduce ethanol production, for example, changing alcohol production metabolism as much as possible to convert sugar into other metabolites instead of ethanol, or inhibiting the fermentation. Those changes mainly come from yeast strains, including *Saccharomyces* and non-*Saccharomyces*. Therefore, the major challenge is to select yeast strains through GMO (genetically modified organism) or non-GMO methods. However, the concentration of other metabolites should be also considered, in case their accumulation will affects the quality of the wine.

Most GMO approaches aimed to increase glycerol or gluconic acid concentration, release glucose repression of respiration and change the NAD^+/NADH ratio (Remize et al., 1999; Malherbe et al., 2003; Henricsson et al., 2005; Cambon et al., 2006; Heux et al., 2006). The carbon metabolism in *S. cerevisiae* is characterized by the Crabtree effect (Pronk et al., 1996; Ribéreau-Gayon et al., 2006). According to the way of regulating respiro-fermentative metabolism, yeast species are generally categorized as Crabtree-positive or Crabtree-negative, or obligate respiratory. As Crabtree positive microorganism, *S. cerevisiae* preferentially chooses glucose as the carbon source in metabolic process. However, the presence of glucose inhibits respiration and gluconeogenesis, which leads to a fermentative metabolism, and a high ethanol production (Kayikci et al., 2015; Alipourfard et al., 2019). The goal of releasing glucose repression as a strategy to lower ethanol production is to activate the respiration of *S. cerevisiae*, or to turn the metabolism target of yeast to other carbon sources, resulting in an ethanol reduction. To modify the TCA cycle, a change of the NAD+/NADH ratio is pursued. A NADH (Nicotinamide adenine dinucleotide) oxidase is introduced to reduce the activity of ADH (Alcohol dehydrogenase), and as consequence, the production of ethanol is decreased (Varela et al., 2015). Therefore, changing the metabolism of *S. cerevisiae* through genetic engineering strategies can be accomplished by improving the relevant genes shown in Figure 8 (Pretorius et al., 2012).

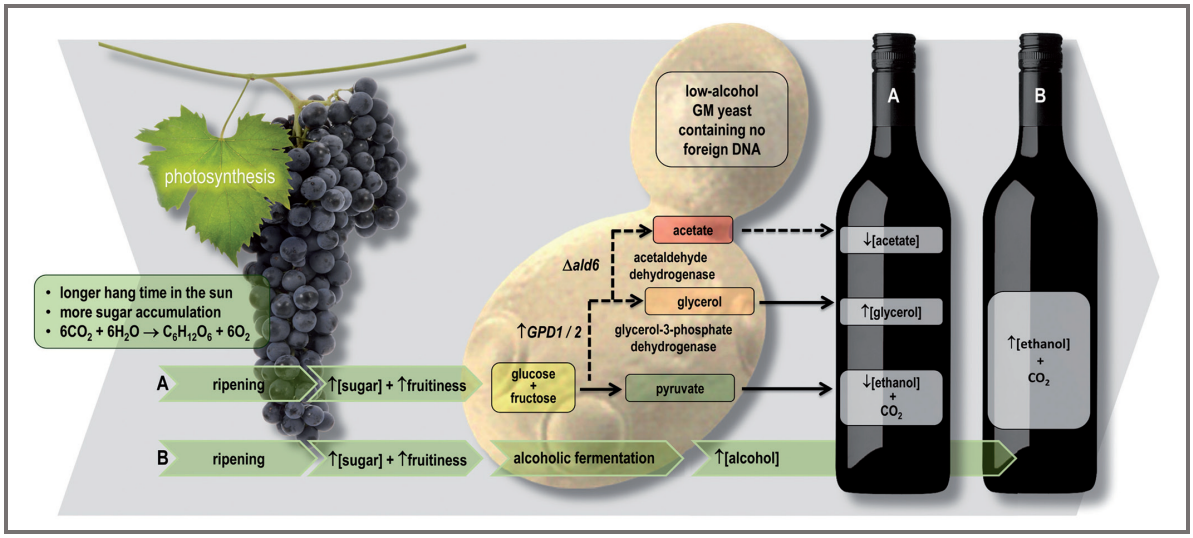

Figure 8. Reducing alcohol levels in wine: several GM-based strategies have been explored to generate wine yeasts that partially divert sugar metabolism away from ethanol production. (A) Two glycerol-3-phosphate dehydrogenase isozymes, GPD1 and GPD2, can be harnessed to divert carbon from glycolysis to glycerol production. However, increased glycerol production was accompanied by undesirable increased concentrations of acetic acid. This problem was alleviated by knocking out ALD6. (B) Wild-type yeast convert most of the sugar they consume into ethanol and CO2 (Pretorius et al., 2012).

Non-GMO strategies to screen *S. cerevisiae* strains for reducing ethanol content are related to improve the metabolic characteristics by selecting the environmental adaptability of yeast species, mainly to screen yeasts from different environmental conditions, such as high sugar conditions or nutrition limited conditions (Ghiaci et al., 2013). The environmental factors, such as sugar abundance or oxygen availability, affect respiratory behavior of yeast species (Rodrigues et al., 2016), and as a consequence, the changes of respiro-fermentative metabolism toward to producing secondary by-products such as glycerol or organic acids, which could lead to ethanol reduction.

One of the non-GMO methods used to reduce ethanol yield has been based on adaptive laboratory evolution (ALE), also called evolutionary engineering. As mentioned above, ALE can drive the redirection of carbon flux in the yeast metabolism by exerting selective pressure. A remarkable feature of microorganisms is that they can adapt to different environmental conditions. Therefore, this method is based on

growing yeast cells for several generations under stress environmental conditions in order to obtain the desired genetic and phenotypic variation/adaption. For example, this method has been conducted to transfer carbon metabolism to the pentose phosphate pathway, and to glycerol through the adaptive evolution of sulfites (Cadière et al., 2011; Kutyna et al., 2012). In another study, *S. cerevisiae* strains were cultivated under high osmotic pressure to achieve the effect of enhancing glycerol and reducing ethanol production, with the reduction of 1.3% (v/v) (Tilloy et al., 2014). Therefore, the use of ALE is a reasonable and effective solution to reshape the pathway of yeast metabolism.

Non-*Saccharomyces* strains have also been used in alcoholic fermentation for lowering alcohol in wine. The application of non-*Saccharomyces* is mainly to use different inoculation strategies, such as sequential inoculation and simultaneous inoculation with *S. cerevisiae*, to complete the ethanol reduction. Different yeast species consume sugars by respiration rather than fermentation and produce the desired concentration of secondary metabolites according to the degree of aerobic respiration, including aerobic, semi-aerobic and anaerobic conditions (Gonzalez et al., 2013; Quirós et al., 2014; Contreras et al., 2015). In the sequential fermentation, the performance of non-*Saccharomyces* yeasts in aerated conditions before inoculating *S. cerevisiae* was the main reason of the ethanol reduction compared to the pure culture *S. cerevisiae* (Figure 9) (Gonzalez et al., 2013).

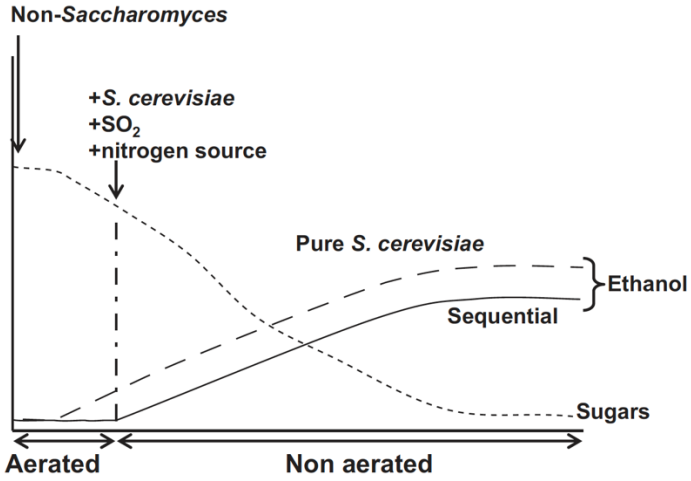

Figure 9. Idealized representation of the expected evolution of ethanol production during grape must fermentation in a sequential inoculation with a Crabtree-negative non-*Saccharomyces* yeast strain, followed by *S. cerevisiae* when indicated (continuous line). Aeration would be restricted to the first stages of alcoholic fermentation. 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 (Gonzalez et al., 2013).

Studies have shown that some non-*Saccharomyces* species, such as *C. sake*, *M. pulcherrima*, *K. lactis*, *K. exigua*, *T. delbrueckii* and *Z. bailii*, exhibit the ability to reduce ethanol content under aerated conditions, and it has found that *M. pulcherrima* achieved an ethanol reduction of 1.6% (v/v) in sequential fermentation (Contreras et al., 2014a; Quirós et al. 2014; Contreras et al., 2015). Then Morales et al. (2015) studied the potential contribution of respiration to ethanol reduction in coinoculated fermentation with *M. pulcherrima* and *S. cerevisiae*. This study concluded that the oxygen concentration and ethanol production are negatively correlated, and the concentration of ethanol can be reduced by as much as 3.7% (v/v) at the end of fermentation, however, this ideal reduction in ethanol content is accompanied by unacceptable levels of volatile acidity. In addition, the temperature of fermentation can also affect ethanol production. In 2019, Maturano et al. found the lowest ethanol production in sequential fermentation by *H. uvarum* or *C. membranaefaciens* with *S. cerevisiae* when the fermentation was performed at 25 °C rather than those at 15 °C and 20 °C.

3.3.4 Post-alcoholic fermentation interventions

The post-fermentation techniques mainly include the blending of high and low alcohol wines, and the physical removal of alcohol. In details, the removal of ethanol can be accomplished by different techniques, such as membrane filtration, distillation, rotating cone column, adsorption, freeze concentration, evaporation and supercritical extraction (Pickering, 2000; Schmidtke et al., 2012).

Blending high-alcohol and low-alcohol wine is a direct strategy to reduce ethanol content in wine. However, the low-alcohol wine is made with grapes harvested in advance, and thus, it may affect the sensory profile of wine. To improve the flavor of this kind of wine, Kontoudakis et al. (2011) used charcoal and bentonite to remove the phenolic substances and green flavor in the low-alcohol wine, and then the resultant colorless and tasteless wine is mixed with high-alcohol wine, reducing the ethanol content by 0.9 – 3% (v/v). The sensory analysis concluded that two of the wines from three different varieties had no significantly differences compared with the control. Regardless of this, this strategy still needs more research to confirm its potential.

Semi-permeable membranes can be used to remove alcohol from wine. Massot et al. (2008) used semi-permeable membranes made of 0.65 μm and 0.45 μm filter cartridges to filter ethanol in wine. Reverse osmosis is a commonly used technique, where wine is filtered in pressure through a fine porous membrane permeable to water and ethanol at low temperatures. This method has less negative impact on wine, however, the applied pressure can cause the temperature increase of the membrane surface. Although most dissolved substances are not able to pass through the semi-permeable membrane, some aroma compounds could be released along with the alcohol (Pickering, 2000).

The rotating cone column, as a new technology for removing ethanol, mainly includes three steps. Firstly, the aroma compound in wine are recovered under low temperature conditions. Subsequently, ethanol is removed under vacuum conditions. Finally, the resultant dealcoholized and deodorized wine is mixed with the recovered aroma compounds. This technology is capable to reduce heat damage and the loss of

aroma compounds in wine. However, the high investment cost of this technology is not conducive to the small-scale wine production (Pickering, 2000; Belisario-Sánchez et al., 2009; Schmidtke et al., 2012).

In addition, supercritical extraction uses CO_2 to separate ethanol and aroma compounds after precipitation of extracted wine components under specific pressure and temperature conditions (Pickering, 2000). Although this method does not cause any degradation (Fornari et al., 2009), due to its high cost, supercritical extraction cannot be widely used.

3.4 Selection of yeast strains to reduce ethanol content in wine

As described above, different strategies can be applied to reduce ethanol content in wine at different stages of fermentation. However, the negative impact of these methods on wine fermentation is obviously, such as high cost, time-consuming, legal prohibition or off-flavor. Compared with the strategies listed above, non-GMO microbial approaches are efficient, inexpensive and easy to implement.

As described above, *S. cerevisiae* is the main yeast in the alcoholic fermentation process. In the case of low glucose concentration and aerobic conditions, respiratory metabolism occurs, while under high sugar concentration conditions, alcoholic fermentation is the main metabolism. With the addition of oxygen or aeration, *S. cerevisiae* can reduce the ethanol content. However, it usually increases the concentration of acetic acid, which may negatively affect wine flavor (Quirós et al., 2014; Contreras et al., 2015).

Some strains belonging to *Saccharomyces* clade, such as *S. kudriavzevii*, *S. uvarum* or *S. paradoxus*, can reduce ethanol content (Magyar and Tóth, 2011; Contreras et al., 2015; Varela et al., 2016). A study has shown that *S. uvarum* decreased the ethanol content by 0.7% (v/v) with a higher concentration of acetic acid during the fermentation of Malvasia delle Lipari wines, compared to the fermentation with *S. cerevisiae* (Muratore et al., 2007). Another study found that, *S. uvarum* produced an ethanol reduction of 1.7% (v/v) while increasing the concentration of glycerol in

fermented Merlot wine (Varela et al., 2017b). However, wines produced by *S. uvarum* usually increased the concentration of compounds considered as off-flavors to wine (Varela et la., 2018). Recently, Alonso-del-Real et al. (2017 & 2019) studied *S. kudriavzevii* species for its ability to reduce ethanol and to absorb and utilize nutrients. *S. kudriavzevii* could achieve an ethanol reduction of 1% (v/v) accompanied by an increase concentration of glycerol in the mixed fermentation inoculated with *S. cerevisiae*, compared to the single inoculation of *S. cerevisiae* (Alonso-del-Real et al., 2017).

Non-*Saccharomyces* yeasts have been reported to improve wine aroma and flavor (Ciani et al., 2010; Padilla et al., 2016; Belda et al., 2017). Due to the diversity of non-*Saccharomyces* species, their ability to reduce alcohol is being studied (Contreras et al., 2014a; Quirós et al., 2014; Ciani et al., 2016). The application of non-*Saccharomyces* yeasts to reduce alcohol is presented in single or mixed fermentations, and the latter is mainly combined with *S. cerevisiae* which is added sequentially or simultaneously because most of non-*Saccharomyces* yeasts would lead to fermentations sluggish or stuck (Bisson, 1999). Some reports have discussed that certain non-*Saccharomyces* yeasts are able to consume carbon sources and reduce the conversion of carbon to ethanol (Contreras et al., 2015; Morales et al., 2015). Table 4 lists the application of non-*Saccharomyces* species to reduce ethanol content in wine and their impact on by-products of after alcoholic fermentation. Strains from *Candida*, *Hanseniaspora*, *Lachancea*, *Metschnikowia*, *Meyerozyma*, *Pichia*, *Schizosaccharomyces*, *Starmerella*, *Torulaspora*, *Wickerhamomyces* and *Zygosaccharomyces* species can be used for reducing ethanol content in wines. being sequential inoculation mostly used by researchers. Among those species, *M. pulcherrima* is presented as the most suitable strain to reduce ethanol in wine, with the maximal ethanol reduction of 3.8% (v/v) as single inoculation (Röcker et al., 2016), and up to 2% (v/v) in sequential fermentations (Aplin and Edward, 2020). Additionally, ethanol reductions above 3% (v/v) could be achieved in fermentations inoculated with *C. membranaefaciens*, *H. uvarum* or *T. delbrueckii* (Brou et al., 2018; Maturano et al., 2019). *C. sake* and *P. kluyveri* have the potential to reduce

ethanol more than 2% (v/v) (Röcker et al., 2016; Ballester-Tomás et al., 2017; Aplin and Edwards, 2020).

The increase in oxygen supplement can enhance the growth and persistence of non-*Saccharomyces* yeasts, however, it may have a negative impact on wine sensory and increase off-flavor of wine (Röcker et al., 2016; Shekhawat et al., 2017; Tronchoni et al., 2018). In addition, both the inoculum size of non-*Saccharomyces* yeasts and the inoculation time of *S. cerevisiae* in sequential fermentation can affect the ethanol reduction. Generally, the inoculum size of non-*Saccharomyces* yeast is negatively correlated with the ethanol production (Maturano et al., 2019). In the sequential fermentation of *H. uvarum* and *S. cerevisiae*, the ethanol reduction of *S. cerevisiae* inoculated at 72 h was less than that at 48 h, with the reduced concentration of 0.78 and 1.06% (v/v), respectively (Canonico et al., 2016). The effect of this inoculation time on ethanol reduction was adapted to *H. uvarum*, and was also confirmed by Maturano et al. (2019). On the contrary, for *H. osmophila*, *M. pulcherrima*, *S. bombicola* yeast species, the ethanol reduction was higher when *S. cerevisiae* was inoculated at 72 h than that at 48 h (Canonico et al., 2016).

Table 4. Summary of non-*Saccharomyces cerevisiae* in reducing ethanol content and their impact on by-products of alcoholic fermentation.

Non-*Saccharomyces* yeast	Must	Inoculation	Ethanol reduction (%, v/v)	Impact on wine composition	References
Candida membranaefaciens	NM	Co-I	3.0	Increased 1-Propanol content; reduced acetic acid content	García et al., 2010
Candida membranaefaciens	NM	Sequential	3.11	Increased acetic acid content	Maturano et al., 2019
Candida oleophila	NM	Sequential	0.7	Increased acetic acid content	Aplin and Edwards, 2020
Candida sake	NM	Single	2.4	Increased sorbitol; low production of glycerol	Ballester-Tomás et al., 2017
Candida stellata	NM	Sequential	0.64	Increased concentration of glycerol and succinic acid	Ferraro et al., 2000
Candida zemplinina	SM	Co-I	0.24	Increased glycerol content	Comitini et al., 2011
Candida zemplinina	NM	Single	1.04	Increased acetic acid content	Sadoudi et al., 2012
Candida zemplinina	NM	Single	0.84	Increased concentration of acetic acid and glycerol	Röcker et al., 2016
Hanseniaspora opuntiae	NM	Sequential	0.6	Increased glycerol content	Rossouw and Bauer, 2016
Hanseniaspora osmophila	SM	Sequential	1.33	Increased concentration of acetic acid and succinic acid	Canonico et al., 2016
Hanseniaspora uvarum	SM	Sequential	0.78	Increased concentration of acetic acid and glycerol	Canonico et al., 2016
Hanseniaspora uvarum	NM	Sequential	3.29	Increased glycerol content	Maturano et al., 2019
Lachancea thermotolerans	NM	Sequential	0.35	Increased lactic acid content	Binati et al., 2020
Lachancea thermotolerans	NM	Sequential	0.68	Increased concentration of lactic acid and glycerol	Gobbi et al., 2013
Metschnikowia pulcherrima	NM	Co-I	1.9	Increased concentration glycerol; decreased acetic acid content	Morales et al., 2015
Metschnikowia pulcherrima	SM	Sequential	1.35	Increased concentration of acetic acid and succinic acid	Canonico et al., 2016
Metschnikowia pulcherrima	NM	Single	3.76	Increased glycerol content	Röcker et al., 2016
Metschnikowia pulcherrima	NM	Sequential	0.6 – 1.3	Increased glycerol content	Tronchoni et al., 2018
Metschnikowia pulcherrima	NM	Sequential	0.9	Increased glycerol content	Puškaš et al., 2019
Metschnikowia pulcherrima	NM	Sequential	1.38	Increased concentration of esters and alcohols	Canonico et al., 2019a
Metschnikowia pulcherrima	NM	Sequential	1.6	Excessive ethyl acetate content	Canonico et al., 2019b
Metschnikowia pulcherrima	NM	Sequential	1.7 – 2.5	Increased glycerol and succinic acid. Reduced acetic acid content	Hranilovic et al., 2020
Metschnikowia pulcherrima	NM	Sequential	2.0	Reduced acetic acid content	Aplin and Edwards, 2020
Meyerozyma guilliermondii	NM	Sequential	1.4	Reduced acetic acid content	Aplin and Edwards, 2020

Non-*Saccharomyces* yeast	Must	Inoculation	Ethanol reduction (%, v/v)	Impact on wine composition	References
Pichia guilliermodii	NM	Single	2.0	Increased concentration of acetic acid and glycerol	Röcker et al., 2016
Pichia kluyveri	NM	Single	3.03	Increased concentration of acetic acid and glycerol	Röcker et al., 2016
Pichia kluyveri	NM	Sequential	0.4 – 1.6	Reduced acetic acid content	Aplin and Edwards, 2020
Pichia kudriavzevii	SM	Sequential	1.0 – 1.6	Decreased succinic acid content	Contreras et al., 2015
Schizosaccharomyces pombe	NM	Single	0.65	Increased glycerol content; decreased total acidity	Benito et al., 2013
Starmerella bacillaris	NM	Sequential	0.7	Increased concentration of glycerol and acetic acid	Englezos et al., 2016
Starmerella bacillaris	NM	Sequential	0.6	Increased glycerol content	Englezos et al., 2019
Starmerella bombicola	SM	Sequential	1.28	Increased glycerol, acetic acid and succinic acid content	Canonico et al., 2016
Torulaspora delbrueckii	SM	Sequential	1.0	Increased concentration of glycerol and succinic acid	Contreras et al., 2015
Torulaspora delbrueckii	SM	Single	3.29	Increased glycerol content	Brou et al., 2018
Torulaspora delbrueckii	NM	Sequential	0.5 – 1.9	Increased acetic acid content	Tronchoni et al., 2018
Torulaspora delbrueckii	NM	Sequential	0.9	Increased glycerol content	Canonico et al., 2019b
Torulaspora delbrueckii	NM	Sequential	0.52	Increased glycerol content	Puškaš et al., 2019
Wickerhamomyces anomalus	NM	Sequential	1.0	Increased acetic acid content	Aplin and Edwards, 2020
Zygosaccharomyces bailli	NM	Sequential	1.0	Increased higher alcohols and volatile acids content	Canonico et al., 2019b
Zygosaccharomyces bailli	SM	Sequential	1.0 – 1.8	Decreased glycerol content	Contreras et al., 2015

NM: Natural must; SM: Synthetic must; Co-I: Coinoculation.

CHAPTER 1

A rapid method for selecting non-*Saccharomyces* strains with a low ethanol yield

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Abstract

The alcohol content in wine has increased due to external factors in recent decades. In recent reports, some non-*Saccharomyces* yeast species have been confirmed to reduce ethanol during the alcoholic fermentation process. Thus, an efficient screening of non-*Saccharomyces* yeasts with low ethanol yield is required due to the broad diversity of these yeasts. In this study, we proposed a rapid method for selecting strains with a low ethanol yield from forty-five non-*Saccharomyces* yeasts belonging to eighteen species. Single fermentations were carried out for this rapid selection. Then, sequential fermentations in synthetic and natural must were conducted with the selected strains to confirm their capacity to reduce ethanol compared with that of *Saccharomyces cerevisiae*. The results showed that ten non-*Saccharomyces* strains were able to reduce the ethanol content, namely, *Hanseniaspora uvarum* (2), *Issatchenkia terricola* (1), *Metschnikowia pulcherrima* (2), *Lachancea thermotolerans* (1), *Saccharomycodes ludwigii* (1), *Torulaspora delbrueckii* (2) and *Zygosaccharomyces bailii* (1). Compared with *S. cerevisiae*, the ethanol reduction of the selected strains ranged from 0.29 to 1.39% (v/v). Sequential inoculations of *M. pulcherrima* (Mp51 and Mp FA) and *S. cerevisiae* reduced the highest concentration of ethanol by 1.17 to 1.39% (v/v) in synthetic or natural must. Second, sequential fermentations with *Z. bailii* (Zb43) and *T. delbrueckii* (Td Pt) performed in natural must yielded ethanol reductions of 1.02 and 0.84% (v/v), respectively.

Keywords: alcoholic fermentation; wine yeast; sequential inoculation; ethanol reduction; *Metschnikowia pulcherrima*; *Torulaspora delbrueckii*; *Zygosaccharomyces bailii*

Introduction

Global climate change has caused an increase in the alcohol content of wines in recent decades (Godden, 2000; Jones et al., 2005; Alston et al., 2011). Specifically, global warming has accelerated maturation, increased the total soluble solids content and pH, and unbalanced the maturation of phenolic compounds and the increase in sugar concentration (Jones et al., 2005). If grapes are harvested when phenolic compounds are mature, the grape must will have high concentration of sugars and low acidity, which produces wines with a high ethanol content. Otherwise, if the harvest occurs before that point, when sugar accumulation and pH are appropriate, wines will present a reduction in several characteristics (aroma, taste and astringency) due to insufficient phenolic maturation. Regarding alcoholic fermentation, a high concentration of ethanol may lead to sluggish and stuck fermentations (Bisson, 1999; Buescher et al., 2001; Coulter et al., 2008). In addition, it can break the balance among acids, sugars and tannins and develop unpleasant characteristics due to the enhancement of bitterness and burning sensation during tasting (Escudero et al., 2007). There are other reasons to achieve a lower ethanol content in wines, from their reduction in aromatic profile to the tax increase that will impact the final price of wines.

Previous studies about the reduction of alcohol in wines have focused on viticulture management, prefermentation and postfermentation treatments and microbiological strategies during fermentation (García-Martín et al., 2010; Kontoudakis et al., 2011; Diban et al., 2013; Varela et al., 2015). Specifically, from the point of viticulture management, a reduction in leaf area and removal of functional leaves were tested to reduce sugar accumulation, which could lead to a reduction in anthocyanins and soluble solids, delay maturity and significantly reduce the yield of grapes (De Toda et al., 2013). Another strategy for viticulture management was sequential harvesting, which aside from influencing phenolic maturity, could also affect the balance between fruity and vegetal aromas (Bindon et al., 2013). As a prefermentation treatment, García-Martín et al. (2010) and Mihnea et al. (2012) used

membrane technologies, especially nanofiltration, to remove sugars from grape must. However, this method led to a reduction in wine color and flavor compounds. In addition, the removal of ethanol in wine was mainly considered during the postfermentation process. Aguera et al. (2010) reported that removing 2% (v/v) ethanol had a significant effect on the concentration of volatile compounds, such as fusel alcohol and esters, which were reduced by 25% and 45%, respectively.

In recent years, microbiological strategies have garnered interest as alternatives to reduce ethanol concentrations (Kutyna et al., 2010; Gonzalez et al., 2013; Varela and Varela, 2019). For instance, the selection of evolved or modified strains from *Saccharomyces cerevisiae*, as well as low-ethanol producer strains from non-*Saccharomyces* yeasts, have been considered. In terms of *S. cerevisiae*, non-GMO strategies, such as evolutionary engineering, including experimental evolution under selective cultivation conditions or quantitative trait loci (QTL) mapping followed by breeding, have been used to improve industrial yeasts (Swinnen et al., 2013; Steensels et al., 2014). However, evolutionary engineering could affect some strain features under the conditions of industrial production and fermentation and lead to a distinct response of evolved strains to environmental factors that is different than that of ancestral strains (Rollero et al., 2015). The other strategy was genetic modification (GM), which has focused on changing the carbon metabolic conversion of sugar into other byproducts (Kutyna et al., 2010; Varela et al., 2012; Rossouw et al., 2013). However, the application of GM methods in food and beverage production is forbidden due to poor public acceptance and regulations. Based on this limitation, screening non-*Saccharomyces* yeasts with alcohol-lowering abilities has become a consistent proposal to maintain wine quality and reduce the ethanol content (Contreras et al., 2014; Quirós et al., 2014; Ciani et al., 2016; Varela et al., 2016 & 2017; Canonico et al., 2019). The use of some non-*Saccharomyces* strains from *Candida*, *Hanseniaspora*, *Lachancea*, *Metschnikowia*, *Picha*, *Schizosaccharomyces*, *Starmerella* and *Torulaspora* species has been shown to reduce ethanol in wines (Varela and Varela, 2019).

Non-*Saccharomyces* yeasts have been used in fermentations to reduce ethanol as a single or mixed inoculation. For example, *Candida sake* H14Cs reduced 2.4% (v/v) ethanol in natural must fermentations with a single inoculation (Ballester-Tomás et al., 2017). Varela et al. (2017) reported that, compared with *S. cerevisiae*, single fermentation by *Saccharomyces uvarum* AWRI2846 reduced the ethanol content by 1.7% (v/v), and coinoculated fermentation by *M. pulcherrima*/*S. cerevisiae* (10:1) reduced the ethanol content by 1% (v/v) in Merlot wines. Strains from *Hanseniaspora uvarum*, *Zygosaccharomyces sapae*, *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus* species used as pure cultures in fermentations also showed a significant ethanol reduction in ethanol yield compared with *S. cerevisiae* (Gobbi et al., 2014). However, the growth and metabolism of non-*Saccharomyces* yeasts will be affected by the presence of *S. cerevisiae*, especially in simultaneous fermentations (Ciani and Comitini, 2015; Lleixà et al., 2016). Thus, sequential inoculation strategies, where *S. cerevisiae* is inoculated 24 or 48 h after the beginning of fermentation with non-*Saccharomyces* yeast, have been adopted by researchers and wine producers (Canonico et al., 2016; Padilla et al., 2017; Maturano et al., 2019). Englezos et al. (2016) proposed a protocol to reduce ethanol based on the sequential fermentation of *S. bacillaris* and *S. cerevisiae*, showing higher ethanol reduction inoculating *S. cerevisiae* at 48 h than at 24 h.

In the present work, we proposed a rapid method to select non-*Saccharomyces* strains with a low ethanol yield. The ethanol production and yield of 45 non-*Saccharomyces* yeasts, belonging to 18 species, were evaluated. After an initial screening on optimal medium for 3 days and on synthetic must for 48 h (to set the beginning of alcoholic fermentation), we reduced the number to 10 strains with a high potential to reduce the ethanol content. Afterwards, this ability was verified by complete fermentations in synthetic and natural must using sequential inoculations with a commercial *S. cerevisiae* wine yeast. In addition, all final samples were subjected to an in-depth chemical analysis to characterize the resulting wines.

Materials and Methods

Strains and culture conditions

One commercial wine yeast *Saccharomyces cerevisiae* (Lalvin QA23®, Lallemand Inc. Montreal, Canada, used as a control and referred to as Sc23) and forty-five non-*Saccharomyces* strains used in this study are listed in Table 1. Yeasts grew at 28 °C in YPD Agar (2% (w/v) glucose, 2% (w/v) yeast extract, 1% (w/v) peptone, and 1.7% (w/v) agar; Cultimed, Barcelona, Spain) and Wallerstein laboratory nutrient (WLN) agar (Becton, Dickinson and Company, Isère, France) from frozen stocks at – 80 °C. Before starting fermentations, strains were identified at species level by PCR-RFLP analysis of 5.8S-ITS rDNA according to Esteve-Zarzoso et al. (1999).

Propagation of strains was performed by picking a single colony from YPD plates. Strains grew in YPD liquid medium (2% (w/v) glucose, 2% (w/v) yeast extract, and 1% (w/v) peptone) for 24 h (Sc23) or 48 h (non-*Saccharomyces* strains) at 28 °C. After incubation, cells were counted in a Neubauer chamber (Leica Microsystems GMS QmbH, Leica, Germany), and 2×10^6 cells/mL were inoculated into the appropriate fermentation medium. In all cases, the identity at the species level was confirmed by growth on differential WLN agar, and molecular identification by PCR-RFLP of 5.8S-ITS rDNA (Esteve-Zarzoso et al., 1999) was used to distinguish the non-*Saccharomyces* yeasts that presented similar morphological profiles with Sc23 on the WLN agar.

Table 1. Yeast strains used in this study (CECT, Spanish Type Culture Collection; URV, our group yeast collection, some of them isolated in Priorat Appelation of origin (Padilla et al., 2016); UdlaR, Universidad de la República yeast collection, Uruguay; Agrovin S.A, Ciudad Real, Spain; Lallemand, Lallemand Inc. Montreal, Canada).

Yeast species	Strain designation	Collections	Isolation source	Abbreviations in this paper
Saccharomyces cerevisiae	QA23	Lallemand	Commercial	Sc23
Candida boidinii	10029	CECT	Milk	Cb29
	10035	CECT	Frass on *Amygdalus communis*	Cb35
	1014 [T]	CECT	Tanning fluid	Cb14
Candida mesenterica	1025	CECT	Brewery	Cm25
Candida sake	10034	CECT	Feces of sheep	Cs34
	1044	CECT	Lambic beer	Cs44
Candida stellata	11918 [T]	CECT	Wine grape	Cs18
Starmerella bacillaris	4	URV	Grape must (Priorat)	Sb4
	11046	CECT	Grape juice	Sb46
	11109	CECT	Wine	Sb09
	NS c	URV	Grape must	Sb Nc
	NS d	URV	Grape must	Sb Nd
Hanseniaspora guilliermondii	11027	CECT	Grape must	Hg27
	11029 [T]	CECT	Infected nail	Hg19
	11102	CECT	Grape juice	Hg02
Hanseniaspora osmophila	11206	CECT	Ripe Riesling grape	Ho06
	11207	CECT	Grape	Ho07
Hanseniaspora uvarum	11106	CECT	Wine grape	Hu06
	13130	CECT	Grape must (Priorat)	Hu4
	3	URV	Grape must (Priorat)	Hu3
	34	URV	Grape must (Priorat)	Hu34
Hanseniaspora vineae	11.24	UdlaR	Grapes (Uruguay)	Hv24
	12.219	UdlaR	Grapes (Uruguay)	Hv19
	13714	CECT	Nd [a]	Hv14
	1471	CECT	Grape juice	Hv71
Issatchenkia terricola	11139	CECT	Dregs of pressed grapes	It39
	11176	CECT	Soil	It76
Lachancea thermotolerans	1	Agrovin	Nd [a]	Lt1
	2	Agrovin	Nd [a]	Lt2
Meyerozyma guilliermondii	1020	CECT	Nd [a]	Mg20
Metschnikowia pulcherrima	51	URV	Grape must (Priorat)	Mp51
	52	URV	Grape must (Priorat)	Mp52
	FLAVIA	Lallemand	Commercial	Mp FA
Saccharomycodes ludwigii	1235 [T]	CECT	Nd [a]	Sl35
	1371	CECT	Nd [a]	Sl71
Schizosaccharomyces pombe	1379	CECT	Nd [a]	Sp79
Torulaspora delbrueckii	10558	CECT	White wine	Td58
	13135	CECT	Grape must (Priorat)	Td35
	1880	CECT	Wine of Airen grape	Td80
	Priorat	URV	Grape must (Priorat)	Td Pt
	BIODIVA	Lallemand	Commercial	Td BA
Zygosaccharomyces bailii	11042	CECT	Grape must	Zb42
	11043	CECT	Cloudy wine	Zb43
Zygosaccharomyces rouxii	1230	CECT	Honey	Zr30
	1232	CECT	Concentrate must	Zr32

[T] presents Type strain; [a] presents No description.

Fermentations

Three different media were used in fermentations, namely, YPD liquid medium, synthetic must (SM) and natural must (NM). SM was prepared according to Beltran et al. (2004) (200 g/L sugars), and NM was obtained from Muscat grapes from Finca Experimental Mas dels Frares of Rovira i Virgili University (Constantí, Spain) during the 2019 vintage (219.2 g/L sugars, 4.52 g/L total acidity (as tartaric acid), 77.8 mg/L assimilable nitrogen, and pH 3.27). The nitrogen concentration in NM was corrected with diammonium phosphate (Panreac Quimica SA, Barcelona, Spain) until a final concentration of 240 mg N/L. Before the start of fermentation, dimethyl dicarbonate (0.2 mL/L) (ChemCruz™ Biochemicals, Dallas, TX, USA) was added to NM, and kept at 4 °C for 24 h to eliminate the endogenous microorganisms. The absence of endogenous microorganisms was confirmed by plating a sample of the must on YPD Agar. Different fermentation procedures were performed in single and sequential fermentations. All fermentations were performed in triplicate, and single fermentations by Sc23 were used as a control.

In the first screening, strains were inoculated as single cultures in 5 mL YPD liquid medium in 12 mL tubes and incubated at 28 °C and 120 rpm for 3 days. Samples were taken daily to evaluate yeast growth, and after 3 days, extracellular media was kept to determine sugar and ethanol content. In the next step, strains were inoculated in 40 mL SM in 50 mL Falcon tubes, and fermentations were performed at 22 °C and 120 rpm and monitored over 48 h to evaluate yeast growth. Samples were taken at 48 h and centrifuged at 12000 rpm for 5 min, and the supernatant was kept at – 20 °C until chemical compound analysis.

For sequential fermentations, experiments were carried out either in SM or NM. Non-*Saccharomyces* strains (2×10^6 cells/mL) were used to start the fermentation, and 48 h later, Sc23 was inoculated (2×10^6 cells/mL). Fermentations were conducted in 250 mL glass bottles with 230 mL of SM or NM (bottle caps were not tightly screwed in order to allow the release of CO_2) and incubated at 22 °C with stirring at 120 rpm. SM and NM fermentations were monitored by evaluating yeast growth, and must

density which was determined with an electronic densitometer (Densito 30PX Portable Density Meter, Mettler Toledo, Hospitalet de Llobregat, Spain). The fermentation was considered finished when residual sugars were below 2 g/L, which was confirmed by enzymatic analysis in a Miura autoanalyzer (EE-MIURAONE Rev., I.S.E. S.r.l., Italy). Samples were centrifuged at 7800 rpm for 5 min, and the supernatants were frozen at – 20 °C until analysis.

Population dynamics

In single fermentation samples, the total population was assessed by microscope counting using a Neubauer chamber after 48 h of fermentation. Viability was also determined in sequential fermentations. Briefly, samples were serially diluted in sterilized Milli-Q water from a Milli-Q water purification system (Millipore S.A.S., Molsheim, France). The number of colony-forming units per milliliter (CFU/mL) was determined by plating 100 μL of three appropriately chosen dilutions on YPD, WLN or lysine agar (11.75 g/L yeast carbon base, 2.5 g/L L-lysine monohydrochloride, and 20 g/L agar, Cultimed, Barcelona, Spain). Plates were incubated at 28 °C for 2 or 3 days.

Chemical analysis

The glucose and ethanol contents of the samples from YPD cultures were determined with D-glucose and ethanol enzymatic bioanalysis kits (r-biopharm, Darmstadt, Germany), respectively. Residual sugars of samples at the end of fermentation in both SM and NM fermentations were quantified by D-glucose/D-fructose assays (Biosystems S.A., Barcelona, Spain).

Ethanol, glycerol and organic acids (citric acid, malic acid, tartaric acid, acetic acid, lactic acid and succinic acid) in samples after 48 h of single fermentation and at the end of sequential fermentation and the sugars (glucose and fructose) after 48 h of single fermentation were determined by high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC (Agilent Technologies, Germany) as previously described by Quirós et al. (2010). The HPLC was equipped with a Hi-Plex H column

(300 mm × 7.7 mm) inside a 1260 MCT column compartment (Infinity II Multicolumn Thermostat) connected to MWD (G1365B multiwavelength detector) and RID detectors (1260 Infinity II refractive index detector) (Agilent Technologies, Waldbronn, Germany). The temperature of the column was maintained at 60 °C for a 30 min run time, and the mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min. The sample injection volume was 10 μL. Before injection, samples were filtered through 0.22 μm filters (Dominique Dutscher, Brumath, France). OpenLAB CDS (Agilent Technologies, Santa Clara, CA, USA) was used to analyze HPLC chromatographs.

Statistical analysis

All graphs were generated using GraphPad Prism® version 8 (GraphPad Software, San Diego, CA, USA). The results are expressed as the mean ± standard deviation (SD). Statistically significant differences (one-way ANOVA) were analyzed by IBM SPSS Statistics version 23.0 (IBM, NY, USA). The ethanol yield was calculated with the formula "Ethanol yield (g/g) = ethanol production (g/L)/sugar consumption (g/L)". Ethanol reduction was calculated by the formula "Δethanol (%, v/v) = Δethanol yield (g/g) × T sugars (g/L)/10 × 0.78924 (g/mL)", where T sugars is the initial sugar concentration in the must and 0.78924 is the density of ethanol at room temperature.

Results

Rapid screening of non-*Saccharomyces* strains with a low ethanol yield

A first screening with forty-five non-*Saccharomyces* strains was performed under low sugar fermentation conditions (YPD liquid medium), to evaluate the capacity of some yeast species and strains to consume sugars with a limited production of ethanol (fermentation vs. respiration capacity) (Figure 1, Table S1). *Saccharomyces cerevisiae* QA23 (Sc23), inoculated as a control, was able to consume all glucose (20 g/L) and produced 0.84% (v/v) ethanol, with an ethanol yield of 0.33 g ethanol/g glucose. The selection criteria for lower ethanol-producing yeast were established according to this result, taking into account their ability to consume glucose. Based on this, fourteen

non-*Saccharomyces* strains were selected due to a high glucose consumption (> 19.90 g/L), and a lower ethanol production than that of the control with ethanol yields below 0.30 g/g (< 0.76%, v/v ethanol, 10% ethanol reduction compared with Sc23) (Figure 1). These strains belonged to the species *Hanseniaspora uvarum* (2), *Issatchenkia terricola* (1), *Lachancea thermotolerans (2), Metschnikowia pulcherrima* (2), *Saccharomycodes ludwigii* (1), *Starmerella bacillaris* (1), *Torulaspora delbrueckii* (3) and *Zygosaccharomyces bailii* (2).

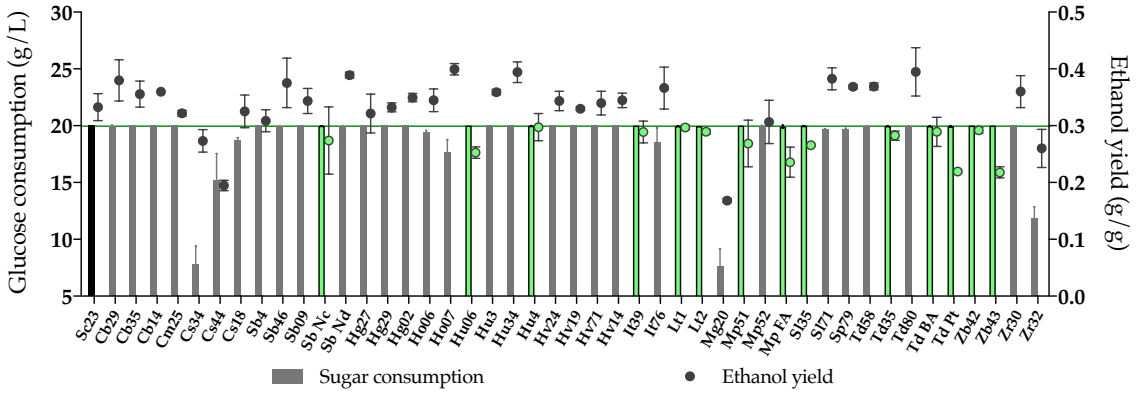

Figure 1. Glucose consumption (g/L) and ethanol yield (ethanol production (g/L)/sugar consumption (g/L), g/g) of 45 non-*Saccharomyces* yeasts and Sc23 (control yeast) after 3 days fermentation in 5 mL YPD medium. The non-*Saccharomyces* yeasts selected for the next step are colored in green (glucose consumption > 19.90 g/L and ethanol yield ≤ 0.30 g/g). The value of the green line is 0.30 g/g (10% ethanol reduction of Sc23).

As non-*Saccharomyces* yeasts are commonly used in sequential fermentations, inoculating *S. cerevisiae* after 24 – 48 h, in the next step we analyzed the performance of the selected non-*Saccharomyces* strains during the first 48 h of fermentation. Therefore, we tested the 14 selected non-*Saccharomyces* strains on fermentation media (synthetic must) using Sc23 as a control. Must density, total yeast population, ethanol production, sugar consumption and other main organic compounds were measured at 48 h of fermentation (Figure 2, Table S2). We observed that all selected strains were able to start fermentation in 48 h, consuming some of the sugars present in the must (with a corresponding decrease in must density, Figure 2a), although in lesser amount than that of Sc23 (Table S2). The total yeast population showed that all strains were able to grow in fermentation media, and two of them, Sb Nc and Zb42, grew

significantly higher than the control strain at 48 h (Figure 2b). The single fermentation with Sc23 was able to consume 47% (93.68 g/L) of total sugars in 48 h and produced the highest concentration of ethanol (5.26%, v/v), with an ethanol yield of 0.44 g/g (Table S2, Figure 2c). Most non-*Saccharomyces* strains consumed more glucose than fructose during 48 h, similar to the control Sc23 strain. However, three of the strains, Sb Nc, Sl35 and Zb43, consumed more fructose than glucose, and the two *H. uvarum* strains, Hu06 and Hu4, consumed equal quantities of glucose and fructose (Table S2). Ten out of 14 strains produced lower ethanol contents and lower ethanol yields than Sc23 (< 0.44 g/g), Hu06, Hu4, It39, Mp51, Mp FA, Lt2, Sl35, Td35, Td BA and Zb43, and they were selected for subsequent experiments (Figure 2c).

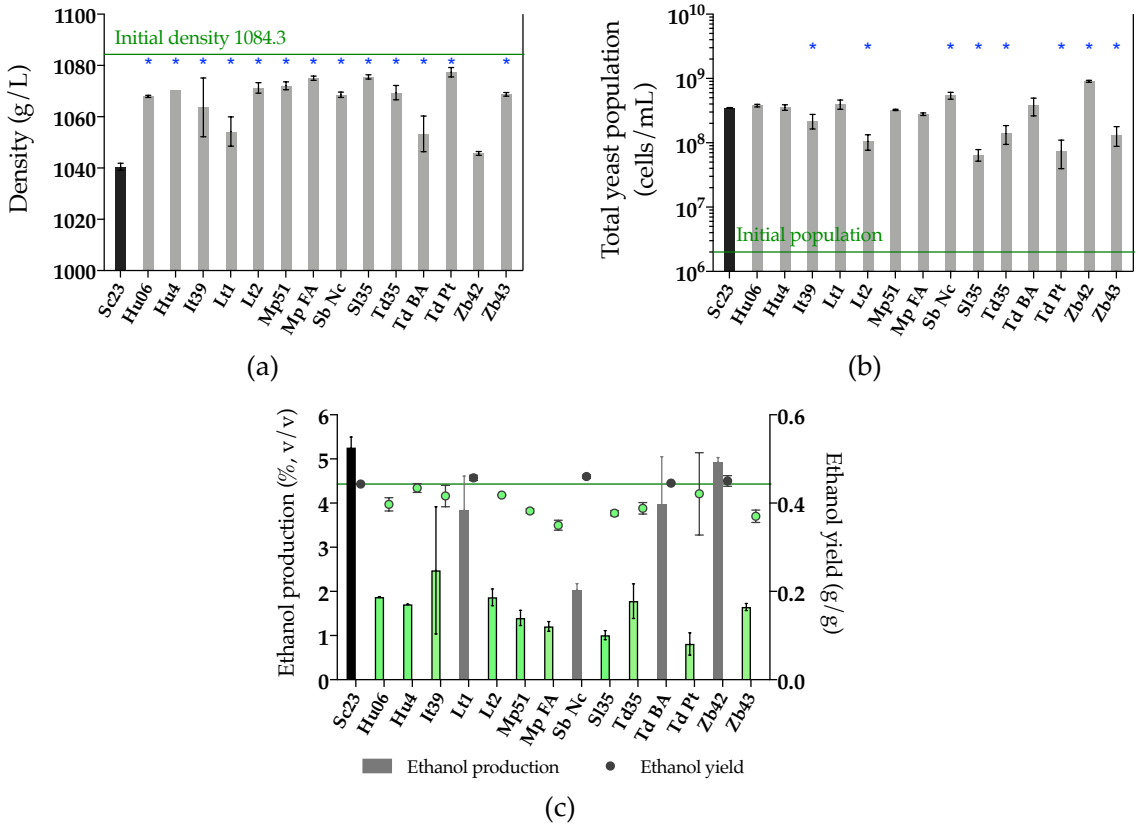

Figure 2. (a) Density (g/L); (b) Total yeast population (cells/mL); (c) Ethanol production (%, v/v) and ethanol yield (g/g) at 48 h of single fermentation in synthetic must. Non-*Saccharomyces* yeasts selected for the next step are colored in green, with the ethanol yield below that of Sc23 (0.44 g/g, the value of the green line in Figure (c)). Asterisk (*) means the significant difference compared with Sc23 (LSD, $p < 0.05$).

Sequential inoculation in synthetic must (SM) and natural must (NM)

To verify the ability of the 10 selected strains to reduce ethanol, sequential fermentations were performed. In the sequential fermentations, Sc23 was inoculated at 48 h in both SM and NM fermentations.

In the SM fermentation, Sc23 completed fermentation in 6 days, and the density of sequential fermentation trials with Lt2 showed the fastest reduction among the non-*Saccharomyces* strains. Nevertheless, more than 9 days were necessary to complete sequential fermentations by the other strains (Figure 3a). Interestingly, all non-*Saccharomyces* strains were detected during the fermentation process, with Hu06 and It39 being the strains with the fastest decrease in viability and Td Pt and Td35 maintaining relatively high viability until the end of fermentation (Figure 3b). Correspondingly, the Sc23 population reached a significant increase after inoculation at 48 h, with final viable populations between 3.5×10^7 and 1.1×10^8 CFU/mL, except in the Lt2/Sc23 fermentation, where Sc23 grew poorly (up to 6.7×10^6 CFU/mL) (Figure 3c). Ethanol production decreased by 0.08 to 1.23% (v/v) in all sequential fermentations compared with that of the single fermentation by Sc23 (Figure 3d, Table 2), although this decrease was significant only with 7 of the non-*Saccharomyces* strains (Lt2, Mp51, Mp FA, Sl35, Td35, Td Pt and Zb43). Higher concentrations of residual sugars were observed in the fermentation of Zb43/Sc23 and Hu06/Sc23. Our results (Table 2) showed that the sequential fermentations with *M. pulcherrima* strains Mp51/Sc23 and Mp FA/Sc23 had the highest ethanol reduction with the lowest ethanol yields (both are 0.43 g/g compared to 0.48 g/g for Sc23).

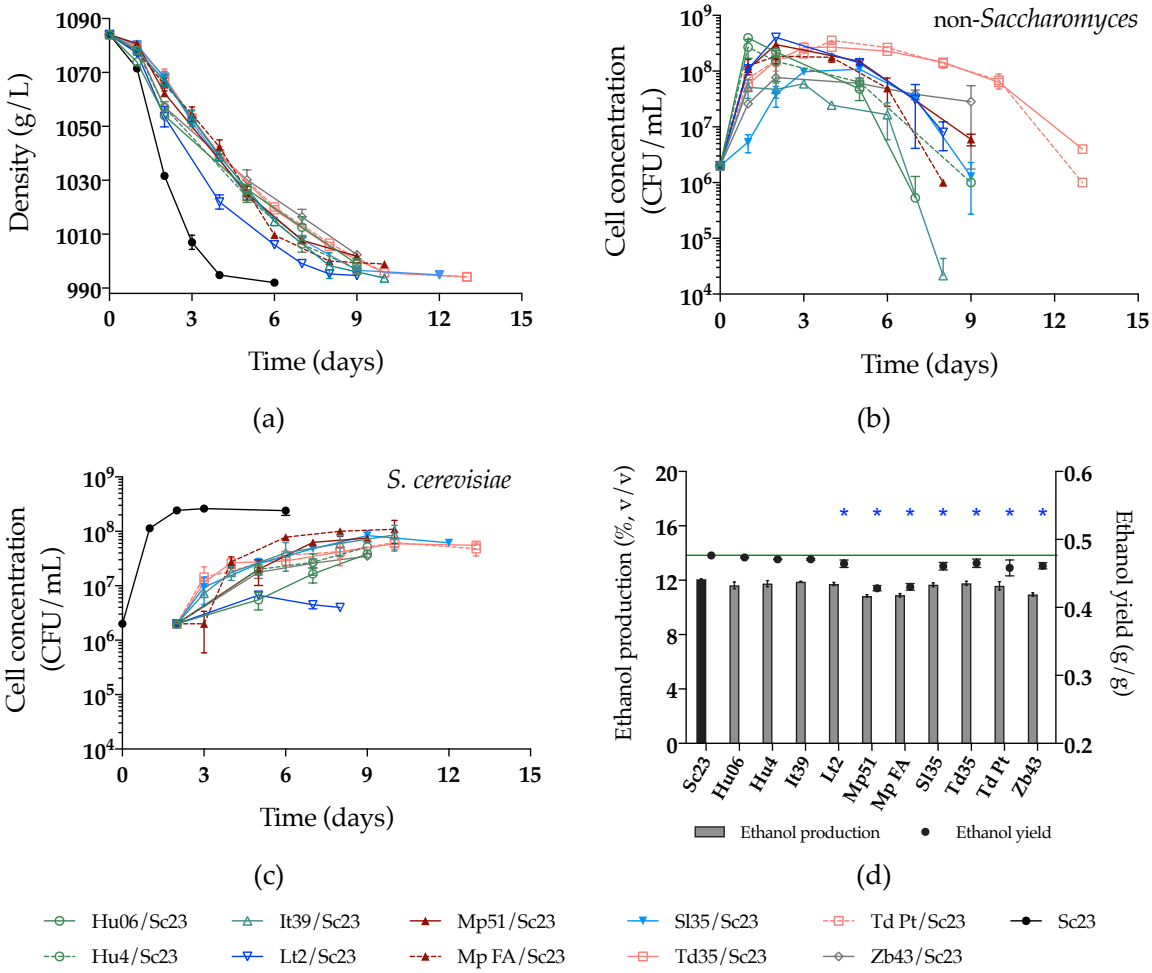

Figure 3. (a) Density (g/L); (b) Yeast population of non-*Saccharomyces*; (c) Yeast population of Sc23 and (d) Ethanol production (%, v/v) and yield (g/g) during sequential fermentations in synthetic must. The value of the green line in Figure (d) is 0.48 g/g (ethanol yield of Sc23). Asterisks (*) show the significant difference of ethanol yield compared with Sc23 (LSD, $p < 0.05$).

In the NM fermentation, all fermentations were delayed, probably due to the higher concentration of sugars in the natural must (219.2 g/L), especially fermentations that involved non-*Saccharomyces* strains, with Mp FA, Td Pt and Zb43 taking the longest time, up to 14 days (Figure 4a). Noteworthily, the fermentation with Lt2/Sc23 was slower in NM, differing from the behavior observed in SM. In NM, the growth of Sc23 in sequential fermentations (Figure 4c) was higher than in SM (Figure 3c), and consequently, the growth of some non-*Saccharomyces* was hampered (Figure 4b). Only five non-*Saccharomyces* strains could be counted on WLN at the end of NM fermentations (Lt2, Mp51, Td35, Td Pt and Zb43) (Figure 4b), which were also the ones

observed at the end of SM fermentations (Figure 3b). The ethanol production of all selected strains was reduced compared to the control fermentation with Sc23 (13.48%, v/v). The sequential fermentation by Mp51/Sc23 again showed the highest ethanol reduction, followed by Zb43/Sc23, Td Pt/Sc23 and Mp FA/Sc23 (Figure 4d, Table 2).

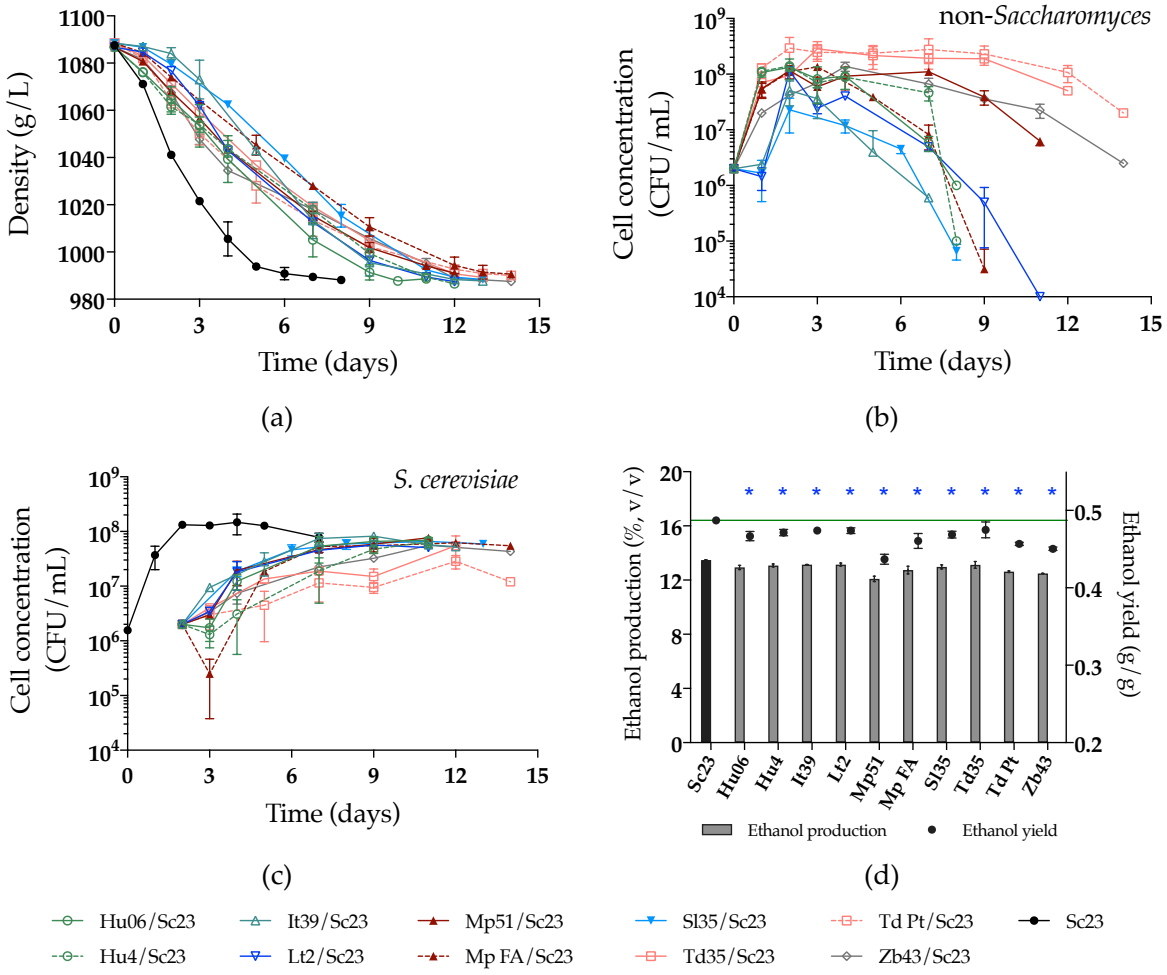

Figure 4. (a) Density (g/L); (b) Yeast population of non-*Saccharomyces*; (c) Yeast population of Sc23 and (d) Ethanol production (%, v/v) and yield (g/g) during sequential fermentations in natural must. The value of the green line in Figure (d) is 0.49 g/g (ethanol yield of Sc23). Asterisks (*) show the significant difference of ethanol yield compared with Sc23 (LSD, $p < 0.05$).

Table 2. Analysis of sugars, ethanol, organic acids, and glycerol from samples at the end of sequential fermentations.

Samples	Residual sugar (g/L)	Sugar consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)	Ethanol reduction (%, v/v)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)
Synthetic must fermentation									
Sc23	0.12 ± 0.10	199.88 ± 0.10	12.07 ± 0.02	0.48 ± 0	0 ± 0	0.56 ± 0.04	0.23 ± 0	0.30 ± 0.03	5.76 ± 0.16
Hu06	6.24 ± 3.83 *	193.76 ± 3.83 *	11.62 ± 0.25 *	0.47 ± 0	0.08 ± 0.02	0.54 ± 0.08	0.18 ± 0.01	0.24 ± 0.04	6.60 ± 0.22
Hu4	3 ± 3.94	197 ± 3.94	11.75 ± 0.23	0.47 ± 0	0.14 ± 0.01	0.41 ± 0.01	0.16 ± 0.01	0.28 ± 0.02	6.90 ± 0.03
It39	0.71 ± 1	199.29 ± 1	11.89 ± 0.03	0.47 ± 0	0.15 ± 0.03	0.46 ± 0.06	0.23 ± 0.02	0.33 ± 0.01	7.65 ± 0.84 *
Lt2	0.82 ± 0.43	199.18 ± 0.43	11.72 ± 0.11	0.46 ± 0.01 *	0.31 ± 0.14 *	0.56 ± 0	0.55 ± 0.04 *	0.27 ± 0.11	7.44 ± 0.33 *
Mp51	0.03 ± 0.04	199.97 ± 0.04	10.85 ± 0.09 *	0.43 ± 0 *	1.23 ± 0.10 *	0.58 ± 0.01	0.21 ± 0.03	0.28 ± 0.04	10.30 ± 0.45 *
Mp FA	0 ± 0	200 ± 0	10.90 ± 0.12 *	0.43 ± 0 *	1.17 ± 0.12 *	0.59 ± 0.03	0.16 ± 0.01	0.16 ± 0.06 *	9.83 ± 0.67 *
Sl35	0.12 ± 0.06	199.88 ± 0.06	11.67 ± 0.14 *	0.46 ± 0.01 *	0.40 ± 0.14 *	0.65 ± 0.01	0.22 ± 0.11	0.17 ± 0.01 *	7.76 ± 0.93 *
Td35	0.24 ± 0.05	199.77 ± 0.05	11.77 ± 0.15	0.47 ± 0.01 *	0.29 ± 0.15 *	0.53 ± 0.16	0.16 ± 0.04	0.26 ± 0.04	5.31 ± 0.12
Td Pt	0.40 ± 0.15	199.61 ± 0.15	11.59 ± 0.30 *	0.46 ± 0.01 *	0.47 ± 0.29 *	0.53 ± 0.02	0.37 ± 0.05 *	0.24 ± 0.05	5.36 ± 0.10
Zb43	12.62 ± 2.58 *	187.38 ± 2.58 *	10.95 ± 0.12 *	0.46 ± 0 *	0.39 ± 0.11 *	1.35 ± 0.52 *	0.31 ± 0.12	0.12 ± 0.01 *	8.61 ± 0.86 *
Natural must fermentation									
Sc23	0.76 ± 0.10	218.42 ± 0.10	13.48 ± 0.03	0.49 ± 0	0 ± 0	0.77 ± 0.08	0.16 ± 0.08	0.31 ± 0.04	5.56 ± 0.06
Hu06	0.41 ± 0.16	218.76 ± 0.16	12.94 ± 0.16 *	0.47 ± 0.01 *	0.56 ± 0.17 *	0.73 ± 0.04	0.15 ± 0.01	0.48 ± 0.17 *	6.60 ± 0.75 *
Hu4	0.19 ± 0.14	218.98 ± 0.14	13.08 ± 0.11 *	0.47 ± 0 *	0.44 ± 0.11 *	0.55 ± 0.04	0.13 ± 0.01	0.57 ± 0.04 *	6.50 ± 0.08 *
It39	0.13 ± 0.03	218.91 ± 0.03	13.20 ± 0.10	0.47 ± 0 *	0.37 ± 0 *	0.52 ± 0.07	0.24 ± 0.04	0.26 ± 0.08	6.12 ± 0.37
Lt2	0.24 ± 0.06	218.93 ± 0.06	13.15 ± 0.11	0.47 ± 0 *	0.37 ± 0.10 *	0.33 ± 0 *	4.12 ± 0.06 *	0.16 ± 0.01	8.48 ± 0.02 *
Mp51	0.70 ± 0.42	218.48 ± 0.42	12.10 ± 0.20 *	0.44 ± 0.01 *	1.39 ± 0.18 *	0.80 ± 0.05	0.14 ± 0.02	0.20 ± 0.01	5.83 ± 0.31
Mp FA	0.75 ± 0.35	218.29 ± 0.35	12.74 ± 0.28 *	0.46 ± 0.01 *	0.74 ± 0.26 *	0.70 ± 0.02	0.25 ± 0.02	0.12 ± 0.02 *	6.71 ± 0.33 *
Sl35	0.65 ± 0.52	218.39 ± 0.52	12.97 ± 0.15 *	0.47 ± 0 *	0.51 ± 0.12 *	0.72 ± 0.10	0.25 ± 0.06	0.20 ± 0.06	6.18 ± 0.08
Td35	0.87 ± 0.48	218.17 ± 0.48	13.12 ± 0.25 *	0.47 ± 0.01 *	0.34 ± 0.28 *	0.56 ± 0	0.33 ± 0.14 *	0.26 ± 0.01	5.14 ± 0.33
Td Pt	1.01 ± 0.04	218.03 ± 0.04	12.62 ± 0.06 *	0.46 ± 0 *	0.84 ± 0.06 *	0.95 ± 0.43	0.50 ± 0.01 *	0.31 ± 0.09	6.37 ± 0.61 *
Zb43	0.37 ± 0.50	218.80 ± 0.50	12.61 ± 0.21 *	0.45 ± 0 *	1.02 ± 0.05 *	0.83 ± 0.13	0.07 ± 0.04	0.30 ± 0.04	4.67 ± 0.09 *

Values are mean ± standard deviation of three independent replicates; The initial sugar concentration of synthetic and natural must was 200 and 219.2 g/L, respectively; * means statistically significant differences from the control sample in the same column (LSD test, $p < 0.05$).

The production of glycerol differed significantly among the different sequential fermentations (Table 2), with Mp51/Sc23 and Mp FA/Sc23 fermentations having the highest glycerol levels in SM (10.3 and 9.83 g/L, respectively) and Lt2/Sc23 fermentations in NM (8.48 g/L). Indeed, the increase in glycerol of Mp FA/Sc23 and Lt/Sc23 fermentations, compared to single Sc23 fermentation, was significant both in SM and NM. The concentration of acetic acid remained under the recommended values for wines, below 0.35 g/L in SM and below 0.6 g/L in NM (the highest values were for Hu06 and Hu4 strains, 0.48 and 0.57 g/L, respectively). On the other hand, a significant increase in lactic acid was observed in the sequential fermentations performed with the Lt2 strain, both in SM and NM. Noteworthily, the concentration of succinic acid was significantly higher (1.35 g/L) in SM fermentation with Zb43.

Discussion

The selection of non-*Saccharomyces* yeasts to be used as fermentation starters, usually in combination with *S. cerevisiae*, has been mainly focused on improving the aromatic characteristics of wines (Jolly et al., 2006; Varela et al., 2017) and reproducing the microbiota of vineyard or grapes (Mas et al., 2016). In recent years, another reason for screening non-*Saccharomyces* yeasts has been the ability of some species to reduce ethanol content. Researchers have applied different combinations of non-*Saccharomyces* and *S. cerevisiae* yeasts to achieve this goal (Quirós et al., 2014; Contreras et al., 2015; Varela et al., 2016). In this study, we focused on the selection of non-*Saccharomyces* yeasts with low ethanol yield by performing two short-term trials in 5 days. In the first selection step, we used YPD liquid medium, which contains a low concentration of sugar, and analyzed ethanol yield and sugar consumption of the different strains. The metabolic characteristics of non-*Saccharomyces* yeasts will determine ethanol reduction, which implies that their metabolic footprints should be introduced before the inoculation of *S. cerevisiae* (Ciani et al., 2016). Therefore, the second selection step was performed in synthetic must for 48 h, in order to detect their

ability to reduce ethanol before the inoculation of *S. cerevisiae*. With the selected strains, two sequential fermentation trials were performed, in synthetic and natural must, in which *S. cerevisiae* was inoculated after 48 h. Simultaneous inoculations could reduce the contribution of non-*Saccharomyces* yeast in the fermentation process, and periods of longer than two days could jeopardize the imposition of *S. cerevisiae* and, as a consequence, the development of fermentation (Lleixà et al., 2016).

Regarding non-*Saccharomyces* screening strategies to achieve wines with low ethanol concentrations, Contreras et al. (2014) used the fermentation of single yeast species in a defined medium for 4 days under anaerobic conditions to select strains from 50 non-*Saccharomyces* yeasts, followed by a second step with sequential fermentation for 7 days. After 11 days, eleven strains showed lower ethanol yields than *S. cerevisiae*. Another study reported by Contreras et al. (2015) used a similar methodology over 11 days, and the difference was the use of semi-aerobic conditions of the initial fermentation. They selected seven strains out of 48 non-*Saccharomyces* yeasts with lower ethanol yield than *S. cerevisiae*. Quirós et al. (2014) selected fifteen yeasts from 63 non-*Saccharomyces* strains by determining the respiratory quotient under fully aerobic conditions in 4 days, followed by the performance of selected strains in synthetic must for 4 days. However, after 8 days of analysis, several of the selected strains showed a higher ethanol yield than that of *S. cerevisiae*. Thus, compared to previous selection trials, the screening process applied in our study was equally rapid, but the pre-selection of strains was more reliable, and we included an important screening criterion to be considered, that is, the selected non-*Saccharomyces* yeasts were able to finish fermentations under low-sugar conditions.

The ethanol yields of *S. cerevisiae* in YPD liquid medium (0.33 g/g) were lower than those in semi-anaerobic fermentative conditions (approximately 0.48 g/g), which agrees with the results of Quirós et al. (2014) in fully aerobic conditions, where the ethanol yield of *S. cerevisiae* was approximately 0.25 – 0.30 g/g. Instead, when fermentative conditions in synthetic or natural must were used, the ethanol yields for *S. cerevisiae* were close to the expected values (i.e., 0.44-0.48 g/g) (Contreras et al., 2014).

The differences could be due to the importance of respiratory metabolism in YPD liquid medium, where the sugar concentration was low (20 g/L), whereas in synthetic or natural must, with high sugar concentrations (≥ 200 g/L), glucose repression occurred (Barnett and Entian, 2005).

In general, most non-*Saccharomyces* yeasts present weak fermentation capacity and grow slower than *S. cerevisiae* (Fleet and Heard, 1993; Ciani et al., 2006). Similar results were observed in the current study, where all non-*Saccharomyces* yeasts started fermentations slower than Sc23, and eight of the strains had poorer growth than Sc23 during the first 48 h. In the present work, Sc23 consumed almost half of the sugars at 48 h and presented the highest sugar consumption among all fermentations. This is supported by previous studies in which different non-*Saccharomyces* strains, *M. pulcherrima*, *S. bombicola*, *H. uvarum*, *T. delbrueckii* and *Z. bailii*, consumed less sugar than *S. cerevisiae* in a single fermentation before 48 h (Quirós et al., 2014; Contreras et al., 2015; Canonico et al., 2016). On the other hand, four of the strains had faster growth and higher ethanol yields than Sc23 (Lt1, Sb Nc, Td BA and Zb42), and three of them (Lt1, Td BA and Zb42) also had higher sugar consumption at 48 h in SM. Thus, during alcoholic fermentation in synthetic must, growth seemed to be positively correlated with sugar consumption and ethanol yield. In fully aerobic conditions, Quirós et al. (2014) also observed a positive correlation between ethanol yield and sugar consumption in non-*Saccharomyces* strains but a negative correlation with biomass, which may be due to the higher growth capacity in respiratory conditions.

After the proposed screening, we demonstrated that the ten selected non-*Saccharomyces* yeasts reduced the ethanol content, in both synthetic and natural musts, by sequential fermentations. Therefore, the strategy of two short-term trials in 5 days to select the non-*Saccharomyces* strains was appropriate, as the ethanol reduction was confirmed for most strains. Moreover, the timing of *S. cerevisiae* inoculation in the sequential fermentations (48 h) was also appropriate, as most non-*Saccharomyces* species could persist until mid-end of the fermentation, showing an impact on the ethanol content and the final product.

Non-*Saccharomyces* yeasts lose viability during alcoholic fermentation, and are soon replaced by *S. cerevisiae*. This may be due to several factors, such as low resistance to ethanol (Ribéreau-Gayon et al., 2006), nutrient competition (Albergaria et al., 2003; Andorrà et al., 2012; Lleixà et al., 2016), or microbial interactions, either by cell-to-cell contact (Nissen et al., 2003; Arneborg et al., 2005; Wang et al., 2016) or the secretion of antimicrobial compounds by different yeasts (mainly *S. cerevisiae*) (Albergaria et al., 2010; Branco et al., 2014; Wang et al., 2015). The populations of Lt2, Mp51, Td Pt, Td35 and Zb43 were found viable until the end of fermentation (cultivating on WLN agar), although they showed different performance in SM and NM fermentations. This persistence seems to be inconsistent with previous studies that claimed that most non-*Saccharomyces* species cannot tolerate ethanol concentrations above 5 – 7% (v/v) (Arneborg et al., 2005; Branco et al., 2014; Ciani et al., 2016). However, we have recently shown that *L. thermotolerans* and *T. delbrueckii*, used as a single culture, were able to finish a fermentation with 200 g/L of sugars, producing up to 9 – 10% (v/v) of ethanol (Roca-mesa et al., 2020). Even if the presence of *S. cerevisiae* in mixed fermentations could induce the death of other yeast species (Albergaria and Arneborg, 2016), other studies have shown that the presence of both *Saccharomyces* and non-*Saccharomyces* yeasts increased the persistence of non-*Saccharomyces* yeasts during the fermentation process (Ciani et al., 2006; Mendoza et al., 2007). Indeed, interactions between *Saccharomyces* and non-*Saccharomyces* wine yeasts have an effect not only on the persistence of the non-*Saccharomyces* yeasts but also on the behavior of the *Saccharomyces* wine strains (Ciani et al., 2015). Thus, the survival of these non-*Saccharomyces* yeasts until the end of fermentation in the current study might be a result of possible synergistic interactions between yeasts, and also due to their tolerance to a higher alcohol content, although this fact needs to be confirmed by further research.

Our results also showed that yeast performance and survival were influenced by the type of must (SM and NM), which could be due to the different nutrient composition. Indeed, we have previously observed that different sugar and nitrogen

concentrations on the must had a clear effect on the evolution of mixed fermentations done with *H. uvarum*, *S. bacillaris* and *T. delbrueckii* species, both on sugar consumption and population dynamics (Lleixà et al., 2016). Another study showed different sugar consumption profiles between Chardonnay and Shiraz grape must (with 240 and 210 g/L sugars, respectively) in mixed fermentations with *M. pulcherrima*/*S. cerevisiae* (Varela et al., 2016). Moreover, in a previous study we observed that changes in the concentration of some fermentation metabolites had an effect on the cultivability of some non-*Saccharomyces* strains (*H. uvarum*, *S. bacillaris* and *M. pulcherrima*) when used in mixed fermentations (Wang et al., 2016).

In the present work, Mp51/Sc23 fermentation demonstrated the highest ethanol reduction of 1.23% (v/v) in SM and 1.39% (v/v) in NM. The other strain belonging to the *M. pulcherrima* species, Mp FA, reduced the ethanol content by 1.17% (v/v) in SM fermentation. Similar to previous studies, *M. pulcherrima* has been recognized as a strain with a relatively high capacity to reduce ethanol in sequential fermentation with *S. cerevisiae* and had exhibited ethanol reductions by 0.9 to 3.6% (v/v) (Gobbi et al., 2013; Contreras et al., 2014; Röcker et al., 2016). In addition, fermentation by Zb43/Sc23 and Td Pt/Sc23 reduced the ethanol content by 1.02 and 0.84% (v/v) in NM fermentation, respectively. In agreement with previous research, *Z. bailli* and *T. delbrueckii* in sequential fermentation reduced the ethanol content by 1.0 to 1.6% (v/v) (Gobbi et al., 2014; Contreras et al., 2015).

During the fermentation process, the reduction of the ethanol concentration by non-*Saccharomyces* yeasts could be explained not only by their greater accumulation of yeast biomass but also by other byproducts produced after consuming sugars (Ciani et al., 2016). Under sufficient oxygen availability, carbon from sugar metabolism can be diverted towards organic acids and glycerol, resulting in low ethanol production (Giovanelli et al., 1996; Rodrigues et al., 2016). As the present study aims to be a method for screening non-*Saccharomyces*, we only evaluated the concentration of main byproducts after alcoholic fermentation. Interestingly, the content of byproducts was influenced by the type of must used. In the current study,

the highest concentration of glycerol was achieved in fermentations with Mp51 and Mp FA but only in SM. Furthermore, the production of glycerol in Mp51/Sc23 fermentation was affected by the type of must, as no significant increase was observed in NM fermentation, even if the highest ethanol reduction was obtained in this condition. As discussed before, the different nutrient composition of the must could affect the viability and metabolism of some non-*Saccharomyces* strains (Varela et al., 2016; Wang et al., 2016). Thus, the current study reveals that the glycerol production should not be the only metabolic pathway to reduce ethanol content. On the contrary, the highest concentration of glycerol was observed in NM fermentation with Lt2. This was consistent with the results from Gobbi et al. (2013) when fermentations with *L. thermotolerans* generated higher concentrations of glycerol (more than 7 g/L) in natural must. Associated with the overproduction of glycerol caused by ethanol reduction, the concentration of acetic acid might be increased, mainly in aerobic conditions (Ciani and Rosini, 1995; Morales et al., 2015). However, in the present work, performed in semi-anaerobic conditions, the fermentation with Mp FA/Sc23 in SM and NM significantly reduced the concentration of acetic acid, although increased the glycerol content, when achieving an ethanol reduction. The same performance was also observed in sequential fermentations with Sl35 and Zb43 in SM. These results supported those of Morales et al. (2015), where *M. pulcherrima* was able to reduce the concentration of acetic acid while increased glycerol and reduced ethanol content in mixed fermentations, compared with single *S. cerevisiae* inoculation. In the current study, the concentration of acetic acid remained below 0.8 g/L, considered the level when acetic acid may confer unpleasant acidic taste to wine (Fleet and Heard, 1993). Nevertheless, our results showed that both *H. uvarum* strains (Hu4 and Hu06) have significantly increased the acetic acid content in NM wines, confirming its higher production of negative byproducts and its poor oenological performance (Ciani and Maccarelli, 1998). Previous studies have shown that in *T. delbrueckii* and *H. uvarum* species acetic acid production was unrelated to ethanol formation, being *T. delbruecki* a low and constant acetic acid producer, and *H. uvarum* a high acetic acid producer

species (Ciani and Maccarelli, 1998). Additionally, fermentation by *L. thermotolerans* (Lt2/Sc23) produced the highest concentration of lactic acid in SM and NM fermentations, especially in NM fermentation. Strains of *L. thermotolerans* are frequently used for the acidification of low-acidity wines due to their ability of producing lactic acid during wine fermentations (Kapsopoulou et al., 2007; Benito et al., 2015). Our results also agreed with Binati et al. (2020), who reported that sequential fermentation with *L. thermotolerans* followed by inoculation of *S. cerevisiae* at 48 h produced a high concentration of lactic acid and reduced the ethanol content by 0.35% (v/v) in Pinot Grigio must. In our study, Lt2 reduced approximately 0.35% (v/v) ethanol in SM and NM fermentations. Moreover, The highest content of succinic acid was produced in SM fermentation with Zb43. Likewise, sequential fermentation by *Z. bailli* increased the concentration of succinic acid in defined grape must (Contreras et al., 2015).

In conclusion, this was a rapid method for screening yeasts with low ethanol yields. *M. pulcherrima* Mp51 and Mp FA are two appropriate wine yeasts for reducing ethanol in sequential fermentation trials. The potential of *Z. bailii* Zb43 and *T. delbrueckii* Td Pt to reduce ethanol concentrations needs to be explored. In addition, a complete analysis of the aromatic compounds should be analyzed to determine the impact of those sequential fermentations and ethanol reduction on wine quality and flavor. Thus, further research should focus on optimizing the inoculation time of non-*Saccharomyces* strains in sequential fermentation, as well as on the chemical and sensory analysis of the resulting wines. However, the application at the industrial scale is still a challenge to be addressed in the future.

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References

Aguera, E.; Bes, M.; Roy, A.; Camarasa, C.; Sablayrolles, J.M. Partial removal of ethanol during fermentation to obtain reduced-alcohol wines. *Am. J. Enol. Vitic.* **2010**, 61, 53–60, doi:10.1016/B978-0-12-381045-8.00003-5.

Albergaria, H.; Arneborg, N. Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: Role of physiological fitness and microbial interactions. *Appl. Microbiol. Biotechnol.* **2016**, 100, 2035–2046, doi:10.1007/s00253-015-7255-0.

Albergaria, H.; Francisco, D.; Gori, K.; Arneborg, N.; Gírio, F.M. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl. Microbiol. Biotechnol.* **2010**, 86, 965–972, doi:10.1007/s00253-009-2409-6.

Albergaria, H.; Torrão, A.R.; Hogg, T.; Gírio, F.M. Physiological behaviour of *Hanseniaspora guilliermondii* in aerobic glucose-limited continuous cultures. *FEMS Yeast Res.* **2003**, 3, 211–216, doi:10.1016/S1567-1356(02)00187-3.

Alston, J.M.; Fuller, K.B.; Lapsley, J.T.; Soleas, G. Too much of a good thing? Causes and consequences of increases in sugar content of California wine grapes. *J. Wine Econ.* **2011**, 6, 135–159, doi:10.1017/S1931436100001565.

Andorrà, I.; Berradre, M.; Mas, A.; Esteve-Zarzoso, B.; Guillamón, J.M. 2012. Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT Food Sci. Technol.* **2012**, 49, 8–13, doi:10.1016/j.lwt.2012.04.008.

Arneborg, N.; Siegumfeldt, H.; Andersen, G.H.; Nissen, P.; Daria, V.R.; Rodrigo, P.J.; Glückstad, J. Interactive optical trapping shows that confinement is a determinant of growth in a mixed yeast culture. *FEMS Microbiol. Lett.* **2005**, 245, 155–159, doi:10.1016/j.femsle.2005.03.008.

Ballester-Tomás, L.; Prieto, J.A.; Gil, J.V.; Baeza, M.; Randez-Gil, F. The Antarctic yeast *Candida sake*: Understanding cold metabolism impact on wine. *Int. J. Food Microbiol.* **2017**, 245, 59–65, doi:10.1016/j.ijfoodmicro.2017.01.009.

Barnett, J.A.; Entian, K.D. A history of research on yeasts 9: Regulation of sugar metabolism. *Yeast.* **2005**, 22, 835–894, doi:10.1002/yea.1249.

Beltran, G.; Novo, M.; Rozès, N.; Mas, A.; Guillamón, J.M. Nitrogen catabolite repression in during wine fermentations. *FEMS Yeast Res.* **2004**, 4, 625–632, doi:10.1016/j.femsyr.2003.12.004.

Benito, A.; Calderón, F.; Palomero, F.E.; Benito, S. Combine use of selected *Schizosaccharomyces pombe* and *Lachancea thermotolerans* yeast strains as an alternative to the traditional malolactic fermentation in red wine production. *Molecules* **2015**, 20, 9510–9523, doi:10.3390/molecules20069510.

Binati, R.L.; Lemos Junior, W.J.F.; Luzzini, G.; Slaghenaufi, D.; Ugliano, M.; Torriani, S. Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans*, *Metschnikowia spp*. and *Starmerella bacillaris* strains isolated in Italy. *Int. J. Food Microbiol.* **2020**, 318, 108470, doi:10.1016/j.ijfoodmicro.2019.108470.

Bindon, K.; Varela, C.; Kennedy, J.; Holt, H.; Herderich, M. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. cabernet sauvignon 1. Grape and wine chemistry. *Food Chem.* **2013**, 138, 1696–1705 doi:10.1016/j.foodchem.2013.03.014.

Bisson, L.F. Stuck and sluggish fermentations. *Am. J. Enol. Vitic.* **1999**, 50, 107–119, doi:10.1533/9781845698133.5.280.

Branco, P.; Francisco, D.; Chambon, C.; Hébraud, M.; Arneborg, N.; Almeida, M.G.; Caldeira, J.; Albergaria, H. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* **2014**, 98, 843–853, doi:10.1007/s00253-013-5411-y.

Buescher, W.A.; Siler, C.E.; Morris, J.R.; Threlfall, R.T.; Main, G.L.; Cone, G.C. High alcohol wine production from grape juice concentrates. *Am. J. Enol. Vitic.* **2001**, 52, 345–351, doi:10.1002/1097-0010(20010115)81:2<281::AID-JSFA808>3.0.CO;2-B.

Canonico, L.; Comitini, F.; Oro, L.; Ciani, M. Sequential fermentation with selected immobilized non-*Saccharomyces* yeast for reduction of ethanol content in wine. *Front. Microbiol.* **2016**, 7, 278, doi:10.3389/fmicb.2016.00278.

Canonico, L.; Solomonb, M.; Comitinia, F.; Ciani, M.; Varela, C. Volatile profile of reduced alcohol wines fermented with selected non-*Saccharomyces* yeasts under different aeration conditions. *Food Microbiol.* **2019**, 84, 103247, doi:10.1016/j.fm.2019.103247, doi:10.1016/j.ijfoodmicro.2005.11.012.

Ciani, M.; Beco, L.; Comitini, F. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int. J. Food Microbiol.* **2006**, 108, 239–245, doi:10.1016/j.ijfoodmicro.2005.11.012.

Ciani, M.; Comitini, F. Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* **2015**, 1, 1–6, doi:10.1016/j.cofs.2014.07.001.

Ciani, M.; Maccarelli, F. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World J. Microbiol. Biotechnol.* **1998**, 14, 199–203, doi:10.1023/A:1008825928354.

Ciani, M.; Morales, P.; Comitini, F.; Tronchoni, J.; Canonico, L.; Curiel, J.A.; Oro, L.; Rodrigues, A.J.; Gonzalez, R. Non-conventional yeast species for lowering ethanol content of wines. *Front. Microbiol.* **2016**, 7, 642, doi:10.3389/fmicb.2016.00642.

Ciani, M.; Rosini, G. Validity of Genevois' equation in wine yeast selection. *Ann. Microbiol. Enzim.* **1995**, 45, 201–207.

Contreras, A.; Hidalgo, C.; Henschke, P.A.; Chambers, P.J.; Curtin, C.; Varela, C. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* **2014**, 80, 1670–1678, doi:10.1128/aem.03780-13.

Contreras, A.; Hidalgo, C.; Schmidt, S.; Henschke, P.; Curtin, C.; Varela, C. The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* **2015**, 205, 7–15, doi:10.1016/j.ijfoodmicro.2015.03.027.

Coulter, A.D.; Henschke, P.A.; Simos, C.A.; Pretorius, I.S. When the heat is on, yeast fermentation runs out of puff. *Aust. N. Z. Wine Ind.* **2008**, 23, 26–30.

De Toda, F.M.; Sancha, J.C.; Balda, P. Reducing the sugar and pH of the grape (*Vitis vinifera* L. cvs. 'Grenache' and 'Tempranillo') through a single shoot trimming. *S. Afr. J. Enol. Vitic.* **2013**, 34, 246–251, doi:10.21548/34-2-1101.

Diban, N.; Arruti, A.; Barcelo, A.; Puxeu, M.; Urtiaga, A.; Ortiz, I. Membrane dealcoholization of different wine varieties reducing aroma losses. Modeling and experimental validation. *Innov. Food Sci. Emerg.* **2013**, 20, 259–268, doi:10.1016/j.ifset.2013.05.011.

Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Ortiz-Julien, A.; Vincenzo Gerbi, V.; Rolle, L.; Cocolin, L. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Appl. Microbiol. Biotechnol.* **2016**, 100, 5515–5526, doi:10.1007/s00253-016-7413-z.

Escudero, A.; Campo, E.; Fariña, L.; Cacho, J.; Ferreira, V. Analytical characterization of the aroma of five premium red wines. Insight into the role of odor families and the concept of fruitiness of wines. *J. Agric. Food Chem*. **2007**, 55, 4501–4510, doi:10.1021/jf0636418.

Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* **1999**, 49, 329–337, doi:10.1099/00207713-49-1-329.

Fleet, G.H.; Heard, G. Yeasts-growth during fermentation. In *Wine Microbiology and Biotechnology*; Fleet, G.H., Ed.; Harwood Academic Publishers: Chur, Switzerland, 1993; pp. 27–54.

García-Martín, N.; Perez-Magariño, S.; Ortega-Heras, M.; González-Huerta, C.; Mihnea, M.; González-Sanjosé, M.L.; Palacio, L.; Prádanos, P.; Hernández, A. Sugar reduction in musts with nanofiltration membranes to obtain low alcohol-content wines. *Sep. Purif. Technol.* **2010**, 76, 158–170, doi:10.1016/j.seppur.2010.10.002.

Giovanelli, G.; Peri, C.; Parravicini, E. Kinetics of grape juice fermentation under aerobic and anaerobic conditions. *Am. J. Enol. Vitic.* **1996**, 47, 429–434, doi:10.1007/BF00223387.

Gobbi, M.; Comitini, F.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* **2013**, 33, 271–281, doi:10.1016/j.fm.2012.10.004.

Gobbi, M.; De Vero, L.; Solieri, L.; Comitini, F.; Oro, L.; Giudici, P.; Ciani, M. Fermentative aptitude of non-*Saccharomyces* wine yeast for reduction in the ethanol content in wine. *Eur. Food Res. Technol.* **2014**, 239, 41–48, doi:10.1007/s00217-014-2187-y.

Godden, P. Persistent wine instability issues. *Aust. Grapegrow. Winemak.* **2000**, 443, 10–14.

Gonzalez, R.; Quirós, M.; Morales, P. Yeast respiration of sugars by non-*Saccharomyces* yeast species: A promising and barely explored approach to lowering alcohol content of wines. *Trends Food Sci. Tech.* **2013**, 29, 55–61, doi:10.1016/j.tifs.2012.06.015.

Jolly, N.P.; Augustyn, O.H.P.; Pretorius, I.S. The role and use of non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic.* **2006**, 27, 15–39, doi:10.21548/27-1-1475.

Jones, G.V.; White, M.A.; Cooper, O.R.; Storchmann, K. Climate change and global wine quality. *Clim. Chang.* **2005**, 73, 319–343, doi:10.1007/s10584-005-4704-2.

Kapsopoulou, K.; Mourtzini, A.; Anthoulas, M.; Nerantzis, E. Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *World J. Microb. Biot.* **2007**, 23, 735–739, doi:10.1007/s11274-006-9283-5.

Kontoudakis, N.; Esteruelas, M.; Fort, F.; Canals, J.M.; Zamora, F. Use of unripe grapes harvested during cluster thinning as a method for reducing alcohol content and pH of wine. *Aust. J. Grape Wine R.* **2011**, 17, 230–238, doi:10.1111/j.1755-0238.2011.00142.x.

Kutyna, D.R.; Varela, C.; Henschke, P.A.; Chambers, P.J.; Stanley, G.A. Microbiological approaches to lowering ethanol concentration in wine. *Trends Food Sci. Tech.* **2010**, 21, 293–302, doi:10.1016/j.tifs.2010.03.004.

Lleixà, J.; Manzano, M.; Mas, A.; Portillo, M.C. *Saccharomyces* and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions. *Front. Microbiol*. **2016**, 7, 1959, doi:10.3389/fmicb.2016.01959.

Mas, A.; Padilla, B.; Esteve-Zarzoso, B.; Beltran, G.; Reguant, C.; Bordons, A. Taking advantage of natural biodiversity for wine making: The WILDWINE project. *Agric. Agric. Sci. Procedia.* **2016**, 8, 4–9, doi:10.1016/j.aaspro.2016.02.002.

Maturano, Y.P.; Mestre, M.V.; Kuchen, B.; Toro, M.E.; Mercado, L.A.; Vazquez, F.; Combina, M. Optimization of fermentation-relevant factors: A strategy to reduce ethanol in red wine by sequential culture of native yeasts. *Int. J. Food Microbiol.* **2019**, 289, 40–48, doi:10.1016/j.ijfoodmicro.2018.08.016.

Mendoza, L.M.; De Nadra, M.C.M.; Farías, M.E. Kinetics and metabolic behavior of a composite culture of *Kloeckera apiculata* and *Saccharomyces cerevisiae* wine related strains. *Biotechnol. Lett.* **2007**, 29, 1057–1063, doi:10.1007/s10529-007-9355-0.

Mihnea, M.; González-SanJosé, M.L.; Ortega-Heras, M.; Pérez-Magariño, S.; García-Martin, N.; Palacio, L.; Prádanos, P.; Hernández, A. Impact of must sugar reduction by membrane applications on volatile composition of verdejo wines. *J. Agric. Food Chem.* **2012**, 60, 7050–7063, doi:10.1021/jf301433j.

Morales, P.; Rojas, V.; Quirós, M.; Gonzalez, R. The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotech.* **2015**, 99, 3993–4003, doi:10.1007/s00253-014-6321-3.

Nissen, P.; Nielsen, D.; Arneborg, N. Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell–cell contact-mediated mechanism. *Yeast.* **2003**, 20, 331–341, doi:10.1002/yea.965.

Padilla, B.; García-Fernández, D.; González, B.; Izidoro, I.; Esteve-Zarzoso, B.; Beltran, G.; Mas, A. Yeast biodiversity from DOQ priorat uninoculated fermentations. *Front. Microbiol.* **2016**, 7, 930, doi:10.3389/fmicb.2016.00930.

Padilla, B.; Zulian, L.; Ferreres, À.; Pastor, R.; Esteve-Zarzoso, B.; Beltran, G.; Mas, A. Sequential inoculation of native non-*Saccharomyces* and *Saccharomyces cerevisiae* strains for wine making. *Front. Microbiol.* **2017**, 8, 1293, doi:10.3389/fmicb.2017.01293.

Quirós, M.; Gonzalez-Ramos, D.; Tabera, L.; Gonzalez, R. A new methodology to obtain wine yeast strains overproducing mannoproteins. *Int. J. Food Microbiol.* **2010**, 139, 9–14, doi:10.1016/j.ijfoodmicro.2010.02.014.

Quirós, M.; Rojas, V.; Gonzalez, R.; Morales, P. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* **2014**, 181, 85–91, doi:10.1016/j.ijfoodmicro.2014.04.024.

Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. In *Handbook of Enology: The Microbiology of Wine and Vinifications*, 2nd ed.; John Wiley & Sons, Ltd.: Chichester, UK, **2006**; pp. 22–53, doi:10.1002/0470010398.

Roca-mesa, H.; Sendra, S.; Mas, A.; Beltran, G.; Torija, M.J. Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. *Microorganisms* **2020**, 8, 157, doi:10.3390/microorganisms8020157.

Röcker, J.; Strub, S.; Ebert, K.; Grossmann, M. Usage of different aerobic non-*Saccharomyces* yeasts and experimental conditions as a tool for reducing the potential ethanol content in wines. *Eur. Food Res. Technol.* **2016**, 242, 2051–2070, doi:10.1007/s00217-016-2703-3.

Rodrigues, A.J.; Raimbaud, T.; Gonzalez, R.; Morales, P. Environmental factors influencing the efficacy of different yeast strains for alcohol level reduction in wine by respiration. *LWT Food Sci. Technol.* **2016**, 65, 1038–1043. doi:10.1016/j.lwt.2015.09.046.

Rollero, S.; Bloem, A.; Camarasa, C.; Sanchez, I.; Ortiz-Julien, A.; Sablayrolles, J.M.; Dequin, S.; Mouret, J.R. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. *Appl. Microbiol. Biotechnol.* **2015**, 99, 2291–2304, doi:10.1007/s00253-014-6210-9.

Rossouw, D.; Heyns, E.H.; Setati, M.E.; Bosch, S.; Bauer, F.F. Adjustment of trehalose metabolism in wine *Saccharomyces cerevisiae* strains to modify ethanol yields. *Appl. Environ. Microbiol.* **2013**, 79, 5197–5207, doi:10.1128/AEM.00964-13.

Steensels, J.; Snoek, T.; Meersman, E.; Nicolino, M.P.; Voordeckers, K.; Verstrepen, K.J. Improving industrial yeast strains: Exploiting natural and artificial diversity. *FEMS Microbiol. Rev.* **2014**, 38, 947–995, doi:10.1111/1574-6976.12073.

Swinnen, S.; Thevelein, J.M.; Nevoigt, E. Genetic mapping of quantitative phenotypic traits in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **2013**, 12, 215–227, doi:10.1111/j.15671364.2011.00777.x.

Varela, C.; Barker, A.; Tran, T.; Borneman, A.; Curtin, C. Sensory profile and volatile aroma composition of reduced alcohol Merlot wines fermented with *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Int. J. Food Microbiol.* **2017**, 252, 1–9, doi:10.1016/j.ijfoodmicro.2017.04.002.

Varela, C.; Dry, P.R.; Kutyna, D.R.; Francis, I.L.; Henschke, P.A.; Curtin, C.D.; Chambers, P.J. Strategies for reducing alcohol concentration in wine. *Aust. J. Grape Wine R.* **2015**, 21, 670–679, doi:10.1111/ajgw.12187.

Varela, C.; Kutyna, D.R.; Solomon, M.R.; Black, C.A.; Borneman, A.; Henschke, P.A.; Pretorius, I.S.; Chambers, P.J. Evaluation of gene modification strategies for the development of low-alcohol wine yeasts. *Appl. Environ. Microbiol.* **2012**, 78, 6068–6077, doi:10.1128/AEM.01279-12.

Varela, C.; Sengler, F.; Solomon, M.; Curtin, C. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chem.* **2016**, 209, 57–64, doi:10.1016/j.foodchem.2016.04.024.

Varela, J.; Varela, C. Microbiological strategies to produce beer and wine with reduced ethanol concentration. *Curr. Opin. Biotechnol.* **2019**, 56, 88–96, doi:10.1016/j.copbio.2018.10.003.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. *Int. J. Food Microbiol.* **2015**, 206, 67–74, doi:10.1016/j.ijfoodmicro.2015.04.022.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* **2016**, 7, 502, doi:10.3389/fmicb.2016.00502.

Supplementary

Table S1. Glucose consumption (g/L), ethanol production (%, v/v) and ethanol yield (g ethanol/g glucose) after 3 days in 5 mL YPD medium.

Strains	Glucose consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)	Strains	Glucose consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)
Sc23	**20 ± 0**	**0.84 ± 0.06**	**0.33 ± 0.02**	Hv19	20 ± 0	0.84 ± 0.01	0.33 ± 0.00
Cb29	19.90 ± 0.15	0.96 ± 0.08 *	0.38 ± 0.04 *	Hv14	20 ± 0	0.86 ± 0.05	0.34 ± 0.02
Cb35	20 ± 0	0.90 ± 0.06	0.36 ± 0.02	Hv71	19.98 ± 0.01	0.87 ± 0.03	0.34 ± 0.01
Cb14	20 ± 0	0.91 ± 0.01	0.36 ± 0	**It39**	**20 ± 0**	**0.73 ± 0.05 ***	**0.29 ± 0.02 ***
Cm25	20 ± 0	0.82 ± 0.01	0.32 ± 0	It76	18.53 ± 1.28 *	0.86 ± 0.06	0.37 ± 0.04
Cs34	7.79 ± 1.61 *	0.27 ± 0.04 *	0.27 ± 0.02 *	Lt1	19.98 ± 0.01	0.75 ± 0.01 *	0.30 ± 0
Cs44	15.19 ± 2.37 *	0.37 ± 0.05 *	0.19 ± 0.01 *	Lt2	19.97 ± 0	0.73 ± 0.01 *	0.29 ± 0 *
Cs18	18.71 ± 0.24 *	0.77 ± 0.06	0.33 ± 0.03	Mg20	7.58 ± 1.59 *	0.16 ± 0.04 *	0.17 ± 0 *
Sb4	20 ± 0	0.78 ± 0.05	0.31 ± 0.02	**Mp51**	**20 ± 0**	**0.68 ± 0.10 ***	**0.27 ± 0.04 ***
Sb46	19.94 ± 0.03	0.95 ± 0.11 *	0.38 ± 0.04 *	Mp52	19.97 ± 0.05	0.78 ± 0.10	0.31 ± 0.04
Sb09	20 ± 0	0.87 ± 0.06	0.34 ± 0.02	**Mp FA**	**19.92 ± 0.14**	**0.60 ± 0.07 ***	**0.24 ± 0.03 ***
Sb Nc	**19.99 ± 0.01**	**0.69 ± 0.15 ***	**0.27 ± 0.06 ***	**Sl35**	**20 ± 0.01**	**0.67 ± 0.01 ***	**0.27 ± 0 ***
Sb Nd	19.90 ± 0.08	0.98 ± 0.01 *	0.39 ± 0.01 *	Sl71	19.65 ± 0.10	0.95 ± 0.05 *	0.38 ± 0.02 *
Hg27	20 ± 0	0.81 ± 0.09	0.32 ± 0.03	Sp79	19.71 ± 0.08	0.92 ± 0.01	0.37 ± 0
Hg29	19.99 ± 0.01	0.84 ± 0.02	0.33 ± 0.01	Td58	19.95 ± 0.04	0.93 ± 0.01 *	0.37 ± 0.01
Hg02	20 ± 0	0.89 ± 0.02	0.35 ± 0.01	**Td35**	**19.97 ± 0.03**	**0.72 ± 0.02 ***	**0.28 ± 0.01 ***
Ho06	19.45 ± 0.15	0.85 ± 0.06	0.34 ± 0.02	Td80	19.94 ± 0.06	1.00 ± 0.11 *	0.39 ± 0.04 *
Ho07	17.65 ± 1.13 *	0.89 ± 0.04	0.40 ± 0.01 *	**Td Pt**	**19.95 ± 0.07**	**0.55 ± 0.01 ***	**0.22 ± 0 ***
Hu06	**20 ± 0**	**0.64 ± 0.03 ***	**0.25 ± 0.01 ***	**Td BA**	**19.97 ± 0.01**	**0.73 ± 0.06 ***	**0.29 ± 0.03 ***
Hu4	**20 ± 0**	**0.75 ± 0.06**	**0.30 ± 0.02**	**Zb42**	**20 ± 0**	**0.74 ± 0.01 ***	**0.29 ± 0 ***
Hu3	20 ± 0	0.91 ± 0.01	0.36 ± 0	**Zb43**	**20 ± 0**	**0.55 ± 0.02 ***	**0.22 ± 0.01 ***
Hu34	20 ± 0	1.00 ± 0.05 *	0.39 ± 0.02 *	Zr30	19.98 ± 0.01	0.91 ± 0.07	0.36 ± 0.03
Hv24	19.99 ± 0.01	0.87 ± 0.04	0.34 ± 0.02	Zr32	11.80 ± 1.07 *	0.39 ± 0.02 *	0.26 ± 0.03 *

Fermentation by Sc23 was considered as the control; The concentration of initial glucose in YPD was 20 g/L; * means statistically significant differences from the control sample in the same column (LSD test, $p < 0.05$); Selected strains are marked in bold.

Supplementary

Table S2. Analysis of sugars, ethanol, organic acids and glycerol from samples after 48 h of single fermentation.

Samples	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Glucose (g/L)	Fructose (g/L)	Sugar consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)
Sc23	0.59 ± 0.09	2.07 ± 0.44	3.38 ± 0.38	0.14 ± 0.02	0.15 ± 0.01	0.21 ± 0.06	2.90 ± 0.23	40.72 ± 2.25	65.95 ± 1.60	93.68 ± 4.28	5.26 ± 0.24	0.44 ± 0
Hu06	0.58 ± 0.05	1.84 ± 0.02	4.22 ± 0.12 *	0.10 ± 0.02	0.01 ± 0 *	0 ± 0 *	0.79 ± 0.11 *	81.91 ± 0.84 *	81.67 ± 0.72 *	37.28 ± 1.51 *	1.87 ± 0.01 *	0.40 ± 0.02 *
Hu4	0.49 ± 0.04 *	1.98 ± 0.02	5.35 ± 0 *	0.05 ± 0.01 *	0.01 ± 0.01 *	0.01 ± 0 *	0.62 ± 0.04 *	85.72 ± 0.50 *	84.79 ± 0.14 *	31.00 ± 0.64 *	1.71 ± 0.01 *	0.43 ± 0.01
It39	0.50 ± 0.15	1.76 ± 0.17 *	2.98 ± 0.26	0.19 ± 0.09	0.02 ± 0.03 *	0.01 ± 0 *	1.69 ± 1.49 *	65.71 ± 18.42 *	87.23 ± 11.69 *	47.91 ± 30.12 *	2.48 ± 1.44 *	0.42 ± 0.02
Lt1	0.37 ± 0.01 *	1.81 ± 0.12	4.26 ± 0.33 *	0.07 ± 0.04 *	0.01 ± 0.01 *	0 ± 0 *	2.12 ± 0.93 *	62.37 ± 6.60 *	72.83 ± 5.69 *	66.32 ± 12.29 *	3.85 ± 0.76 *	0.46 ± 0.01
Lt2	0.39 ± 0 *	2.18 ± 0.03	4.29 ± 0.19 *	0.10 ± 0.01	0.11 ± 0.01 *	0.11 ± 0.03 *	0.80 ± 0.30 *	80.62 ± 2.12 *	84.10 ± 1.38 *	35.28 ± 3.50 *	1.87 ± 0.19 *	0.42 ± 0
Mp51	0.87 ± 0.03 *	2.25 ± 0.19	4.83 ± 0.05 *	0.09 ± 0.02 *	0 ± 0 *	0 ± 0 *	0.33 ± 0.23 *	81.39 ± 2.01 *	91.26 ± 1.16 *	28.87 ± 3.18 *	1.40 ± 0.17 *	0.38 ± 0.01 *
Mp FA	0.74 ± 0.03 *	2.50 ± 0.04 *	3.53 ± 0.19	0.08 ± 0.01 *	0 ± 0 *	0 ± 0 *	0.02 ± 0.04 *	82.57 ± 2.34 *	90.18 ± 0.98 *	27.25 ± 3.29 *	1.21 ± 0.11 *	0.35 ± 0.01 *
Sb Nc	0.87 ± 0.08 *	2.36 ± 0 *	3.70 ± 0.13	0.01 ± 0 *	0 ± 0 *	0.01 ± 0 *	1.30 ± 0.22 *	93.45 ± 0.56 *	71.61 ± 2.14 *	34.94 ± 2.69 *	2.04 ± 0.14 *	0.46 ± 0
Sl35	0.42 ± 0.02	2.21 ± 0.04	3.37 ± 0.10	0.02 ± 0.01 *	0 ± 0 *	0.01 ± 0 *	0 ± 0 *	93.46 ± 0.49 *	85.43 ± 1.83 *	21.12 ± 2.32 *	1.01 ± 0.10 *	0.38 ± 0.01 *
Td35	0.46 ± 0.02 *	2.09 ± 0.06	4.15 ± 0.09 *	0.08 ± 0.02 *	0.01 ± 0 *	0 ± 0 *	0.25 ± 0.22 *	76.02 ± 4.08 *	88.68 ± 3.23 *	36.15 ± 7.28 *	1.78 ± 0.39 *	0.39 ± 0.01 *
Td BA	0.44 ± 0.06 *	2.47 ± 0.04 *	3.23c ± 0.36	0.11 ± 0.07	0.04 ± 0.02 *	0.01 ± 0 *	1.23 ± 0.96 *	53.95 ± 11.64 *	75.72 ± 7.26 *	70.33 ± 18.89 *	3.96 ± 1.09 *	0.44 ± 0
Td Pt	0.42 ± 0 *	1.96 ± 0.07	4.22 ± 0.42 *	0.01 ± 0.01 *	0 ± 0 *	0 ± 0 *	0 ± 0 *	87.33 ± 4.62 *	97.41 ± 3.67 *	16.12 ± 8.29 *	0.81 ± 0.25 *	0.42 ± 0.09
Zb42	0.88 ± 0.02 *	1.99 ± 0.05	3.26 ± 0.08	0.34 ± 0.05 *	0.03 ± 0.01 *	0 ± 0 *	2.06 ± 0.39 *	45.39 ± 1.37	68.90 ± 1.42	86.56 ± 2.79	4.93 ± 0.10	0.45 ± 0.01
Zb43	0.40 ± 0.01 *	1.92 ± 0.02	4.14 ± 0.35 *	0.08 ± 0.01 *	0.06 ± 0.01 *	0 ± 0 *	0.83 ± 0.16 *	92.36 ± 0.94 *	73.98 ± 1.63 *	35.18 ± 1.50 *	1.65 ± 0.08 *	0.37 ± 0.01 *

* means statistically significant differences from the control sample in the same column (LSD test, $p < 0.05$).

CHAPTER 2

Effect of a multistarter yeast inoculum on ethanol reduction and population dynamics in wine fermentation

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Abstract

Microbiological strategies are currently being considered as methods for reducing the ethanol content of wine. Fermentations started with a multistarter of three non-*Saccharomyces* yeasts (*Metschnikowia pulcherrima* (Mp), *Torulaspora delbrueckii* (Td) and *Zygosaccharomyces bailii* (Zb)) at different inoculum concentrations. *S. cerevisiae* (Sc) was inoculated into fermentations at 0 h (coinoculation), 48 h or 72 h (sequential fermentations). The microbial populations were analyzed by a culture-dependent approach (Wallerstein Laboratory Nutrient (WLN) culture medium) and a culture-independent method (PMA-qPCR). The results showed that among these three non-*Saccharomyces* yeasts, Td became the dominant non-*Saccharomyces* yeast in all fermentations, and Mp was the minority yeast. Sc was able to grow in all fermentations where it was involved, being the dominant yeast at the end of fermentation. We obtained a significant ethanol reduction of 0.48 to 0.77% (*v*/*v*) in sequential fermentations, with increased concentrations of lactic and acetic acids. The highest reduction was achieved when the inoculum concentration of non-*Saccharomyces* yeast was 10 times higher (10^7 cells/mL) than that of *S. cerevisiae*. However, this reduction was lower than that obtained when these strains were used as single non-*Saccharomyces* species in the starter, indicating that interactions between them affected their performance. Therefore, more combinations of yeast species should be tested to achieve greater ethanol reductions.

Keywords: wine; PMA-qPCR; *Metschnikowia pulcherrima*; *Torulaspora delbrueckii*; *Zygosaccharomyces bailii*; mixed fermentation; coinoculation; sequential fermentation

Introduction

In recent years, the average ethanol concentration in wine has increased, mainly due to climate change and consumer preference for wine styles (Godden, 2000; Jones et al., 2005; Contreras et al., 2015). Different strategies have been applied to reduce ethanol production in wine, such as decreasing the leaf area to lower the sugar content in grape berries, reducing the maturity of grapes, removing sugar from the grape must, developing or screening low-alcohol wine yeasts and removing alcohol from wine (reviewed by Varela et al. (2015)). Compared with microbiological strategies, other strategies might have negative effects on wine, such as delaying maturity, reducing the yield of berries, causing a significant reduction in anthocyanins, soluble solids and volatile compounds, and decreasing the wine color (García-Martín et al., 2010; Bindon et al., 2013; De Toda et al., 2016; Longo et al., 2016). Therefore, microbiological strategies were considered to be effective and accompanied by smaller impacts on the wine sensory profile and quality. In particular, the use of non-*Saccharomyces* yeast strains for reducing the alcohol content of wines has been proven to improve the wine aroma complexity and has become a consistent proposal (Padilla et al., 2016; Varela et al., 2017; Canonico et al., 2019a).

Non-*Saccharomyces* yeasts have been applied in fermentations to reduce ethanol using different inoculation strategies. For example, several studies have shown that single-culture fermentations with *Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Starmerella bombicola*, *Starmerella bacillaris*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces sapae* species are able to reduce the ethanol content in wine (Gobbi et al., 2013 & 2014; Englezos et al., 2016; Furlani et al., 2017; Varela et al., 2017; Castrillo et al., 2019; Junior et al., 2019; Binati et al., 2020; Hranilovic et al., 2020). However, most of these single yeast fermentations became stuck. To solve this problem, mixed fermentations with non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* have been proposed, demonstrating that simultaneous inoculation of *S. cerevisiae* with *L. thermotolerans, S.*

bacillaris or *M. pulcherrima* species produced a reduction in ethanol (Gobbi et al., 2013; Morales et al., 2015; Englezos et al., 2016; Varela et al., 2017; Hranilovic et al., 2020). In addition, to achieve this purpose, most researchers preferred to use non-*Saccharomyces* yeasts (*Hanseniaspora osmophila*, *H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, *S. bombicola*, *Torulaspora delbrueckii* and *Z. bailii* species) in sequential fermentations, with *S. cerevisiae* inoculated at 48 h, 72 h, or when 50% of the sugar from grape must was consumed (Comitini et al., 2011; Gobbi et al., 2013; Varela et al., 2016; Canonico et al., 2016, 2019 a & b; Binati et al., 2020).

Due to the growing interest in mixed fermentations, it is necessary to understand the behavior and interactions of strains throughout the fermentation process. Thus, the population dynamics of yeasts have become a key factor in the study of yeast interactions. The traditional microbial counting method usually uses different solid media, such as Wallerstein Laboratory Nutrient (WLN) and lysine agar, to distinguish or isolate different yeast species based on their dissimilar morphological characteristics or selective growth (Pallmann et al., 2001; Wang et al., 2016). However, this is not an effective method of discrimination when two or more non-*Saccharomyces* yeast species are simultaneously inoculated in mixed fermentations because some of them show similar morphological profiles. In addition, for an accurate evaluation of the population dynamics of different yeast species, we have to consider the existence of viable but nonculturable (VBNC) cells caused by the different metabolic statuses of cells. In these cases, the use of the plating method would not be appropriate because those VBNC cells would not be detectable (Díaz et al., 2013).

Compared with the classical method, researchers have confirmed that quantitative real-time polymerase chain reaction (qPCR) is a more sensitive and specific technique for the detection of nucleic acids, and it shows a wide detection range for different cell concentrations, usually from 10 to 10^8 cells/mL (Phister and Mills, 2003; Rawsthorne and Phister, 2006; Forootan et al., 2017). This sensitive and low-detection limit method provides the possibility to detect species present in low quantities in the mixed fermentation process. However, the population of yeasts is

often overestimated from qPCR because the method does not discriminate the DNA of living and dead cells in fermenting must (Hierro et al., 2007; Padilla et al., 2017). To distinguish the population of living cells from that of dead cells, DNA-binding dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA), are applied to must samples as a pretreatment before DNA extraction. The qPCR analysis of these samples quantifies only living cells because the living cell membrane is impermeable to DNA-binding dye, and therefore, it will only bind to free DNA from dead cells, avoiding its amplification. Nocker et al. (2006) showed that PMA is more effective than EMA, which is the reason why PMA has been favored by researchers in the counting of living cells in recent years (Andorrà et al., 2010a; Fittipaldi et al., 2012; Vendrame et al., 2013; Li et al. 2017; Navarro et al., 2020).

Most articles have focused on evaluating the effects of single strains or mixed starters composed of one *S. cerevisiae* strain and one non-*Saccharomyces* species. However, this work aims to test a multistarter culture consisting of several non-*Saccharomyces* species previously selected for their ability to reduce ethanol (Zhu et al., 2020) and a commercial *S. cerevisiae* strain. The use of multiple selected species as the inoculum may improve ethanol reduction, as well as the overall complexity of wines (Padilla et al., 2017).

The aim of the present work was to analyze the effect of the mixed inoculation of *S. cerevisiae* and several non-*Saccharomyces* yeasts on ethanol reduction and population dynamics. Three non-*Saccharomyces* strains belonging to *M. pulcherrima*, *T. delbrueckii* and *Z. bailii* species, which have been demonstrated to have the ability to reduce ethanol in single fermentations (Zhu et al., 2020), were simultaneously inoculated into the must. To determine the best inoculation protocol to achieve ethanol reduction, different mixed inoculation conditions with *S. cerevisiae* were evaluated (sequential or coinoculation at different inoculation ratios). The population dynamics of the different yeasts were assessed by WLN counting and PMA-qPCR analysis during the fermentation process, and the concentrations of the main organic compounds were analyzed by HPLC in the resulting wines.

Materials and Methods

Yeast Strains

Four different yeast strains were used in this study: the commercial wine yeast *S. cerevisiae* (Lalvin QA23®, referred to as Sc) from Lallemand Inc. (Montreal, QC, Canada), *M. pulcherrima* 51 (Mp) and *T. delbrueckii* Priorat (Td), selected from the Priorat Appellation of Origin (URV collection, Tarragona, Spain) (Padilla et al., 2016), and *Z. bailii* CECT 11043 (Zb), obtained from the Spanish Type Culture Collection. These strains were previously selected for their ability to produce low ethanol yield (Zhu et al., 2020) when several strains of each species were tested.

The strains were stored at − 80 °C in YPD liquid medium (2% (*w*/*v*) glucose, 2% (*w*/*v*) yeast extract, and 1% (*w*/*v*) peptone, Cultimed, Barcelona, Spain) with 40% (*v*/*v*) glycerol. Before starting the fermentations, the yeasts were grown at 28 °C in YPD agar (YPD liquid with 1.7% (*w*/*v*) agar) and Wallerstein laboratory nutrient (WLN) agar (Becton, Dickinson and Company, Isère, France). The species identification of the four strains was confirmed by PCR-RFLP analysis of 5.8S-ITS rDNA according to Esteve-Zarzoso et al. (1999).

Natural Must and Starter Cultures

Natural must (NM) was obtained from Muscat grapes harvested from Finca Experimental Mas dels Frares of Rovira i Virgili University (Constantí, Spain) during the 2019 vintage (after treatment with 50 mg/L SO_2 and settling, the must had 220 g/L sugars, 4.49 g/L total acidity (as tartaric acid), 77.8 mg/L assimilable nitrogen and a pH of 3.27). The concentration of assimilable nitrogen was determined in a Miura autoanalyzer (EE-MIURAONE Rev., I.S.E. S.r.l., Italy) using an Ammonia and α-Aminic Nitrogen Enzymatic KIT (Tecnología Difusión Ibérica, S.L., Barcelona, Spain) and corrected with diammonium phosphate (Panreac Quimica SA, E.U.) until reaching a final concentration of 250 mg N/L. Before the start of the fermentations, dimethyl dicarbonate (0.2 mL/L) (ChemCruz, Santa Cruz Biotechnology, America)

was added to NM and kept at 4 °C for 24 h to eliminate endogenous microorganisms. The absence of microorganisms after this treatment was confirmed by plating must samples with no dilution and 10-fold dilution on YPD agar.

The starter cultures were performed by transferring a single colony from YPD agar to YPD liquid medium and incubating it for 24 h (*S. cerevisiae*) or 48 h (non-*Saccharomyces*) at 28 °C with a stirring rate of 120 rpm in an orbital shaker. After incubation, the cells were counted in a Neubauer chamber (Leica Microsystems GMS QmbH, Leica, Germany) and inoculated at the indicated concentrations into the NM.

Fermentation Trials in Natural Must

Eight different mixed fermentations were carried out in this study (Table 1). Initially, all fermentations were simultaneously inoculated with three non-*Saccharomyces* strains at two different concentrations: 10^6 cells/mL (fermentations named 1CN) or 10^7 cells/mL (fermentations named 10CN). Additionally, Sc was inoculated into these two mixed non-*Saccharomyces* starters at the same moment (coinoculated fermentations were named 1CA and 10CA) and at 48 or 72 h later (sequential fermentations named 1S48, 1S72, 10S48 and 10S72, respectively), always at the same concentration (10^6 cells/mL). A single fermentation with Sc (10^6 cells/mL) was used as the control fermentation (C).

Triplicate fermentations were conducted in 250 mL glass bottles with 230 mL of NM and incubated at 22 °C with stirring at 120 rpm. The bottle cap had two ports—one connected to a 0.22 µm filter (Dominique Dutscher, Brumath, France) for gas flow and the other clamped by an iron clip for sampling. Before inoculation with Sc in sequential fermentations, the concentration of assimilable nitrogen in the fermented must was determined and supplemented, if needed, to 100 mg/L. Fermentations were monitored by measuring the must density with an electronic densitometer (Densito 30PX Portable Density Meter, Mettler Toledo, Hospitalet de Llobregat, Spain). Fermentations were considered finished when the residual sugars were below 2 g/L, checked by D-glucose/D-fructose enzymatic assays (Biosystems S.A., Barcelona, Spain)

in the Miura autoanalyzer, or arrested when the must density did not decrease for more than two days. Samples from the end of fermentation were centrifuged at 7800 rpm for 5 min, and supernatants were frozen at − 20 °C until chemical analysis.

Table 1. Fermentation conditions in 230 mL Muscat sterile must. Non-*Saccharomyces* (Mp, Td and Zb) and *S. cerevisiae* (Sc) strains were inoculated at the listed cell concentrations (cells/mL) and times.

Fermentations	Inoculum Procedures	Inoculum Ratios (Mp:Td:Zb:Sc)	Mp	Td	Zb	Sc
1CA	Coinoculation of all strains	1:1:1:1	1×10^6	1×10^6	1×10^6	1×10^6
1S48	Sequential inoculation of Sc at 48 h	1:1:1:1	1×10^6	1×10^6	1×10^6	1×10^6
1S72	Sequential inoculation of Sc at 72 h	1:1:1:1	1×10^6	1×10^6	1×10^6	1×10^6
1CN	Coinoculation of non-*Saccharomyces* strains	1:1:1:0	1×10^6	1×10^6	1×10^6	
10CA	Coinoculation of all strains	10:10:10:1	1×10^7	1×10^7	1×10^7	1×10^6
10S48	Sequential inoculation of Sc at 48 h	10:10:10:1	1×10^7	1×10^7	1×10^7	1×10^6
10S72	Sequential inoculation of Sc at 72 h	10:10:10:1	1×10^7	1×10^7	1×10^7	1×10^6
10CN	Coinoculation of non-*Saccharomyces* strains	10:10:10:0	1×10^7	1×10^7	1×10^7	
C	Single inoculation of Sc					1×10^6

Colony Counting

The yeast population during fermentation was analyzed by colony growth on WLN plates according to the different colony morphologies of *Saccharomyces* and non-*Saccharomyces* strains (Fittipaldi et al., 2012). Briefly, samples were serially diluted in sterilized Milli-Q water from a Milli-Q purification system (Millipore S.A.S., Molsheim, France). The number of colony-forming units per milliliter (CFU/mL) was determined by plating 100 µL of three appropriately chosen dilutions on WLN agar. The plates were incubated at 28 °C for 2 or 3 days.

PMAxx Treatment

To obtain only the DNA of living cells, PMAxx™ viability dye (Biotium Inc., Fremont, CA, USA) was added to must samples as previously described by Navarro et al. (2020). Briefly, selected samples of must (1 mL) were centrifuged, and the pellets were washed with sterilized distilled water and treated with 25 µM PMAxx. After

incubation for 10 min in darkness, PMAxx was permanently linked to the DNA of dead cells by subjecting samples twice to light for 30 s, with an interval of 1 min on ice. Pellets were recovered by centrifugation and frozen until DNA extraction.

DNA Extraction and qPCR Analysis

DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) following the instructions of the manufacturer. Cell quantification of the different yeast species was conducted by species-specific qPCR using the primers CESP-F/SCER-R for *S. cerevisiae* (Hierro et al., 2017), MP2-F/MP2-R for *M. pulcherrima* (García et al., 2017a), Tods L2/Tods R2 for *T. delbrueckii* (Zott et al., 2010) and ZBF1/ZBR1 for *Z. bailii* (Rawsthorne and Phister, 2006) (all primer sequences are shown in Table A1). For all samples, qPCR was performed in triplicate using TB Green™ Premix Ex Taq™ II (2×) (Takara Bio Inc., Kusatsu, Japan) with a final volume of 20 μL (2 μL of DNA, 0.8 μL of each primer, 0.08 μL of ROX Reference Dye (50×), 10 μL TB Green Premix Ex Taq II (2×) and 6.32 μL of sterilized Milli-Q water) on a QuantStudio™ 5 real-time PCR instrument (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). A 96-well nonskirted PCR plate (4titude® Ltd., Wotton, UK) was used for the reaction. The amplification reaction was one cycle of 95 °C for 1 min and 40 cycles of 95 °C for 5 s and 60 °C for 35 s, followed by a dissociation step. Milli-Q water was used as a negative control. Ct (the cycle threshold) was determined using Thermo Fisher Scientific software (Waltham, MA, USA).

Standard curves were calculated for each species with and without PMAxx treatment, as described by Navarro et al. (2020) but slightly modified. After incubating the yeast colony in YPD liquid medium at 28 °C for 24 h, samples with 10^8 cells/mL were collected in triplicate and centrifuged at 10,000 rpm for 2 min. The pellet was washed once with 1 mL sterilized distilled water and was subjected to PMAxx treatment as previously described. The pellet was stored at − 20 °C until DNA extraction. Standard curves were created by plotting the average Ct values of a tenfold serial dilution of DNA from 10^8 to 10 cells/mL against the log of cells/mL.

Chemical Analysis

Residual sugars of samples at the end of fermentation were quantified by D-glucose/D-fructose enzymatic assays. Ethanol, glycerol and organic acids (citric acid, malic acid, tartaric acid, acetic acid, lactic acid and succinic acid) in the samples were determined by high-performance liquid chromatography (HPLC) using an Agilent 1100 (Agilent Technologies, Waldbronn, Germany) as previously described by Quirós et al. (2010) and Zhu et al. (2020).

Statistical Analysis

All graphs were generated using GraphPad Prism® version 8 (GraphPad Software, San Diego, CA, USA). The results are expressed as the mean ± standard deviation (SD). Statistically significant differences (one-way ANOVA) were analyzed by IBM SPSS Statistics version 23.0 (IBM, NY, USA). The ethanol yield was calculated with the formula (1).

$$\text{Ethanol yield (g/g)} = \text{ethanol production (g/L)}/\text{sugar consumption (g/L)} \quad (1)$$

Results

Fermentation Kinetics

The fermentation kinetic profiles obtained under the different inoculation conditions, measured as must density reduction, are shown in Figure 1. The fastest fermentations were those with single inoculation with Sc and with the coinoculation of all the strains at the same time (1CA and 10CA), which showed similar fermentation profiles and completed fermentations in 7 – 8 days (10CA completed the fermentation 1 day earlier than that of Sc alone (C)). The fermentation kinetics were less affected by the inoculum concentration of non-*Saccharomyces* yeasts but were influenced by the inoculation time of Sc. The sequential inoculations, in which only non-*Saccharomyces* strains were fermented for 48 – 72 h, resulted in slower fermentations, finishing all the sugars in 11 (1S48, 10S48, 10S72) or 13 days (1S72) (Figure 1). These fermentations

were slightly slower when Sc was inoculated later, at 72 h, mainly in the lower non-*Saccharomyces* inoculum (1S72). Finally, fermentations with only non-*Saccharomyces* species (1CN and 10CN) showed the slowest fermentation kinetics, getting stuck on the 13th day, at densities of 1.002 and 1.001 g/mL, respectively (Figure 1a,b). In general, the fermentations with non-*Saccharomyces* strains inoculated at 10^7 cells/mL showed slightly faster fermentation kinetics than did those inoculated at 10^6 cells/mL (Figure 1a,b).

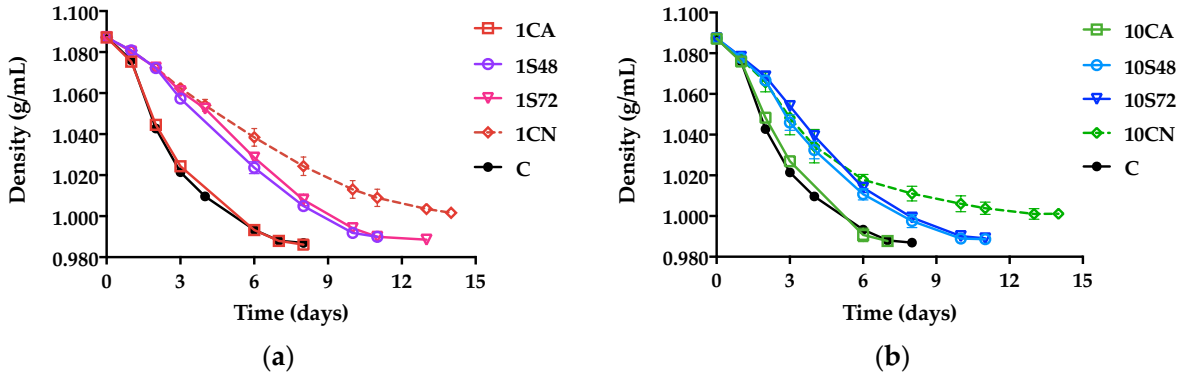
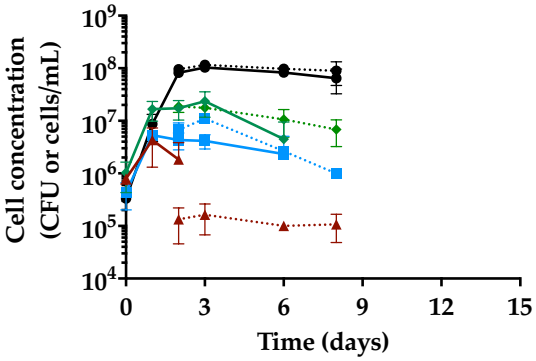


Figure 1. Density of single and mixed fermentations of *S. cerevisiae* and non-*Saccharomyces* yeasts with different inoculation conditions (see Table 1). The initial non-*Saccharomyces* inoculum size was 10^6 cells/mL (**a**) and 10^7 cells/mL (**b**).

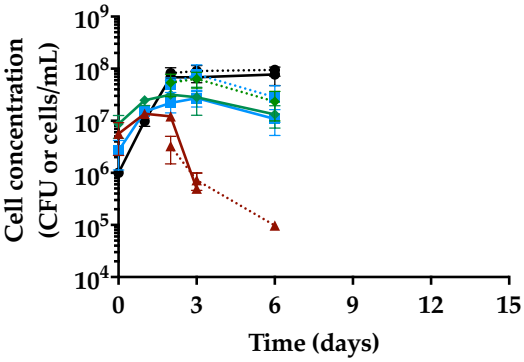
Yeast Population Dynamics

To study the yeast population dynamics during single or mixed fermentations, the growth of different species was obtained by different methodologies (Figure 2). On one hand, viability was determined during the fermentation process by plating the samples on WLN agar. Due to the different colony morphologies of the four yeast species on WLN agar (Fittipaldi et al., 2012), the colonies of these four strains could be counted on the same plate. On the other hand, PMA-qPCR analysis was performed to quantify the living yeast population using specific primers for each species. We applied PMA-qPCR from the second day of fermentation because the adaptation of the cells to the medium may produce an underestimation of the population by qPCR (Navarro et al., 2020).

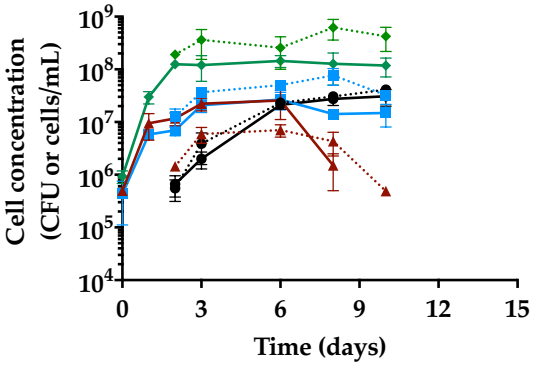
(a) (1CA)



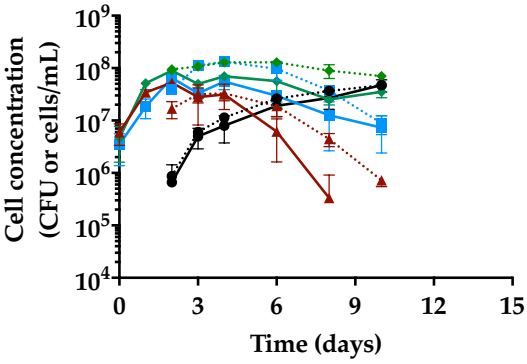
(b) (10CA)



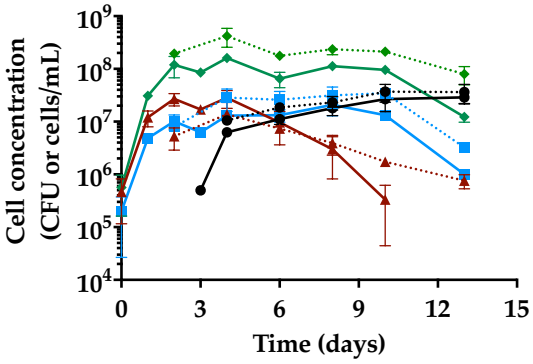
(c) (1S48)



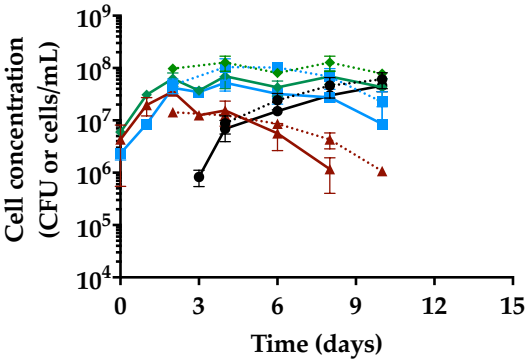
(d) (10S48)



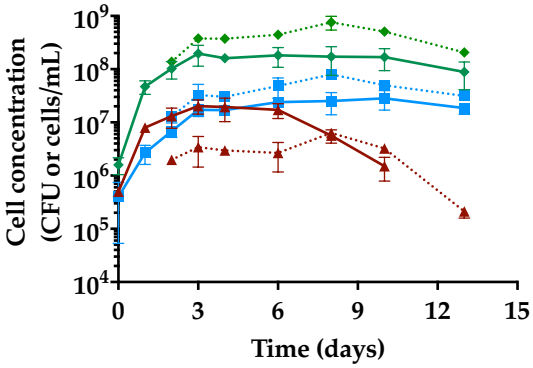
(e) (1S72)



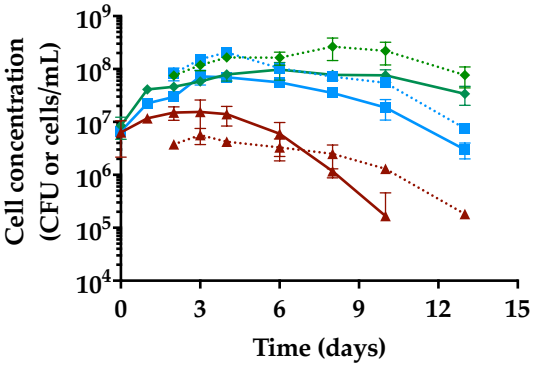
(f) (10S72)



(g) (1CN)



(h) (10CN)



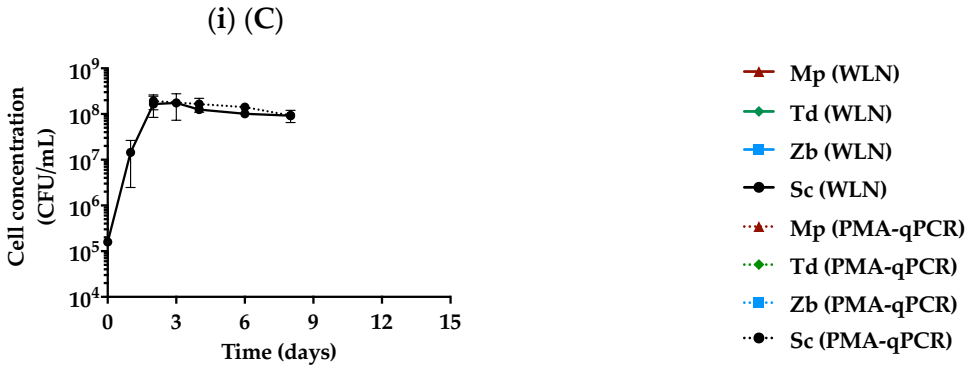


Figure 2. Yeast population analysis based on Wallerstein Laboratory Nutrient (WLN) plates (solid lines —) and qPCR with PMAxx treatment (dotted lines ·····), determined in the different fermentative conditions: non-*Saccharomyces* multistarter coinoculated with (**a**,**b**) and without (**g**,**h**) *S. cerevisiae (Sc)*; non-*Saccharomyces* multistarter with sequential inoculation of Sc at 48 h (**c**,**d**) or 72 h (**e**,**f**) and the single Sc fermentations (**i**). The initial non-*Saccharomyces* inoculum size was 10^6 cells/mL (**a**,**c**,**e**,**g**) and 10^7 cells/mL (**b**,**d**,**f**,**h**). Each species is shown in different colors (Mp in red, Td in green, Zb in blue and Sc in black).

First, the standard curves were calculated by plotting the Ct values (<30) of DNA from 10^3 to 10^8 cells/mL (*M. pulcherrima* was from 10^4 to 10^8 cells/mL) against the log input cells/mL (Table A2), with efficiencies between 87.92% and 98.83%.

After yeast inoculation, all strains were able to grow in fermentation media and showed different population dynamics, as seen in the results obtained from WLN plates. In fermentations in which each non-*Saccharomyces* species was inoculated at 10^6 cells/mL, the population of non-*Saccharomyces* yeasts grew rapidly on the first day (Figure 2a,c,e,g). Td quickly increased the population, reaching up to 2×10^8 CFU/mL on the third day. The Td population was then stable until the end of fermentation, becoming the dominant yeast in all mixed inoculations, except in the coinoculated fermentations with Sc (1CA and 10CA, Figures 2a,b), in which Sc significantly impaired growth, with the fermentation reaching a maximum Td population of only 10^7 CFU/mL under 1CA conditions. Mp reached a maximum population of 2.7×10^7 CFU/mL in the sequential or non-*Saccharomyces* coinoculations, with densities higher than that of Zb within the first days but decreasing after the sixth day under most conditions (the Mp decrease occurred much earlier in the coinoculation with Sc, 1CA).

In the case of Zb, the maximum growth was reached in the late fermentation stage, with a population of 2.8×10^7 CFU/mL, and was maintained rather stably until the end of fermentation.

The population dynamics in fermentations inoculated with 10^7 cells/mL of each non-*Saccharomyces* yeast differed from those inoculated at 10^6 cells/mL (Figures 2b,d,f,h). In those fermentations, Td and Zb presented similar population dynamics, and although Td was again the yeast with the highest population among all non-*Saccharomyces* yeasts, the difference relative to Zb was not as large (Td reached 9.7×10^7 CFU/mL and Zb 7.0×10^7 CFU/mL). Mp achieved the highest population (5.0×10^7 CFU/mL) on the second day and became the minority strain until the end of fermentation.

Regarding the *S. cerevisiae* dynamics, the dominance was only achieved in both coinoculated fermentations (1CA and 10CA), reaching a similar concentration to that of the Sc single inoculation on the third day of fermentation (1.8×10^8 CFU/mL, Figures 2a,b). However, the growth of Sc was restricted in sequential fermentations, where its population increased gradually until the end of fermentation, achieving up to 4.7×10^7 CFU/mL (Figures 2c – 2f). In these sequential fermentations, Sc together with Td were the majority strains at the end of the fermentation. This loss of the full imposition was not due to the lack of assimilable nitrogen, since supplementation to 100 mg/L was carried out in the deficient fermentations (1S72, 10S48 and 10S72) before the inoculation of Sc (as detailed in Table A3).

As already mentioned, during the fermentation process, Td and Zb colonies were observed until the end of fermentation (except in fermentation 1CA, where Td and Zb were absent at the end point), while Mp was absent on WLN agar after mid-fermentation. However, PMA-qPCR analysis allowed the detection of Mp until the end of fermentation, with the population ranging from 1×10^5 to 1.1×10^6 cells/mL, as well as that of Td and Zb at the end of fermentation in 1CA (Figure 2a). After the second day of fermentation, the trends of the yeast populations of Td, Zb and Sc obtained by PMA-qPCR analysis were similar to those of the WLN counting, although

the cell concentrations obtained by PMA-qPCR were higher, reaching a difference of one order of magnitude for most non-*Saccharomyces* yeasts (Figures 2). Specifically, for the Td, Zb and Sc strains, the maximum populations obtained by PMA-qPCR from mixed fermentations reached 7.6×10^8, 1.7×10^8 and 1.2×10^8 cells/mL, respectively. In contrast, the population of Mp obtained by PMA-qPCR analysis before mid-fermentations was lower than that obtained by WLN counting in the first 6 days for fermentations inoculated with 10^6 cells/mL and in the first 3 days for fermentations inoculated with 10^7 cells/mL.

Main Fermentation Byproducts

To detect the ethanol reduction of the different inoculated fermentations and the metabolic characteristics of yeasts under the different conditions, the residual sugar, ethanol production and main byproducts at the end of fermentation were analyzed, as shown in Table 2.

All fermentations with a mixed inoculum of non-*Saccharomyces* and Sc were completed, with less than 2 g/L residual sugars. However, the fermentations with only non-*Saccharomyces* strains, 1CN and 10CN, presented higher concentrations of residual sugars due to the stagnation of these two conditions (Table 2). Among all fermentations, the control fermentation (C) had the highest ethanol production (13.06%, *v*/*v*) and ethanol yield (0.47 g/g) (Figure 3). Mixed fermentations decreased ethanol production by 0.13 to 0.77% (*v*/*v*) compared to single fermentation by Sc (C), and this decrease was significant in 5 fermentations (1S48, 1S72, 10CA, 10S48 and 10S72) (Table 2). Fermentation 10S72 showed the highest ethanol reduction with the lowest ethanol yield (0.44 g/g), followed by 10S48, 1S72, 1S48 and 10CA (Table 2, Figure 3). Among these sequential fermentations, our results show a trend in relation to the inoculum concentration of non-*Saccharomyces* yeast, since a higher ethanol reduction was achieved when a higher inoculum of non-*Saccharomyces* was used (0.65 ± 0.11 and 0.77 ± 0.06 with 10^7 cells/mL vs. 0.48 ± 0.10 and 0.49 ± 0.08 with 10^6 cells/mL).

Table 2. Concentrations of sugars, ethanol, glycerol and organic acids at the end of fermentation.

Compounds	C	1CA	1S48	1S72	1CN	10CA	10S48	10S72	10CN
Residual sugars (g/L)	0.13 ± 0.17	0.39 ± 0.29	1.22 ± 0.62	0.42 ± 0.57	29.61 ± 5.65 *	0.42 ± 0.41	0.37 ± 0.22	0.88 ± 1.03	28.30 ± 6.29 *
Ethanol production (%, *v*/*v*)	13.06 ± 0.03	12.93 ± 0.06	12.58 ± 0.10 *	12.57 ± 0.08 *	11.04 ± 0.13 *	12.77 ± 0.05 *	12.41 ± 0.11 *	12.29 ± 0.06 *	10.91 ± 0.06 *
Ethanol yield (g/g)	0.47 ± 0	0.46 ± 0	0.45 ± 0 *	0.45 ± 0 *	0.46 ± 0.01	0.46 ± 0	0.45 ± 0 *	0.44 ± 0 *	0.45 ± 0.01 *
Ethanol reduction (%, *v*/*v*)	0	0.13 ± 0.06	0.48 ± 0.10 *	0.49 ± 0.08 *	NC	0.29 ± 0.05 *	0.65 ± 0.11 *	0.77 ± 0.06 *	NC
Glycerol (g/L)	6.57 ± 0.08	6.57 ± 0.22	5.41 ± 0.75 *	5.74 ± 0.93	4.96 ± 0.41 *	6.23 ± 0.11	6.70 ± 0.48	6.38 ± 0.18	5.44 ± 0.68 *
Citric acid (g/L)	0.13 ± 0	0.13 ± 0	0.11 ± 0.01 *	0.12 ± 0.01 *	0.12 ± 0 *	0.12 ± 0.01 *	0.12 ± 0.01 *	0.11 ± 0 *	0.11 ± 0 *
Tartaric acid (g/L)	2.79 ± 0.07	3.10 ± 0.14	3.41 ± 0.38	3.30 ± 0.31	3.38 ± 0.34	2.47 ± 1.10	3.23 ± 0.25	3.44 ± 0.14	3.38 ± 0.28
Malic acid (g/L)	0.81 ± 0.13	0.63 ± 0.05 *	0.50 ± 0.03 *	0.45 ± 0.03 *	0.39 ± 0.03 *	0.61 ± 0.02 *	0.54 ± 0.03 *	0.51 ± 0.01 *	0.44 ± 0.02 *
Succinic acid (g/L)	0.44 ± 0	0.43 ± 0.05	0.40 ± 0.05	0.36 ± 0.05	0.37 ± 0.02	0.44 ± 0.06	0.52 ± 0.03	0.52 ± 0.02	0.67 ± 0.05 *
Lactic acid (g/L)	0.20 ± 0.03	0.24 ± 0.02	0.24 ± 0.01	0.30 ± 0.10 *	0.29 ± 0.01 *	0.23 ± 0.05	0.30 ± 0 *	0.33 ± 0.04 *	0.25 ± 0.03
Acetic acid (g/L)	0.10 ± 0.01	0.14 ± 0.03	0.29 ± 0.08 *	0.36 ± 0.05 *	0.32 ± 0.06 *	0.22 ± 0.05 *	0.23 ± 0.05 *	0.22 ± 0.08 *	0.21 ± 0.04 *

Values are mean ± standard deviation of three independent replicates; The initial sugar concentration of synthetic must was 220 g/L; * means statistically significant differences from the control sample of C on the same row (LSD test, $p < 0.05$); NC means no calculate ethanol reduction due to stuck fermentation.

Regarding the other fermentation byproducts, the concentration of glycerol in the mixed fermentations was similar to that of single fermentation with Sc (C), except in the 1S48, 1CN and 10CN fermentations, which had lower glycerol contents, although the latter two conditions had residual sugars left. On the other hand, in all fermentations, the concentrations of lactic and acetic acids were higher in the mixed fermentations, although they remained below 0.33 g/L and 0.36 g/L, respectively (Table 2). Surprisingly, more acetic acid was detected at a lower inoculum concentration of non-*Saccharomyces* yeasts (10^6 cells/mL).

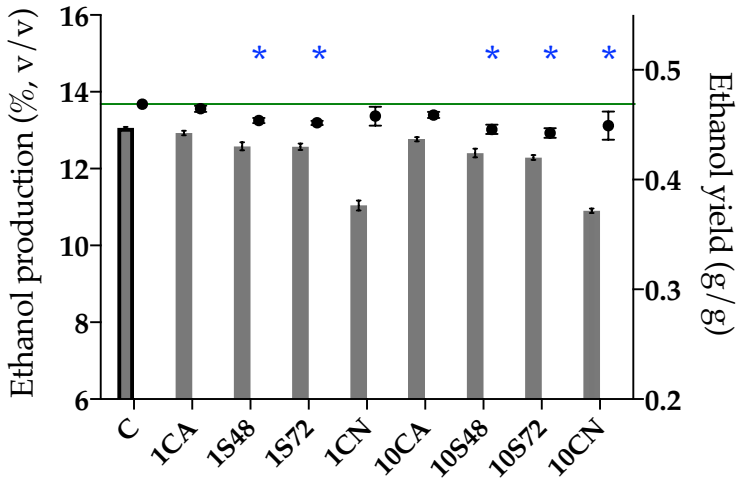


Figure 3. Ethanol production (%, *v*/*v*) and ethanol yield (g/g) at the end of single and mixed fermentations. * indicates statistically significant differences from the control sample (C) (LSD (least significant difference) test, $p < 0.05$). The value of the green line is 0.47 g/g (ethanol yield of C). 1CN and 10CN were stuck fermentations.

Discussion

In recent years, non-*Saccharomyces* yeasts have been proposed for use as starters in alcoholic fermentation to reduce the ethanol content in wine (Contreras et al., 2014a; Gobbi et al., 2014; Quirós et al., 2014; Canonico et al., 2016; Ciani et al., 2016; Junior et al., 2019; Hranilovic et al., 2020; Zhu et al., 2020). Most studies have focused on evaluating the effects of single or mixed starters composed of one *S. cerevisiae* strain and one non-*Saccharomyces* species. Previous studies showed that as single starters, strains of *M. pulcherrima* and *Z. bailii* were not able to complete fermentation or had a

low fermentation capacity (Comitini et al., 2011; Contreras et al., 2014a), while *T. delbrueckii* has been reported to be a strong fermenter and able to finish fermentations (Canonico et al., 2016; Roca-Mesa et al., 2020). However, fermentations inoculated with several non-*Saccharomyces* species as a multistarter have been poorly studied. In the current study, we evaluated the use of a multistarter of three non-*Saccharomyces* strains in Muscat grape fermentation. These strains were selected in a previous study (Zhu et al., 2020), in which several strains of these three species and other non-*Saccharomyces* species were screened for their ability to reduce ethanol. When this multistarter of non-*Saccharomyces* strains was used, we observed stagnation of fermentation, even though Td was the dominant strain throughout the fermentation process and has shown its ability to complete fermentation when used as a single inoculum (Chen et al., 2016; Canonico et al., 2017; Roca-Mesa et al., 2020). This lower performance of Td when used in multistarter fermentation could be due to interspecific microbial interactions, such as competition for nutrients (Curiel et al., 2017; Tronchoni et al., 2017; Rollero et al., 2018), or the production of inhibitory compounds, such as killer toxins or other antimicrobial peptides or vesicles (Branco et al., 2014; Evelázquez et al., 2015; Mencher et al., 2020; Yap et al., 2000), as part of cell-cell interaction mechanisms. The higher population size of the non-S*accharomyces* inoculum sped the consumption of sugars until mid-fermentation, although this was not enough to complete the fermentation, and similar final populations and residual sugars were obtained under both conditions. Mp was the minority strain under all conditions, and its population decreased significantly throughout the fermentation process, which agrees with previous studies (Sadoudi et al., 2012; Wang et al., 2016; Escribano-Viana et al., 2018).

As most non-*Saccharomyces* yeasts are incapable of completing alcoholic fermentation, *S. cerevisiae* is usually added, either simultaneously as a coinoculum or sequentially at 24 – 72 h after non-*Saccharomyces* yeast inoculation. These inoculation strategies reduce the risk of a stuck fermentation (Zironi et al., 1993; Soden et al., 2000). Indeed, in the current study, only the fermentations involving *S. cerevisiae* were

complete. When all strains were coinoculated, Sc was the dominant strain and became the most abundant yeast at the end of fermentation, regardless of the inoculum concentration of non-*Saccharomyces* yeasts. The dominance of Sc during the fermentation process could be explained by its high ability to tolerate different stresses, such as high ethanol levels, especially relevant in the last stage of fermentation (Querol et al., 2003). Additionally, different mechanisms, such as cell-to-cell contact, nutrient competition, secretion of toxic compounds or changes in media (Goddard, 2008; Wang et al., 2015 & 2016; Lleixà et al., 2016) could also be responsible of the dominance. The effect of metabolite production and changes in the fermentative medium produced by *S. cerevisiae* has been proven to reduce the competition and persistence of *M. pulcherrima* (García et al., 2017b), which agrees with the drastic reduction in the Mp population observed in fermentations when Sc was coinoculated. As described above, the fermentation kinetics were mainly affected by the inoculation time of Sc. Specifically, coinoculated fermentations were faster and finished earlier compared to sequential fermentations, which agrees with previous studies (Loira et al., 2015; Lleixà et al., 2016).

Focusing on competition for nutrients, a key factor in wine fermentation is the availability of assimilable nitrogen. On one hand, in pure-culture fermentations, some non-*Saccharomyces* yeasts (*H. uvarum*, *H. vineae*, *S. bacillaris* and *T. delbrueckii*) consume less assimilable nitrogen than does *S. cerevisiae* (Ciani et al., 2006; Medina et al., 2012; Roca-Mesa et al., 2020). On the other hand, studies have shown that in sequential fermentations, some non-*Saccharomyces* species, such as *T. delbrueckii*, *L. thermotolerans* and *S. bacillaris*, are capable of consuming almost all assimilable nitrogen within 48 or 72 h before *S. cerevisiae* is inoculated, which could result in incomplete fermentation due to low growth of *S. cerevisiae* (Taillandier et al., 2014; Roca-Mesa et al., 2020). In the current study, to avoid stuck fermentations due to nitrogen limitation and to promote the growth of Sc, assimilable nitrogen was restored before Sc inoculation, as non-*Saccharomyces* yeasts had been consuming up to 80% of assimilable nitrogen present in the must. As expected, this addition produced a quick increase in the

population of Sc. This conclusion is supported by Medina et al. (2012), who also observed an increase in the percentage of the *Saccharomyce*s strain after assimilable nitrogen addition.

A critical aspect for studying yeast interactions during fermentation is to evaluate the yeast population, especially living cells. The traditional method used for determining the yeast living cells during alcoholic fermentation is based on colony counting in different culture media, which takes 2 or 3 days and might cause some deviations (Díaz et al., 2013; Chambers et al., 2015). In mixed or spontaneous fermentations, to differentiate and count the different yeast species, we need to use selective or differential media (Díaz et al., 2013; García et al., 2017a). One of these media is WLN agar, which is able to distinguish different yeast species according to their colony morphology (Pallmann et al., 2001; Cavazza et al., 2010; Navarro et al., 2020). In our work, the four yeast species had different colony morphologies on this medium, which allowed their differential counting. In comparison, qPCR analysis is a relatively fast and sensitive technique to simultaneously identify and quantify different targeted yeast species (Rawsthorne and Phister, 2006; Andorrà et al., 2010b; García et al., 2017a). However, as described above, this method cannot distinguish the populations of viable and dead cells some DNA-binding dyes, such as PMA, are used and combined with qPCR, allowing the quantification of only the viable cell population (Andorrà et al., 2010a; Vendrame et al., 2014; Navarro et al., 2020).

In the current study, cell counting on WLN plates and PMA-qPCR were used to monitor the viable populations of the different yeast species during the fermentation process. Specific primers for qPCR were available for all species used in the current work (Andorrà et al., 2010a; Navarro et al., 2020), which presented a high efficiency and good quantification limit. As described above, even though yeast population profiles were similar between WLN counting and PMA-qPCR analysis, they still showed some differences. First, in the coinoculated fermentations with all strains (1CA and 10CA), Sc was the dominant species at the end of fermentation, without detection of these species in the WLN counting in 1CA. Indeed, WLN medium has

been described to be useful for quantifying species with similar log populations but is unable to detect species with a population 1 or 2 logs lower than the main species (Navarro et al., 2020), which would explain the inability to detect some species, especially Mp, when its population started decreasing. In contrast, PMA-qPCR sensitively detected the populations of all non-*Saccharomyces* strains until the end of fermentation, even at low concentrations, as observed in previous studies (Andorrà et al., 2011; García et al., 2017a; Padilla et al., 2017; Navarro et al., 2020). Surprisingly, the viable cell population of Mp obtained from PMA-qPCR analysis was lower than that from WLN counting before mid-fermentation. This could be explained by the different membrane compositions and permeabilities of this species. Vázquez et al. (2019) showed that the cellular lipid composition of *M. pulcherrima* during grape must fermentation contains a high percentage of polyunsaturated fatty acids, which results in more fluid membranes. Moreover, the permeability of the cell membrane is known to be increased upon contact with grape must or ethanol (Pérez-Torrado et al., 2002). Thus, this higher permeability could facilitate the penetration of PMAxx into viable Mp cells, resulting in lower quantification by PMA-qPCR analysis. Navarro et al. (2020) described this effect in different strains in the early stage of fermentation.

In recent years, *M. pulcherrima*, *T. delbrueckii* and *Z. bailii* have been proven to be species able to reduce ethanol content, mainly in mixed fermentations with *S. cerevisiae*. For example, *M. pulcherrima* was able to reduce 0.7–1.5% (*v*/*v*) ethanol in sequential fermentations in grape must or defined medium (Contreras et al., 2014a; Canonico et al., 2019a; Hranilovic et al., 2020; Zhu et al., 2020), while *T. delbrueckii* and *Z. bailii* achieved ethanol reductions of 0.6–1.0% (*v*/*v*) and 0.7–1.8% (*v*/*v*), respectively, in sequential fermentations with *S. cerevisiae* compared to single *S. cerevisiae* fermentation (Contreras et al., 2015; Canonico et al., 2019a; Zhu et al., 2020). All these ethanol reductions were achieved with fermentations initiated by a single non-*Saccharomyces* strain; however, this reduction changed when a multistarter inoculation was used. For example, according to the results from Varela et al. (2016), in sequential fermentations with *M. pulcherrima* or *S. uvarum*, the ethanol content decreased by 1.09 and 1.01%

(*v*/*v*), respectively, while when those species were simultaneously inoculated as mixed starters with *S. cerevisiae*, the ethanol content decreased by 1.85% (*v*/*v*), showing an additive effect of coinoculation. In the current study, we used a mixed starter of three non-*Saccharomyces* species selected based on their ability to reduce ethanol. Our previous studies showed that the selected Mp, Td and Zb strains were able to reduce the ethanol content by 1.39, 0.84 and 1.02% (*v*/*v*), respectively, in sequential fermentations with Sc (inoculated at 48 h), using the same natural must (Zhu et al., 2020). In this research, the mixed inoculation of these three species in sequential fermentations with Sc resulted in a lower ethanol reduction (less than 0.77% (*v*/*v*)) compared to sequential fermentation with a single non-*Saccharomyces* strain. Thus, we did not observe an additive effect, as did Contreras et al. (2014b) or Varela et al. (2016). Instead, those species showed a dissipative impact on reducing ethanol production when used together. Similar results were also observed in the study of Contreras et al. (2014b), in which sequential fermentations by the single starter of *M. pulcherrima* achieved an ethanol reduction of 1.16–1.76% (*v*/*v*), while sequential fermentations initiated by mixed starters of *M. pulcherrima*, *H. uvarum*, *P. kluyveri* and *T. delbrueckii* reduced the ethanol content by only 0.38% (*v*/*v*). Therefore, Contreras et al. (2014b) demonstrated that ethanol reduction is very dependent on the yeast combination used in the starter, since a yeast strain with a high ability to reduce ethanol, such as *M. pulcherrima*, was affected by the presence of other yeasts, and as a result, its ability to reduce ethanol could be weakened or strengthened. Moreover, the relative proportion between yeast species during the fermentation could affect the metabolite production. Indeed, comparisons between pure cultures and sequentially inoculated cultures revealed changes in the distribution of carbon fluxes during fermentation (Seguinot et al., 2020). Finally, the initial must composition and nutrient availability can also have an impact in the metabolite production, as previously demonstrated by Seguinot et al. (2002).

In addition, our results showed that the inoculum size of non-*Saccharomyces* yeasts also has an impact on ethanol production, with a lower ethanol content in wines

inoculated with higher populations of non-*Saccharomyces* yeasts. This agrees with the results obtained by Maturano et al. (2019), in which a higher inoculum size of non-*Saccharomyces* yeasts (5×10^6 cells/mL vs. 1×10^6 cells/mL) produced wines with a lower ethanol content. Thus, more combinations of yeast species in terms of both the number and diversity of strains and the amount of each strain inoculated should be tested to find the best combination for ethanol reduction.

Non-*Saccharomyces* yeasts usually differ from *S. cerevisiae* in the distribution of their metabolic flux during fermentation (Ciani et al., 2006 & 2016; Minebois et al., 2020; Seguinot et al., 2020). Indeed, several non-*Saccharomyces* species are able to aerobically respire sugar, which results in altered formation of the main metabolites produced during fermentation, including ethanol, glycerol and organic acids (Quirós et al., 2014; Contreras et al., 2015; Morales et al., 2015; Ciani et al., 2016). However, although ethanol production can be decreased when providing non-*Saccharomyces* yeasts with oxygen, this sometimes has undesirable side effects, such as higher acetic acid or ethyl acetate levels (Contreras et al., 2015; Röcker et al., 2016). In this study, a significant decrease in ethanol levels was observed in mixed fermentations, even without the addition of oxygen and without a detrimental increase in acetic acid. Indeed, higher levels of acetic and lactic acids were obtained in the presence of non-*Saccharomyces* yeast, mainly in sequential inoculations, but the levels were kept below 0.33 g/L for lactic acid and 0.36 g/L for acetic acid, with nondetrimental concentrations for the final wines. On the other hand, the concentration of glycerol did not increase in our reduced-ethanol wines, which implied that the production of glycerol was not the main pathway of ethanol reduction in mixed culture fermentations, as occurred in other studies (Rodrigues et al., 2016; Zhu et al., 2020). Nevertheless, the final amounts of acetic and lactic acids are not enough to counterbalance the decrease in ethanol and glycerol. Previous studies had also observed that the reduction of the ethanol yield by some non-*Saccharomyces* strains, could not be fully explained by the overproduction of glycerol or organic acids, suggesting that respiration would be responsible, at least in part, of the poor ethanol yield observed for these strains (Magyar et al., 2011;

Gonzalez et al., 2013). However, the analysis of the volatile composition of wines (higher alcohols, volatile fatty acids, esters, carbonyl compounds) would help the understanding of the effect of this type of inoculation on the overall complexity of wines (Tronchoni et al., 2018; Canonico et al., 2019a).

In summary, our results confirm that PMA-qPCR analysis is a fast and sensitive method for monitoring the viable cell population dynamics in mixed fermentations of *T. delbrueckii*, *M. pulcherrima*, *Z. bailii* and *S. cerevisiae*. *T. delbruecckii* was the dominant non-*Saccharomyces* species under all conditions, and *M. pulcherrima* was the minority species, being detected by PMA-qPCR throughout fermentation but at lower and decreasing concentrations. The use of a multistarter culture consisting of several non-*Saccharomyces* species previously selected for their ability to reduce ethanol and a *S. cerevisiae* strain resulted in reduced-alcohol wines, even if no aeration was applied. The sequential fermentations obtained ethanol reductions from 0.48 – 0.77% (*v*/*v*), which were accompanied by increases in the lactic acid and acetic acid contents. Among all fermentations, the highest reduction was obtained in the sequential inoculation with a higher inoculum size (10^7 cells/mL), when *S. cerevisiae* was added at 72 h. Nevertheless, the ethanol reduction obtained when using a multistarter of non-*Saccharomyces* species was lower than that when using each non-*Saccharomyces* species separately (Zhu et al., 2020), which indicates no additive effect among them but a lower efficiency to deliver this outcome due to microbial interactions. Although not as efficient in reducing ethanol, a multistarter inoculation strategy could have additional benefits, such as improving the aroma profile and overall complexity of wines (Varela et al., 2016; Padilla et al., 2017). Nevertheless, the use of several species can also be challenged by winemaking conditions and the initial yeast population; therefore, special care has to be taken in the winery for this kind of procedure to be applied (Contreras et al., 2014b; Padilla et al., 2017).

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References

Andorrà, I.; Esteve-Zarzoso, B.; Guillamon, J.M.; Mas, A. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. *Int. J. Food Microbiol.* **2010a**, 144, 257–262, doi:10.1016/j.ijfoodmicro.2010.10.003.

Andorrà, I.; Landi, S.; Mas, A.; Esteve-Zarzoso, B.; Guillamon, J.M. Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques. *Food Res. Int.* **2010b**, 43, 773–779, doi:10.1016/j.foodres.2009.11.014.

Andorrà, I.; Monteiro, M.; Esteve-Zarzoso, B.; Albergaria, H.; Mas, A. Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence in situ hybridization, flow cytometry and quantitative PCR. *Food Microbiol.* **2011**, 28, 1483–1491, doi:10.1016/j.fm.2011.08.009.

Binati, R.L.; Junior, W.J.L.; Luzzini, G.; Slaghenaufi, D.; Ugliano, M.; Torriani, S. Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans*, *Metschnikowia spp*. and *Starmerella bacillaris* strains isolated in Italy. *Int. J. Food Microbiol.* **2020**, 318, 108470, doi:10.1016/j.ijfoodmicro.2019.108470.

Bindon, K.; Varela, C.; Kennedy, J.; Holt, H.; Herderich, M. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. *Food Chem.* **2013**, 138, 1696–1705, doi:10.1016/j.foodchem.2013.03.014.

Branco, P.; Francisco, D.; Chambon, C.; Hébraud, M.; Arneborg, N.; Almeida, M.G.; Caldeira, J.; Albergaria, H. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* **2014**, 98, 843–853, doi:10.1007/s00253-013-5411-y.

Canonico, L.; Comitini, F.; Ciani, M. *Torulaspora delbrueckii* contribution in mixed brewing fermentations with different *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* **2017**, 259, 7–13, doi:10.1016/j.ijfoodmicro.2017.07.017.

Canonico, L.; Ecomitini, F.; Eoro, L.; Eciani, M. Sequential fermentation with selected immobilized non-*Saccharomyces* yeast for reduction of ethanol content in wine. *Front. Microbiol.* **2016**, 7, 278, doi:10.3389/fmicb.2016.00278.

Canonico, L.; Solomon, M.; Comitini, F.; Ciani, M.; Varela, C. Volatile profile of reduced alcohol wines fermented with selected non-*Saccharomyces* yeasts under different aeration conditions. *Food Microbiol.* **2019a**, 84, 103247, doi:10.1016/j.fm.2019.103247.

Canonico, L.; Comitini, F.; Ciani, M. *Metschnikowia pulcherrima* selected strain for ethanol reduction in wine: influence of cell immobilization and aeration condition. *Foods* **2019b**, 8, 378, doi:10.3390/foods8090378.

Castrillo, D.; Rabuñal, E.; Neira, N.; Blanco, P. Oenological potential of non-*Saccharomyces* yeasts to mitigate effects of climate change in winemaking: Impact on aroma and sensory profiles of Treixadura wines. *FEMS Yeast Res.* **2019**, 19, doi:10.1093/femsyr/foz065.

Cavazza, A.; Poznanski, E.; Guzzon, R. Must treatments and wild yeast growth before and during alcoholic fermentation. *Ann. Microbiol.* **2010**, 61, 41–48, doi:10.1007/s13213-010-0132-2.

Chambers, P.; Borneman, A.; Varela, C.; Cordente, A.; Bellon, J.; Tran, T.; Henschke, P.; Curtin, C. Ongoing domestication of wine yeast: Past, present and future. *Aust. J. Grape Wine Res.* **2015**, 21, 642–650, doi:10.1111/ajgw.12190.

Chen, D.; Liu, S.Q. Impact of simultaneous and sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on non-volatiles and volatiles of lychee wines. *LWT* **2016**, 65, 53–61, doi:10.1016/j.lwt.2015.07.050.

Ciani, M.; Beco, L.; Comitini, F. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int. J. Food Microbiol.* **2006**, 108, 239–245, doi:10.1016/j.ijfoodmicro.2005.11.012.

Ciani, M.; Morales, P.; Comitini, F.; Tronchoni, J.; Canonico, L.; Curiel, J.A.; Oro, L.; Rodrigues, A.J.; Gonzalez, R. Non-conventional yeast species for lowering ethanol content of wines. *Front. Microbiol.* **2016**, 7, 642, doi:10.3389/fmicb.2016.00642.

Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, 28, 873–882, doi:10.1016/j.fm.2010.12.001.

Contreras, A.; Hidalgo, C.; Henschke, P.A.; Chambers, P.J.; Curtin, C.; Varela, C. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* **2014a**, 80, 1670–1678, doi:10.1128/aem.03780-13.

Contreras, A.; Curtin, C.; Varela, C. Yeast population dynamics reveal a potential 'collaboration' between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Appl. Microbiol. Biotechnol.* **2014b**, 99, 1885–1895, doi:10.1007/s00253-014-6193-6.

Contreras, A.; Hidalgo, C.; Schmidt, S.; Henschke, P.; Curtin, C.; Varela, C. The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* **2015**, 205, 7–15, doi:10.1016/j.ijfoodmicro.2015.03.027.

Curiel, J.A.; Morales, P.; Gonzalez, R.; Tronchoni, J. Different non-*Saccharomyces* yeast species stimulate nutrient consumption in *S. cerevisiae* mixed cultures. *Front. Microbiol.* **2017**, 8, 2121, doi:10.3389/fmicb.2017.02121.

De Toda, F.M.; Sancha, J.; Balda, P. Reducing the sugar and pH of the grape (*Vitis vinifera* L. cvs. 'Grenache' and 'Tempranillo') through a single shoot trimming. *S. Afr. J. Enol. Vitic.* **2016**, 34, 246–251, doi:10.21548/34-2-1101.

Díaz, C.; Molina, A.M.; Nähring, J.; Fischer, R. Characterization and dynamic behavior of wild yeast during spontaneous wine fermentation in steel tanks and amphorae. *BioMed Res. Int.* **2013**, 2013, 1–13, doi:10.1155/2013/540465.

Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Ortiz-Julien, A.; Gerbi, V.; Rolle, L.; Cocolin, L. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Appl. Microbiol. Biotechnol.* **2016**, 100, 5515–5526, doi:10.1007/s00253-016-7413-z.

Escribano-Viana, R.; González-Arenzana, L.; Portu, J.; Garijo, P.; López-Alfaro, I.; López, R.; Santamaría, P.; Gutiérrez, A.R. Wine aroma evolution throughout alcoholic fermentation sequentially inoculated with non- *Saccharomyces*/*Saccharomyces yeasts*. *Food Res. Int.* **2018**, 112, 17–24, doi:10.1016/j.foodres.2018.06.018.

Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Evol. Microbiol.* **1999**, 49, 329–337, doi:10.1099/00207713-49-1-329.

Evelázquez, R.; Ezamora, E.; Álvarez, M.L.; Hernández, L.M.; Eramírez, M. Effects of new *Torulaspora delbrueckii* killer yeasts on the must fermentation kinetics and aroma compounds of white table wine. *Front. Microbiol.* **2015**, 6, 1222, doi:10.3389/fmicb.2015.01222.

Fittipaldi, M.; Nocker, A.; Codony, F. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *J. Microbiol. Methods* **2012**, 91, 276–289, doi:10.1016/j.mimet.2012.08.007.

Forootan, A.; Sjöback, R.; Björkman, J.; Sjögreen, B.; Linz, L.; Kubista, M. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomol. Detect. Quantif.* **2017**, 12, 1–6, doi:10.1016/j.bdq.2017.04.001.

Furlani, M.V.M.; Maturano, Y.P.; Combina, M.; Mercado, L.A.; Toro, M.E.; Vazquez, F. Selection of non-*Saccharomyces* yeasts to be used in grape musts with high alcoholic potential: A strategy to obtain wines with reduced ethanol content. *FEMS Yeast Res.* **2017**, 17, 1–11, doi:10.1093/femsyr/fox010.

García, M.; Esteve-Zarzoso, B.; Crespo, J.; Cabellos, J.M.; Arroyo, T. Yeast monitoring of wine mixed or sequential fermentations made by native strains from D.O. "Vinos de Madrid" using real-time quantitative PCR. *Front. Microbiol.* **2017a**, 8, 2520, doi:10.3389/fmicb.2017.02520.

García, M.; Arroyo, T.; Crespo, J.; Cabellos, J.M.; Esteve-Zarzoso, B. Use of native non-*Saccharomyces* strain: A new strategy in D.O. "Vinos de Madrid" (Spain) wines elaboration. *Eur. J. Food Sci. Technol*. **2017b**, 5, 1–31.

García-Martín, N.; Perez-Magariño, S.; Ortega-Heras, M.; González-Huerta, C.; Mihnea, M.; González-Sanjosé, M.L.; Palacio, L.; Prádanos, P.; Hernández, A. Sugar reduction in musts with nanofiltration membranes to obtain low alcohol-content wines. *Sep. Purif. Technol.* **2010**, 76, 158–170, doi:10.1016/j.seppur.2010.10.002.

Gobbi, M.; Comitini, F.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* **2013**, 33, 271–281, doi:10.1016/j.fm.2012.10.004.

Gobbi, M.; De Vero, L.; Solieri, L.; Comitini, F.; Oro, L.; Giudici, P.; Ciani, M. Fermentative aptitude of non-*Saccharomyces* wine yeast for reduction in the ethanol content in wine. *Eur. Food Res. Technol.* **2014**, 239, 41–48, doi:10.1007/s00217-014-2187-y.

Goddard, M.R. Quantifying the complexities of *Saccharomyces cerevisiae*'s ecosystem engineering via fermentation. *Ecology*. **2008**, 89, 2077–2082, doi:10.1890/07-2060.1.

Godden, P. Persistent Wine Instability Issues. *Aust. Grapegrow. Winemak.* **2000**, 443, 10–14.

Gonzalez, R.; Quirós, M.; Morales, P. Yeast respiration of sugars by non-*Saccharomyces* yeast species: A promising and barely explored approach to lowering alcohol content of wines. *Trends Food Sci. Technol.* **2013**, 29, 55–61, doi:10.1016/j.tifs.2012.06.015.

Hierro, N.; Esteve-Zarzoso, B.; Mas, A.; Guillamã, N.J.M. Monitoring of *Saccharomyces* and *Hanseniaspora* populations during alcoholic fermentation by real-time quantitative PCR. *FEMS Yeast Res.* **2007**, 7, 1340–1349, doi:10.1111/j.1567-1364.2007.00304.x.

Hranilovic, A.; Gambetta, J.M.; Jeffery, D.W.; Grbin, P.R.; Jiranek, V. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The

effect of sequential inoculation timing. *Int. J. Food Microbiol.* **2020**, 329, 108651, doi:10.1016/j.ijfoodmicro.2020.108651.

Jones, G.V.; White, M.A.; Cooper, O.R.; Storchmann, K. Climate Change and Global Wine Quality. *Clim. Chang.* **2005**, 73, 319–343, doi:10.1007/s10584-005-4704-2.

Junior, W.J.F.L.; Nadai, C.; Crepalde, L.T.; de Oliveira, V.S.; de Matos, A.D.; Giacomini, A.; Corich, V. Potential use of *Starmerella bacillaris* as fermentation starter for the production of low-alcohol beverages obtained from unripe grapes. *Int. J. Food Microbiol.* **2019**, 303, 1–8, doi:10.1016/j.ijfoodmicro.2019.05.006.

Li, Y.; Yang, L.; Fu, J.; Yan, M.; Chen, D.; Zhang, L. The novel loop-mediated isothermal amplification based confirmation methodology on the bacteria in Viable but Non-Culturable (VBNC) state. *Microb. Pathog.* **2017**, 111, 280–284, doi:10.1016/j.micpath.2017.09.007.

Lleixà, J.; Manzano, M.; Mas, A.; Portillo, M.D.C. *Saccharomyces* and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions. *Front. Microbiol.* **2016**, 7, 1959, doi:10.3389/fmicb.2016.01959.

Loira, I.; Morata, A.; Comuzzo, P.; Callejo, M.J.; González, C.; Calderón, F.; Suárez-Lepe, J.A. Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality. *Food Res. Int.* **2015**, 76, 325–333, doi:10.1016/j.foodres.2015.06.030.

Longo, R.; Blackman, J.W.; Torley, P.J.; Rogiers, S.Y.; Schmidtke, L.M. Changes in volatile composition and sensory attributes of wines during alcohol content reduction. *J. Sci. Food Agric.* **2016**, 97, 8–16, doi:10.1002/jsfa.7757.

Magyar, I.; Tóth, T. Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, 28, 94–100, doi:10.1016/j.fm.2010.08.011.

Maturano, Y.P.; Mestre, M.V.; Kuchen, B.; Toro, M.E.; Mercado, L.A.; Vazquez, F.; Combina, M. Optimization of fermentation-relevant factors: A strategy to reduce ethanol in red wine by sequential culture of native yeasts. *Int. J. Food Microbiol.* **2019**, 289, 40–48, doi:10.1016/j.ijfoodmicro.2018.08.016.

Medina, K.; Boido, E.; Dellacassa, E.; Carrau, F. Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int. J. Food Microbiol.* **2012**, 157, 245–250, doi:10.1016/j.ijfoodmicro.2012.05.012.

Mencher, A.; Morales, P.; Valero, E.; Tronchoni, J.; Patil, K.R.; Gonzalez, R. Proteomic characterization of extracellular vesicles produced by several wine yeast species. *Microb. Biotechnol.* **2020**, 13, 1581–1596, doi:10.1111/1751-7915.13614.

Minebois, R.; Pérez-Torrado, R.; Querol, A. A time course metabolism comparison among *Saccharomyces cerevisiae*, *S. uvarum* and *S. kudriavzevii* species in wine fermentation. *Food Microbiol.* **2020**, 90, 103484, doi:10.1016/j.fm.2020.103484.

Morales, P.; Rojas, V.; Quirós, M.; Gonzalez, R. The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotechnol.* **2015**, 99, 3993–4003, doi:10.1007/s00253-014-6321-3.

Navarro, Y.; Torija, M.-J.; Mas, A.; Beltran, G. Viability-PCR allows monitoring yeast population dynamics in mixed fermentations including viable but non-culturable yeasts. *Foods* **2020**, 9, 1373, doi:10.3390/foods9101373.

Nocker, A.; Cheung, C.-Y.; Camper, A.K. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods* **2006**, 67, 310–320, doi:10.1016/j.mimet.2006.04.015.

Padilla, B.; García-Fernández, D.; González, B.; Izidoro, I.; Esteve-Zarzoso, B.; Beltran, G.; Mas, A. Yeast biodiversity from DOQ Priorat uninoculated fermentations. *Front. Microbiol.* **2016**, 7, 930, doi:10.3389/fmicb.2016.00930.

Padilla, B.; Zulian, L.; Ferreres, Àngela; Pastor, R.; Esteve-Zarzoso, B.; Beltran, G.; Mas, A. Sequential inoculation of native non-*Saccharomyces* and *Saccharomyces cerevisiae* strains for wine making. *Front. Microbiol.* **2017**, 8, 1293, doi:10.3389/fmicb.2017.01293.

Pallmann, C.L.; Brown, J.A.; Olineka, T.L.; Cocolin, L.; Mills, D.A.; Bisson, L.F. Use of WL medium to profile native flora fermentations. *Am. J. Enol. Vitic.* **2001**, 52, 198–203, doi:10.1016/S0065-2164(01)49013-7

Pérez-Torrado, R.; Carrasco, P.; Aranda, A.; Gimeno-Alcañiz, J.; Pérez-Ortín, J.E.; Matallana, E.; Del Olmo, M.L. Study of the first hours of microvinification by the use of osmotic stress-response genes as probes. *Syst. Appl. Microbiol.* **2002**, 25, 153–161, doi:10.1078/0723-2020-00087.

Phister, T.G.; Mills, D.A. Real-Time PCR Assay for detection and enumeration of *Dekkera bruxellensis* in Wine. *Appl. Environ. Microbiol.* **2003**, 69, 7430–7434, doi:10.1128/aem.69.12.7430-7434.2003.

Querol, A.; Fernández-Espinar, M.T.; Del Olmo, M.; Barrio, E. Adaptive evolution of wine yeast. *Int. J. Food Microbiol.* **2003** 86, 3–10, doi:10.1016/S0168-1605(03)00244-7.

Quirós, M.; Gonzalez-Ramos, D.; Tabera, L.; Gonzalez, R. A new methodology to obtain wine yeast strains overproducing mannoproteins. *Int. J. Food Microbiol.* **2010**, 139, 9–14, doi:10.1016/j.ijfoodmicro.2010.02.014.

Quirós, M.; Rojas, V.; Gonzalez, R.; Morales, P. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* **2014**, 181, 85–91, doi:10.1016/j.ijfoodmicro.2014.04.024.

Rawsthorne, H.; Phister, T.G. A real-time PCR assay for the enumeration and detection of *Zygosaccharomyces bailii* from wine and fruit juices. *Int. J. Food Microbiol.* **2006**, 112, 1–7, doi:10.1016/j.ijfoodmicro.2006.05.003.

Roca-Mesa, H.; Sendra, S.; Mas, A.; Beltran, G.; Torija, M.-J. Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. *Microorganisms* **2020**, 8, 157, doi:10.3390/microorganisms8020157.

Röcker, J.; Strub, S.; Ebert, K.; Grossmann, M. Usage of different aerobic non-*Saccharomyces* yeasts and experimental conditions as a tool for reducing the potential ethanol content in wines. *Eur. Food Res. Technol.* **2016**, 242, 2051–2070, doi:10.1007/s00217-016-2703-3.

Rodrigues, A.J.; Raimbaud, T.; Gonzalez, R.; Morales, P. Environmental factors influencing the efficacy of different yeast strains for alcohol level reduction in wine by respiration. *LWT Food Sci. Technol.* **2016**, 65, 1038–1043. doi:10.1016/j.lwt.2015.09.046.

Rollero, S.; Bloem, A.; Ortiz-Julien, A.; Camarasa, C.; Divol, B. Altered fermentation performances, growth, and metabolic footprints reveal competition for nutrients between yeast species inoculated in synthetic grape juice-like medium. *Front. Microbiol.* **2018**, 9, 196, doi:10.3389/fmicb.2018.00196.

Sadoudi, M.; Tourdot-Maréchal, R.; Rousseaux, S.; Steyer, D.; Gallardo-Chacón, J.-J.; Ballester, J.; Vichi, S.; Guérin-Schneider, R.; Caixach, J.; Alexandre, H. Yeast–yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol.* **2012**, 32, 243–253, doi:10.1016/j.fm.2012.06.006.

Seguinot, P.; Ortiz-Julien, A.; Camarasa, C. Impact of Nutrient Availability on the fermentation and production of aroma compounds under sequential inoculation With *M. pulcherrima* and *S. cerevisiae*. *Front. Microbiol.* **2020**, 11, 305, doi:10.3389/fmicb.2020.00305.

Soden, A.; Francis, I.; Oakey, H.; Henschke, P. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonnay wine. *Aust. J. Grape Wine Res.* **2000**, 6, 21–30, doi:10.1111/j.1755-0238.2000.tb00158.x.

Taillandier, P.; Lai, Q.P.; Julien-Ortiz, A.; Brandam, C. Interactions between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* in wine fermentation: Influence of inoculation and nitrogen content. *World J. Microbiol. Biotechnol.* **2014**, 30, 1959–1967, doi:10.1007/s11274-014-1618-z.

Tronchoni, J.; Curiel, J.A.; Morales, P.; Torres-Pérez, R.; Gonzalez, R. Early transcriptional response to biotic stress in mixed starter fermentations involving *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*. *Int. J. Food Microbiol.* **2017**, 241, 60–68, doi:10.1016/j.ijfoodmicro.2016.10.017.

Tronchoni, J.; Curiel, J.A.; Sáenz-Navajas, M.P.; Morales, P.; Blanco, A.D.L.F.; Fernández-Zurbano, P.; Ferreira, V.; Gonzalez, R. Aroma profiling of an aerated fermentation of natural grape must with selected yeast strains at pilot scale. *Food Microbiol.* **2018**, 70, 214–223, doi:10.1016/j.fm.2017.10.008.

Varela, C.; Barker, A.; Tran, T.; Borneman, A.; Curtin, C. Sensory profile and volatile aroma composition of reduced alcohol Merlot wines fermented with *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Int. J. Food Microbiol.* **2017**, 252, 1–9, doi:10.1016/j.ijfoodmicro.2017.04.002.

Varela, C.; Dry, P.; Kutyna, D.; Francis, I.; Henschke, P.; Curtin, C.; Chambers, P. Strategies for reducing alcohol concentration in wine. *Aust. J. Grape Wine Res.* **2015**, 21, 670–679, doi:10.1111/ajgw.12187.

Varela, C.; Sengler, F.; Solomon, M.; Curtin, C. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chem.* **2016**, 209, 57–64, doi:10.1016/j.foodchem.2016.04.024.

Vázquez, J.; Grillitsch, K.; Daum, G.; Mas, A.; Beltran, G.; Torija, M.J. The role of the membrane lipid composition in the oxidative stress tolerance of different wine yeasts. *Food Microbiol.* **2019**, 78, 143–154, doi:10.1016/j.fm.2018.10.001.

Vendrame, M.; Iacumin, L.; Manzano, M.; Comi, G. Use of propidium monoazide for the enumeration of viable O*enococcus oeni* in must and wine by quantitative PCR. *Food Microbiol.* **2013**, 35, 49–57, doi:10.1016/j.fm.2013.02.007.

Vendrame, M.; Manzano, M.; Comi, G.; Bertrand, J.; Iacumin, L. Use of propidium monoazide for the enumeration of viable *Brettanomyces bruxellensis* in wine and beer by quantitative PCR. *Food Microbiol.* **2014**, 42, 196–204, doi:10.1016/j.fm.2014.03.010.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. *Int. J. Food Microbiol.* **2015**, 206, 67–74, doi:10.1016/j.ijfoodmicro.2015.04.022.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. The Interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* **2016**, 7, 502, doi:10.3389/fmicb.2016.00502.

Yap, N.; Lopes, M.D.B.; Langridge, P.; Henschke, P. The incidence of killer activity of non-*Saccharomyces* yeasts towards indigenous yeast species of grape must: Potential application in wine fermentation. *J. Appl. Microbiol.* **2000**, 89, 381–389, doi:10.1046/j.1365-2672.2000.01124.x.

Zhu, X.; Navarro, Y.; Mas, A.; Torija, M.J.; Beltran, G. A rapid method for selecting non-*Saccharomyces* strains with a low ethanol yield. *Microorg.* **2020**, 8, 658, doi:10.3390/microorganisms8050658.

Zironi, R.; Romano, P.; Suzzi, G.; Battistutta, F.; Comi, G. Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **1993**, 15, 235–238, doi:10.1007/bf00128311.

Zott, K.; Claisse, O.; Lucas, P.; Coulon, J.; Lonvaud-Funel, A.; Masneuf-Pomarede, I. Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol.* **2010**, 27, 559–567, doi:10.1016/j.fm.2010.01.006.

Appendix A

Table A1. Primer sequences used for quantitative PCR analysis.

Target species	Primer name	Primer sequence 5′-3′	References
S. cerevisiae	CESP-F	ATCGAATTTTTGAACGCACATTG	Hierro et al., 2007
	SCER-R	CGCAGAGAAACCTCTCTTTGGA	
M. pulcherrima	MP2-F	AGACACTTAACTGGGCCAGC	García et al., 2017a
	MP2-R	GGGGTGGTGTGGAAGTAAGG	
T. delbrueckii	Tods L2	CAAAGTCATCCAAGCCAGC	Zott et al., 2010
	Tods R2	TTCTCAAACAATCATGTTTGGTAG	
Z. bailii	ZBF1	CATGGTGTTTTGCGCC	Rawsthorne and Phister, 2006
	ZBR1	CGTCCGCCACGAAGTGGTAGA	

Table A2. Slopes, Y-Intersections, correlation coefficients (R^2), efficiencies (%), limits of quantification (LoQ) and limits of detection (LoD) of standard curves obtained from serially diluted DNA of *S. cerevisiae*, *M. pulcherrima*, *T. delbrueckii* and *Z. bailii* with PMAxx treatment. Efficiency was estimated by the formula $E = (10^{-1/slope}) - 1$.

Yeasts	Slope	Y-Intersection	R^2	Efficiency (%)	LoQ	LoD
S. cerevisiae	– 3.45 ± 0.04	40.38 ± 0.20	0.9985	94.80	10^3	10
M. pulcherrima	– 3.40 ± 0.03	43.51 ± 0.19	0.9991	97.01	10^4	10^2
T. delbrueckii	– 3.35 ± 0.04	40.02 ± 0.25	0.9978	98.83	10^3	10
Z. bailii	– 3.65 ± 0.03	41.81 ± 0.17	0.9986	87.92	10^3	10

Table A3. The concentration of assimilable nitrogen and the added concentration on the 2nd and 3rd day of fermentation.

Days	Fermentation	Nitrogen (mg/L)	Added nitrogen (mg/L)
2nd day	1S48	102 ± 5.66	0
	10S48	79.67 ± 9.02	20 ± 5.50
3rd day	1S72	70.67 ± 13.05	31 ± 8.54
	10S72	48 ± 5.29	53.33 ± 5.77

CHAPTER 3

Evaluation of different inoculation strategies, using selected non-*Saccharomyces* and non-GMO enhanced *Saccharomyces cerevisiae* yeasts, on the reduction of the ethanol content in wines

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Abstract

The increasing content of ethanol has a negative effect on the high quality wines that consumers require. Therefore, microbiological strategies have garnered interest as alternatives to reduce ethanol content. One of the approaches is to perform fermentations using *Saccharomyces cerevisiae* and non-*Saccharomyces* strains. In this study, we performed mixed fermentations using two selected non-*Saccharomyces* (NS) yeasts, *Metschnikowia pulcherrima* (Mp51) and *Lachancea thermotolerans* (Lt2), a wine commercial *S. cerevisiae* strain, three non-GMO *S. cerevisiae* strains, enhanced by adaptative laboratory evolution (ALE), and their respective parental strains. The fermentations were carried out using different inoculation strategies: single inoculation by the different *S. cerevisiae* and NS strains, simultaneous inoculation (coinoculation) of NS/*S. cerevisiae* at 1:1 and 9:1 ratios, and sequential inoculation, starting the fermentation with the non-*Saccharomyces* strains, and inoculating *S. cerevisiae* after 24 – 48 h (in natural or synthetic must, respectively). When used as single inoculum, ALE *S. cerevisiae* strains showed slower fermentation kinetics than their parental strains in synthetic must fermentation, but similar fermentative profile in natural must fermentation, and no significant difference on ethanol production, except for the strain adapted on alcohol tolerance, which had a significant lower ethanol yield than its parental on natural must fermentation. However, all ALE strains had a lower ethanol production than the commercial wine strain, mainly in natural high sugar must. Regarding the mixed inoculum fermentations, the results showed that Mp51 is the most efficient strain to reduce ethanol content, especially when used in sequential fermentations, in which the ethanol reduction ranged from 1.22 to 1.44% (v/v) in synthetic must, and from 0.38 to 1.18% (v/v) in natural must. The inoculation protocol influenced the resilience of the non-*Saccharomyces* strains during the fermentation process, as well as the ethanol reduction, with the sequential inoculation resulting in the lowest ethanol yield and the highest non-*Saccharomyces* persistence, followed by 9:1 coinoculation. In fermentations with Lt2, a significant ethanol

reduction up to 0.24% (v/v) and a lactic acid increase was detected only in some sequential fermentations, mainly in synthetic must. Overall our results confirm that the use of sequential inoculations of *M. pulcherrrima* and *S. cerevisiae* are the best strategy for reducing ethanol content in wines, even when used in high sugar must.

Keywords: Evolved *Saccharomyces cerevisiae*, sequential fermentation, coinoculated fermentation, ethanol yield, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*.

Introduction

In recent decades, global warming has not only negatively affected vinifications, wine microbiology and wine sensory profile, but also increased the ethanol content of wines up to 2% (v/v) (Mira de Orduña et al., 2010; Ubeda et al., 2020). However, a higher ethanol content leads to sluggish fermentations, breaks the balance of wine structure, weakens aromatic sensory impact and increases tax load (Bisson et al., 1999; Buescher et al., 2001; Escudero et al., 2007). Therefore, the reduction of ethanol content in wine is considered as a main target in most winemaking areas around the world. To achieve this objective, some researchers have chosen microbial strategies to generate *Saccharomyces cerevisiae* or select non-*Saccharomyces* wine yeasts with a low ethanol yield (Kutyna et al., 2010; Gonzalez et al., 2013; Varela et al., 2019). The strategies focus on modification of the central carbon pathway and change the redox metabolism, which could be interpreted as the transfer of carbons to other by-products than ethanol and the metabolism of sugar into unavailable compounds for producing ethanol, respectively (Kutyna et al., 2010; Tilloy et al., 2015; Goold et al., 2017).

As far as the metabolic engineering is concerned, an effective strategy for developing *S. cerevisiae* with a low ethanol yield is gene modification (GM) technologies. Most GM approaches aim to increase glycerol or gluconic acid concentration, releasing glucose repression of respiration and diverting carbon to the tricarboxylic acids (TCA) cycle (Remize et al., 1999; Malherbe et al., 2003; Henricsson et al., 2005; Cambon et al., 2006; Heux et al., 2006). However, due to the consumer rejection of the use of GMO (Genetically Modified Organism) in food production, non-GMO strategies are preferred by researchers in recent years. One approach is adaptive laboratory evolution (ALE) which involved a long-term and multi-generational cultivation of yeasts under selective conditions, that finally lead to mutations that improve yeast fitness in the corresponding selective environment (Dragosits and Mattanovich, 2013). Several researchers had used ALE strategy to redirect the yeast carbon metabolism to reduce the ethanol yield by increasing glycerol production. The

most commonly used are osmotic stress inducers (i.e., potassium chloride or sodium chloride), and sulfites, such as potassium sulfite or sodium sulfite (Kutyna et al., 2012; Vejarano et al., 2013; Tilloy et al., 2014 & 2015; Goold et al., 2017), however, this redirection of the metabolic flux to glycerol might affect wine flavor and quality (Kutyna et al., 2010; Abalos et al., 2011). Another approach is to isolate natural variants of *S. cerevisiae* strains during fermentation process. However, due to the adaptability of natural yeasts to the environment under the natural selection, it is difficult that *S. cerevisiae* spontaneously acquires the required characteristics (Varela et al., 2015).

Recently, screening of low-yielding ethanol yeasts seems to be challenging due to the large proportion of sugar that some strains needed to be metabolized to other by-products instead of ethanol, while maintaining the positive properties of yeast and preventing excessive accumulation of by-products resulting off-flavor to wine (Tilloy et al., 2015; Varela et al., 2015). Thereby, most non-*Saccharomyces* yeasts were used to enhance wine sensory profiles (Cañas et al., 2011; Loira et al., 2015; Renault et al., 2015; Padilla et al., 2016a) and to reduce ethanol content in wine. Several researches have screened non-*Saccharomyces* yeasts with low ethanol yields under anaerobic or aerobic conditions (Quirós et al., 2014; Contreras et al., 2014 & 2015; Zhu et al., 2020). However, as most of the non-*Saccharomyces* used are not able to complete alcoholic fermentation, their application has been always combined with *S. cerevisiae*. For example, Englezos et al. (2016) investigated the alcohol-lowering potential of *Starmerella bacillaris* in combination with *S. cerevisiae* in coinoculated and sequential fermentations, which reduced up to 0.7% (v/v) of ethanol in sequential fermentation inoculated *S. cerevisiae* at 48 h. In the same year, Canonico et al. (2016) performed sequential fermentations by *Starmerella bombicola* and *Metschnikowia pulcherrima* with *S. cerevisiae* inoculated after 72 h of fermentation, obtaining ethanol reductions of 1.6 and 1.4% (v/v), respectively. Recently, Canonico et al. (2019a) achieved an ethanol reduction of 1.38% (v/v) using *M. pulcherrima* in sequential fermentation under aeration conditions compared to *S. cerevisiae.* Our previous studies evaluated the ethanol reduction of wines produced by *M. pulcherrima*, *Torulaspora delbrueckii* or *Zygosaccharomyces bailii*,

as well as a multistarter of these three non-*Saccharomyces* yeasts, in sequential fermentations with *S. cerevisiae*, achieving and ethanol reduction that ranged from 0.48 to 1.39% (v/v) (Zhu et al., 2020 & 2021). These studies applied different inoculation strategies, therefore, the application of coinoculation and sequential inoculation should be considered to explore the best inoculation strategy. In addition, none of the studies performed so far with mixed inoculation strategies combined the use of evolved *S. cereviciae* strains with the use of low-producing non-*Sacharomyces* species. Therefore, the use of evolved *S. cerevisiae* and selected non-*Saccharomyces* yeasts might be a solution for lowering ethanol content in wines.

In the present work, two selected non-*Saccharomyces* yeasts and four *S. cerevisiae* (one commercial and three evolved strains) were used to ferment synthetic and natural must in coinoculation or sequential inoculation, with the aim of reducing ethanol content of wines. The non-*Saccharomyces* yeasts strains (*M. pulcherrima* and *L. thermotolerans)* were selected due to different metabolic characteristics during the alcoholic fermentation, and their alcohol-lowering ability in sequential fermentations (Zhu et al., 2020). The evolved strains were obtained in a previous study (manuscript in preparation) using high glucose and alcohol as challenges for adaptive evolution. In this work, we first analyzed two different coinoculation ratios of non-*Saccharomyces*/*Saccharomyces* yeast in synthetic must, as well as a sequential inoculation, using the selected yeast strains, in order to explore the ability of these mixed fermentations to reduce ethanol. Then, the sequential inoculation strategy was also validated in high sugar natural must. In addition, the by-products after the alcoholic fermentation were also analyzed to evaluate the impact of the different inoculation strategies on the final wines.

Materials and Methods

Yeast strains and culture media

Two non-*Saccharomyces* yeasts and four *Saccharomyces cerevisiae* strains were used in this study. *Metschnikowia pulcherrima* (Mp51) was selected from Priorat Appellation of Origin (URV collection, Tarragona, Spain) (Padilla et al., 2016b) and *Lachancea thermotolerans* (Lt2) was provided from Agrovin S.A. (Ciudad Real, Spain). Commercial *Saccharomyces cerevisiae* (Lalvin QA23®, Lallemand Inc. Montreal, Canada) was refereed as Sc23, and used as a control. Evolved *S. cerevisiae* strains, SeSH1 and SeSH2 (evolved in high sugar concentration) and SeIB1 (evolved Sc23 in presence of iso-butanol to select for tolerance to alcohol), and their respective parental strains (SH1, SH2 and IB1) were selected from a previous study (manuscript in preparation).

The strains were stored at − 80 °C in YPD liquid medium (2% (w/v) glucose, 2% (w/v) yeast extract, and 1% (w/v) peptone, Cultimed, Barcelona, Spain) with 40% (v/v) glycerol. Before use, yeasts grew at 28 °C in YPD agar (YPD liquid with 1.7% (w/v) agar) and Wallerstein laboratory nutrient (WLN) agar (Becton, Dickinson and Company, Isère, France). Before starting fermentations, the strains identification was confirmed by PCR-RFLP analysis of 5.8S-ITS rDNA according to Esteve-Zarzoso et al. (1999).

Propagation of strains was performed by picking single colony from YPD plates to YPD liquid medium, and incubated for 24 h (*S. cerevisiae*) or 48 h (non-*Saccharomyces*) at 28 °C. After incubation, cells were counted by a Neubauer chamber (Leica Microsystems GMS QmbH, Leica, Germany) and inoculated at the corresponding initial population in synthetic must with 200 g/L reducing sugars, prepared according to Beltran et al. (2004), or in sterile concentrated must (65.4° Brix; Mostos Españoles S.A., Tomelloso, Spain) diluted to a concentration of 240 g/L of sugars (Martín-García et al., 2020).

Adaptative laboratory evolution (ALE) of *S. cerevisiae* and selection of evolved strains

High throughout ALE has been used in a previous research project to evolve a set of wine yeast strains onto several selection environments related to wine fermentation,

such as high sugar (synthetic must with 35% sugar) and tolerance to alcohol (synthetic must with 1.3% 1-butanol, to simulate the presence of ethanol, avoiding evaporation). All experiments were performed using the high-resolution platform Scan-o-matic (Zackrisson et al., 2016). Cell doubling times for each ALE population in each environment were calculated (Zackrisson et al., 2016), and the populations with the best growth improvement were preselected for further validations. Some preselected ALE populations and their parental strains were validated at lab scale, and the evolved strains selected for this study had been chosen for their better performance either on growth, fermentation kinetics, or ethanol yield, compared to their respective parental strains (data not shown).

Single and Mixed Inoculum Fermentations

A first trial was performed in synthetic must with 200 g/L sugar, using different inoculation strategies. Single fermentations of the *S. cerevisiae* (Sc23, the evolved SeSH1, SeSH2 and SeIB1, and their respective parental strains SH1, SH2 and IB1), and the non-*Saccharomyces* strains (Mp51 and Lt2) were inoculated with 2 x 10^6 cells/mL. Coinoculated fermentations were performed using two different inoculation ratios of non-*Saccharomyces*/*Saccharomyces* cells (Table 1), 1:1 and 9:1, keeping the total initial population to 2 x 10^6 cells/mL. Sequential fermentations were inoculated with 2 x 10^6 non-*Saccharomyces* cells/mL and, after 48 h of fermentation, 2 x 10^6 *S. cerevisiae* cells/mL were inoculated.

Single and sequential fermentations were also performed using natural must with high sugar concentration (concentrated must diluted to a concentration of 240 g/L sugars) (Table 1). Single fermentations of each of the *S. cerevisiae* strains (Sc23, the parentals SH1, SH2 and IB1, and the evolved SeSH1, SeSH2 and SeIB1), as well as the non-*Saccharomyces* strains (Mp51 and Lt2) were inoculated with 2 x 10^6 cells/mL. Sequential fermentations were inoculated with 2 x 10^6 cells/mL of the non-*Saccharomyces* (Mp51 or Lt2) and, after 24 h of fermentation, 2 x 10^6 cells/mL of *S. cerevisiae* (Sc23, SeSH1, SeSH2 or SeIB1) were inoculated.

Table 1. Fermentations performed in synthetic must (200 g/L sugars) or natural must (240 g/L sugars) with different inoculation strategies (single, sequential or coinoculations at different ratios). Non-*Saccharomyces* and *S. cerevisiae* strains were inoculated at the listed cell concentrations (cells/mL).

Inoculation strategies	Non-*Saccharomyces*	*S. cerevisiae*
	Lt2 or Mp51	Sc23 (1), parental (SH1, SH2, IB1) (2) or evolved (SeSH1, SeSH2, SeIB1) (3)
Synthetic must		
Single inoculation	2×10^6	
Single inoculation		2×10^6 (1, 2, 3)
Coinoculation (1:1)	1×10^6	1×10^6 (1, 3)
Coinoculation (9:1)	1.8×10^6	2×10^5 (1, 3)
Sequential inoculation (48 h)	2×10^6	2×10^6 (1, 3)
Natural must		
Single inoculation	2×10^6	
Single inoculation		2×10^6 (1, 2, 3)
Sequential inoculation (24 h)	2×10^6	2×10^6 (1, 3)

Fermentations were conducted in 250 mL glass bottles with 230 mL of synthetic or natural must, incubated at 22 °C with stirring 120 rpm, and in triplicate for each condition. The bottle cap had two ports, one connected with a 0.22 μm filter (Dominique Dutscher, Brumath, France) for gas flow and the other clamped by an iron clip for taking samples. Fermentation kinetics were monitored by measuring must density, determined with an electronic densitometer (Densito 30PX Portable Density Meter, Mettler Toledo, Hospitalet de Llobregat, Spain), and yeast growth, determined by plating samples during fermentation in YPD agar plates. Yeast viable population of each species was confirmed by growth in differential WLN or lysine agar (11.75 g/L yeast carbon base, 2.5 g/L L-lysine monohydrochloride, and 20 g/L agar, Cultimed, Barcelona, Spain) according to the different morphological profile between *Saccharomyces* and non-*Saccharomyces* strains. Briefly, samples were serially diluted in sterilized Milli-Q water from a Milli-Q purification system (Millipore S.A.S., Molsheim, France). The number of colony forming units per milliliter (CFU/mL) was

determined by plating 100 μL of three appropriately chosen dilutions on WLN or lysine agar. The plates were incubated at 28 °C for 2 or 3 days.

Fermentations were considered to be finished when residual sugars were below 2 g/L, checked by enzymatic analysis in a Miura autoanalyzer (EE-MIURAONE Rev., I.S.E. S.r.l., Italy), or when density of must was not decreasing for more than two days. Samples were centrifugated at 7800 rpm for 5 min and supernatants were frozen at –20 °C until the chemical analysis.

Chemical analysis

Residual sugars of samples at the end of fermentation were quantified by D-glucose/D-fructose assays (Biosystems S.A., Barcelona, Spain). Ethanol, glycerol and organic acids (citric acid, malic acid, tartaric acid, acetic acid, lactic acid and succinic acid) of samples were determined by high-performance liquid chromatography (HPLC) using an Agilent 1100 (Aglient Technologies, Waldbronn, Germany) as previously described by Quirós et al. (2010) and Zhu et al. (2020).

Statistical analysis

All graphs were generated using GraphPad Prism® version 8 (GraphPad Software, San Diego, CA, USA). Results expressed as mean ± standard deviation (SD). Statistically significant differences (one-way ANOVA) were analyzed by IBM SPSS Statistics version 23.0 (IBM, NY, USA). The ethanol yield was calculated with the formula "Ethanol yield (g/g) = ethanol production (g/L)/sugar consumption (g/L)".

Results

1. Selection of the strains to be used in mixed fermentations

In a previous study, several wine *S. cerevisiae* strains were exposed to different selective environments, using a high throughput ALE platform, with the aim to improve their fermentative ability in different wine-related stressful conditions (WineSys project, manuscript in preparation). The challenges that wine strains had to

face were different nutritional environments tailored to impose several selective pressures that put forward evolutionary enhancements of industrial relevance, such as, high sugars, high ethanol, nutrient limitation, etc. Fermentative performance, ethanol production and ethanol yield of some of the preselected ALE strains was analyzed, and two of the evolved strains on high sugar condition (35% sugars), SeSH1 and SeSH2, and one strain adapted on iso-butanol (to emulate the presence of ethanol, avoiding evaporation), SeIB1, were selected for showing a better performance to their parental strains, including a slight decrease of the ethanol yield (data not shown). A commercial wine yeast broadly used in wine industry, was also used as a control strain (Sc23).

The two non-*Saccharomyces* strains, *M. pulcherrima* (Mp51) and *L. thermotolerans* (Lt2), were selected from the previous study (Zhu et al., 2020), for their lower ethanol yield in lab-scale fermentations.

2. Effect of different inoculation strategies on fermentation, population kinetics and ethanol yield

The evolved *S. cerevisiae* and the non-*Saccharomyces* strains were used in sequential and coinoculated fermentations of synthetic must, to evaluate their ability to reduce ethanol, as well as the effect on fermentation kinetics and population dynamics. After alcoholic fermentation, all final samples were subjected to an in-depth chemical analysis to characterize the resulting wines. Fermentations with single inoculum of each *S. cerevisiae* and non-*Saccharomyces* strains were also monitored.

Fermentation kinetics

The fermentation kinetics obtained with the different inoculations are shown in Figure 1. Among the different single fermentations with *S. cerevisiae* strains, Sc23 showed the fastest fermentation kinetics (5 days), and surprisingly, the evolved strains completed the fermentations slower than their parental strains (7 – 15 days), being SeSH1 and SeSH2 the slowest strains (Figure 1h). Focusing on non-*Saccharomyces*

yeasts, single fermentations with Mp51 were stuck, with more than 60 g/L of residual sugars (Figure 1, Table S1), while single fermentations with Lt2 were able to complete the fermentation in 13 – 16 days (Figure 1, Table S2).

On the other hand, most mixed fermentations showed slower fermentation kinetics than those with single *S. cerevisiae* inoculum, being the coinoculated fermentations faster than the corresponding sequential ones. Sequential fermentations with Mp51/SeSH1 and Mp51/SeSH2 were the slowest, and required almost 30 days to finish, which was much longer than the respective fermentations with Lt2 (16 days) (Figures 1c – 1f). The coinoculated fermentations with the ratio of 1:1 (non-*Saccharomyces*/*S. cerevisiae*) were faster than those with 9:1 ratio (Figures 1a – 1g).

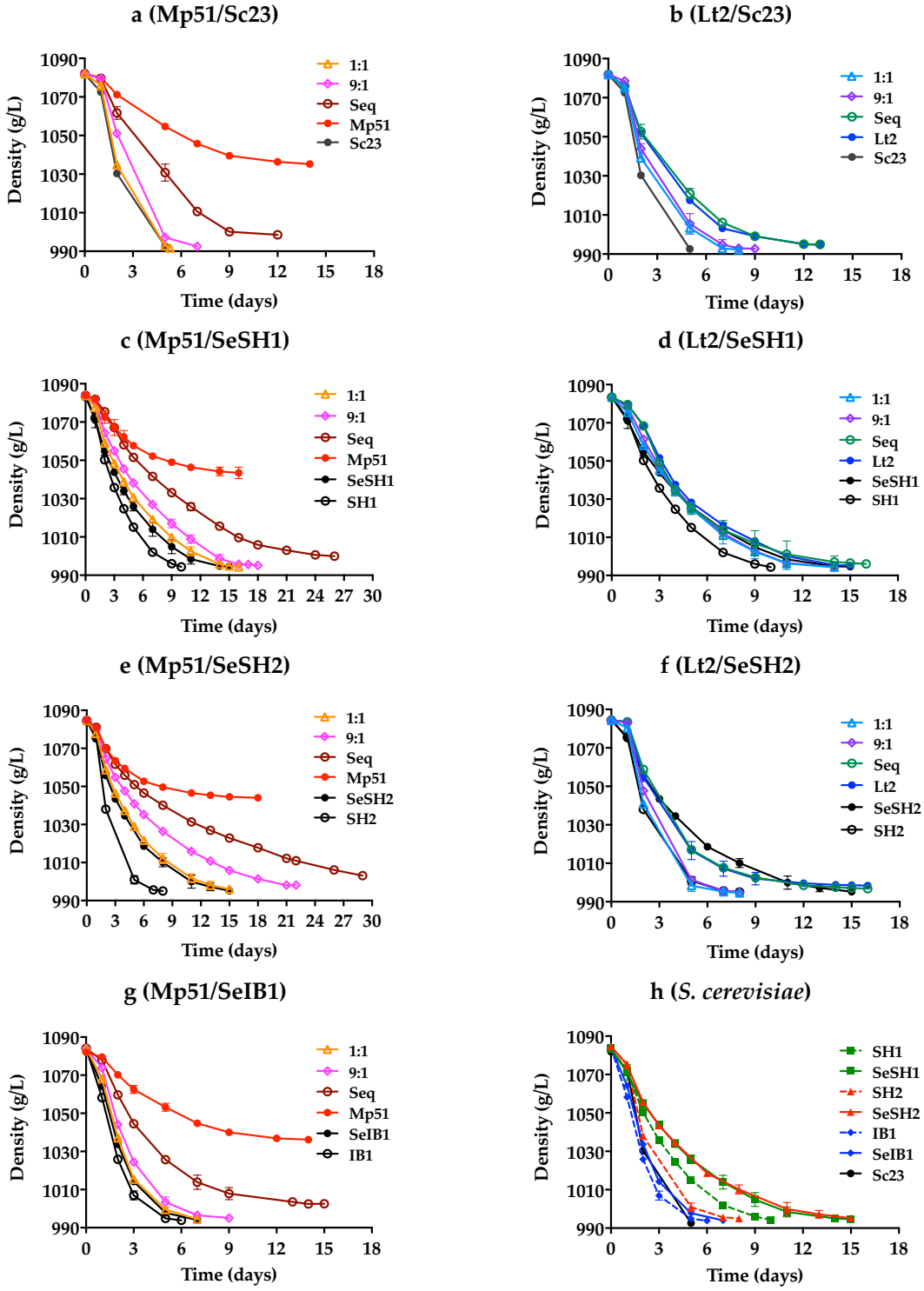

Figure 1. Density of single and mixed fermentations in synthetic must using the non-*Saccharomyces* (Lt2, Mp51), and different *S. cerevisiae* (Sc23, parental strains (SH1, SH2, IB1), and evolved strains (SeSH1, SeSH2, SeIB1)). Sequential (Seq, inoculating *S. cerevisiae* at 48 h) and coinoculated fermentations (inoculation ratios non-*Saccharomyces*/*S. cerevisiae*, 1:1 and 9:1,) were performed with non-*Saccharomyces* and *S. cerevisiae* strains.

Population dynamics

The population dynamics of *S. cerevisiae* and non-*Saccharomyces* yeasts during single or mixed fermentations is summarized in Figure 2. All the *S. cerevisiae* strains reached a cell population above 10^8 CFU/mL on the second day of their single fermentations, among them, SeIB1 showed the highest population, reaching to 2.8 × 10^8 CFU/mL (Figure 2g). The population of non-*Saccharomyces* in pure cultures also reached around 2 – 3 × 10^8 CFU/mL on the second or third day of fermentation (Figure 2).

Focusing on coinoculated and sequential fermentations, Mp51 was not able to compete with *S. cerevisiae*, and it was replaced by *S. cerevisiae* strains in all conditions, and even not detected at the end of fermentation (after mid-fermentation in the coinoculations) (Figures 2a,c,e,g). On the contrary, the growth of Lt2 was observed on WLN plates until the end of fermentation (Figures 2b,d,f). Indeed, when used in sequential fermentations Lt2 was the dominant strain until at least 3 days before the end of fermentation with SeSH2 and Sc23 strains, and till the end of the fermentation with SeSH1 strain. As expected, the persistence of non-*Saccharomyces* strains was longer in sequential inoculations than in the coinoculated fermentations. In the latter the persistence was longer with 9:1 inoculum ratio than with 1:1 inoculum ratio. Specifically, in the 1:1 ratio, the maximal Mp51 population was lower than 2.5 × 10^7 CFU/mL and had a rapid decrease after 3 days of fermentation, while in the 9:1 ratio, Mp51 reached higher populations, above 8 x 10^7 CFU/mL, and slightly longer persistence. Similar results were observed in coinoculated fermentations with Lt2: higher populations and persistence with 9:1 ratios of inoculation. It is worth noting that the fermentations with Lt2/SeSH1, in which Lt2 persistence was higher, were also the ones with slower fermentation kinetics (Figure 2b). In addition, the yeast population of Sc23 in sequential inoculation with Mp51 was higher than that with Lt2, with the number of 1.0 x 10^8 vs 3.4 x 10^7 CFU/mL at the end of fermentation (Figures 2a,b).

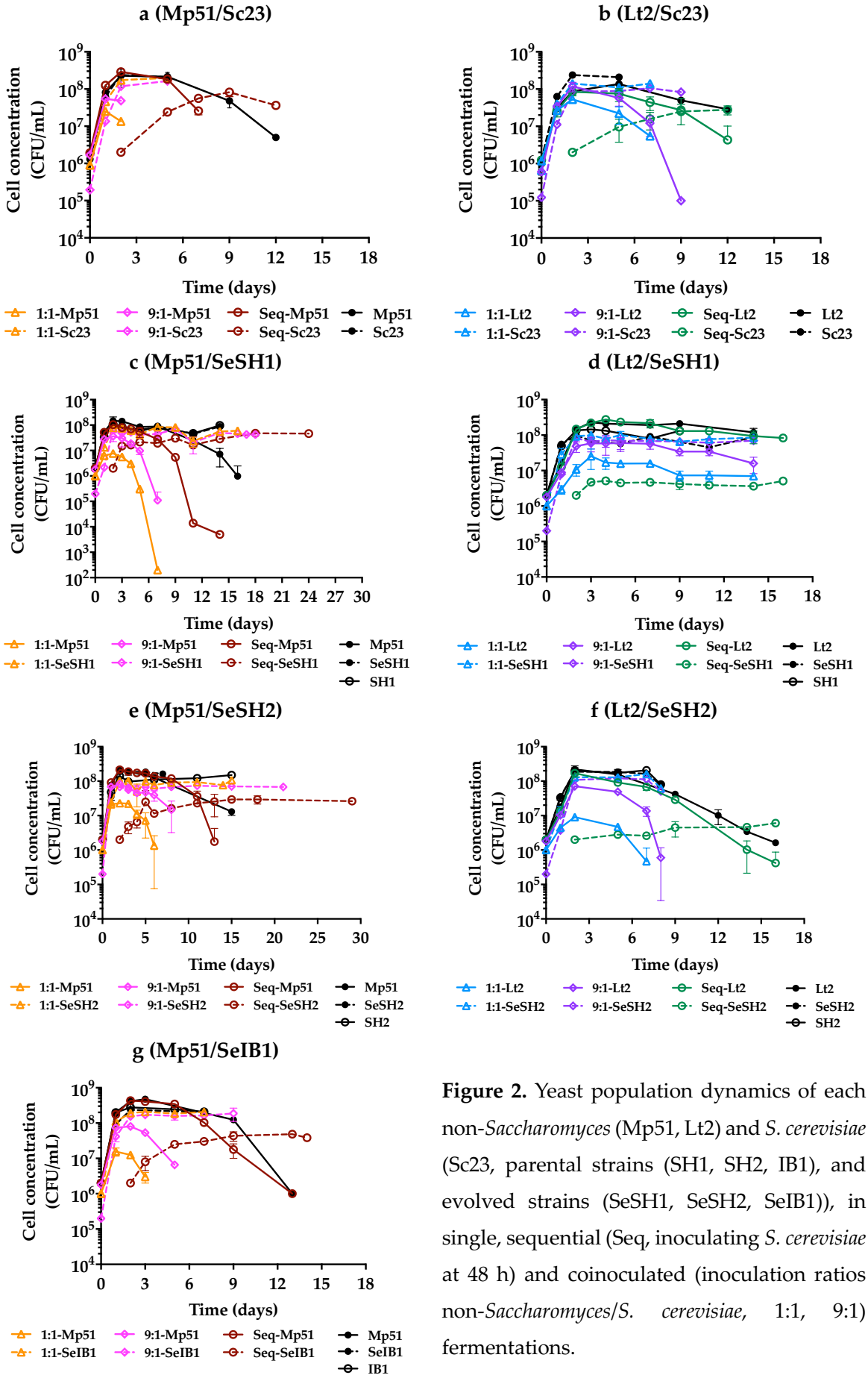

Figure 2. Yeast population dynamics of each non-*Saccharomyces* (Mp51, Lt2) and *S. cerevisiae* (Sc23, parental strains (SH1, SH2, IB1), and evolved strains (SeSH1, SeSH2, SeIB1)), in single, sequential (Seq, inoculating *S. cerevisiae* at 48 h) and coinoculated (inoculation ratios non-*Saccharomyces*/*S. cerevisiae*, 1:1, 9:1) fermentations.

Main analytical characteristics

To demonstrate the alcohol-lowering ability of the different inoculation strategies, as well as their metabolic characteristics, residual sugar, ethanol production and main by-products were analyzed at the end of different fermentations, and shown in Table S1 (fermentations with Mp51) and Table S2 (fermentations with Lt2). The end point of most fermentations was based on the concentration of residual sugar (< 2 g/L). However, in single non-*Saccharomyces* fermentations and sequential fermentation with Mp51/SeSH2, samples were analyzed with a higher concentration of residual sugars (single fermentation by Mp51 (> 60 g/L) and Lt2 (4.25 g/L), and Mp51/SeSH2 (3.61 g/L)), because these fermentations showed slow or stuck fermentation kinetics.

Focusing on the ethanol production of *S. cerevisiae* (Figure 3h), when used as single inoculum, Sc23 showed the highest ethanol production of 11.81% (v/v) with the ethanol yield of 0.47 g/g. The ethanol production between parental and its evolved strains showed no significant differences except for the strains evolved on high sugar conditions, SeSH2 and SeSH1, that presented higher ethanol production (higher ethanol yield) than their parental strains in some case, although not always significant (Tables S1 and S2, Figure 3d).

Ethanol production and ethanol yield were clearly influenced by the strategy of inoculation in Mp51 fermentations (Table S1, Figure 3). We observed that sequential inoculations with Mp51 produced the lowest ethanol content and ethanol yield (0.41 – 0.42 g/g), achieving an ethanol reduction of 1.44, 1.42, 1.40 and 1.22% (v/v), compared to single fermentation with their respective *S. cerevisiae*. Following, coinoculated fermentations with 9:1 ratio reduced the content of ethanol from 0.18 to 0.95% (v/v), achieving a significant reduction in Mp51/SeSH2 and Mp51/SeSH2 fermentation (0.41 and 0.95% (v/v), respectively), compared to their respective single *S. cerevisiae* inoculation (Table S1, Figures 3a,c,e,g). Lastly, the ethanol reduction obtained with the increase of the *S. cerevisiae* inoculation (1:1 ratio) was not significant in any fermentation. Fermentations performed with Lt2 did not present a high alcohol-lowering ability in any condition, except in sequential fermentation by Lt2/Sc23,

where the ethanol production was significantly reduced by 0.28% (v/v) (Table S2, Figure 3b).

For the other analyzed by-products, they were influenced by the yeasts used and the inoculation strategy. Compared to single *S. cerevisiae* fermentations, sequential inoculations with Mp51 produced the highest concentration of glycerol (6.90 – 8.63 g/L), among them, the fermentation with Mp51/Sc23 had the highest glycerol content of 8.63 g/L (Table S1). Equally, Lt2 was able to increase the concentration of glycerol in all sequential fermentations (8.55 – 9.40 g/L), as well as in single Lt2 fermentations (7.17 to 8.19 g/L) (Table S2). Noteworthy, the concentration of lactic acid was significantly increased in Lt2 fermentations, both in single (from 2.41 to 2.59 g/L) and sequential fermentations (reaching to 3.45 – 4.40 g/L). On the other hand, the acetic acid concentration was below 0.8 g/L at the end of all fermentation. Both Lt2 and Mp51 single fermentations showed lower content of acetic acid than that of *S. cerevisiae*. However, the concentration of acetic acid was significantly increased in their mixed fermentations compared to single fermentations (Tables S1 and S2). Moreover, single fermentations with the evolved strains had a higher concentration of succinic acid than Sc23, consequently, mixed fermentations with both Mp51 and Lt2 showed also a higher concentration of succinic acid.

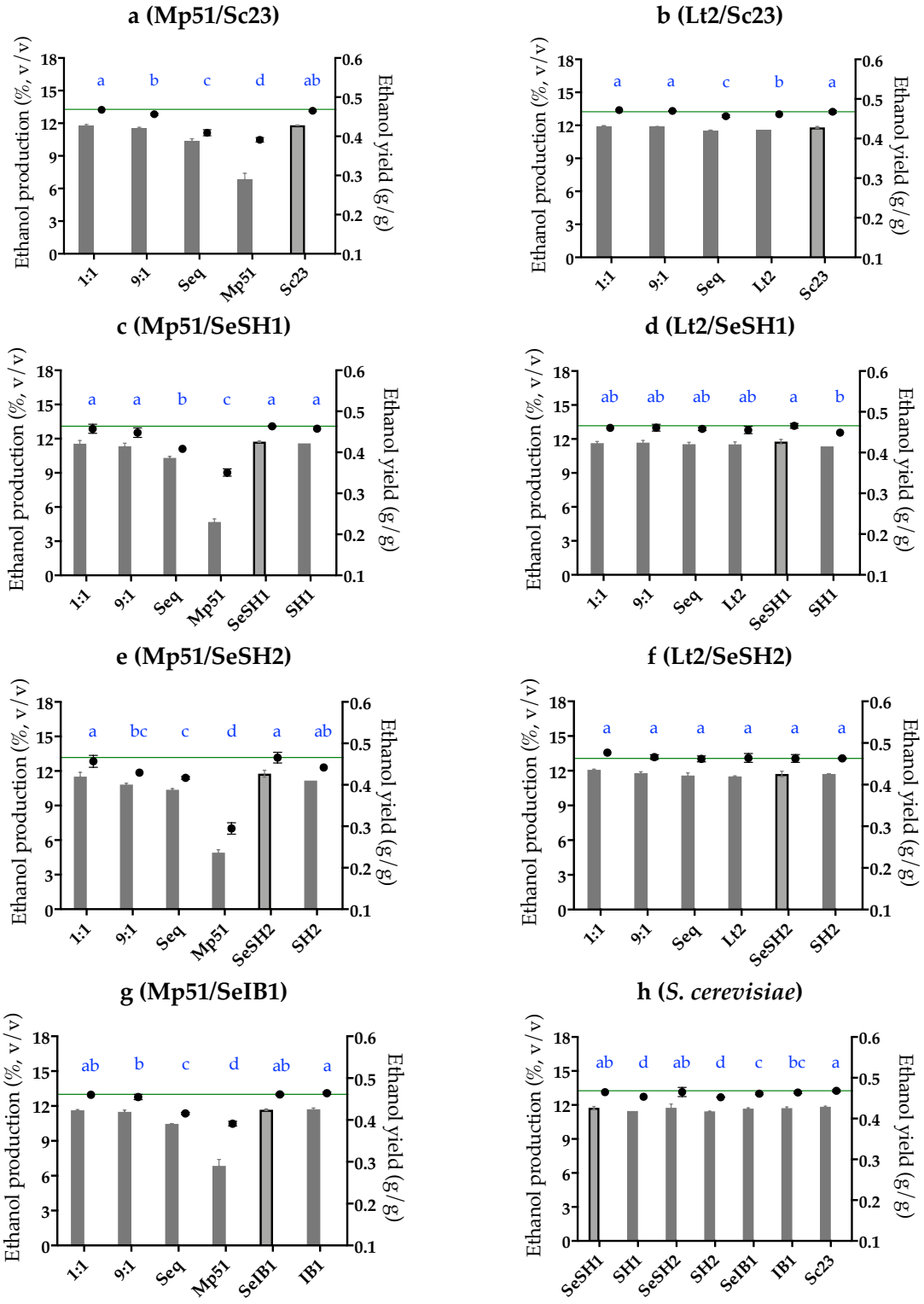

Figure 3. Ethanol production (%, v/v) and ethanol yield (g/g) at the end of single, using non-*Saccharomyces* (Mp51, Lt2) or *S. cerevisiae* (Sc23, parental strains (SH1, SH2, IB1), and evolved strains (SeSH1, SeSH2, SeIB1)), sequential (Seq, inoculating *S. cerevisiae* at 48 h) and coinoculated (inoculation ratios non-*Saccharomyces*/*S. cerevisiae*, 1:1, 9:1) fermentations in synthetic must. Letters (a,b,c,d) in each figure mean the statistic significant difference (Duncan, $p < 0.05$). The value of the green line is the ethanol yield of Sc23 or evolved *S. cerevisiae* strains.

3. Effect of single and sequential inoculated fermentations on high sugar natural must

Once determined that the best inoculation strategy for reducing the ethanol yield, and therefore, the ethanol levels of the wines, was the sequential inoculation, we wanted to validate those results using single and sequential inoculations on natural must with high sugar concentration (Table 1), as an approach to wine production conditions. In real industrial conditions, the inoculation of *S. cerevisiae* in sequential fermentations cannot be delayed, to avoid the imposition of other wild yeast. For that reason, *S. cerevisiae* is usually inoculated after 24 h of the start of the fermentation, and not later. Therefore, in this validation trial we also used 24 h for inoculating *S. cerevisiae* strains in the sequential fermentations.

Fermentation kinetics

When used the pure culture of these *S. cerevisiae* in fermentations of natural must, the Sc23 took 7 days to complete the fermentation, showing the fastest fermentation kinetics. However, different from what was observed in synthetic must, the fermentation kinetics of parental and their evolved strains was similar, being SeIB1 the slowest strain (Figure 4a). The sequential fermentations were again slower than the single inoculations, except for Lt/SeIB1, that finished in 16 days, like single SeIB1 inoculation. In natural must, sequential fermentations inoculated with Lt2 were finished in 14 – 17 days (Figure 4c), similar to synthetic must, while with Mp51 finished in 10 – 20 days, faster than in synthetic must. Among all sequential fermentations, Mp/SeSH1 was the fastest one to complete the fermentation, in 10 days, and the slowest Mp/SeIB1, in 20 days (Figure 4b).

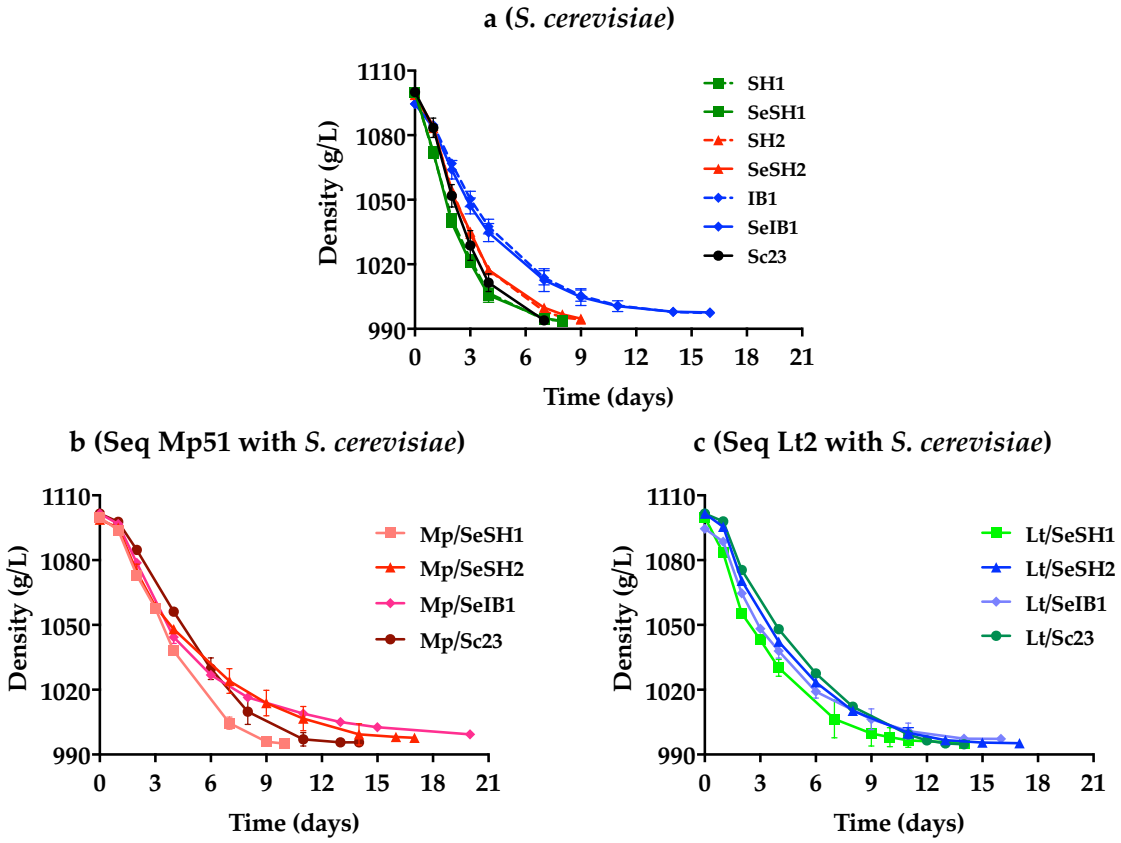

Figure 4. Density of single (a), using different *S. cerevisiae* (Sc23, parental strains (SH1, SH2, IB1), and evolved strains (SeSH1, SeSH2, SeIB1)), and sequential (Seq, using non-*Saccharomyces* yeasts Mp51 (b) or Lt2 (c), inoculating *S. cerevisiae* at 24 h) fermentations in natural must.

Population dynamics

To evaluate the yeast growth and dynamics in single and sequential fermentations, the yeast population was monitored during alcoholic fermentation. In single fermentations, Sc23 showed the highest growth, reaching 2.4 x 10^8 CFU/mL in the middle of the fermentation (Figure 5d). Evolved strains had similarly population dynamics than their parental strains, over 10^8 CFU/mL after 24 h of fermentation (Figures 5a,b,c).

In sequential fermentations, the performance between Mp51 and Lt2 was totally different. Specifically, Lt2 could reach the largest population of 2.5 x 10^8 CFU/mL in the third day of fermentation, and was maintain quite stable (Figure 5c), however, the population dynamics of Mp51 dropped sharply after *S. cerevisiae* inoculation. Similar to the fermentation in synthetic must, Lt2 could persist until the end of the

fermentation, being the dominant strain in most cases, while Mp51 almost could not be observed at the end stage of fermentation, being *S. cerevisiae* the dominant strain. Indeed, the *S. cerevisiae* strains showed a steady growth trend in fermentations with Mp51, reaching 10^8 CFU/mL at the end of fermentation. However, in the sequential fermentations with Lt2, the population of *S. cerevisiae* was maintained low, around at 10^7 CFU/mL, until the end of fermentation.

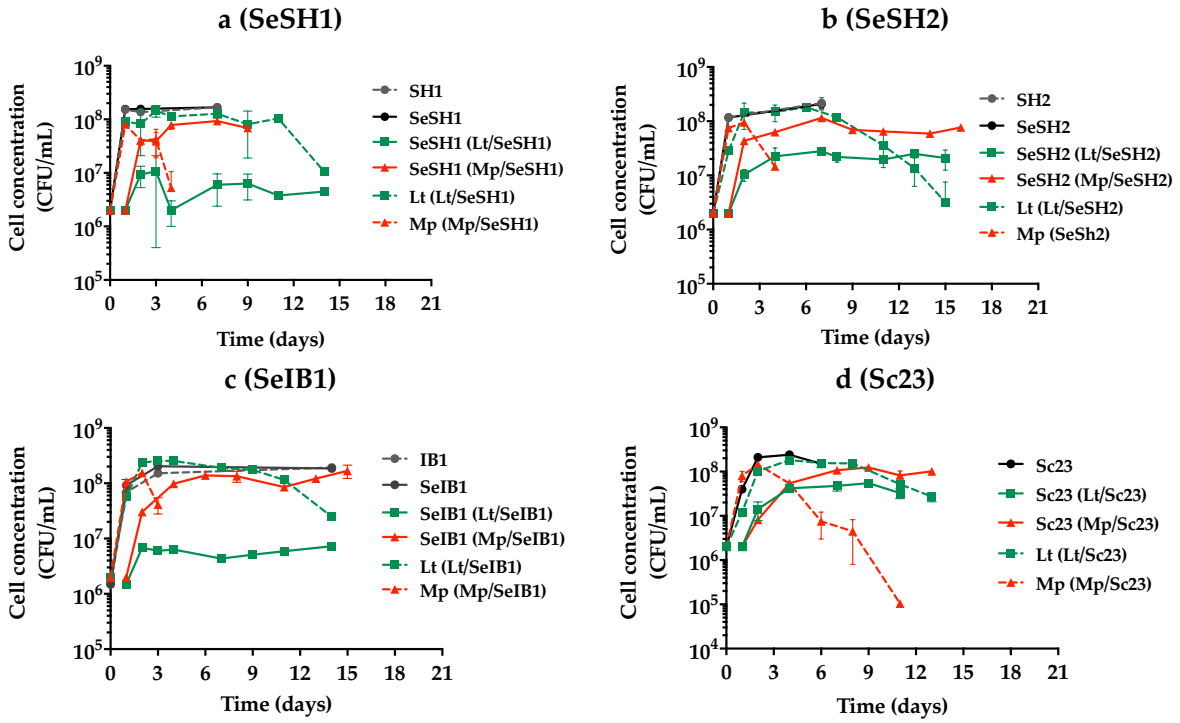

Figure 5. Yeast population (CFU/mL) of each non-*Saccharomyces* (Mp51, Lt2) and *S. cerevisiae* (Sc23, parental strains (SH1, SH2, IB1), and evolved strains (SeSH1, SeSH2, SeIB1)), in single and sequential (Seq, inoculating *S. cerevisiae* at 24 h) fermentations in natural must.

Main analytical characteristics

To verify the ethanol reduction, as well as other metabolic characteristic of Mp51 or Lt2 when used in sequential fermentations of natural must, the residual sugar, ethanol production and the main by-products at the end of the fermentations were analyzed (Table S3). All fermentations were able to consume most of the sugar present in the must (more than 236 g/L), except the one with Mp/SeIB1, which had 5.77 g/L of

residual sugars after 20 days (it was considered stuck, as the density did not decrease for more than two days).

The ethanol production of evolved strains was similar than their parental strains, except the SeIB1, which presented a significant ethanol reduction of 0.46% (v/v) compared to the parental strain IB1 (Figure 6, Table S3). Sequential fermentations inoculated with Mp51 showed lower ethanol production and yield, compared to the single fermentation of the corresponding *S. cerevisiae* strains, reducing ethanol by 0.38, 0.44, 0.75 and 1.18% (v/v), being the ethanol reduction of Mp/SeIB1 inoculation the highest one. However, in sequential fermentations with Lt2, only Lt/Sc23 achieved a significant ethanol reduction of 0.23% (v/v), lower than with Mp51 inoculation (Table S3).

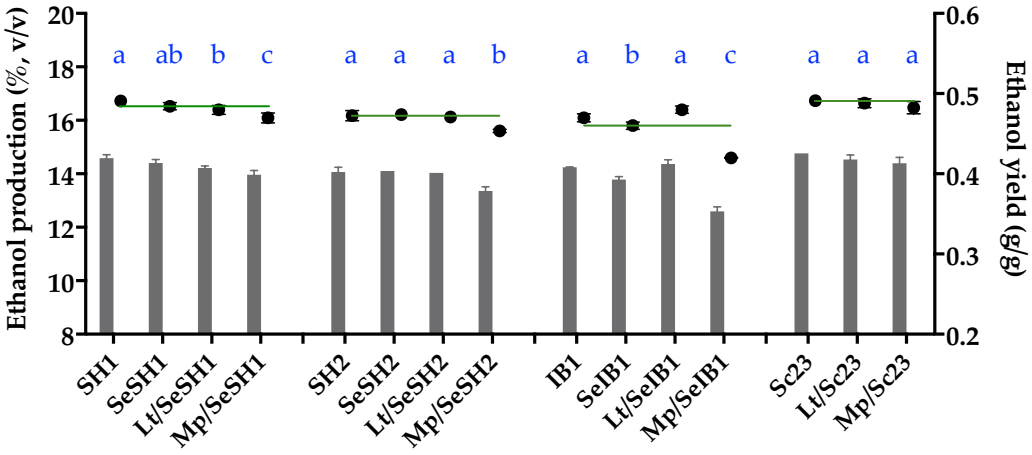

Figure 6. Ethanol production (%, v/v) and yield (g/g) at the end of single, using different *S. cerevisiae* (Sc23, parental strains (SH1, SH2, IB1), and evolved strains (SeSH1, SeSH2, SeIB1)), and sequential (Seq, using non-*Saccharomyces* yeasts Mp51 or Lt2, inoculating *S. cerevisiae* at 24 h) fermentations in natural must. Shared superscript letters (a,b,c) within each group of fermentation by different *S. cerevisiae* mean statistically significant differences (Duncan, $p < 0.05$). The value of the green line is the ethanol yield of Sc23 or evolved *S. cerevisiae* strains.

The other by-products produced were influenced by the inoculation strategy and the yeasts used. The concentration of glycerol was significantly increased in sequential fermentations with Mp51, which was a resemblance with fermentations in synthetic must, however, no glycerol increased was observed in fermentations with Lt2. The

lactic acid was significantly higher in some sequential fermentations with Lt2, in Lt/SeIB1 and Lt/SeSH1, being the later the one with the highest concentration, 0.58 g/L.

Discussion

The approach of physiological adaptations has been adopted by researchers to select strains better adapted to nutrient limitations, with better fermentative performance and the ability to reduce ethanol content. Several researchers had used ALE strategy to reduce the ethanol yield by redirecting the yeast carbon metabolism to increased glycerol production (Kutyna et al., 2012; Vejarano et al., 2013; Tilloy et al. 2015; Goold et al., 2017). In this study we used some ALE *S. cerevisiae* strains previously selected from high sugar and tolerance to 2-butanol (as an approach to ethanol tolerance) as evolution challenges. We used high concentration of sugar as an adaptive pressure environment to isolate strains that could have shift part of their sugar consumption to other metabolites than ethanol as an adaptation strategy, thereby reducing ethanol production of wines. Indeed, the exposure to high osmotic stress is known to increase glycerol production, and has been used as selective pressure on ALE strategy for lowering ethanol (Vejarano et al., 2013; Tilloy et al., 2014). On the other hand, the strains evolved to become more tolerant to 2-butanol had been proved to be also more tolerant to ethanol, and to increase their glycerol production, suggesting that the tolerance mechanisms could lead to higher flow of carbon to glycerol and likely also to respiration (Ghiaci et al., 2013). Therefore, a lower ethanol production could benefit the cell survival on high sugar and high alcohol environment.

Our results showed that strains isolated from high sugar conditions took longer to complete fermentation in synthetic must than their parental, or that of Sc23. Instead, the strain isolated from isobutanol, SeIB1, had a slower fermentation rate in natural must, compared to the other evolved strains. It could be because the evolution in iso-butanol reduced sugar consumption rate resulting in a reduction of fermentation rate (Albers et al., 2009; Ghiaci et al., 2013). In any case, the ethanol yields of parental and

evolved *S. cerevisiae* strains were very similar, and between the expected values reported by Contreras et al. (2014) (0.44 – 0.48 g/g), and in most cases slightly inferior to the ethanol yields obtained with the commercial wine strain used as reference *S. cerevisiae* (Sc23, 0.47 – 0.49 g/g). For instance, the ALE strains and their parental produced more glycerol than Sc23 in synthetic must, which could explain the lower ethanol yields of those strains. In a previous study, Ghiaci et al. (2013) observed that *S. cerevisiae* strains evolved to butanol tolerance could increase the synthesis of glycerol and decrease the synthesis of ethanol. However, our results showed no big differences on ethanol production between the selected ALE and their parental strains, suggesting that the adaptation process of yeasts to these environments did not directly affect the carbon or fermentative metabolism.

On the other hand, the use of non-*Saccharomyces* yeasts has also been confirmed to reduce ethanol yield, both by single and mixed fermentations (Quirós et al., 2014; Contreras et al., 2015; Zhu et al., 2020). Yeasts can be classified according to the way they regulate their respiro-fermentative metabolism. It is well known that *S. cerevisiae* exhibits strong fermentation metabolism, even in presence of oxygen, when sugar concentration is above a certain threshold, due to glucose catabolite repression and Crabtree effect, producing ethanol as the main metabolite (Carlson, 1999; Barnett et al., 2005). Therefore, Crabtree-positive yeasts, such as *S. cerevisiae*, show a strong preference towards fermentative metabolism, and this characteristic seems to have played a key role in the adaptation of *S. cerevisiae* to sugar rich environments (Piskur et al., 2006, González et al., 2013). In contrast, Crabtree-negative species would favor sugar respiration whenever enough oxygen is available. Therefore, a recent strategy for lowering ethanol production in wines has been the search for Crabtree-negative yeast species or species that would preferentially consume sugars by respiration rather than fermentation, and produce wines with lower ethanol content (Quirós et al., 2014; Contreras et al., 2015). However, most low-ethanol yield non-*Saccharomyces* yeasts showed slow growth and were not capable of completing fermentation during alcoholic process (Fleet et al., 1993; Ciani et al., 2006).

In our hands, both *L. thermotolerans* (Lt) and *M. pulcherrima* (Mp) single fermentations were able to reduce ethanol yield, compared to *S. cerevisiae*. In agreement with a previous study (Zhu et al., 2020), the extent of ethanol reduction was higher for Mp, but showed slower fermentation kinetics than *S. cerevisiae*, being not able to complete the alcoholic fermentation. This is consistent with previous studies where slow or sluggish fermentations appeared in *M. pulcherrima* pure inoculations (Medina et al., 2012; Morales et al., 2015). On the other hand, the Lt pure cultures presented higher fermentation kinetics and could complete the fermentation, which agreed also with previous studies (Comitini et al., 2011; Gobbi et al., 2013).

To avoid the possibility of having sluggish or stuck fermentations, the non-*Saccharomyces* strains are usually used in mixed inoculations with *S. cerevisiae* (Bisson, 1999; Ciani et al., 2006; Jolly et al., 2006). Nevertheless, the different inoculation protocols could affect the population dynamics of the different yeast species during fermentation process, and therefore, their metabolic contribution to the final product (Contreras et al., 2014; Canonico et al., 2016; Zhu et al., 2021). In this study we explored the impact of different mixed inoculation strategies (coinoculation at different rations and sequential inoculation) on yeast growth and ethanol reduction, and we observed that non-*Saccharomyces* strains had higher competitiveness and longer persistence in sequential fermentations, than in coinoculated fermentations. In coinoculation, the yeast growth and viability depended on the inoculation ratio of non-*Saccharomyces* and *S. cerevisiae*, resulting the fermentation with 9:1 ratio (non-*Saccharomyces*/*S. cerevisiae*) on a longer persistence of both non-*Saccharomyces* species, compared to 1:1 ratio, where the growth of *S. cerevisiae* was not affected by any of non-*Saccharomyces* yeasts. Those results agree with those previously reported by Comitini et al. (2011) and Morales et al. (2015). Comitini et al. (2011) also confirmed that *M. pulcherrima* was able to persist longer in co-cultures at ratio 100:1 and 10000:1 (non-*Saccharomyces*/*S. cerevisiae*). This could be explained by the lower ethanol tolerance of non-*Saccharomyces* yeasts, nutrient competition, cell-to-cell contact, or the interaction of antimicrobial compounds secreted by different yeasts (mostly *S. cerevisiae*) (Nissen et

al., 2003; Ribéreau-Gayon et al., 2006; Albergaria et al., 2010; Andorrà et al., 2012; Branco et al., 2014; Wang et al., 2015 & 2016; Lleixà et al., 2016). In addition, the behavior of *S. cerevisiae* in the current study was also different depending on the inoculation strategy. The *S. cerevisiae* population at the end of sequential fermentation, in which non-*Saccharomyces* reached higher growth, was lower than in coinoculated fermentations. This points out that interactions between non-*Saccharomyces* and *S. cerevisiae* could affect the growth of both non-*Saccharomyces* and *S. cerevisiae* strains, as previously described by Ciani et al. (2015). Moreover, Lt could persist until the end of sequential fermentations, probably due to its high ethanol tolerance and synergistic interactions with *S. cerevisiae*. This was also confirmed by Gobbi et al. (2013), in which *L. thermotolerans* showed a high competitiveness in sequential fermentations combined with *S. cerevisiae*. On the other hand, the fermentation kinetics of the sequential inoculations were the slowest followed by the coinoculations at 9:1 ratio, and finally at 1:1 ratio. This indicated that the higher proportion of non-*Saccharomyces* strains slowed the rate of sugar consumption.

As we have commented, mixed fermentations are being explored for its ability to reduce ethanol content in wines, which we had mainly observed in mixed fermentations with Mp. However, the ethanol reduction was directly related to the proportion and persistence of *M. pulcherima* along the fermentation. Accordingly, sequential fermentations achieved the lowest ethanol content, with a reduction from 1.22 to 1.44% (v/v), agreeing with a previous study, where an ethanol reduction of 1.6% (v/v) was achieved (Contreras et al., 2014). Secondly, the inoculum ratio of 9:1 (*M. pulcherrima*/*S. cerevisiae*) showed a higher ethanol reduction than that of 1:1. Likewise, a previous study showed how that high inoculum proportion of non-*Saccharomyces* could achieve higher ethanol reduction (*M. pulcherrima*/*S. cerevisiae:* 10000:1 > 100:1 > 1:1) (Comitini et al., 2011). With Lt2, only in sequential fermentations we observed a significant ethanol reduction, but lower than with Mp51 (0.28%, v/v). Previous studies had observed an ethanol reduction between 0.14 and 0.35% (v/v) when using sequential fermentations with *L. thermotolerans* and *S. cerevisiae* (Binati et al., 2020), and

higher ethanol reductions in sequential fermentations rather than those of co-culture fermentations (Gobbi et al., 2013).

The production of other by-products from sugar metabolism was also a criterion to explain the reduction of ethanol (Ciani et al., 2016). As previously described, the most efficient strategy is to reroute sugar metabolism towards increased glycerol production (Tilloy et al., 2015). During fermentation process, when carbons are metabolized into more glycerol, it causes a substantial reduction in ethanol (Michnick et al., 1997; Remize et al., 1999). In sequential inoculations, where ethanol was further reduced, a significant increase in glycerol content was obtained. This fact was supported by previous reports where *M. pulcherrima* used in sequential fermentations increased the content of glycerol from 1.6 to 4.5 g/L (Contreras et al., 2014; Canonico et al., 2019b; Zhu et al., 2020), while other previous studies showed that the increase in glycerol content was less than 1.1 g/L in coinoculated fermentations (Comitini et al., 2011; Varela et al., 2016).

Acetic acid is also one of the important metabolites that might be overproduced accompanied by ethanol reduction, mainly in aerobic conditions (Ciani et al., 1995; Morales et al., 2015; Canonico et al., 2019b). In the present work, fermentations were performed in semi-aerobic conditions, and acetic acid was below 0.8 g/L in synthetic must, which is considered the limit to prevent unpleasant acidic taste to wine (Fleet et al., 1993). Noteworthy, the evolved strains, SeSH1 and SeSH2, increased the acetic acid in mixed fermentations. This might be due to the fact that they were isolated from stressful conditions, and their low fermentation kinetics led to the accumulation of acetic acid.

Finally, *L. thermotolerans* has been proven to be a lactic acid producer, with the potential to reduce ethanol content (Comitini et al., 2011; Gobbi et al., 2013; Binati et al., 2020). In the present work, in synthetic must, the lactic acid content in single or sequential fermentations with Lt was increased by 2.40 and 4.08 g/L, respectively, compared to the single fermentation of *S. cerevisiae*. The production of lactic acid is redirecting part of the sugar to that metabolism, therefore, decreasing slightly the

ethanol content (Morata et al., 2018). Surprisingly, in natural must fermentation, the concentration of lactic acid was much lower than in synthetic must (less than 0.6 g/L), but still increased in some of the mixed fermentations with Lt. The different composition between natural and synthetic must might affect the production of lactic acid. A previous study confirmed that the production of lactic acid by *L. thermotolerans* was more affected by assimilable nitrogen content than by sugar (Hernández, 2018).

In conclusion, when used as single inoculum, ALE *S. cerevisiae* strains showed slower fermentation kinetics than their parental strains in synthetic must fermentation, but similar fermentative profile in natural must fermentation, with no significant difference on ethanol production, except for the strain adapted on alcohol tolerance, which had a significant lower ethanol yield than its parental on natural must fermentation, and a significant increase of glycerol production. On the other hand, *M. pulcherrima* was the most efficient strain for reducing ethanol production, especially when used in sequential fermentations, both in synthetic and high sugar natural must, which had noteworthy reduction of ethanol content along with higher glycerol production. In coinoculated fermentations, the inoculation ratio of non-*Saccharomyces* yeast was related to their persistence during fermentation process, and higher inoculation ratios (9:1, Mp51/*S. cerevisiae*) caused longer viability of Mp51 and a higher ethanol reduction. For *L. thermotolerans*, a significant ethanol reduction could only be detected in sequential inoculations of synthetic must, which was accompanied by an increase of lactic acid and glycerol content. The persistence of Lt2 in mixed fermentations was much longer than that of Mp51, mainly in sequential inoculations, in which ended being the dominant strain, above *S. cerevisiae* populations. However, no ethanol reduction was observed in mixed fermentations with Lt2 on high sugar natural must, indicating that the ability of this strain to reduce ethanol is more limited and affected by the media composition and fermentative conditions.

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References

Abalos, D.; Vejarano, R.; Morata, A.; Gonzalez, C.; Suarez-Lepe, J.A. The use of furfural as a metabolic inhibitor for reducing the alcohol content of model wines. *Eur. Food Res. Technol.* **2011**, 232, 663–669, doi:10.1007/s00217-011-1433-9.

Albergaria, H.; Francisco, D.; Gori, K.; Arneborg, N.; Gírio, F.M. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl. Microbiol. Biotechnol.* **2010**, 86, 965–972, doi:10.1007/s00253-009-2409-6.

Albers, E.; Larsson, C. A comparison of stress tolerance in YPD and industrial lignocellulose-based medium among industrial and laboratory yeast strains. *J. Ind. Microbiol. Biotechnol.* **2009**, 36, 1085–1091, doi:10.1007/s10295-009-0592-1.

Andorrà, I.; Berradre, M.; Mas, A.; Esteve-Zarzoso, B.; Guillamón, J.M. Effect of mixed culture fermentations on yeast populations and aroma profile. *LWT Food Sci. Technol.* **2012**, 49, 8–13, doi:10.1016/j.lwt.2012.04.008.

Barnett, J.A.; Entian, K.D. A history of research on yeasts 9: regulation of sugar metabolism. *Yeast*. **2005**, 22, 835–894, doi:10.1002/yea.1249.

Beltran, G.; Novo, M.; Rozès, N.; Mas, A.; Guillamón, J.M. Nitrogen catabolite repression in during wine fermentations. *FEMS Yeast Res.* **2004**, 4, 625–632, doi:10.1016/j.femsyr.2003.12.004.

Binati, R.L.; Lemos Junior, W.J.F.; Luzzini, G.; Slaghenaufi, D.; Ugliano, M.; Torriani, S. Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans*, *Metschnikowia* spp. and *Starmerella bacillaris* strains isolated in Italy. *Int. J. Food Microbiol.* **2020**, 318, 108470, doi:10.1016/j.ijfoodmicro.2019.108470.

Bisson, L.F. Stuck and sluggish fermentations. *Am. J. Enol. Vitic.* **1999**, 50, 107–119, doi:10.1533/9781845698133.5.280

Branco, P.; Francisco, D.; Chambon, C.; Hébraud, M.; Arneborg, N.; Almeida, M.G.; Caldeira, J.; Albergaria, H. Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. *Appl. Microbiol. Biotechnol.* **2014**, 98, 843–853, doi:10.1007/s00253-013-5411-y.

Buescher, W.A.; Siler, C.E.; Morris, J.R.; Threlfall, R.T.; Main, G.L.; Cone, G.C. High alcohol wine production from grape juice concentrates. *Am. J. Enol. Vitic.* **2001**, 52, 345–351, doi:10.1002/1097-0010(20010115)81:2<281::AID-JSFA808>3.0.CO;2-B.

Cambon, B.; Monteil, V.; Remize, F.; Camarasa, C.; Dequin, S. Effects of GPD1 overexpression in *Saccharomyces cerevisiae* commercial wine yeast strains lacking ALD6 genes. *Appl. Environ. Microbiol.* **2006**, 72, 4688–4694, doi:10.1128/AEM.02975-05.

Cañas, P.M.I.; García, A.T.P.; Romero, E.G. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starter. *Vitis.* **2011**, 50, 177–182.

Canonico, L.; Comitini, F.; Oro, L.; Ciani, M. Sequential fermentation with selected immobilized non-*Saccharomyces* yeast for reduction of ethanol content in wine. *Front. Microbiol.* **2016**, 7, 278, doi:10.3389/fmicb.2016.00278.

Canonico, L.; Comitini, F.; Ciani, M. *Metschnikowia pulcherrima* selected strain for ethanol reduction in wine: Influence of cell immobilization and aeration condition. *Foods*. **2019a**, 8, 378, doi:10.3390/foods8090378.

Canonico, L.; Solomonb, M.; Comitinia, F.; Ciani, M.; Varela, C. Volatile profile of reduced alcohol wines fermented with selected non-*Saccharomyces* yeasts under different aeration conditions. *Food Microbiol.* **2019b**, 84, 103247, doi:10.1016/j.fm.2019.103247.

Carlson, M. Glucose repression in yeast. *Curr. Opin. Microbiol.* **1999**, 2, 202–207, doi:10.1016/S1369-5274(99)80035-6.

Ciani, M.; Rosini, G. Validity of Genevois' equation in wine yeast selection. *Ann. Microbiol. Enzim.* **1995**, 45, 201–207.

Ciani, M.; Beco, L.; Comitini, F. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int. J. Food Microbiol.* **2006**, 108, 239–245, doi:10.1016/j.ijfoodmicro.2005.11.012.

Ciani, M.; Comitini, F. Yeast interactions in multi-starter wine fermentation. *Curr. Opin. Food Sci.* **2015**, 1, 1–6, doi:10.1016/j.cofs.2014.07.001.

Ciani, M.; Morales, P.; Comitini, F.; Tronchoni, J.; Canonico, L.; Curiel, J.A.; Oro, L.; Rodrigues, A.J.; Gonzalez, R. Non-conventional yeast species for lowering ethanol content of wines. *Front. Microbiol.* **2016**, 7, 642, doi:10.3389/fmicb.2016.00642.

Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, 28, 873–882, doi:10.1016/j.fm.2010.12.001.

Contreras, A.; Hidalgo, C.; Henschke, P.A.; Chambers, P.J.; Curtin, C.; Varela, C. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* **2014**, 80, 1670–1678, doi:10.1128/AEM.03780-13.

Contreras, A.; Hidalgo, C.; Schmidt, S.; Henschke, P.; Curtin, C.; Varela, C. The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* **2015**, 205, 7–15, doi:10.1016/j.ijfoodmicro.2015.03.027.

Dragosits, M.; Mattanovich, D. Adaptive laboratory evolution–principles and applications for biotechnology. *Microb Cell Fact.* **2013**, 12, 64, doi:10.1186/1475-2859-12-64.

Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Ortiz-Julien, A.; Vincenzo Gerbi, V.; Rolle, L.; Cocolin, L. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Appl. Microbiol. Biotechnol.* **2016**, 100, 5515–5526, doi:10.1007/s00253-016-7413-z.

Escudero, A.; Campo, E.; Fariña, L.; Cacho, J.; Ferreira, V. Analytical characterization of the aroma of five premium red wines. Insight into the role of odor families and the concept of fruitiness of wines. *J. Agric. Food Chem.* **2007**, 55, 4501–4510, doi:10.1021/jf0636418.

Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* **1999**, 49, 329–337, doi:10.1099/00207713-49-1-329.

Fleet, G.H.; Heard, G. Yeasts-growth during fermentation. In *Wine Microbiology and Biotechnology*, Fleet, G.H. (Ed.) Harwood Academic Publishers, Chur, Switzerland, **1993**; pp. 27–54.

García-Martín, N.; Perez-Magariño, S.; Ortega-Heras, M.; González-Huerta, C.; Mihnea, M.; González-Sanjosé, M.L.; Palacio, L.; Prádanos, P.; Hernández, A. Sugar reduction in musts with nanofiltration membranes to obtain low alcohol-content wines. *Sep. Purif. Technol.* **2010**, 76, 158–170, doi:10.1016/j.seppur.2010.10.002.

Ghiaci, P., Norbeck, J., Larsson, C. Physiological adaptations of *Saccharomyces cerevisiae* evolved for improved butanol tolerance. *Biotechnol. Biofuels.* **2013**, 6, 101, doi:10.1186/1754-6834-6-101.

Gobbi, M.; Comitini, F.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* **2013**, 33, 271–281, doi:10.1016/j.fm.2012.10.004.

Gonzalez, R.; Quirós, M.; Morales, P. Yeast respiration of sugars by non-*Saccharomyces* yeast species: A promising and barely explored approach to lowering alcohol content of wines. *Trends Food Sci. Tech.* **2013**, 29, 55–61, doi:10.1016/j.tifs.2012.06.015.

Goold, H.D.; Kroukamp, H.; Williams, T.C.; Paulsen, I.T.; Varela, C.; Pretorius, I.S. Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. *Microbial Biotechnol.* **2017**, 10, 264–278, doi:10.1111/1751-7915.12488.

Henricsson, C.; Ferreira, M.C.D.J.; Hedfalk, K.; Elbing, K.; Larsson, C.; Bi, R.M.; Norbeck, J.; Hohmann, S.; Gustafsson, L. Engineering of a novel *Saccharomyces cerevisiae* wine strain with a respiratory phenotype at high external glucose concentrations. *Appl. Environ. Microbiol.* **2005**, 71, 6185–6192, doi:10.1128/AEM.71.10.6185-6192.2005.

Hernández, P. Use of *Lachancea thermotolerans* to Improve pH in Red Wines. Effect of the coinoculation with *Oenococcus oeni*. Master's Thesis, Universidad Politécnica de Madrid, Madrid, Spain, **2018**.

Heux, S.; Sablayrolles, J.M.; Cachon, R.; Dequin, S. Engineering a *Saccharomyces cerevisiae* wine yeast that exhibits reduced ethanol production during fermentation under controlled microoxygenation conditions. *Appl. Environ. Microbiol.* **2006**, 72, 5822–5828, doi:10.1128/AEM.00750-06.

Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The role and use of non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic.* **2006**, 27, 15–39, doi:10.21548/27-1-1475.

Kutyna, D.R.; Varela, C.; Henschke, P.A.; Chambers, P.J.; Stanley, G.A. Microbiological approaches to lowering ethanol concentration in wine. *Trends Food Sci. Tech.* **2010**, 21, 293–302, doi:10.1016/j.tifs.2010.03.004.

Kutyna, D.R.; Varela, C.; Stanley, G.A.; Borneman, A.R.; Henschke, P.A.; Chambers, P.J. Adaptive evolution of *Saccharomyces cerevisiae* to generate strains with enhanced glycerol production. *Appl. Microbiol. Biotechnol.* **2012**, 93, 1175–1184, doi:10.1007/s00253-011-3622-7.

Lleixà, J.; Manzano, M.; Mas, A.; Portillo, M.C. *Saccharomyces* and non-*Saccharomyces* Competition during Microvinification under Different Sugar and Nitrogen Conditions. *Front. Microbiol.* **2016**, 7, 1959, doi:10.3389/fmicb.2016.01959.

Loira, I.; Morata, A.; Comuzzo, P.; Callejo, M.J.; González, C.; Calderón, F.; Suárez-Lepe, J.A. Use of *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* strains in mixed and sequential fermentations to improve red wine sensory quality. *Food Res. Int.* **2015**, 76, 325–333, doi:10.1016/j.foodres.2015.06.030.

Malherbe, D.F.; du Toit, M.; Otero, R.R.C.; van Rensburg, P.; Pretorius, I.S. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. *Appl. Microbiol. Biotechnol.* **2003**, 61, 502–511, doi:10.1007/s00253-002-1208-0.

Martín-García, A.; Balmaseda, A.; Bordons, A.; Reguant, C. Effect of the inoculation strategy of non-*Saccharomyces* yeasts on wine malolactic fermentation. *OENO One.* **2020**, 54, 101–108, doi:10.20870/oeno-one.2020.54.1.2906.

Medina, K.; Boido, E.; Dellacassa, E.; Carrau, F. Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int. J. Food Microbiol.* **2012**, 157, 245–250, doi:10.1016/j.ijfoodmicro.2012.05.012.

Michnick, S.; Roustan, J.L.; Remize, F.; Barre, P.; Dequin, S. Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for GPD1 encoding glycerol 3-phosphate dehydrogenase. *Yeast Chichester Engl.* **1997**, 13, 783–793, doi:10.1002/(SICI)1097-0061(199707)13:9<783::AID-YEA128>3.0.CO.

Mira de Orduña, R. Climate change associated effects on grape and wine quality and production. *Food Res. Int.* **2010**, 43, 1844–1855, doi:10.1016/j.foodres.2010.05.001.

Morales, P.; Rojas, V.; Quirós, M.; Gonzalez, R. The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotech.* **2015**, 99, 3993–4003, doi:10.1007/s00253-014-6321-3.

Morata, A.; Loira, I.; Tesfaye, W.; Bañuelos, M.A.; González, C.; Suárez Lepe, J.A. *Lachancea thermotolerans* applications in wine technology. *Ferment.* **2018**, 4, 53, doi:10.3390/fermentation4030053.

Nissen, P.; Nielsen, D.; Arneborg, N. Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell–cell contact-mediated mechanism. *Yeast*. **2003**, 20, 331–341, doi:10.1002/yea.965.

Padilla, B.; Gil, J.V.; Manzanares, P. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front. Microbiol.* **2016a**, 7, 411, doi:10.3389/fmicb.2016.00411.

Padilla, B.; García-Fernández, D.; González, B.; Izidoro, I.; Esteve-Zarzoso, B.; Beltran, G.; Mas, A. Yeast biodiversity from DOQ Priorat uninoculated fermentations. *Front. Microbiol.* **2016b**, 7, 930, doi:10.3389/fmicb.2016.00930.

Quirós, M.; Gonzalez-Ramos, D.; Tabera, L.; Gonzalez, R. A new methodology to obtain wine yeast strains overproducing mannoproteins. *Int. J. Food Microbiol.* **2010**, 139, 9–14, doi:10.1016/j.ijfoodmicro.2010.02.014.

Quirós, M.; Rojas, V.; Gonzalez, R.; Morales, P. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* **2014**, 181, 85–91, doi:10.1016/j.ijfoodmicro.2014.04.024.

Remize, F.; Roustan, J.L.; Sablayrolles, J.M.; Barre, P.; Dequin, S. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. *Appl. Environ. Microbiol.* **1999**, 65, 143–149.

Renault, P.; Coulon, J.; de Revel, G.; Barbe, J.C.; Bely, M. Increase of fruity aroma during mixed *T. delbrueckii*/*S. cerevisiae* wine fermentation is linked to specific esters enhancement. *Int. J. Food Microbiol.* **2015**, 207, 40–48, doi:10.1016/j.ijfoodmicro.2015.04.037.

Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. In *Handbook of Enology: The Microbiology of Wine and Vinifications*, 2nd ed.; Jhon Wiley & Sons, Ltd: Chichester, UK, **2006**; pp. 22–53, doi:10.1002/0470010398.

Tilloy, V.; Ortiz-Julien, A.; Dequin, S. Reduction of ethanol yield and improvement of glycerol formation by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions. *Appl. Environ. Microbiol.* **2014**, 80, 2623–2632, doi:10.1128/AEM.03710-13.

Tilloy, V.; Cadière, A.; Ehsani, M.; Dequin, S. Reducing alcohol levels in wines through rational and evolutionary engineering of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2015**, 213, 49-58, doi:10.1016/j.ijfoodmicro.2015.06.027.

Ubeda, C.; Hornedo-Ortega, R.; Cerezo, A.B.; Garcia-Parrilla, M.C.; Troncoso, A.M. Chemical hazards in grapes and wine, climate change and challenges to face. *Food Chem.* **2020**, 314, 126222, doi:10.1016/j.foodchem.2020.126222.

Varela, C.; Dry, P.R.; Kutyna, D.R.; Francis, I.L.; Henschke, P.A.; Curtin, C.D.; Chambers, P.J. Strategies for reducing alcohol concentration in wine. *Aust. J. Grape Wine R.* **2015**, 21, 670–679, doi:10.1111/ajgw.12187.

Varela, C.; Sengler, F.; Solomon, M.; Curtin, C. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chem.* **2016**, 209, 57–64, doi:10.1016/j.foodchem.2016.04.024.

Varela, J.; Varela, C. Microbiological strategies to produce beer and wine with reduced ethanol concentration. *Curr. Opin. Biotechnol.* **2019**, 56, 88–96, doi:10.1016/j.copbio.2018.10.003.

Vejarano, R.; Morata, A.; Loira, I.; Gonzalez, M.C.; Suarez-Lepe, J.A. Theoretical considerations about usage of metabolic inhibitors as possible alternative to reduce alcohol content of wines from hot areas. *Eur. Food Res. Technol.* **2013**, 237, 281–290, doi:10.1007/s00217-013-1992-z.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. *Int. J. Food Microbiol.* **2015**, 206, 67–74, doi:10.1016/j.ijfoodmicro.2015.04.022.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* **2016**, 7, 502, doi:10.3389/fmicb.2016.00502.

Zackrisson, M.; Hallin, J.; Ottosson, L.G.; Dahl, P.; Parada, E.F.; Ländström, E.; Ricaud, L.F.; Kaferle, P.; Skyman, A.; Stenberg, S.; Omholt, S.; Petrovič, U.; Warringer, J.; Blomberg, A. Scan-o-matic: High-Resolution Microbial Phenomics at a Massive Scale. G3 (Bethesda). **2016**, 8, 6, 3003-3014. doi:10.1534/g3.116.032342. PMID: 27371952; PMCID: PMC5015956.

Zhu, X.; Navarro, Y.; Mas, A.; Torija, M.J.; Beltran, G. A rapid method for selecting non-*Saccharomyces* strains with a low ethanol yield. *Microorg.* **2020**, 8, 658, doi:10.3390/microorganisms8050658.

Zhu, X.; Torija, M.J.; Mas, A.; Beltran, G.; Navarro, Y. Effect of a multistarter yeast Inoculum on ethanol reduction and population dynamics in wine fermentation. *Foods.* **2021**, 10, 623, doi:10.3390/foods10030623.

Supplementary

Table S1. Analysis of sugars, ethanol, organic acids and glycerol at final fermentation in synthetic must by Mp51 and *S. cerevisiae*.

Fermentations	Treatment	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Residual sugar (g/L)	Sugar consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)
Mp51/Sc23	1:1	0.63 ± 0.05^{b}	2.34 ± 0.23^{a}	2.64 ± 0.14^{a}	0.22 ± 0.04^{c}	0.21 ± 0.04^{a}	0.41 ± 0.03^{a}	5.14 ± 0.07^{b}	0.58 ± 0.56^{b}	199.42 ± 0.56^{a}	11.81 ± 0.07^{a}	0.47 ± 0.00^{a}
	9:1	0.61 ± 0.00^{b}	2.28 ± 0.04^{a}	2.29 ± 0.07^{ab}	0.27 ± 0.02^{c}	0.22 ± 0.07^{a}	0.28 ± 0.13^{a}	5.64 ± 0.02^{b}	0.00 ± 0.00^{b}	200.00 ± 0.00^{a}	11.57 ± 0.06^{ab}	0.46 ± 0.00^{b}
	Seq	1.62 ± 0.47^{a}	2.39 ± 0.02^{a}	2.03 ± 0.46^{b}	0.57 ± 0.09^{a}	0.26 ± 0.17^{a}	0.30 ± 0.00^{a}	8.63 ± 1.23^{a}	0.03 ± 0.02^{b}	199.98 ± 0.02^{a}	10.37 ± 0.20^{b}	0.41 ± 0.01^{c}
	Mp51	1.04 ± 0.46^{ab}	1.87 ± 0.10^{b}	2.70 ± 0.25^{a}	0.40 ± 0.07^{b}	0.18 ± 0.07^{a}	0.09 ± 0.06^{b}	5.74 ± 0.29^{b}	61.5 ± 3.20^{a}	138.50 ± 3.20^{b}	6.85 ± 0.55^{c}	0.39 ± 0.01^{d}
	Sc23	0.64 ± 0.13^{b}	2.38 ± 0.08^{a}	2.73 ± 0.06^{a}	0.24 ± 0.00^{c}	0.15 ± 0.07^{a}	0.32 ± 0.04^{a}	4.88 ± 0.17^{b}	0.16 ± 0.23^{b}	199.84 ± 0.23^{a}	11.81 ± 0.04^{a}	0.47 ± 0.00^{ab}
Mp51/SeSH1	1:1	1.03 ± 0.05^{ab}	2.59 ± 0.10^{ab}	2.47 ± 0.12^{b}	0.95 ± 0.16^{a}	0.09 ± 0.01^{ab}	0.38 ± 0.03^{ab}	6.68 ± 0.27^{ab}	0.46 ± 0.34^{b}	199.54 ± 0.34^{a}	11.56 ± 0.27^{ab}	0.46 ± 0.01^{a}
	9:1	1.00 ± 0.10^{b}	2.61 ± 0.11^{a}	2.30 ± 0.10^{b}	1.23 ± 0.31^{a}	0.08 ± 0.01^{bc}	0.42 ± 0.17^{ab}	6.51 ± 0.46^{ab}	0.47 ± 0.43^{b}	199.53 ± 0.43^{a}	11.33 ± 0.27^{b}	0.45 ± 0.01^{a}
	Seq	0.97 ± 0.04^{b}	2.60 ± 0.09^{ab}	2.03 ± 0.29^{c}	1.27 ± 0.04^{a}	0.06 ± 0.02^{c}	0.54 ± 0.13^{a}	8.36 ± 0.12^{a}	0.67 ± 0.50^{b}	199.33 ± 0.50^{a}	10.32 ± 0.13^{c}	0.41 ± 0.01^{b}
	Mp51	1.12 ± 0.16^{ab}	2.39 ± 0.11^{b}	3.64 ± 0.02^{a}	0.27 ± 0.05^{b}	0.02 ± 0.00^{d}	0.00 ± 0.00^{c}	4.91 ± 2.83^{b}	94.47 ± 8.97^{a}	105.53 ± 8.97^{b}	4.68 ± 0.28^{d}	0.35 ± 0.01^{c}
	SeSH1	1.00 ± 0.10^{b}	2.59 ± 0.03^{ab}	2.31 ± 0.02^{b}	1.10 ± 0.10^{a}	0.10 ± 0.00^{a}	0.31 ± 0.05^{b}	6.44 ± 0.14^{ab}	0.22 ± 0.31^{b}	199.78 ± 0.31^{a}	11.74 ± 0.06^{a}	0.46 ± 0.00^{a}
	SH1	1.24 ± 0.00^{a}	2.69 ± 0.00^{a}	2.23 ± 0.00^{bc}	1.29 ± 0.00^{a}	0.09 ± 0.00^{ab}	0.32 ± 0.00^{b}	6.17 ± 0.00^{ab}	0.12 ± 0.00^{b}	199.88 ± 0.00^{a}	11.59 ± 0.00^{ab}	0.46 ± 0.00^{a}
Mp51/SeSH2	1:1	1.61 ± 0.11^{ab}	2.61 ± 0.19^{a}	2.46 ± 0.15^{b}	1.03 ± 0.14^{c}	0.07 ± 0.03^{a}	0.28 ± 0.06^{b}	6.20 ± 1.10^{ab}	1.18 ± 0.78^{c}	198.82 ± 0.78^{a}	11.51 ± 0.39^{ab}	0.46 ± 0.01^{a}
	9:1	1.52 ± 0.11^{ab}	2.55 ± 0.07^{ab}	2.27 ± 0.03^{b}	1.35 ± 0.07^{b}	0.03 ± 0.03^{ab}	0.62 ± 0.03^{a}	5.61 ± 0.08^{ab}	1.14 ± 0.38^{c}	198.86 ± 0.38^{a}	10.82 ± 0.12^{cd}	0.43 ± 0.00^{bc}
	Seq	1.25 ± 0.26^{b}	2.32 ± 0.01^{c}	2.35 ± 0.02^{b}	1.13 ± 0.26^{bc}	0.01 ± 0.01^{b}	0.42 ± 0.09^{b}	6.90 ± 0.42^{a}	3.61 ± 0.54^{b}	196.40 ± 0.54^{b}	10.37 ± 0.11^{d}	0.42 ± 0.00^{c}
	Mp51	1.26 ± 0.38^{b}	2.39 ± 0.06^{bc}	3.49 ± 0.30^{a}	0.35 ± 0.10^{d}	0.01 ± 0.01^{b}	0.00 ± 0.00^{c}	3.16 ± 0.30^{c}	68.47 ± 0.9^{a}	131.53 ± 0.90^{c}	4.92 ± 0.26^{e}	0.29 ± 0.01^{d}
	SeSH2	1.64 ± 0.17^{ab}	2.63 ± 0.06^{a}	2.40 ± 0.22^{b}	1.43 ± 0.18^{b}	0.06 ± 0.01^{a}	0.41 ± 0.13^{b}	5.46 ± 0.47^{b}	0.47 ± 0.55^{c}	199.53 ± 0.55^{a}	11.77 ± 0.30^{a}	0.47 ± 0.01^{a}
	SH2	1.94 ± 0.00^{a}	2.73 ± 0.00^{a}	2.41 ± 0.00^{b}	1.80 ± 0.00^{a}	0.04 ± 0.00^{ab}	0.52 ± 0.00^{ab}	5.36 ± 0.00^{b}	0.58 ± 0.00^{c}	199.42 ± 0.00^{a}	11.16 ± 0.00^{bc}	0.44 ± 0.00^{ab}
Mp51/SeIB1	1:1	0.63 ± 0.07^{c}	2.51 ± 0.02^{a}	2.73 ± 0.08^{a}	0.70 ± 0.18^{ab}	0.10 ± 0.03^{ab}	0.10 ± 0.07^{b}	6.43 ± 0.22^{bc}	0.56 ± 0.59^{bc}	199.44 ± 0.59^{ab}	11.63 ± 0.07^{ab}	0.46 ± 0.00^{ab}
	9:1	0.72 ± 0.11^{c}	2.52 ± 0.04^{a}	2.58 ± 0.12^{a}	0.69 ± 0.06^{ab}	0.11 ± 0.04^{ab}	0.10 ± 0.03^{b}	6.73 ± 0.40^{b}	0.38 ± 0.31^{bc}	199.62 ± 0.31^{ab}	11.50 ± 0.14^{b}	0.46 ± 0.01^{b}
	Seq	1.42 ± 0.10^{b}	2.38 ± 0.38^{a}	2.38 ± 0.61^{a}	0.56 ± 0.01^{bc}	0.05 ± 0.01^{bc}	0.00 ± 0.00^{b}	7.68 ± 0.09^{a}	1.38 ± 0.94^{b}	198.62 ± 0.94^{b}	10.46 ± 0.03^{c}	0.42 ± 0.00^{c}
	Mp51	1.77 ± 0.02^{a}	2.34 ± 0.18^{a}	2.28 ± 0.56^{a}	0.46 ± 0.03^{c}	0.05 ± 0.00^{c}	0.00 ± 0.00^{b}	5.79 ± 0.23^{d}	61.5 ± 3.20^{a}	138.50 ± 3.20^{c}	6.85 ± 0.55^{d}	0.39 ± 0.01^{d}
	SeIB1	0.62 ± 0.01^{c}	2.54 ± 0.09^{a}	2.81 ± 0.07^{a}	0.88 ± 0.09^{a}	0.09 ± 0.02^{abc}	0.23 ± 0.08^{a}	6.31 ± 0.35^{bcd}	0.03 ± 0.05^{c}	199.97 ± 0.05^{a}	11.68 ± 0.07^{ab}	0.46 ± 0.00^{ab}
	IB1	0.56 ± 0.07^{c}	2.42 ± 0.02^{a}	2.69 ± 0.08^{a}	0.79 ± 0.10^{a}	0.11 ± 0.03^{a}	0.09 ± 0.01^{b}	6.09 ± 0.09^{cd}	0.56 ± 0.74^{bc}	199.45 ± 0.74^{ab}	11.72 ± 0.10^{a}	0.46 ± 0.00^{a}

Values are mean ± standard deviation of three independent replicates; The initial sugar concentration of synthetic must was 200 g/L; Shared superscript letters (a,b,c, etc.) within each column of fermentation by different *S. cerevisiae* mean statistically significant differences (Duncan, $p < 0.05$).

Supplementary

Table S2. Analysis of sugars, ethanol, organic acids and glycerol at final fermentation in synthetic must by Lt2 and *S. cerevisiae*.

Fermentations	Treatment	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Residual sugar (g/L)	Sugar consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)
Lt2/Sc23	1:1	0.61 ± 0.10^{b}	2.43 ± 0.19^{ab}	2.44 ± 0.37^{ab}	0.49 ± 0.21^{a}	0.41 ± 0.20^{c}	0.52 ± 0.20^{b}	5.58 ± 0.22^{b}	0.16 ± 0.23^{b}	199.84 ± 0.23^{a}	11.95 ± 0.02^{a}	0.47 ± 0.00^{a}
	9:1	0.65 ± 0.15^{a}	1.94 ± 0.30^{b}	2.34 ± 0.10^{b}	0.50 ± 0.10^{a}	0.66 ± 0.41^{c}	0.34 ± 0.05^{bc}	5.83 ± 0.81^{b}	0.33 ± 0.36^{ab}	199.67 ± 0.36^{ab}	11.88 ± 0.02^{ab}	0.47 ± 0.00^{a}
	Seq	0.32 ± 0.02^{b}	2.24 ± 0.06^{ab}	2.26 ± 0.08^{b}	0.36 ± 0.01^{a}	3.45 ± 0.55^{a}	0.41 ± 0.09^{b}	9.40 ± 0.75^{a}	0.44 ± 0.67^{ab}	199.56 ± 0.67^{ab}	11.54 ± 0.04^{c}	0.46 ± 0.00^{c}
	Lt2	0.22 ± 0.00^{b}	1.95 ± 0.00^{b}	2.70 ± 0.00^{a}	0.42 ± 0.00^{a}	2.59 ± 0.00^{b}	0.19 ± 0.00^{c}	8.19 ± 0.00^{a}	1.17 ± 0.00^{a}	198.83 ± 0.00^{b}	11.62 ± 0.00^{c}	0.46 ± 0.00^{b}
	Sc23	0.79 ± 0.06^{a}	2.59 ± 0.30^{a}	2.70 ± 0.08^{a}	0.32 ± 0.03^{a}	0.19 ± 0.01^{c}	0.80 ± 0.07^{a}	5.53 ± 0.16^{b}	0.54 ± 0.05^{ab}	199.47 ± 0.05^{ab}	11.82 ± 0.09^{b}	0.47 ± 0.00^{a}
Lt2/SeSH1	1:1	1.13 ± 0.05^{b}	2.51 ± 0.18^{ab}	2.45 ± 0.05^{ab}	1.22 ± 0.02^{c}	0.08 ± 0.02^{c}	0.74 ± 0.04^{a}	5.91 ± 0.46^{b}	0.59 ± 0.19^{ab}	199.42 ± 0.19^{a}	11.64 ± 0.14^{a}	0.46 ± 0.01^{ab}
	9:1	1.16 ± 0.17^{b}	2.48 ± 0.24^{ab}	2.39 ± 0.04^{ab}	1.28 ± 0.15^{bc}	0.08 ± 0.02^{c}	0.66 ± 0.13^{a}	5.86 ± 0.52^{b}	0.21 ± 0.11^{b}	199.79 ± 0.11^{a}	11.67 ± 0.20^{a}	0.46 ± 0.01^{ab}
	Seq	0.51 ± 0.04^{c}	2.28 ± 0.19^{b}	2.20 ± 0.43^{b}	1.47 ± 0.00^{b}	3.68 ± 0.27^{a}	0.24 ± 0.01^{b}	8.08 ± 0.74^{a}	0.98 ± 1.02^{a}	199.02 ± 1.02^{a}	11.55 ± 0.16^{a}	0.46 ± 0.00^{ab}
	Lt2	0.51 ± 0.01^{c}	2.52 ± 0.17^{ab}	1.97 ± 0.04^{b}	0.38 ± 0.02^{d}	2.52 ± 0.44^{b}	0.19 ± 0.00^{b}	7.84 ± 0.18^{a}	0.27 ± 0.35^{b}	199.74 ± 0.35^{a}	11.52 ± 0.21^{a}	0.46 ± 0.01^{ab}
	SeSH1	1.29 ± 0.10^{b}	2.69 ± 0.03^{ab}	2.75 ± 0.16^{a}	1.43 ± 0.16^{bc}	0.15 ± 0.07^{c}	0.69 ± 0.16^{a}	6.58 ± 0.66^{b}	0.69 ± 0.91^{ab}	199.32 ± 0.91^{a}	11.76 ± 0.20^{a}	0.47 ± 0.01^{a}
	SH1	1.52 ± 0.00^{a}	2.83 ± 0.00^{a}	2.75 ± 0.00^{a}	2.12 ± 0.00^{a}	0.16 ± 0.00^{c}	0.60 ± 0.00^{a}	5.81 ± 0.00^{b}	0.50 ± 0.00^{ab}	199.50 ± 0.00^{a}	11.35± 0.00^{b}	0.45 ± 0.00^{b}
Lt2/SeSH2	1:1	0.97 ± 0.01^{bc}	2.57 ± 0.03^{a}	2.56 ± 0.13^{ab}	0.52 ± 0.00^{b}	0.24 ± 0.00^{c}	0.38 ± 0.02^{a}	5.74 ± 0.03^{d}	0.00 ± 0.00^{b}	200.00 ± 0.00^{a}	12.08 ± 0.06^{a}	0.48 ± 0.00^{a}
	9:1	0.90 ± 0.02^{c}	2.49 ± 0.05^{a}	2.81 ± 0.42^{a}	0.45 ± 0.03^{bc}	0.52 ± 0.11^{c}	0.33 ± 0.00^{ab}	6.39 ± 0.19^{c}	0.26 ± 0.40^{b}	199.74 ± 0.40^{a}	11.80 ± 0.13^{ab}	0.47 ± 0.01^{a}
	Seq	0.46 ± 0.02^{d}	2.30 ± 0.12^{b}	2.48 ± 0.17^{ab}	0.35 ± 0.00^{c}	4.40 ± 0.10^{a}	0.37 ± 0.01^{ab}	8.55 ± 0.01^{a}	1.84 ± 0.69^{ab}	198.17 ± 0.69^{ab}	11.59 ± 0.22^{b}	0.46 ± 0.01^{a}
	Lt2	0.43 ± 0.09^{d}	2.13 ± 0.07^{c}	2.58 ± 0.58^{ab}	0.34 ± 0.07^{c}	2.41 ± 0.70^{b}	0.12 ± 0.05^{c}	7.17 ± 0.05^{b}	4.25 ± 3.53^{a}	195.76 ± 3.53^{b}	11.51 ± 0.05^{b}	0.46 ± 0.01^{a}
	SeSH2	1.05 ± 0.07^{ab}	2.32 ± 0.00^{b}	1.89 ± 0.03^{b}	0.56 ± 0.10^{b}	0.32 ± 0.04^{c}	0.32 ± 0.01^{b}	6.30 ± 0.39^{c}	0.05 ± 0.03^{b}	199.95 ± 0.03^{a}	11.73 ± 0.24^{b}	0.46 ± 0.01^{a}
	SH2	1.15 ± 0.02^{a}	2.47 ± 0.04^{a}	2.01 ± 0.26^{ab}	0.77 ± 0.08^{a}	0.32 ± 0.06^{c}	0.27 ± 0.02^{b}	6.35 ± 0.04^{c}	0.21 ± 0.18^{b}	199.79 ± 0.18^{a}	11.72 ± 0.03^{b}	0.46 ± 0.00^{a}

Values are mean ± standard deviation of three independent replicates; The initial sugar concentration of synthetic must was 200 g/L; Shared superscript letters (a,b,c, etc.) within each column of fermentation by different *S. cerevisiae* mean statistically significant differences (Duncan, $p < 0.05$).

Supplementary

Table S3. Analysis of sugars, ethanol, organic acids and glycerol at final fermentation of single *S. cerevisiae* and sequential inoculations done with natural must.

Fermentations	Treatment	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Glycerol (g/L)	Residual sugar (g/L)	Sugar consumption (g/L)	Ethanol production (%, v/v)	Ethanol yield (g/g)
Sc23	Sc23	0.02 ± 0.00^{a}	2.16 ± 0.00^{b}	3.43 ± 0.00^{c}	2.76 ± 0.00^{a}	0.31 ± 0.00^{a}	7.29 ± 0.00^{ab}	1.45 ± 0.00^{b}	238.56 ± 0.00^{a}	14.77 ± 0.01^{a}	0.49 ± 0.01^{a}
	Lt/Sc23	0.01 ± 0.00^{a}	1.98 ± 0.06^{a}	4.02 ± 0.15^{b}	2.89 ± 0.05^{a}	0.19 ± 0.06^{b}	6.86 ± 0.39^{b}	2.75 ± 0.24^{a}	237.25 ± 0.24^{b}	14.54 ± 0.17^{ab}	0.49 ± 0.01^{a}
	Mp/Sc23	0.01 ± 0.00^{a}	2.28 ± 0.16^{ab}	4.60 ± 0.13^{a}	2.45 ± 0.12^{b}	0.12 ± 0.02^{b}	7.88 ± 0.16^{a}	2.75 ± 0.13^{a}	237.25 ± 0.13^{b}	14.39 ± 0.23^{b}	0.48 ± 0.01^{a}
SeSH1	SH1	0.03 ± 0.01^{a}	2.73 ± 0.09^{a}	4.36 ± 0.39^{a}	2.47 ± 0.26^{a}	0.36 ± 0.09^{b}	7.32 ± 0.16^{b}	2.55 ± 0.11^{b}	237.45 ± 0.10^{a}	14.59 ± 0.12^{a}	0.49 ± 0.00^{a}
	SeSH1	0.04 ± 0.01^{a}	2.24 ± 0.04^{b}	3.82 ± 0.17^{b}	2.49 ± 0.17^{a}	0.31 ± 0.02^{b}	7.29 ± 0.27^{b}	2.11 ± 0.25^{b}	237.89 ± 0.24^{a}	14.40 ± 0.14^{ab}	0.48 ± 0.00^{ab}
	Lt/SeSH1	0.05 ± 0.02^{a}	1.41 ± 0.04^{d}	3.74 ± 0.20^{b}	2.33 ± 0.10^{a}	0.58 ± 0.09^{a}	7.12 ± 0.56^{b}	3.26 ± 1.28^{a}	236.74 ± 1.28^{b}	14.21 ± 0.08^{b}	0.48 ± 0.01^{b}
	Mp/SeSH1	0.03 ± 0.02^{a}	1.76 ± 0.14^{c}	4.45 ± 0.67^{a}	2.23 ± 0.12^{a}	0.16 ± 0.03^{c}	9.18 ± 0.29^{a}	2.31 ± 0.85^{b}	237.69 ± 0.84^{a}	13.96 ± 0.17^{c}	0.47 ± 0.01^{c}
SeSH2	SH2	0.01 ± 0.01^{b}	2.46 ± 0.06^{a}	3.49 ± 0.10^{b}	3.42 ± 0.05^{a}	0.40 ± 0.01^{a}	7.39 ± 0.40^{b}	2.31 ± 0.31^{b}	237.69 ± 0.31^{a}	14.07 ± 0.18^{a}	0.47 ± 0.01^{a}
	SeSH2	0.03 ± 0.01^{ab}	2.37 ± 0.17^{a}	3.85 ± 0.20^{ab}	3.25 ± 0.23^{ab}	0.40 ± 0.05^{a}	7.32 ± 0.62^{b}	2.10 ± 0.24^{b}	237.90 ± 0.24^{a}	14.11 ± 0.07^{a}	0.47 ± 0.01^{a}
	Lt/SeSH2	0.00 ± 0.00^{b}	2.18 ± 0.58^{ab}	4.05 ± 0.64^{a}	2.90 ± 0.01^{b}	0.28 ± 0.06^{b}	7.02 ± 0.15^{b}	2.55 ± 0.81^{b}	237.45 ± 0.11^{a}	14.04 ± 0.01^{a}	0.47 ± 0.00^{a}
	Mp/SeSH2	0.04 ± 0.02^{a}	1.86 ± 0.23^{b}	3.63 ± 0.32^{b}	2.81 ± 0.27^{b}	0.18 ± 0.01^{c}	8.02 ± 0.32^{a}	3.81 ± 1.92^{a}	236.19 ± 1.91^{b}	13.36 ± 0.16^{b}	0.45 ± 0.00^{b}
SeIB1	SIB	0.00 ± 0.00^{a}	2.30 ± 0.13^{a}	2.69 ± 0.16^{a}	4.09 ± 0.16^{b}	0.05 ± 0.02^{b}	7.23 ± 0.35^{b}	2.99 ± 0.63^{b}	237.01 ± 0.62^{a}	14.24 ± 0.02^{a}	0.47 ± 0.00^{a}
	SeIB1	0.00 ± 0.00^{a}	2.70 ± 0.06^{a}	2.52 ± 0.17^{ab}	4.53 ± 0.15^{a}	0.02 ± 0.02^{b}	6.59 ± 0.21^{b}	3.17 ± 0.91^{b}	236.83 ± 0.91^{a}	13.78 ± 0.11^{b}	0.46 ± 0.01^{b}
	Lt/SeIB1	0.00 ± 0.00^{a}	1.29 ± 0.11^{b}	2.16 ± 0.03^{c}	3.13 ± 0.16^{c}	0.51 ± 0.21^{a}	6.50 ± 0.55^{b}	2.61 ± 1.02^{b}	237.38 ± 1.02^{a}	14.36 ± 0.16^{a}	0.48 ± 0.00^{a}
	Mp/SeIB1	0.00 ± 0.00^{a}	2.33 ± 0.54^{a}	2.23 ± 0.27^{bc}	3.99 ± 0.10^{b}	0.02 ± 0.04^{b}	9.46 ± 1.03^{a}	5.77 ± 1.11^{a}	234.32 ± 1.11^{b}	12.60 ± 0.16^{c}	0.42 ± 0.00^{c}

Values are mean ± standard deviation of three independent replicates; The initial sugar concentration of concentrate must was 240 g/L; Shared superscript letters (a,b,c, etc.) within each column of fermentation by different *S. cerevisiae* mean statistically significant differences (Duncan, $p < 0.05$).

GENERAL DISCUSSION

As stated in the introduction, climate instability in the past decades has derived in new challenges in wine making, especially oriented to reduce an increasing sugar concentration, which results in higher alcoholic degree. The response from researchers have included many different strategies (reviewed by Varela et al., 2015). A microbiological approach has been also proposed taking advantage of the different metabolism among wine yeast species, that can maintain or improve the wine quality and eliminate the possible negative effects of ethanol reduction (González et al., 2013; Ciani et al., 2020). The search for some new starters composed by non-*Saccharomyces* yeasts or mixed non-*Saccharomyces* and *S. cerevisiae* yeasts might contribute to ethanol reduction in wine (Pérez-Torrado et al., 2017).

Non-*Saccharomyces* yeasts

The strategy of using non-*Saccharomyces* yeasts to reduce ethanol is due on the one hand to the metabolic diversity of the ethanol production, and on the other hand to the fact that some non-*Saccharomyces* yeast species exhibit respiratory metabolism of sugar consumption (González et al., 2013). Therefore, the selection of non-*Saccharomyces* yeasts with a low ethanol production and their application in fermentation process are often carried out under aerated and semi-aerated conditions (Quirós et al., 2014; Contreras et al., 2015; Rodrigues et al., 2016). However, the selection of non-*Saccharomyces* yeasts with lower ethanol production is a complex and time-consuming task. This process requires multiple cultivation trials and fermentation experiments with different yeast species.

In Chapter 1, we proposed a rapid method for screening and selecting non-*Saccharomyces* yeasts with low ethanol yields under semi-aerobic conditions, thus, with the ability to reduce ethanol content in wines. The proposed screening method allows the selection of the strains in 5 days, taking in account two main criteria, namely glucose consumption and low ethanol yield. In a first screening step, we cultivated the yeast strains in a low sugar concentration (YPD liquid medium, 20 g/L glucose) for three days, and initially selected fourteen out of forty-five non-

Saccharomyces yeast strains for having lower ethanol yields than *S. cerevisiae*. In a second screening step, we cultivated the yeast strains in synthetic must (200 g/L sugars) for two days, to test the behavior of the strains in fermentation conditions, and we monitored their growth, sugar consumption and ethanol yield at 48 h. The metabolic characteristics of the non-*Saccharomyces* yeasts that could impact on the wine and determine the ethanol reduction are mainly traceable before inoculation with *S. cerevisiae*, which uses to be at 24 – 48 h in sequential fermentations. Longer times may impair the imposition of *S. cerevisiae* strains, and lead to problematic and stuck fermentations (Ciani et al., 2016; Lleixà et al., 2016).

After the second step we selected ten strains able to grow and consume part of the sugar present in the must in 48 h, and with lower ethanol yield than *S. cerevisiae*. The differences between the ethanol yields obtained in low or high sugar media could be explained by the Crabtree effect. This is the capacity of some yeast species to ferment sugars into ethanol, even in the presence of oxygen, when sugar concentrations are sufficiently high. Yeast can then be classified as Crabtree-positive or Crabtree-negative according their respiro-fermentative metabolism (Hagman et al., 2013; Pfeiffer and Morley, 2014). In the current study, some non-*Saccharomyces* strains showed lower ethanol yields in low glucose, but not in high sugar media (grape must), suggesting that some non-*Saccharomyces* species may use more efficiently the respiratory metabolism at low sugar concentrations, compared to *S. cerevisiae*, exhibiting lower ethanol yields. This could be due to a relaxation of the Crabtree effect/glucose repression at higher glucose concentration than *S. cerevisiae*. However, when the sugar concentration is high, the differences on the ethanol yield between yeast strains and species was lower.

From our results, strains from *M. pulcherrima* species stood out among the 19 yeast species tested as the strains with the highest ability for ethanol reduction (Chapters 1 and 3). Several studies have confirmed the outstanding capacity of this non-*Saccharomyces* yeast to reduce ethanol content when used under aerobic conditions, due to the respiratory metabolism of *M. pulcherrima* (Contreras et al., 2014b; Mestre

Furlani et al., 2017; Morata et al., 2019). For example, a previous study concluded that the fermentation with *M. pulcherrima* can obtain an ethanol reduction of 3.8% (v/v) under the aeration at pilot-scale (Röcker et al., 2016). It is worth noting that *M. pulcherrima*, as a kind of yeast ubiquitous in spontaneous fermentation, can enhance the release of various aromatic compounds and improve wine flavor when used in alcoholic fermentations (Zott et al., 2011; Jolly et al., 2017; Zhang et al., 2018). The enhanced aroma characteristics of *M. pulcherrima* species may neutralize the volatile metabolites that are harmful to wine flavor and aroma while reducing ethanol production (Varela et al., 2016).

In our study, in addition to *M. pulcherrima* yeast, *T. delbrueckii* and *Z. bailii* species were selected for the advantage of lower ethanol production. A previous study has also confirmed that the yeast strains from these two species are suitable for reducing ethanol content (Contreras et al., 2015). It has reported that strains from *T. delbrueckii* have a positive impact on wine quality, improving the composition of volatile compounds and affecting foam characteristics in production of traditional sparkling wine (Loira et al., 2014; González-Royo et al., 2015; Arslan et al., 2018; Canonico et al., 2018). In addition, a balance between ethanol reduction and aromatic composition was found when *T. delbrueckii* yeast was used in sequential fermentations under restricted aerobic conditions, compared to the *S. cerevisiae* pure culture fermentation (Canonico et al., 2019b). Therefore, the monitoring of the aromatic composition of wines fermented by the *T. delbrueckii* strains used in this study is worthy further research. Similarly, Contreras et al. (2015) also reported that when *Z. bailii* was used a significant reduction of acetic acid was produced at the same time that ethanol concentration decreased. Our study also observed similar results, however, the difference was that we obtained a significant increase of the succinic acid concentration. The use of *Z. bailii* to reduce ethanol content can also improve the volatile components (Canonico et al., 2019b).

Another strain from *L. thermotolerans* species was also used in mixed fermentations to reduce ethanol content. This selection was also based on its high level

of competitiveness, and its ability to produce lactic acid and glycerol. This was in agreement with previous studies which also confirmed that the produced wine by *L. thermotolerans* significantly increased the spicy notes and total acidity (Gobbi et al., 2013; Binati et al., 2020). In our results, this strain is a high lactic acid and glycerol producer; however, the taste of resultant wine needs to be verified by sensory analysis. Therefore, the use of this yeast species cannot be considered neutral to the volatile composition, so it should be taken into account when use to reduce the ethanol in wine.

Use of evolved *S. cerevisiae* strains

A remarkable feature of microorganisms is that they can quickly adapt to different environmental conditions. Based on the long-term adaptation of yeast under environmental or metabolic constraints, the method of adaptive evolution can be used to improve the characteristics of yeast strains related to the application in winemaking (Tolly et al., 2014). Some studies have assessed the potential of adaptative evolution strategies under hyperosmotic stress to obtain evolved yeasts with lower ethanol yield. This method drives carbon flux to generate glycerol, 2,3-butanediol, acetic acid and other metabolites, thereby reducing ethanol production (Ghiaci et al., 2013; Tolly et al., 2015). The evolved *S. cerevisiae* strains used in this study were obtained under restrictive conditions of high alcohol and high sugar. Compared to the commercial *S. cerevisiae*, which had the advantages of better environmental adaptability and strong fermentation capacity, in our hands the evolved *S. cerevisiae* strains had shortcomings in fermentation capacity and slowed down the fermentation. In addition, our results showed that the ALE *S. cerevisiae* strains isolated from high sugar and high alcohol conditions achieved an equal level of ethanol reduction. Strains evolved in presence of high sugar levels produced high concentrations of acetic and succinic acids, and also high concentrations of succinic acid were produced by strains evolved in high alcohol. Therefore, these results confirmed the previous arguments.

Therefore, the selective pressures used to improve *S. cerevisiae* wine strains by laboratory adaptive evolution did not seem to have an impact on ethanol production,

although it might have improved other characteristics on yeast and wines. Current research is performed in the group, through an Era-CoBiotech project (Project CoolWine, PCI2018-092962) to test other adaptive environments on *Saccharomyces* and non-*Saccharomyces* yeast strains and to test their impact on ethanol reduction.

Inoculation strategies

As mentioned above, non-*Saccharomyces* yeasts are often used in winemaking as mixed inoculum (in simultaneous or sequential inoculations) with *S. cerevisiae* (Gobbi et al., 2013; Englezos et al., 2016). In the sequential inoculation, we have used as a control a commercial strain of *S. cerevisiae*, as well as the above-mentioned *S. cerevisiae* evolved strains. When tested simultaneous and sequential inoculations, the latter achieved higher ethanol reduction, probably due to the higher populations achieved and longer persistence of the non-*Saccharomyces* strains in this inoculation strategy (Chapters 2 and 3). Considering that non-*Saccharomyces* yeasts grow slower than *S. cerevisiae* yeast, some studies found that a significant ethanol reduction was usually achieved in sequential fermentations, by inoculating *S. cerevisiae* at 24 – 48 h of fermentation, in order to prolong the growth and activity of the non-*Saccharomyces* strains in grape must fermentations (Canonico et al., 2016; Varela et al., 2016; Hranilovic et al., 2020). In our study, when the selected non-*Saccharomyces* strains were used in sequential inoculations, *S. cerevisiae* was added to the must at 48 h or 72 h of fermentation, in synthetic or natural must, respectively. We concluded that the inoculation at 72 h of fermentation obtained a higher ethanol reduction than that at 48 h (Chapter 2). Thus, we speculate that the delayed inoculation of *S. cerevisiae* in sequential fermentations may obtain higher ethanol reductions, although it might risk the development of the fermentation, as it has been observed that longer periods may lead to stuck or sluggish fermentations due to lack of *S. cerevisiae* imposition (Lleixà et al., 2016).

The other inoculation strategy, coinoculation, is slightly inferior to sequential inoculation in terms of ethanol reduction, while its advantage is that it shows similar

fermentation rates to that of pure cultures of *S. cerevisiae* (Mendoza and Farías, 2010; Mendoza et al., 2011; Tristezza et al., 2016). In coinoculated fermentations, both *S. cerevisiae* and non-*Saccharomyces* yeasts can be chosen at different inoculation ratios. In this study, when the non-*Saccharomyces* yeast was inoculated in similar populations as *S. cerevisiae*, the fermentation rate was similar to the single *S. cerevisiae* fermentation. However, a higher population of non-*Saccharomyces* yeast could extend the fermentation length. Regarding the ethanol production, a 9:1 inoculation ratio of *M. pulcherrima*/*S. cerevisiae* yeast could achieve a higher ethanol reduction than that of 1:1 inoculation ratio, similarly to the results of Comitini et al. (2011), in which a high proportion of non-*Saccharomyces* yeast got a higher ethanol reduction. Therefore, we suggest that for some non-*Saccharomyces* yeasts with low ethanol yield and weak fermentation, such as *M. pulcherrima*, their inoculum ratio in the coinoculated fermentation can be adjusted to compensate for the risk of slow fermentation.

Multi species starter

Most studies used a single non-S*accharomyces* yeast (in sequential or coinoculation with *S. cerevisiae*) as a starter of fermentation to verify their impact on ethanol reduction (Quirós et al., 2014; Contreras et al., 2015; Varela et al., 2017a). However, less common has been the application of multi-species starters of several non-*Saccharomyces* yeasts for alcoholic fermentation. It is very well known that yeast interaction occurs during alcoholic fermentation and their effect could be antagonic, additive or synergic. In Chapter 2, we used the previously selected non-*Saccharomyces* yeasts in a mixed multi non-*Saccharomyces* species starter to try to achieve greater ethanol reduction than with single non-*Saccharomyces* starter. However, our results showed that, although we observed a significant ethanol redaction compared to single *S. cerevisiae* fermentation, the ethanol reduction of the multistarter inoculation was lower than that of single non-*Saccharomyces* starter inoculation (Chapters 1 and 2). Therefore, neither additive nor synergic effect was observed in terms of ethanol reduction. Thus, yeast interaction may affect the ethanol reduction, although the

lowest ethanol yield is achieved with single inoculations of yeast strains with low yield.

Must type

In addition to the diversity of non-*Saccharomyces* species being one of the reasons for the wide range of ethanol yields, the grape must used in the fermentation also affected the ethanol production, in agreement with previous observations (Varela et al., 2016; Tronchoni et al., 2018). In the current study, the ethanol production of the same non-*Saccharomyces* yeast was different between synthetic and natural must, and the ethanol reduction in natural must was often higher than that in synthetic must (Chapter 1). Similar results were confirmed by Canonico et al. (2016) that *M. pulcherrima*, *S. bombicola* and *H. uvarum* species could obtain a higher ethanol reduction in sequential fermentations when fermented in natural must rather than that in synthetic must.

In many different studies done on alcoholic fermentation, both natural and synthetic musts have been used. Synthetic must has the advantages of being a completely defined medium from the chemical point of view, and the analysis of the different compounds can be analyzed by changing the concentrations of the parameters of study. Instead, the natural must is a more complex medium, more variable (variety, origin, year, ripening, etc.) which makes it more restricted for research. In fact, wine produced from natural must have some varietal compounds and precursors which could be incorporated to yeasts and modify some aspects of its metabolism and physiology (such as lipid membranes), and the final wine composition (Beltran et al., 2008; Seguinot et al., 2020a). Therefore, the fact that the concentration of nutrients in natural must is higher and more diverse than the synthetic must may explain the differences of ethanol yields observed in the present study.

The type of grape juice not only modified the ethanol production, but also affected the content of other fermentative by-products, as previously reported (Beltran

et al., 2008; Seguinot et al., 2020b). This study confirmed that strains from the *M. pulcherrima* increased the glycerol content (greater than 4 g/L) in fermentations of synthetic must, but there was no significant increase in natural must. Similarly, strains from *Z. bailii* species increased the content of succinic acid in the fermentation of synthetic must. *H. uvarum* species produced higher content of acetic acid in natural must fermentation. Although we could speculate about the reasons for these differences, we do not have any evidence about the origin, so the specific causes need further research to be explained.

The growth and metabolism of yeasts are important indicators for monitoring the alcoholic fermentation process. The growth of yeasts is affected by environmental factors, such as osmotic pressure, temperature, oxygen, SO_2, pH, etc., and nutrient content (sugar, nitrogen, etc.) in winemaking (Ribéreau-Gayon et al., 2006). Our study indirectly explored the differences between the synthetic and natural must in fermentations on the growth of yeasts. For example, in the early stage of alcoholic fermentation, yeasts from the *T. delbrueckii* species grew quicker in natural than in synthetic must. On the contrary, *H. uvarum* and *L. thermotolerans* yeasts grew faster in the synthetic must during the initial stages of fermentation. In addition, the interaction among yeasts in the multistarter fermentation produced an antagonistic effect resulting in the restriction of non-*Saccharomyces* growth compared to the single starter inoculated fermentation (Chapters 1 and 3).

Viability qPCR

The evaluation of yeast population dynamics is an important direction for exploring the interaction between yeasts. The methods for monitoring yeast populations during the fermentation process can be divided into two categories: the traditional counting methods, using selective or differential media (e.g. Lysine or WLN agar), based on the ability to grow in specific media and the morphology of the different yeast colonies; and the molecular biotechnology techniques (e.g. real-time quantitative PCR), based on the amplification of species-specific gene regions, which

allows to distinguish and quantify the different yeast species in a culture-independent manner (Ribéreau-Gayon et al., 2006; Hierro et al., 2006 & 2007; García et al., 2017; Navarro et al., 2020).

We used both methods to monitor the yeast population dynamics during fermentations. For instance, Lysine can successfully distinguish between non-*Saccharomyces* and *S. cerevisiae* yeasts, and WLN can even distinguish some non-*Saccharomyces* among them. However, we could not recover on plates *M. pulcherrima* at the end of fermentation, whereas we could detect it with molecular methods (Chapter 2). This confirmed the activity of some non-*Saccharomyces* yeasts until the end of alcoholic fermentation, even if they could not be detected by plating. Therefore, the application of molecular methods to monitor yeast population can improve the accuracy of the results (Wang et al., 2014 & 2015a; Portillo and Mas, 2016; Navarro et al., 2020). This fact was also observed by Andorra et al. (2011), in the mixed fermentations of *H. guilliermondii* and *S. cerevisiae*, in which the population of the non-*Saccharomyces* yeasts was significantly reduced after 24 h of fermentation in WLN medium, compared to that from the qPCR and FISH methods. At the same time, these two molecular detection methods allowed the detection and quantification of non-*Saccharomyces* yeasts until the end of fermentation, which did not the WLN medium at. In our study, the growth of non-*Saccharomyces* yeasts fermented as a multistarter was limited, especially *M. pulcherrima* which could not achieve the population that could be observed as a single starter. Indeed, when the population differences among strains is too high (more than 10^2 cells/mL), it is difficult to obtain colonies and quantify the minority yeasts using the WLN medium alone, because they are hidden by the high number of other strains. Therefore, the qPCR method is a good alternative to detect yeast population dynamics. This method has been confirmed by many studies to obtain accurate estimations, especially for those non-*Saccharomyces* yeast species that present weak fermentation ability (Hierro et al., 2006; García et al., 2017; Navarro et al., 2020; Wang et al., 2020).

The qPCR method alone cannot obtain an accurate comparison with the WLN counting method, because WLN agar provides the number of viable cells, or better, the cells that are able to grow on a specific media (culturable), while the qPCR method estimates the total number of cells, which could include viable and dead yeast, as it amplifies the total DNA of the sample. The use of a viability dye, such as PMA pretreatment, can enumerate the viable cells of the sample, because this dye enters and binds the DNA of dead cells, not allowing its amplification, whereas the living cells do not allow the entrance of the dye, and the DNA can be amplified by qPCR (Andorrà et al., 2010a; Vendrame et al., 2014; Navarro et al., 2020). In this study we used PMA treatment combined with qPCR technology and WLN counting method to detect the number of living yeast cells (Chapter 2). It can also be concluded from the current study that the PMA-qPCR analysis technology has high accuracy, especially for monitoring of those strains (such as *M. pulcherrima*) with weak competitiveness in mixed fermentations.

Conclusion and future perspectives

In summary, the results of the present study confirm the hypothesis that the reduction of the ethanol content in wines can be achieved by microbial methods, especially through the use of non-*Saccharomyces* species in the alcoholic fermentation in a sequential inoculation with *S. cerevisiae*.

Although in this study we achieved a light ethanol reduction between 1.39 – 1.44% (v/v), if a higher ethanol reduction of 2 – 3% (v/v) is required, further studies should be conducted. Future investigation should focus on exploring other sequential inoculation strategies of non-*Saccharomyces* yeasts, *M. pulcherrima*, *T. delbrueckii* or *Z. bailii* combined with *S. cerevisiae*, to increase the ethanol reduction. However, it will also be crucial to evaluate if the required ethanol reduction (2 – 3%, v/v) can be achieved by the use of the proposed microbial strategies in industrial conditions. In addition, the screening of non-*Saccharomyces* yeasts with the ability to reduce ethanol has also derived some interesting research directions. For example, why some yeast

have prominent ability to reduce ethanol? How their glucose repression mechanism affects ethanol production? Is the weaker growth ability of *M. pulcherrima* due to the poor resistance to the increasing ethanol content during fermentation, or metabolites from other yeasts?

GENERAL CONCLUSIONS

1. The alcohol reduction can be achieved by a combined strategy of inoculation of selected non-*Saccharomyces* yeast and *Saccharomyces cerevisiae* strains.

2. The rapid method we proposed for screening non-*Saccharomyces* yeasts with a low ethanol yield can be successfully performed under laboratory conditions.

3. Strains from *M. pulcherrima*, *T. delbrueckii and Z. bailii* species achieved higher ethanol yield reductions (1.39, 0.84 and 1.02% (v/v), respectively) when used in fermentation with *S. cerevisiae*, being *M. pulcherrima* the species with the highest ethanol reduction (1.44%, v/v) obtained in all tested conditions.

4. The sequentially inoculated fermentations achieved higher ethanol reductions than coinoculated fermentations.

5. Higher inoculation ratios of non-*Saccharomyces* yeasts resulted on higher persistence of those species along the fermentation, and higher ethanol yield reduction.

6. The use of a multistarter of selected non-*Saccharomyces* yeasts in sequential fermentations with *S. cerevisiae* achieved a significant ethanol reduction, although lower than the single non-*Saccharomyces* yeast starter. The multistarter was also accompanied by an increased concentration of lactic and acetic acids.

7. Along with the ethanol reduction in sequential fermentations *M. pulcherrima* increased glycerol content, *T. delbrueckii* increased the concentration of lactic acid and *Z. bailii* increased succinic acid content.

8. *L. thermotolerans* shows a significant ethanol reduction when used as single inoculum or in sequential fermentations of synthetic must, which was accompanied by an increase of lactic acid and glycerol content.

9. The *S. cerevisiae* strains submitted to Adaptative Laboratory Evolution (ALE) on high sugar and isobutanol show slower fermentation kinetics than their parental

strains in synthetic must fermentation, but similar fermentative profile in natural must fermentation.

10. The *S. cerevisiae* strains submitted to ALE on presence of isobutanol show a significant decrease of the ethanol yield compared to its parental strain, on natural must fermentation. No differences were observed on the strains adapted on high sugar must.

11. PMA-qPCR analysis is a rapid and sensitive method to accurately monitor the viable cell population of four strains in fermentation at the same time, which is a relevant parameter to estimate the participation of the different species in the ethanol reduction.

REFERENCES

References

Aa Kühle, A.V.D.; Jespersen, L. Detection and identification of wild yeasts in lager breweries. *Int. J. Food Microbiol.* **1998**, 43, 205–213, doi:10.1016/S0168-1605(98)00113-5.

Albertin, W.; Sertier, C.M.; Bely, M.; Marullo, P.; Coulon, J.; Moine, V.; Ceccaldi, B.C.; Pomarede, I.M. Oenological prefermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. *Inter. J. Food Microbiol.* **2014**, 178, 87–97, doi:10.1016/j.ijfoodmicro.2014.03.009.

Alessandria, V.; Giacosa, S.; Campolongo, S.; Rolle, L.; Rantsiou, K.; Cocolin. L. Yeast population diversity on grapes during on-vine withering and their dynamics in natural and inoculated fermentations in the production of icewines. *Food Res. Inter.* **2013**, 54, 139–147, doi:10.1016/j.foodres.2013.06.018.

Albergaria, H.; Francisco, D.; Gori, K.; Arneborg, N.; Gírio, F.M. *Saccharomyces cerevisiae* CCMI 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl. Microbiol. Biotechnol.* **2010**, 86, 965–972, doi:10.1007/s00253-009-2409-6.

Albergaria, H.; Arneborg, N. Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: Role of physiological fitness and microbial interactions. *Appl. Microbiol. Biotechnol.* **2016**, 100, 2035–2046, doi:10.1007/s00253-015-7255-0.

Alipourfard, I.; Datukishvili, Nelly.; Bakhtiyari, S.; Haghani, K.; Di Renzo, L.; De Miranda, R.C.; Mikeladze, D. MIG1 glucose repression in metabolic processes of *Saccharomyces cerevisiae*: Genetics to metabolic engineering. *Avicenna J. Medical Biotechnol.* **2019**, 11, 215–220.

Alonso-del-Real, J.; Contreras-Ruiz, A.; Castiglioni, G.L., Barrio, E.; Querol, A. The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: Limited aeration, inoculum proportions, and sequential inoculation. *Front. Microbiol.* **2017**, 8, 2087, doi:10.3389/fmicb.2017.02087.

Alonso-del-Real, J.; Pérez-Torrado, R.; Querol, A.; Barrio, E. Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization. *Environ. Microbiol.* **2019**, 21, 1627–1644, doi:10.1111/1462-2920.14536.

Andorrà, I.; Esteve-Zarzoso, B.; Guillamón, J.M.; Mas, A. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. Int. J. *Food Microbiol.* **2010a**, 144, 257–262, doi:10.1016/j.ijfoodmicro.2010.10.003.

Andorrà, I.; Landi, S.; Mas, A.; Esteve-Zarzoso, B.; Guillamón, J.M. Effect of fermentation temperature on microbial population evolution using culture-independent and dependent techniques. *Food Res. Int.* **2010b**, 43, 773–779, doi:10.1016/j.foodres.2009.11.014.

Andorrà, I.; Monteiro, M.; Esteve-Zarzoso, B.; Albergaria, H.; Mas, A. Analysis and direct quantification of *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentation by fluorescence in situ hybridization, flow cytometry and quantitative PCR. *Food Microbiol.* **2011**, 28, 1483–1491, doi:10.1016/j.fm.2011.08.009.

Aplin, J.J.; Edwards, C.G. Impacts of non-*Saccharomyces* species and aeration on sequential inoculation with *Saccharomyces cerevisiae* to produce lower alcohol merlot wines from Washington State. *J. Sci. Food Agri.* **2020**, doi:10.1002/jsfa.10769.

Arslan, E.; Çelik, Z.D.; Cabaroglu, T. Effects of pure and mixed autochthonous *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on fermentation and volatile compounds of narince wines. *Foods*. **2018**, 7, 147, doi:10.3390/foods7090147.

Bachmann, H.; Pronk, J.T.; Kleerebezem, M.; Teusink, B. Evolutionary engineering to enhance starter culture performance in food fermentations. *Curr. Opin. Biotechnol.* **2015**, 32, 1–7, doi:10.1016/j.copbio.2014.09.003.

Ballester-Tomás, L.; Prieto, J. A.; Gil, J.V.; Baeza, M.; Randez-Gil, F. The Antarctic yeast Candida sake: Understanding cold metabolism impact on wine. *Int .J. Food Microbiol.* **2017**, 245, 59–65, doi:10.1016/j.ijfoodmicro.2017.01.009.

Bankar, S.B.; Bule, M.V.; Singhal, R.S.; Ananthanarayan, L. Glucose oxidase — An overview. *Biotechnol. Adv.* **2009**, 27, 489–501, doi:10.1016/j.biotechadv.2009.04.003.

Barnett, J.A. Some controls on oligosaccharide utilization by yeasts: the physiological basis of the *Kluyver* effect. *FEMS Microbiol. Let.* **1992**, 100, 371–378, doi:10.1111/j.1574-6968.1992.tb14065.x.

Barnett, J.A. A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850–1880. *Yeast.* **2000**, 16, 755–771. doi:10.1002/1097-0061(20000615)16:8<755::AID-YEA587>3.0.CO;2-4.

Bauer, F.F.; Rossouw, D.; Franken, J. Finding novel carbon sinks in *S. cerevisiae*. ALCOHOL LEVEL REDUCTION IN WINE - OENOVITI INTERNATIONAL NETWORK. **2013**, 38–46.

Belda, I.; Ruiz, J.; Esteban-Fernández, A.; Navascués, E.; Marquina, D.; Santos, A.; Moreno-Arribas, M.V. Microbial contribution to wine aroma and its intended use for wine quality improvement. *Molecules.* **2017**, 22, 189, doi:10.3390/molecules22020189.

Belisario-Sánchez, Y.Y.; Taboada-Rodríguez, A.; Marín-Iniesta, F.; López-Gómez, A. Dealcoholized wines by spinning cone column distillation: phenolic compounds and antioxidant activity

measured by the 1,1-diphenyl-2-picrylhydrazyl method. *J. Agric. Food Chem.* **2009**, 57, 6770–6778, doi:10.1021/jf900387g.

Beltran, G.; Torija, M.J.; Novo, M., Ferrer, N.; Poblet, M.; Guillamon, J.M.; Rozes, N.; Mas, A. Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Sys. Appl. Microbiol.* **2002**. 25, 287-293, doi:10.1078/0723-2020-00097.

Beltran, G.; Esteve-Zarzoso, B.; Rozès, N.; Mas, A.; Guillamón, J.M. Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *J. Agric. Food Chem.* **2005**, 53, 996–1002, doi:10.1021/jf0487001.

Beltran, G.; Novo, M.; Guillamón, J.M.; Mas, A.; Rozès, N. Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *Int. J. Food Microbiol.* **2008**, 121, 169–177, doi:10.1016/j.ijfoodmicro.2007.11.030.

Benito, S.; Palomero, F.; Morata, A.; Calderón, F.; Palmero, D.; Suárez-Lepe, J.A. Physiological features of *Schizosaccharomyces pombe* of interest in making of white wines. *Eur Food Res Technol.* **2013**, 236, 29–36, doi:10.1007/s00217-012-1836-2.

Binati, R.L.; Junior, W.J.L.; Luzzini, G.; Slaghenaufi, D.; Ugliano, M.; Torriani, S. Contribution of non-*Saccharomyces* yeasts to wine volatile and sensory diversity: A study on *Lachancea thermotolerans*, *Metschnikowia spp.* and *Starmerella bacillaris* strains isolated in Italy. *Int. J. Food Microbiol.* **2020**, 318, 108470, doi:10.1016/j.ijfoodmicro.2019.108470.

Bindon, K.; Varela, C.; Kennedy, J.; Holt, H. Herderich, M. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 1. Grape and wine chemistry. *Food Chem.* **2013**, 138, 1696–1705, doi:10.1016/j.foodchem.2013.03.014.

Bindon, K.; Holt, H.; Williamson, P.O.; Varela, C.; Herderich, M.; Francis, I.L. Relationships between harvest time and wine composition in *Vitis vinifera* L. cv. Cabernet Sauvignon 2. Wine sensory properties and consumer preference. *Food Chem.* **2014**, 154, 90–101, doi:10.1016/j.foodchem.2013.12.099.

Bisson, L.F. Stuck and sluggish fermentations. *Am. J. Enol. Vitic.* **1999**, 50, 107–119, doi:10.1533/9781845698133.5.280.

Biyela, B.N.E.; du Toit, W.J.; Divol, B.; Malherbe, D.F.; van Rensburg, P. The production of reduced-alcohol wines using Gluzyme Mono® 10.000 BG-treated grape juice. *S. Afr. J. Enol. Vitic.* **2009**, 30, 124–132, doi:10.21548/30-2-1432.

Borneman, A.R.; Pretorius, I.S. Genomic insights into the *Saccharomyces sensu* stricto complex. *Genetics.* **2015**, 199, 281–291, doi:10.1534/genetics.114.173633.

Bottcher, C.; Harvey, K.; Forde, C.G.; Boss, P.K.; Davies, C. Auxin treatment of pre-veraison grape (*Vitis vinifera* L.) berries both delays ripening and increases the synchronicity of sugar accumulation. *Aust. J. Grape Wine Res.* **2011**, 17, 1–8, doi:10.1111/j.1755-0238.2010.00110.x.

Bovo, B.; Andrighetto, C.; Carlot, M.; Corich, V.; Lombardi, A.; Giacomini, A. Yeast population dynamics during pilot-scale storage of grape marcs for the production of Grappa, a traditional Italian alcoholic beverage. *Int. J. Food Microbiol.* **2009**, 129, 221–28, doi:10.1016/j.ijfoodmicro.2008.11.025.

Brou, P.; Taillandier, P.; Beaufort, S.; Brandam, C. Mixed culture fermentation using *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* with direct and indirect contact: impact of anaerobic growth factors. *Eur. Food Res. Technol.* **2018**, 244, 1699–1710, doi:10.1007/s00217-018-3095-3.

Cadière, A.; Ortiz-Julien, A.; Camarasa, C.; Dequin, S. Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased in vivo flux through the pentose phosphate pathway. *Metab. Eng.* **2011**, 13, 263–271, do:10.1016/j.ymben.2011.01.008.

Cambon, B.; Monteil, V.; Remize, F.; Camarasa, C.; Dequin, S. Effects of GPD1 overexpression in *Saccharomyces cerevisiae* commercial wine yeast strains lacking ALD6 genes. *Appl. Environ Microbiol.* **2006**, 72, 4688–4694, doi:10.1128/AEM.02975-05.

Cañas, P.M.I.; García, A.T.P.; Romero, E.G. Enhancement of flavour properties in wines using sequential inoculations of non-*Saccharomyces* (*Hansenula* and *Torulaspora*) and *Saccharomyces* yeast starter. *Vitis.* **2011**, 50, 177–182.

Canonico, L.; Comitini, F.; Ciani, M. *Metschnikowia pulcherrima* selected strain for ethanol reduction in wine: Influence of cell immobilization and aeration condition. *Foods.* **2019a**, 8, 378, doi:10.3390/foods8090378.

Canonico, L.; Ecomitini, F.; Eoro, L.; Eciani, M. Sequential fermentation with selected immobilized non-*Saccharomyces* yeast for reduction of ethanol content in wine. *Front. Microbiol.* **2016**, 7, 278, doi:10.3389/fmicb.2016.00278.

Canonico, L.; Comitini, F.; Ciani, M. *Torulaspora delbrueckii* for secondary fermentation in sparkling wine production. *Food Microbiol.* **2018**, 74, 100–106, doi:10.1016/j.fm.2018.03.009.

Canonico, L.; Solomon, M.; Comitini, F.; Ciani, M.; Varela, C. Volatile profile of reduced alcohol wines fermented with selected non-*Saccharomyces* yeasts under different aeration conditions. *Food Microbiol.* **2019b**, 84, 103247, doi:10.1016/j.fm.2019.103247.

Capozzi, V.; Garofalo, C.; Chiriatti, M.A.; Grieco, F.; Spano, G. Microbial terroir and food innovation: The case of yeast biodiversity in wine. *Microbiol. Res.* **2015**, 181, 75–83, doi:10.1016/j.micres.2015.10.005.

Childs, B.C.; Bohlscheid, J.C.; Edwards, C.G. Impact of available nitrogen and sugar concentration in musts on alcoholic fermentation and subsequent wine spoilage by *Brettanomyces bruxellensis*. *Food Microbiol.* **2015**, 46, 604–609. doi:10.1016/j.fm.2014.10.006.

Ciani, M.; Comitini, F.; Mannazzu, I.; Domizio, P. Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* **2010**, 10, 123–133, doi:10.1111/j.1567-1364.2009.00579.x.

Ciani, M.; Comitini, F. Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. *Ann. Microbiol.* **2011**, 61, 25–32, doi:10.1007/s13213-010-0069-5.

Ciani, M.; Morales, P.; Comitini, F.; Tronchoni, J.; Canonico, L.; Curiel, J.A.; Oro, L.; Rodrigues, A.J.; Gonzalez, R. Non-conventional yeast species for lowering ethanol content of wines. *Front. Microbiol.* **2016**, 7, 642, doi:10.3389/fmicb.2016.00642.

Ciani, M.; Canonico, L.; Oro, L.; Comitini, F. Footprint of nonconventional yeasts and their contribution in alcoholic fermentations. *Biotechnol. Prog. Bever. Consum.* **2020**, 19, 435–465, doi:10.1016/B978-0-12-816678-9.00014-X.

Clingeleffer, P.R. Viticultural practices to moderate wine alcohol content. Proceedings ASVO Seminar: Towards best practice through innovation in winery processing. Tanunda (SA), Australia, 17 Oct., **2007**.

Comitini, F.; Gobbi, M.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. Selected non-*Saccharomyces* wine yeasts in controlled multistarter fermentations with *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, 28, 873–882, doi:10.1016/j.fm.2010.12.001.

Contreras, A.; Hidalgo, C.; Schmidt, S.; Henschke, P.; Curtin, C.; Varela, C. The application of non-*Saccharomyces* yeast in fermentations with limited aeration as a strategy for the production of wine with reduced alcohol content. *Int. J. Food Microbiol.* **2015**, *205*, 7–15, doi:10.1016/j.ijfoodmicro.2015.03.027.

Contreras, A.; Hidalgo, C.; Henschke, P.A.; Chambers, P.J.; Curtin, C.; Varela, C. Evaluation of non-*Saccharomyces* yeasts for the reduction of alcohol content in wine. *Appl. Environ. Microbiol.* **2014a**, 80, 1670–1678, doi:10.1128/aem.03780-13.

Contreras, A.; Curtin, C.; Varela, C. Yeast population dynamics reveal a potential 'collaboration' between *Metschnikowia pulcherrima* and *Saccharomyces uvarum* for the production of reduced alcohol wines during Shiraz fermentation. *Appl. Microbiol. Biotechnol.* **2014b**, 99, 1885–1895, doi:10.1007/s00253-014-6193-6.

David, V.; Terrat, S.; Herzine, K.; Claisse, O.; Rousseaux, S.; Tourdot-Maréchal, R.; Masneuf-Pomarede, I.; Ranjard, L.; Alexandre, H. High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation. *J. Indust. Microbiol. Biotechnol.* **2014**, 41, 811–821, doi:10.1007/s10295-014-1427-2.

Díaz, M.; Herrero, M.; García, L.A.; Quirós, C. Application of flow cytometry to industrial microbial bioprocesses. *Biochem. Eng. J.* **2010**, 48, 385–407, doi:10.1016/j.bej.2009.07.013.

Di Profio, F.; Reynolds, A.G.; Kasimos, A. Canopy Management and Enzyme Impacts on Merlot, Cabernet franc, and Cabernet Sauvignon. I. Yield and Berry Composition. *Am. J. Enol. Vitic.* **2011**, 62, 139–151, doi:10.5344/ajev.2010.10024.

Domizio, P.; Lencioni, L.; Ciani, M.; Di Blasi, S.; Pontremolesi, C.; Sabatelli, M.P. Spontaneous and inoculated yeast population dynamics and their effect on organoleptic characters of Vinsanto wine under different process conditions. *Int. J. Food Microbiol.* **2007**, 115, 281–289, doi:10.1016/j.ijfoodmicro.2006.10.052.

Dujon, B.A.; Louis, E.J.. Genome diversity and evolution in the budding yeasts (*Saccharomycotina*). *Genetics.* **2017**, 206, 717–750, doi:10.1534/genetics.116.199216.

Dutraive, O.; Benito, S.; Fritsch, S.; Beisert, B.; Patz, C.D.; Rauhut, D. Effect of sequential inoculation with non-*Saccharomyces* and *Saccharomyces* yeasts on Riesling wine chemical composition. *Ferment.* **2019**, 5, 79. doi:10.3390/fermentation5030079.

Efriwati, A.S.; Rahayu, G.; Nuraida, L. Population dynamics of yeasts and lactic acid bacteria (LAB) during Tempeh production. *HAYATI J. Biosci.* **2013**, 20, 57–64, doi:10.4308/hjb.20.2.57.

Emerson, J.B.; Adams, R.I.; Román, C.M.B.; Brooks, B.; Coil, D.A.; Dahlhausen, K.; Ganz, H.H.; Hsu, T.; Justice, N.B.; Lima, I.G.P.; Luongo, J.C.; Lymperopoulou, D.S.; Silvan, C.G.; Mancinelli, B.R.; Balk, M.; Huttenhower, C.; Nocker, A.; Vaishampayan, P.; Rothschild, L.J.

Schrödinger's microbes: Tools for distinguishing the living from the dead in microbial ecosystems. *Microbiome.* **2017**, 5, 86, doi:10.1186/s40168-017-0285-3.

Englezos, V.; Rantsiou, K.; Cravero, F.; Torchio, F.; Ortiz-Julien, A.; Gerbi, V.; Rolle, L.; Cocolin, L. *Starmerella bacillaris* and *Saccharomyces cerevisiae* mixed fermentations to reduce ethanol content in wine. *Appl. Microbiol. Biotechnol.* **2016**, 100, 5515–5526, doi:10.1007/s00253-016-7413-z.

Englezos, V.; Rantsiou, K.; Giacosa, S.; Segade, S.R.; Rolle, L.; Cocolin, L. Cell-to-cell contact mechanism modulates *Starmerella bacillaris* death in mixed culture fermentations with *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2019**, 289, 106–114, doi:10.1016/j.ijfoodmicro.2018.09.009.

Escribano-Viana, R.; González-Arenzana, L.; Portu, J.; Garijo, P.; López-Alfaro, I.; López, R.; Santamaría, P.; Gutiérrez, A.R. Wine aroma evolution throughout alcoholic fermentation sequentially inoculated with non-*Saccharomyces*/*Saccharomyces* yeasts. *Food Res. Int.* **2018**, 112, 17–24, doi:10.1016/j.foodres.2018.06.018.

Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.8S RRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* **1999**, 49, 329–337, doi:10.1099/00207713-49-1-329.

Fernández-Espinar, M.T.; Martorell, P.; de Llanos, R.; Querol, A. Molecular methods to identify and characterize yeasts in food and beverages. In: Yeasts in Food and Beverages. Querol, A., Fleet, G. H., (eds). **2006**, pp. 55–82. Springer Science+Business Media LLC, New York, USA, doi:10.1007/978-3-540-28398-0_3.

Ferraro, L.; Fatichenti, F.; Ciani, M. Pilot scale vinification process using immobilized *Candida stellata* cells and *Saccharomyces cerevisiae*. *Process Biochem.* **2000**, 35, 1125–1129, doi:10.1016/S0032-9592(00)00148-5.

Filippetti, I.; Movahed, N.; Allegro, G.; Valentini, G.; Pastore, C.; Colucci, E.; Intrieri, C. Effect of post-veraison source limitation on the accumulation of sugar, anthocyanins and seed tannins in *Vitis vinifera* cv. Sangiovese berries. *Aust. J. Grape Wine Res.* **2015**, 21, 90–100, doi:10.1111/ajgw.12115.

Fittipaldi, M.; Nocker, A.; Codony, F. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *J Microbiol. Methods.* **2012**, 91, 276–289, doi:10.1016/j.mimet.2012.08.007.

Fischer, U.; Noble, A.C. The effect of ethanol, catechin concentration, and pH on sourness and bitterness of wine. *Am. J. Enol. Vitic.* **1994**, 45, 6–10, doi:10.1016/0924-2244(94)90074-4.

Fleet, G. Yeast interactions and wine flavour. *Int J Food Microbiol.* **2003**, 86, 11–22, doi:10.1016/S0168-1605(03)00245-9.

Fleet, G.H. Yeasts: growth during fermentation. *FEMS Yeast Res.* **2008**, 8, 979–995.

Fleet, G.H.; Heard, G.M. Yeasts: growth during fermentation. In Fleet GH (ed.). Wine Microbiology and Biotechnology, Harwood Academic: Chur, Switzerland; **1993**, 27–54.

Fleet, G.H. The microorganisms of winemaking – isolation, enumeration and identification. In: Fleet, G.H. (ed). Wine microbiology and biotechnology. Harwood Academic Publishers, Switzerland. **1993**. pp. 1–25.

Fontoin, H.; Saucier, C.; Teissedre, P.L.; Glories, Y. Effect of pH, ethanol and acidity on astringency and bitterness of grape seed tannin oligomers in model wine solution. *Food Qual. Prefer.* **2008**, 19, 286–291, doi:10.1016/j.foodqual.2007.08.004.

Fornari, T.; Hernández, E.J.; Ruiz-Rodriguez, A.; Señorans, F.J.; Reglero, G. Phase equilibria for the removal of ethanol from alcoholic beverages using supercritical carbon dioxide. *J. Supercritical Fluids.* **2009**, 50, 91–96, doi:10.1016/j.supflu.2009.05.012.

Fugelsang, K.C. Wine Microbiology. Chapman and Hall Pub. Co. New York, N.Y. **1997**.

Gamero, A.; Dijkstra, A.; Smit, B.; de Jong, C. Aromatic potential of diverse non-conventional yeast species for winemaking and brewing. *Ferment.* **2020**, 6, 50, doi:10.3390/fermentation6020050.

Gao, C.; Fleet. G.H. The effects of temperature and pH on the ethanol tolerance of wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. *J. Appl. Bacteriol.* **1988**, 65, 405–409, doi:10.1111/j.1365-2672.1988.tb01909.x.

García, V.; Vásquez, H.; Fonseca, F.; Manzanares, P.; Ganga, M.A. Effects of using mixed wine yeast cultures in the production of Chardonnay wines. *Rev. Argent. Microbiol.* **2010**, 42, 226–229, doi:10.1097/MRM.0b013e32833bb93a.

García, M.; Esteve-Zarzoso, B.; Crespo, J.; Cabellos, J.M.; Arroyo, T. Yeast monitoring of wine mixed or sequential fermentations made by native strains from D.O. "Vinos de Madrid" using real-time quantitative PCR. *Front. Microbiol.* **2017**, *8*, 2520, doi:10.3389/fmicb.2017.02520.

Ghiaci, P.; Norbeck, J.; Larsson, C. Physiological adaptations of *Saccharomyces cerevisiae* evolved for improved butanol tolerance. *Biotechnol. Biofuels.* **2013**, 6, 101, doi:10.1186/1754-6834-6-101.

Gibson, B.; Liti, G. *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. *Yeast.* **2015**, 32, 17–27, doi:10.1002/yea.3033.

Gobbi, M.; Comitini, F.; Domizio, P.; Romani, C.; Lencioni, L.; Mannazzu, I.; Ciani, M. *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: A strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol.* **2013**, 33, 271–281, doi:10.1016/j.fm.2012.10.004.

Godden, P.; Wilkes, E.; Johnson, D. Trends in the composition of Australian wine 1984–2014. *Aust. J. Grape Wine R.* **2015**, 21, 741–753, doi:10.1111/ajgw.12195.

Goldner, M.C.; Zamora, M.C.; Lira, P.D.L.; Gianninoto, H.; Bandoni, A. Effect of ethanol level in the perception of aroma attributes and the detection of volatile compounds in red wine. *J. Sens. Stud.* **2009**, 24, 243–257, doi:10.1111/j.1745-459X.2009.00208.x.

Gonzalez, R.; Quirós, M.; Morales, P. Yeast respiration of sugars by non-*Saccharomyces* yeast species: A promising and barely explored approach to lowering alcohol content of wines. *Trends Food Sci. Tech.* **2013**, 29, 55–61, doi:10.1016/j.tifs.2012.06.015.

González-Royo, E.; Pascual, O.; Kontoudakis, N.; Esteruelas, M.; Esteve-Zarzoso, B.; Mas, A.; Canals, J.M.; Zamora, F. Oenological consequences of sequential inoculation with non-*Saccharomyces* yeasts (*Torulaspora delbrueckii* or *Metschnikowia pulcherrima*) and *Saccharomyces cerevisiae* in base wine for sparkling wine production. *Eur. Food Res. Technol*. **2015**, 240, 999–1012, doi:10.1007/s00217-014-2404-8.

Goold, H.D.; Kroukamp, H.; Williams, T.C.; Paulsen, I.T.; Varela, C.; Pretorius, I.S. Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. *Microbial Biotechnol.* **2017**, 10, 264–278, doi:10.1111/1751-7915.12488.

Guriérrez, A.R.; Epifanio, S.; Garijo, P.; Lopez, R.; Santamaria, P. Killer yeasts: incidence in the ecology of spontaneous fermentation. *Am. J. Enol. Vitic.* **2001**, 52, 352–356, doi:10.1016/S0065-2911(01)45006-5.

Hagman, A.; Säll, T.; Compagno, C.; Piskur, J. Yeast "make-accumulate-consume" life strategy evolved as a multi-step process that predates the whole genome duplication. *PLoS ONE.* **2013**, 8, e68734, doi:10.1371/journal.pone.0068734.

Hansen, E.H.; Nissen, P.; Sommer, P.; Nielsen, J.C.; Arneborg, N. The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* **2001**, 91, 541–547, doi:10.1046/j.1365-2672.2001.01426.x.

Heard, G.M.; Fleet, G.H. The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *J. Appl. Bacteriol.* **1988**, 65, 23–28, doi:10.1111/j.1365-2672.1988.tb04312.x.

Heard, G. Novel yeasts in winemaking – looking to the future. *Food Aus.* **1999**, 51, 347–352, doi:10.1016/S0278-6915(99)00071-X.

Henricsson, C.; Ferreira, M.C.D.J.; Hedfalk, K.; Elbing, K.; Larsson, C.; Bi, R.M.; Norbeck, J.; Hohmann, S.; Gustafsson, L. Engineering of a novel *Saccharomyces cerevisiae* wine strain with a respiratory phenotype at high external glucose concentrations. *Appl. Environ. Microbiol.* **2005**, 71, 6185–6192, doi:10.1128/AEM.71.10.6185-6192.2005.

Heux, S.; Sablayrolles, J.M.; Cachon, R.; Dequin, S. Engineering a *Saccharomyces cerevisiae* wine yeast that exhibits reduced ethanol production during fermentation under controlled microoxygenation conditions. *Appl. Environ. Microbiol.* **2006**, 72, 5822–5828, doi:10.1128/AEM.00750-06.

Hierro, N.; Esteve-Zarzoso, B.; González, Á.; Mas, A.; Guillamón, J.M. Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* **2006**, 72, 7148–7155, doi:10.1128/AEM.00388-06.

Hierro, N.; Esteve-Zarzoso, B.; Mas, A.; Guillamã, N.J.M. Monitoring of *Saccharomyces* and *Hanseniaspora populations* during alcoholic fermentation by real-time quantitative PCR. *FEMS Yeast Res.* **2007**, 7, 1340–1349, doi:10.1111/j.1567-1364.2007.00304.x.

Hranilovic, A.; Gambetta, J.M.; Schmidtke, L.; Boss, P.K.; Grbin, P.R.; Masneuf-Pomarede, I.; Bely, M.; Albertin, W.; Jiranek. V. Oenological traits of *Lachancea thermotolerans* show signs of domestication and allopatric differentiation. *Sci. Rep.* **2018**, 8, 1–13, doi:10.1038/s41598-018-33105-7.

Hranilovic, A.; Gambetta, J.M.; Jeffery, D.W.; Grbin, P.R.; Jiranek, V. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing. *Int. J. Food Microbiol.* **2020**, 329, 108651, doi:10.1016/j.ijfoodmicro.2020.108651.

Jemec, K.P.; Cadez, N.; Zagorc, T.; Bubic, V.; Zupec, A.; Raspor, P. Yeast population dynamics in five spontaneous fermentations of Malvasia must. *Food Microbiol.* **2001**, 18, 247–259, doi:10.1006/fmic.2001.0396.

Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine. *S. Afr. J. Enol. Vitic.* **2003**, 24, 63–69, doi:10.21548/24-2-2641.

Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **2014**, 14, 215–237, doi:10.1111/1567-1364.12111.

Jolly, N.P.; Augustyn, O.P.H.; Pretorius, I.S. The role and use of non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic.* **2006**, 27, 15–39, doi:10.21548/27-1-1475.

Jones, G.V.; White, M.A.; Cooper, O.R.; Storchmann, K. Climate change and global wine quality. *Clim. Change.* **2005**, 73, 319–343, doi:10.1007/s10584-005-4704-2.

Kayikci, Ö.; Nielsen, J. Glucose repression in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **2015**, 15, 1–8, doi:10.1093/femsyr/fov068.

Kliewer, W.M.; Dokoozlian, N.K. Leaf area/crop weight ratios of grapevines: Influence on fruit composition and wine quality. *Am. J. Enol. Vitic.* **2005**, 56, 170–181, doi:10.1016/j.scienta.2004.06.014.

Kontoudakis, N.; Esteruelas, M.; Fort, F.; Canals, J.M.; Zamora, F. Use of unripe grapes harvested during cluster thinning as a method for reducing alcohol content and pH of wine. *Aust. J. Grape Wine R.* **2011**, 17, 230–238, doi:10.1111/j.1755-0238.2011.00142.x.

Korkutal, I.; Bahar, E. Influence of different soil tillage and leaf removal treatments on yield, cluster and berry characteristics in cv. Syrah (*Vitis vinifera* L.). *Bul. J. Agr. Sci.* **2013**, 19, 652–663.

Koufos, G.; Mavromatis, T.; Koundouras, S.; Fyllas, N.M.; Jones, G.V. Viticulture-climate relationships in Greece: The impacts of recent climate trends on harvest date variation. *Int. J. Climatol.* **2014**, 34, 1445–1459, doi:10.1002/joc.3775.

Kurtzman, C.P.; Fell, J.W. The yeasts, a taxonomic study (4th ed). Elsevier Science Publishers, Amsterdam. **1998a**.

Kurtzman, C. P.; Robnett, C. J. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek.* **1998b**, 73, 331–371, doi:10.1023/A:1001761008817.

Kutyna, D,R.; Varela, C.; Stanley, G.A.; Borneman, A.R.; Henschke, P.A.; Chambers, P.J. Adaptive evolution of *Saccharomyces cerevisiae* to generate strains with enhanced glycerol production. *Appl. Microbiol. Biotechnol.* **2012**, 93, 1175–1184, doi:10.1007/s00253-011-3622-7.

Labanda, J.; Vichi, S.; Llorens, J.; López-Tamames, E. Membrane separation technology for the reduction of alcoholic degree of a white model wine. *LWT Food Sci. Technol.* **2009**, 42, 1390–1395, doi:10.1016/j.lwt.2009.03.008.

Li, J.; Hu, W.; Huang, X.; Xu, Y. Investigation of yeast population diversity and dynamics in spontaneous fermentation of Vidal Blanc icewine by traditional culture-dependent and high-throughput sequencing methods. *Food Res. Inter.* **2018**, 112, 66–77, doi:10.1016/j.foodres.2018.06.011.

Lin, M.M.H.; Boss, P.K.; Walker, M.E.; Sumby, K.M.; Grbin, P.R.; Jiranek, V. Evaluation of indigenous non-*Saccharomyces* yeasts isolated from a South Australian vineyard for their potential as wine starter cultures. *Int. J. Food Microbiol.* **2020**, 108373, doi:10.1016/j.ijfoodmicro.2019.108373.

Lleixà, J.; Manzano, M.; Mas, A.; Portillo, M.C. *Saccharomyces* and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions. *Front. Microbiol.* **2016a**, 7, 1959, doi:10.3389/fmicb.2016.01959.

Lleixà, J.; Martín, V.; Portillo, M. del C.; Carrau, F.; Beltran, G.; Mas, A. Comparison of fermentation and wines produced by inoculation of *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. *Front. Microbiol.* **2016b**, 7, doi:10.3389/fmicb.2016.00338.

Longo, R.; Blackman, J.W.; Antalick, G.; Torley, P.J.; Rogiers, S.Y.; Schmidtke, L.M. Volatile and sensory profiling of Shiraz wine in response to alcohol management: Comparison of harvest timing versus technological approaches. *Food Res. Int.* **2018**, 109, 561–571, doi:10.1016/j.foodres.2018.04.057.

Loira, I.; Vejarano, R.; Bañuelos, M.; Morata, A.; Tesfaye, W.; Uthurry, C.; Villa, A.; Cintora, I.; Suárez-Lepe, J. Influence of sequential fermentation with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT-Food Sci. Technol.* **2014**, 59, 915–922, doi: 10.1016/j.lwt.2014.06.019.

Ludovico, P.; Sousa, M.J.; Silva, M.T.; Leao, C.; Corte-Real, M. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiol.* **2001**, 147, 2409–2415, doi:10.1099/00221287-147-9-2409.

Magyar, I.; Tóth, T. Comparative evaluation of some oenological properties in wine strains of *Candida stellata*, *Candida zemplinina*, *Saccharomyces uvarum* and *Saccharomyces cerevisiae*. *Food Microbiol.* **2011**, 28, 94–100, doi:10.1016/j.fm.2010.08.011.

Malherbe, D.F.; du Toit, M.; Otero, R.R.C.; van Rensburg, P.; Pretorius, I.S. Expression of the *Aspergillus niger* glucose oxidase gene in *Saccharomyces cerevisiae* and its potential applications in wine production. *Appl. Microbiol. Biotechnol.* **2003**, 61, 502–511, doi:10.1007/s00253-002-1208-0.

Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., and Beltran, G. Biomass production and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrogen source. *FEMS Yeast Res.* **2012**, 12, 477–482, doi:10.1111/j.1567-1364.2012.00802.x.

Mas, A.; Padilla, B.; Esteve-Zarzoso, B.; Beltran, G.; Reguant, C.; Bordons, A.. Taking advantage of natural biodiversity for wine making: The WILDWINE Project. *Agric. Agric. Sci. Procedia.* **2016**, 8, 4–9, doi:10.1016/j.aaspro.2016.02.002.

Masson, J.; Aurier, P.; d' hauteville, F. Effects of non-sensory cues on perceived quality: the case of low-alcohol wine. *Int. J. Wine Bus. Res.* **2008**, 20, 215–229, doi:10.1108/17511060810901037.

Massot, A.; Mietton-Peuchot, M.; Peuchot, C.; Milisic, V. Nanofiltration and reverse osmosis in winemaking. *Desalination.* **2008**, 231, 283-289, doi:10.1016/j.desal.2007.10.032.

Maturano, Y.P.; Mestre, M.V.; Esteve-Zarzoso, B.; Nally, M.C.; Lerena, M.C.; Toro, M.E.; Vazquez, F.; Combina, M. Yeast population dynamics during prefermentative cold soak of Cabernet Sauvignon and Malbec Wines. *Int. J. Food Microbiol.* **2015**, 199, 23–32, doi:10.1016/j.ijfoodmicro.2015.01.005.

Maturano, Y.P.; Mestre, M.V.; Kuchen, B.; Toro, M.E.; Mercado, L.A.; Vazquez, F.; Combina, M. Optimization of fermentation-relevant factors: A strategy to reduce ethanol in red wine by sequential culture of native yeasts. *Int. J. Food Microbiol.* **2019**, 289, 40–48, doi:10.1016/j.ijfoodmicro.2018.08.016.

Medina, K.; Carrau, F.M.; Gioia, O.; Bracesco, N. Nitrogen availability of grape juice limits killer yeast growth and fermentation activity during mixed-culture fermentation with sensitive commercial yeast strains. *Appl. Environ. Microbiol.* **1997**, 63, 2821–2825, doi:10.2105/AJPH.87.7.1131.

Meillon, S.; Urbano, C.; Schlich, P. Contribution of the Temporal Dominance of Sensations (TDS) method to the sensory description of subtle differences in partially dealcoholized red wines. *Food Qual. Prefer.* **2009**, 20, 490–499, doi:10.1016/j.foodqual.2009.04.006.

Mendoza, L.; Farías, M.E. Improvement of wine organoleptic characteristics by non-*Saccharomyces* yeasts. Curr. Res. Technol. Ed. Top. Appl. Microbiol. *Microb. Biotechnol.* **2010**, 2, 908–919.

Mendoza, L.M.; Merín, M.G.; Morata, V.I.; Farías, M.E. Characterization of wines produced by mixed culture of autochthonous yeasts and *Oenococcus oeni* from the northwest region of Argentina. *J. Ind. Microbiol. Biotechnol.* **2011**, 38, 1777–1785, doi:10.1007/s10295-011-0964-1.

Mihnea, M.; Gonzalez-SanJose, M.L.; Ortega-Heras, M.; Perez-Magarino, S.; Garcia-Martin, N.; Palacio, L.; Pradanos, P.; Hernandez, A. Impact of must sugar reduction by membrane applications on volatile composition of Verdejo wines. *J. Agri. Food Chem.* **2012**, 60, 7050–7063, doi:10.1021/jf301433j.

Millet, V.; Lonvaud-Funel, A. The viable but non-culturable state of wine micro-organisms during storage. *Let. Appl. Microbiol.* **2000**, 30, 136-141, doi:10.1046/j.1472-765x.2000.00684.x.

Mitrakul, C.M.; Henick-Kling, T.; Egli, C.M. Discrimination of *Brettanomyces/Dekkera* yeast isolates from wine by using various DNA finger-printing methods. *Food Microbiol.* **1999**, 16, 3–14, doi:10.1006/fmic.1998.0217.

Morales, P.; Rojas, V.; Quirós, M.; Gonzalez, R. The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. *Appl. Microbiol. Biotechnol.* **2015**, 99, 3993–4003, doi:10.1007/s00253-014-6321-3.

Morata, A.; Loira, I.; Escott, C.; Fresno, J.M.; Bañuelos, M.A.; Suárez-Lepe, J.A. Applications of *Metschnikowia Pulcherrima* in wine biotechnology. *Ferment.* **2019**, 5, 63, doi:10.3390/fermentation5030063.

Morrison-Whittle, P.; Goddard, M.R. Quantifying the relative roles of selective and neutral processes in defining eukaryotic microbial communities. *ISME J.* **2015**, 9, 2003–2011, doi:10.1038/ismej.2015.18.

Muratore, G.; Asmundo, C.N.; Lanza, C.M.; Caggia, C.; Licciardello, F.; Restuccia, C. Influence of *Saccharomyces uvarum* on volatile acidity, aromatic and sensory profile of Malvasia delle Lipari wine. *Food Technol. Biotechnol.* **2007**, 45, 101–106, doi:10.1080/08905430701707802.

Musmanno, R.A.; Maggio, T.; Coratza, G. Studies on strong and weak killer phenotypes of wine yeasts: production, activity of toxin in must, and its effect in mixed culture fermentation. *J. Appl. Microbiol.* **1999**, 87, 932–938, doi:10.1046/j.1365-2672.1999.00954.x.

Navarro, Y.; Torija, M.J.; Mas, A.; Beltran, G. Viability-PCR allows monitoring yeast population dynamics in mixed fermentations including viable but non-culturable yeasts. *Foods.* **2020**, 9, 1373, doi:10.3390/foods9101373.

Naumov, G.I.; James, S.A.; Naumova, E.S.; Louis, E.J.; Roberts, I.N. Three new species in the *Saccharomyces sensu* stricto complex: *Saccharomyces cariocanus*, *Saccharomyces kudriavzevii* and *Saccharomyces mikatae*. I*nt. J. System. Evolution. Microbiol.* **2000**, 50, 1931–1942, doi:10.1099/00207713-50-5-1931.

Nisiotou, A.A.; Spiropoulos, A.E.; Nychas, G.J.E. Yeast community structures and dynamics in healthy and botrytis-affected grape must fermentations. *Appl. Envir. Microbiol.* **2007**, 73, 6705–6713, doi:10.1128/AEM.01279-07.

Nissen, P.; Nielsin, D.; Arneborg, N. Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast.* **2003**, 20, 331-341, doi:10.1007/BF00251119.

Noble, A.C.; Bursick, G.F. 1984. The contribution of glycerol to perceived viscosity and sweetness in white wine. *Am. J. Enol. Vitic.* **1984**, 35, 110–112.

Nocker, A.; Cheung, C.Y.; Camper, A.K. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J. Microbiol. Methods.* **2006**, 67, 310–320, doi:10.1016/j.mimet.2006.04.015.

Novello, V.; de Palma, L. Viticultural strategy to reduce alcohol levels in wine. In: Teissedre PL, editor. Alcohol reduction in wine. Oenoviti International Network. Merignac: Vigne et Vin Publications Internationales. **2013**, pp. 3–8.

Nurgel. C.; Pickering, G. Contribution of glycerol, ethanol and sugar to the perception of viscosity and density elicited by model white wines. *J. Texture Stu.* **2005**, 36, 303–323, doi:10.1111/j.1745-4603.2005.00018.x.

Padilla, B.; Gil, J.V.; Manzanares, P. Past and future of non-*Saccharomyces* yeasts: From spoilage microorganisms to biotechnological tools for improving wine aroma complexity. *Front. Microbiol.* **2016**, 7, 411, doi:10.3389/fmicb.2016.00411.

Pallmann, C.L.; Brown, J.A.; Olineka, T.L.; Cocolin, L.; Mills, D.A.; Bisson, L.F. Use of WL medium to profile native flora fermentations. *Am. J. Enol. Vitic.* **2001**, 52, 198–203, doi:10.1016/S0065-2164(01)49013-7.

Pfeiffer, T.; Morley, A. An evolutionary perspective on the Crabtree effect. *Front. Mol. Biosci.* **2014**, 1, 17, doi:10.3389/fmolb.2014.00017.

Pickering, G.J. Low-and reduced-alcohol wine: a review. *J. Wine Res.* **2000**, 11, 129-144, doi:10.1080/09571260020001575.

Pickering, G.J.; Heatherbell, D.A. Barnes, M.F. The production of reduced-alcohol wine using glucose oxidase-treated juice II–SO2-binding and stability. *Am. J. Enol. Vitic.* **1999a**, 50, 299–306, doi:0002-9254(1999)L:3<299:TPORAW>2.0.TX;2-U.

Pickering, G.J.; Heatherbell, D.A.; Barnes, M.F. The production of reduced-alcohol wine using glucose oxidase-treated juice III–sensory. *Am. J. Enol. Vitic.* **1999b**, 50, 307–316.

Pickering, G.J.; Heatherbell, D.A.; Barnes, M.F. GC-MS Analysis of reduced-alcohol Müller-Thurgau wine produced using glucose oxidase-treated juice. *LWT Food Sci. Technol.* **2001**, 34, 89–94, doi:10.1006/fstl.2000.0744.

Pickering, G.J.; Heatherbell, D.A.; Vanhanen, L.P.; Barnes, M.F. The effect of ethanol concentration on the temporal perception of viscosity and density in white wine. *Am. J. Enol. Vitic.* **1998a**, 49, 306–318, doi:10.1016/S0168-1605(97)00113-X.

Pickering, G. J.; Heatherbell, D.A.; Barnes, M.F. Optimising glucose conversion in the production of reduced alcohol wine using glucose oxidase. *Food Res. Int.* **1998b**, 31, 685–692, doi:10.1016/S0963-9969(99)00046-0.

Poni, S. The impact of leaf removal and the management of crop load on fruit quality – a European perspective. *Wine Vitic. J.* **2014**, 29, 40, 42, 44–48, doi:10.1016/j.juro.2012.11.174.

Poni, S.; Gatti, M.; Bernizzoni, F.; Civardi, S.; Bobeica, N.; Magnanini, E.; Palliotti, A. Late leaf removal aimed at delaying ripening in cv. Sangiovese: physiological assessment and vine performance. *Aust. J. Grape Wine Res.* **2013**, 19, 378–387, doi:10.1111/ajgw.12040.

Pretorius, I.S.; Curtin, C.D.; Chambers, P.J. The winemaker's bug: From ancient wisdom to opening new vistas with frontier yeast science. *Bioengineered Bugs.* **2012**, 3, 147–56, doi:10.4161/bbug.19687.

Pronk, J.T.; Steensma, H.Y.; van Dijken, J.P. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast.* **1996**, 12, 1607-1633, doi:10.1002/(SICI)1097-0061(199612)12:16.

Portillo, M.D.C.; Mas, A. Analysis of microbial diversity and dynamics during wine fermentation of Grenache grape variety by high-throughput barcoding sequencing. *LWT-Food Sci. Technol.* **2016**, 72, 317–321, doi:10.1016/j.lwt.2016.05.009.

Puškaš V.S.; Miljić, U.D.; Djuran J.J.; Vučurović V.M. The aptitude of commercial yeast strains for lowering the ethanol content of wine. *Food Sci Nutri.* **2019**, 8, 1489–1498, doi:10.1002/fsn3.1433.

Quirós, M.; Rojas, V.; Gonzalez, R.; Morales, P. Selection of non-*Saccharomyces* yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* **2014**, 181, 85–91, doi:10.1016/j.ijfoodmicro.2014.04.024.

Ramírez, M.; Velázquez, R.; Maqueda, M.; López-Piñeiro, A.; Ribas, J.C. A new wine *Torulaspora delbrueckii* killer strain with broad antifungal activity and its toxin-encoding double-stranded RNA virus. *Front. Microbiol.* **2015**, 6, doi:10.3389/fmicb.2015.00983.

Rawsthorne, H.; Phister, T.G. A real-time PCR assay for the enumeration and detection of *Zygosaccharomyces bailli* from wine and fruit juices. *Int. J. Food Microbiol.* **2006**, 112, 1–7, doi:10.1016/j.ijfoodmicro.2006.05.003.

Rektor, A.; Kozak, A.; Vatai, G.; Bekassy-Molnar, E. Pilot plant RO-filtration of grape juice, *Sep. Purif. Technol.* **2007**, 57, 473–475, doi:10.1016/j.seppur.2006.07.007.

Rementeria, A.; Rodriguez, J.A.; Cadaval, A.; Amenabar, R.; Muguruza, J.R.; Hernando, F.L.; Sevilla, M.J. Yeast associated with spontaneous fermentations of white wines from the "Txakoli de Bizkaia" region (Basque Country, North Spain). *Int. J. Food Microbiol.* **2003**, 86, 201-207, doi:10.1016/S0168-1605(03)00289-7.

Remize, F.; Roustan, J.L.; Sablayrolles, J.M.; Barre, P.; Dequin, S. Glycerol overproduction by engineered *Saccharomyces cerevisiae* wine yeast strains leads to substantial changes in by-product formation and to a stimulation of fermentation rate in stationary phase. *Appl. Environ. Microbiol.* **1999**, 65, 143–149, doi:10.1002/abio.370190413.

Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. (Eds.), Cytology, Taxonomy and Ecology of Grape and Wine Yeast, Handbook of Enology, vol. 1. John Wiley and Sons, West Sussex (England). **2000**, pp. 1–49, doi:10.1002/0470010363.ch1.

Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. In Handbook of Enology: The Microbiology of Wine and Vinifications, 2nd ed.; Jhon Wiley & Sons, Ltd: Chichester, UK, **2006**, pp. 22–53, doi:10.1002/0470010398.

Roca-Mesa, H.; Sendra, S.; Mas, A.; Beltran, G.; Torija, M.-J. Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. *Microorganisms.* **2020**, 8, 157, doi:10.3390/microorganisms8020157.

Röcker, J.; Strub, S.; Ebert, K.; Grossmann, M. Usage of different aerobic non-*Saccharomyces* yeasts and experimental conditions as a tool for reducing the potential ethanol content in wines. *Eur. Food Res. Technol.* **2016**, 242, 2051–2070, doi:10.1007/s00217-016-2703-3.

Rodrigues, A.J.; Raimbaud, T.; Gonzalez, R.; Morales, P. Environmental factors influencing the efficacy of different yeast strains for alcohol level reduction in wine by respiration. *LWT Food Sci. Technol.* **2016**, 65, 1038–1043. doi:10.1016/j.lwt.2015.09.046.

Romano, P.; Fiore, C.; Paraggio, M.; Caruso, M.; Capece, A. Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* **2003**, 86, 169–180, doi:10.1016/S0168-1605(03)00290-3.

Rossouw, D.; Bauer, F.F. Exploring the phenotypic space of non-*Saccharomyces* wine yeast biodiversity. *Food Microbiol.* **2016**, 55, 32–46, doi:10.1016/j.fm.2015.11.017.

Saha, B.; Torley, P.; Blackmann, J.W.; Schmidtke, L.M. Review of technological strategies to reduce alcohol levels in wines, ALCOHOL LEVEL REDUCTION IN WINE - OENOVITI INTERNATIONAL NETWORK. **2013**, 78-86.

Salamon, A. Techniques to achieve moderate alcohol levels in South African wine. Assignment submitted in partial requirement for the Cape Wine Master Diploma. capewineacademy.co.za. **2006**.

Schmidtke, L.M.; Blackman, J.W.; Agboola, S.O. Production technologies for reduced alcoholic wines. *J. Food Sci.* **2012**, 71, 25–41, doi:10.1111/j.1750-3841.2011.02448.x.

Seguinot, P.; Ortiz-Julien, A.; Camarasa, C. Impact of nutrient availability on the fermentation and production of aroma compounds under sequential inoculation with *M. pulcherrima* and *S. cerevisiae*. *Front. in Microbiol.* **2020a**, 11, 305, doi:10.3389/fmicb.2020.00305.

Seguinot, P.; Bloem, A.; Brial, P.; Meudec, E.; Ortiz-Julien, A.; Camarasa, C. Analysing the impact of the nature of the nitrogen source on the formation of volatile compounds to unravel the

aroma metabolism of two non-*Saccharomyces* strains. *Int. J. Food Microbiol.* **2020b**, 316, 108441, doi:10.1016/jijfoodmicro.2019.108441.

Smart, R.; Robinson, M. Sunlight into Wine: A Handbook for Winegrape Canopy Management. Winetitles, Adelaide. **1991**.

Stoll, M.; Scheidweiler, M,; Lafontaine, M.; Schultz, H.R. Possibilities to reduce the velocity of berry maturation through various leaf area to fruit ratio modifications in *Vitis vinifera* L. *Progres Agricole et Viticole.* **2010**, 7, 68–71.

Sunyer-Figueres, M.; Wang, C.; Mas, A. Analysis of ribosomal RNA stability in dead cells of wine yeast by quantitative PCR. *Int. J. Food Microbiol.* **2018**, 270, 1–4, doi:10.1016/j.ijfoodmicro.2018.01.020.

Taylor, M.W.; Tsai, P.; Anfang, N.; Ross, H.A.; Goddard, M.R. Pyrosequencing reveals regional differences in fruit-associated fungal communities. *Environ. Microbiol.* **2014**, 16, 2848-2858, doi:10.1111/1462-2920.12456.

Tilloy, V.; Ortiz-Julien, A.; Dequin, S. Reduction of ethanol yield and improvement of glycerol formation by adaptive evolution of the wine yeast *Saccharomyces cerevisiae* under hyperosmotic conditions. *Appl. Environ. Microbiol.* **2014**, 80, 2623–2632, doi:10.1128/AEM.03710-13.

Tristezza, M.; Tufariello, M.; Capozzi, V.; Spano, G.; Mita, G.; Grieco, F. The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol.* **2016**, 7. doi:10.3389/fmicb.2016.00670.

Thornton, R.J.; Rodriguez. S.B. Deacidification of red and white wines by a mutant of *Schizosaccharomyces malidevorans* under commercial winemaking conditions. *Food Microbiol.* **1996**, 13, 475–482, doi:10.1006/fmic.1996.0054.

Tronchoni, J.; Curiel, J.A.; Sáenz-Navajas, M.P.; Morales, P.; Blanco, A.D.L.F.; Fernández-Zurbano, P.; Ferreira, V.; Gonzalez, R. Aroma profiling of an aerated fermentation of natural grape must with selected yeast strains at pilot scale. *Food Microbiol.* **2018**, 70, 214–223, doi:10.1016/j.fm.2017.10.008.

VanGuilder, H.D.; Vrana, K.E.; Freeman, W.M. Twenty-five years of quantitative PCR for gene expression analysis. *BioTechniques.* **2008**, 44, 619–626, doi:10.2144/000112776.

Varela, C.; Borneman, A.R. Yeasts of vineyards and wineries. *Yeast.* **2017a**, 34, 111–128, doi:10.1002/yea.3219.

Varela, C.; Barker, A.; Tran, T.; Borneman, A.; Curtin, C. Sensory profile and volatile aroma composition of reduced alcohol Merlot wines fermented with *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Int. J. Food Microbiol.* **2017b**, 252, 1–9, doi:10.1016/j.ijfoodmicro.2017.04.002.

Varela, C.; Dry, P.R.; Kutyna, D.R.; Francis, I.L.; Henschke, P.A.; Curtin, C.D.; Chambers, P.J. Strategies for reducing alcohol concentration in wine. *Aust. J. Grape Wine R.* **2015**, 21, 670–679, doi:10.1111/ajgw.12187.

Varela, C.; Sengler, F.; Solomon, M.; Curtin, C. Volatile flavour profile of reduced alcohol wines fermented with the non-conventional yeast species *Metschnikowia pulcherrima* and *Saccharomyces uvarum*. *Food Chem.* **2016**, 209, 57–64, doi:10.1016/j.foodchem.2016.04.024.

Vendrame, M.; Manzano, M.; Comi, G.; Bertrand, J.; Iacumin, L. Use of propidium monoazide for the enumeration of viable *Brettanomyces bruxellensis* in wine and beer by quantitative PCR. *Food Microbiol.* **2014**, 42, 196–204, doi:10.1016/j.fm.2014.03.010.

Versari, A.; Ferrarini, R.; Parpinello, G.P.; Galassi, S. Concentration of grape must by nanofiltration membranes. *Food Bioprod. Proc.* **2003**, 81, 275–278, doi:10.1205/096030803322438045.

Viana, F.; Gil, J.V.; Vallés, S.,; Manzanares, P. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2009**, 135, 68–74, doi:10.1016/j.ijfoodmicro.2009.07.025.

Vincenzini, M.; Romano, P.; Farris, G.A. Microbiologia del vino. Casa Editrice Ambrosiana. **2005**.

Wang, C.; Esteve-Zarzoso, B.; Mas, A. Monitoring of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, and *Starmerella bacillaris* (Synonym *Candida zemplinina*) populations during alcoholic fermentation by fluorescence in Situ hybridization. *Int. J. Food Microbiol.* **2014**, 191, 1–9, doi:10.1016/j.ijfoodmicro.2014.08.014.

Wang, C.; Esteve-Zarzoso, B.; Cocolin, L.; Mas, A.; Rantsiou, K. Viable and culturable populations of *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (synonym *Candida zemplinina*) during Barbera must fermentation. *Food Res. Int.* **2015a**, 78, 195–200, doi:10.1016/j.foodres.2015.10.014.

Wang, C.; García-Fernández, D.; Mas, A.; Esteve-Zarzoso, B. Fungal diversity in grape must and wine fermentation assessed by massive sequencing quantitative PCR and DGGE. *Front Microbiol.* **2015b**, 6, 1156, doi:10.3389/fmicb.2015.01156.

Wang, C.; Mas, A.; Esteve-Zarzoso, B. The interaction between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast during alcoholic fermentation is species and strain specific. *Front. Microbiol.* **2016**, 7, 502, doi:10.3389/fmicb.2016.00502.

Wang, X.; Glawe, D.A.; Weller, D.M.; Okubara, P.A. Real-Time PCR assays for the quantification of native yeast DNA in grape berry and fermentation extracts. *J. Microbiol. Methods.* **2020**, 168,105794, doi:10.1016/j.mimet.2019.105794.

Yarrow, D. Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman, C.P., Fell, J.W. (Eds.), The Yeasts, a Taxonomic Study. Elsevier, Amsterdam. **1998**, pp. 77–100, doi: 10.1016/B978-044481312-1/50014-9.

Zhang, K.; Wells, P.; Liang, Y.; Love, J.; Parker, D.A.; Botella, C. Effect of diluted hydrolysate as yeast propagation medium on ethanol production. *Bioresour. Technol.* **2019**, 271, 1–8, doi:10.1016/j.biortech.2018.09.080.

Zhang, B.; Shen, J.; Duan, C.; Yan, G. Use of indigenous *Hanseniaspora vineae* and *Metschnikowia pulcherrima* co-fermentation with *Saccharomyces cerevisiae* to improve the aroma diversity of Vidal Blanc icewine. *Front. Microbiol.* **2018**, 9, 2303, doi:10.3389/fmicb.2018.02303.

Zott, K.; Claisse, O.; Lucas, P.; Coulon, J.; Lonvaud-Funel, A.; Masneuf-Pomarede, I. Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol.* **2010**, 27, 559–567, doi:10.1016/j.fm.2010. 01.006.

Zott, K.; Thibon, C.; Bely, M.; Lonvaud-Funel, A.; Dubourdieu, D.; Masneuf-Pomarede, I. The grape must non-*Saccharomyces* microbial community: Impact on volatile thiol release. *Int. J. Food Microbiol.* **2011**, 151, 210–215, doi:10.1016/j.ijfoodmicro.2011.08.02.

ANNEX I

Materials and Methods

Contents

1. Culture media

YPD

YPD (Yeast Extract Peptone Dextrose) is a general medium to grow yeast.

	YPD liquid medium	YPD solid medium
Glucose	20 g/L	20 g/L
Yeast Extract	10 g/L	10 g/L
Bacteriological Peptone	20 g/L	20 g/L
Agar		17 g/L

Mixed well in distilled water and then autoclaved at 121 °C for 15 mins.

WLN agar

WLN (Wallerstein Laboratory Nutrient) agar is a medium to identify yeasts with different morphological profile and colors (Cavazza et al., 1992).

WL Nutrient agar	80 g/L

Bring WL Nutrient agar (Becton, Dickinson and Company, France) to the distilled water to dissolve and then autoclaved at 121 °C for 15 mins.

Lysine agar

Lysine agar is a selective medium that allows non-*Saccharomyces* yeasts to grow.

Yeast Carbon Base	11.75 g/L
L-lysine monohydrochloride	2.5 g/L
Agar	20 /L

Mixed well in distilled water and then autoclaved at 121 °C for 15 mins.

2. Strains store medium

Strains were stored in YPD liquid medium with 40% sterilized glycerol at – 80 °C before inoculation.

3. Must

Synthetic must

Synthetic must is prepared according to Beltran et al. (2004).

Sugar	glucose	100 g/L
	fructose	100 g/L
Acid	DL-malic acid	5 g/L
	citric acid	0.5 g/L
	tartaric acid	3 g/L
Mineral salts	KH_2PO_4	0.75 g/L
	K_2SO_4	0.5 g/L
	$MgSO_4 \cdot 7H_2O$	0.25 g/L
	$CaCl_2 \cdot 2H_2O$	0.16 g/L
	NaCl	0.2 g/L
Ammonium salt	NH_4Cl	0.46 g/L
Anaerobic factors stock solution		1 ml/L
Vitamins stock solution		10 ml/L
Amino acid stock solution		10 ml/L
Oligo-elements stock solution		1 ml/L

Dissolved sugar, acid, mineral salts and ammonium salt in distilled water and then autoclaved at 121 °C for 15 mins. After autoclave, added the four stock solutions previously prepared. Adjusted pH to 3.3 with NaOH and then added the final volume until the required level by distilled water. Finally, sterilized by a 0.2 μm filtration system.

Anaerobic factors stock solution (100 mL)

Ergosterol	1.5 g
Oleic acid	0.5 mL
Tween 80	50 mL
Ethanol (dissolving solution)	Until 100 mL

Dissolved the solution at 70 °C, and stored at 4 °C.

Vitamins stock solution

Myo-inositol	2 g/L
Pantothenate calcium	0.15 g/L
Thiamine hydrochloride	0.025 g/L
Nicotinic acid	0.2 g/L
Pyridoxine	0.025 g/L
Biotine	3 mL (100mg/mL solution)

Dissolved in distilled water, then sterilized by a 0.2 μm filter and stored at – 20 °C.

Amino acid stock solution

Tyrosine (Tyr)	1.95 g/L (heated at 100 °C)
Tryptophan (Trp)	17.42 g/L (70 °C)
Isoleucine (Ile)	3.25 g/L (70 °C)
Aspartic acid (Asp)	4.42 g/L (degas CO_2)
Glutamic acid (Glu)	11.96 g/L (degas CO_2)
Arginine (Arg)	36.79 g/L
Leucine (Leu)	4.81 g/L (heated to dissolve)
Threonine (Thr)	7.54 g/L
Glycine (Gly)	1.82 g/L
Glutamine (Gln)	49.92 g/L
Alanine (Ala)	14.56 g/L
Valine (Val)	4.42 g/L
Methionine (Met)	3.12 g/L
Phenylalanine (Phe)	3.77 g/L
Serine (Ser)	7.8 g/L
Histidine (His)	3.38 g/L
Lysine (Lys)	1.69 g/L
Cysteine (Cys)	2.08 g/L
Proline (Pro)	59.93 g/L

Dissolved in 20 g/L $NaHCO_3$ solution, then sterilized by a 0.2 μm filter and stored at – 20 °C.

Oligo-elements stock solution

$MnSO_4 \cdot H_2O$	4 g/L
$ZnSO_4 \cdot 7H_2O$	4 g/L
$CuSO_4 \cdot 5H_2O$	1 g/L
KI	1 g/L
$CoCl_2 \cdot 6H_2O$	0.4 g/L
H_3BO_3	1 g/L
$(NH_4)_6Mo_7O_{24}$	1 g/L

Dissolved in distilled water, then sterilized by a 0.2 μm filter and stored at 4 °C.

Natural must

Natural must was obtained from Muscat grapes from Finca Experimental Mas dels Frares of Rovira i Virgili University (Constantí, Spain) during the 2019 vintage.

Sugar	219.6 ± 0.57 g/L
Total acidity (as tartaric acid)	4.53 ± 0.05 g/L
Assimilable nitrogen	77.8 mg/L
pH	3.27

Used diammonium phosphate (Panreac Quimica SA, E.U.) to correct the nitrogen concentration. Added dimethyl dicarbonate (0.2 mL/L) (ChemCruz, Santa Cruz Biotechnology, America) to natural must, and kept at 4 °C for 24 h to eliminate the endogenous microorganisms.

4. Monitoring alcoholic fermentation

Density

Density analysis is a common method to monitor fermentation kinetics, due to the decrease in density is directly related to sugar consumption during alcoholic fermentation. An electronic densitometer (Densito 30PX Portable Density Meter, Mettler Toledo, Hospitalet de Llobregat, Spain) was used for measuring density.

Microscope counting

The total cells population is assessed by microscope counting using a Neubauer chamber (Leica Microsystems GMS QmbH, Leica, Germany). Hemocytometer Counting method is used for counting the cells number.

Plate counting

The living cells population during fermentation is analyzed by colony growth on plates. Samples were serially diluted in sterilized Milli-Q water from a Milli-Q purification system (Millipore S.A.S., Molsheim, France). The number of colony forming units per milliliter (CFU/mL) is determined by plating 100 μL of three appropriately chosen dilutions on solid media. The plates are incubated at 28 °C for 2 or 3 days.

5. Enzyme KIT analysis

Glucose, ethanol and glucose/fructose

Glucose, ethanol and glucose/fructose of the samples from the end of fermentation (in YPD liquid medium, synthetic must or natural must) are detected by a Ultrospec 2100 pro UV/Visible Spectrophotometer (Biotech Ltd. Cambridge, England) using D-Glucose, Ethanol and D-glucose/D-fructose Enzymatic BioAnalysis KIT (r-biopharm, Darmstadt, Germany), respectively.

Residual sugars

Residual sugars of samples from the end of fermentation to evaluate whether the fermentation is complete (in synthetic or natural must) are analyzed in a Miura autoanalyzer (EE-MIURAONE Rev., I.S.E. S.r.l., Italy) using D-glucose/D-fructose Enzymatic KIT (Biosystems S.A., Barcelona, Spain).

Assimilable nitrogen

The concentration of assimilable nitrogen is analyzed in the Miura autoanalyzer using Ammonia and α-Aminic nitrogen Enzymatic KIT (Tecnología Difusión Ibérica, S.L., Barcelona, Spain).

6. HPLC analysis

Ethanol, glycerol, and organic acids (acetic acid, citric acid, malic acid, tartaric acid, lactic acid and succinic acid) in samples and sugars (glucose and fructose) after 48 h of fermentation are determine by high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) as described by Quirós et al. (2010). The HPLC is equipped with a Hi-PlexHcolumn (300mm X 7.7 mm) inside a 1260 MCT column compartment (Infinity II Multicolumn Thermostat) connected to MWD (G1365B multiwavelength detector) and RID detectors (1260 Infinity II refractive index detector) (Agilent Technologies, Waldbronn, Germany). The temperature of the column is maintained at 60 °C for a 30 min run time, and the mobile phase is 5 mM H2SO4 with a flow rate of 0.6 mL/min. The sample injection volume is 10 μL. Before injection, samples are filtered through 0.22 μm filters (Dominique Dutscher, Brumath, France). OpenLAB CDS (Agilent Technologies, Santa Clara, CA, USA) is used to analyze HPLC chromatographs.

7. DNA extraction and pretreatment

DNA extraction

According to the DNeasy Plant Mini Kit (Qiagen, USA) and Hierro et al. (2006).

1. Wash cells pellet using 1mL sterilized distilled water. Centrifuge 10 mins at 12000 rpm.
2. Resuspend cells in 700 µL lysis solution of **Buffer AP1**. Transfer the solution into a microcentrifuge tube (2 mL) containing 1 g glass beads with 0.5 mm of diameter (previously sterilized).
3. Lysate cells in a mini bead-beater (Biospec Products Inc., Bartlesville, Okla) by subjecting samples three times for 1 min at maximum agitation with an interval of 1 min on ice.
4. Centrifuge for 1 min at 4 °C and 10000 rpm.
5. Transfer 400 µL the solution to a new 1.5 mL microcentrifuge tube for RNA treatment.
6. Add 4 µL **RNase A** in cells suspension and incubate for 10 mins at 65 °C. Mix 2 – 3 times during incubation by inverting tube.
7. Add 130 µL **Buffer P3**. Mix and incubate for 5 mins on ice. Centrifuge for 5 mins at 4 °C and 14000 rpm.
8. Pipet the lysate 500 µL into a **QIAshredder Mini spin column**. Centrifuge for 2 mins at 14000 rpm.
9. Transfer the flow-through fraction 450 µL into a new 1.5 mL microcentrifuge tube and add 675 µL **Buffer AW1**. Mix well by pipetting.
10. Pipet the mixture 650 µL into a **DNeasy Mini spin column**. Centrifuge for 1 min at 10000 rpm. Discard the flow-through. Repeat this step once with the remining sample.

11. Place the **Dneasy Mini spin column** into a new 2 mL **collection tube**, add 500 μL **Buffer AW2**. Centrifuge for 1 min at 10000 rpm and discard the flow-through.
12. Add the other 500 μL **Buffer AW2**. Centrifuge for 2 min at 14000 rpm.
13. Transfer the **Dneasy Mini spin column** to a new 1.5 mL microcentrifuge tube. Add 50 μL **Buffer AE** for elution. Incubate for 5 mins at room temperature and centrifuge for 1 min at 8000 rpm.
14. Add 50 μL **Buffer AE** for elution again. Incubate for 5 mins at room temperature and centrifuge for 1 min at 8000 rpm.

    * The purity DNA should be stored at – 20 °C.

    * Reagents and materials indicated in bold are provided by the commercial kit.

PMA treatment

The PMAxx™ viability dye (Biotium Inc., Fremont, CA, USA) is used to process cells before the DNA extraction. The cell membrane-impermeable PMAxx™ selectively enters dead cells with compromised membranes and after light treatment, covalently modifies the DNA. Subsequent PCR amplification of PMAxx™-modified DNA templates is inhibited, allowing selective quantitation of DNA from viable cells. The procedure is according to Andorrà et al. (2010) and Navarro et al. (2020).

1. Collect 500 μL must homogenates during fermentation. Centrifuge at 10000 rpm for 2 mins. Remove the supernatant.
2. Wash pallets once with 1 mL sterilized distilled water. Centrifuge at 10000 rpm for 2 mins. Remove the supernatant.
3. Resuspend the pallets into 500 μL sterilized distilled water. Add 25 μM PMAxx™. Incubate in the dark for 10 mins.

4. Expose samples twice to light for 30 s with an interval of 1 min on ice. Centrifuge at 10000 rpm for 2 mins. Remove the supernatant.
5. Add 1 mL sterilized distilled water to eliminate the excess of the PMAxx™ unbound. Centrifuge for 2 mins at 12000 rpm. Discard the supernatant.
6. Extract the DNA directly by DNeasy Plant Mini Kit (Qiagen) and proceed with qPCR analysis.

   * The pellets should be stored at – 20 °C.

The standard curve of species with PMAxx™ treatment in qPCR requires a preculture of yeasts.

a) Inoculate one yeast colony from YPD agar to YPD liquid medium. Incubate at 28 °C for 24 h.
b) Take a certain amount of sample containing 10^8 cells. Centrifuge for 2 mins at 12000 rpm. Remove the supernatant.
c) Wash pellets once with 1 mL sterilized distilled water. Centrifuge for 2 mins at 12000 rpm. Remove the supernatant.
d) Process pellets with PMAxx™. According to the steps 3 – 6 mentioned above.

8. Molecular techniques

5.8s-ITS-RFLP analysis

5.8s-ITS-RFLP analysis is used for the identification of yeast species based on Esteve-Zarzoso et al. (1999). This method includes amplifying the region comprised between the 18S and 26S rDNA genes, digesting the latter with several restriction enzymes, and performing restriction profile analysis.

The main reaction parameters are listed as follows.

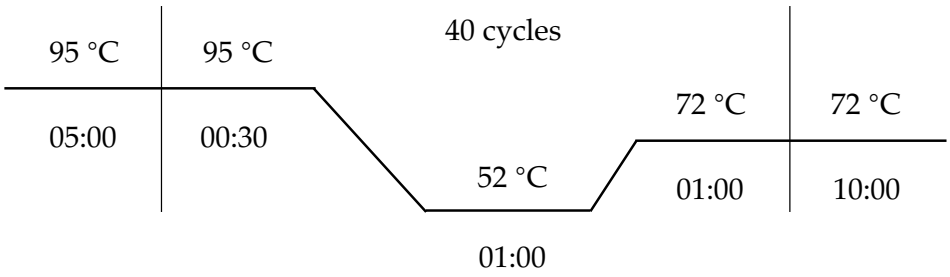
Primers

Primers	Sequence
ITS1	5′-TCCGTACGTGAACCTGCGG-3′
ITS4	5′-TCCTCCGCTTATTGATATGC-3′

The mixture for the amplification

Primer ITS1 (10 μM)	1 μL
Primer ITS4 (10 μM)	1 μL
dNTPs (10 mM each dNTP)	1 μL
$MgCl_2$ (50 mM)	3 μL
Buffer 10x without Mg^{2+}	5 μL
Taq DNA polymerase (5 U/μL)	0.5 μL
DNA	1 μL
Sterilized Milli-Q water	37.5 μL
Total	50 μL

PCR program



After obtained PCR products, the products are visualized by 1.5% agarose gel electrophoresis containing ethidium bromide.

qPCR analysis

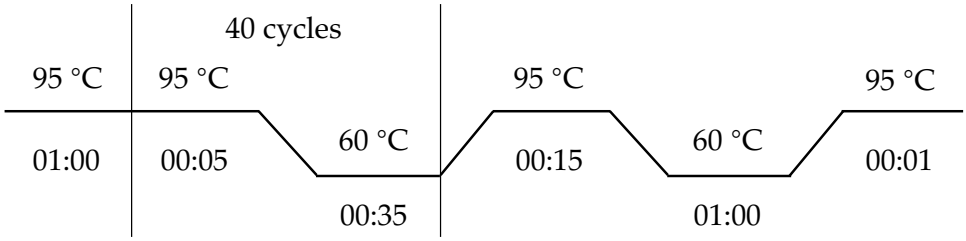
This technique is used for the quantification of yeast species. Standard curves are established for each species between Ct value and a series dilution (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10 cells/mL) of purified DNA. The primers designed for qPCR analysis are listed.

Species	Primer name	Primer sequence 5′-3′	References
S. cerevisiae	CESP-F	ATCGAATTTTTGAACGCACATTG	Hierro et al., 2007
	SCER-R	CGCAGAGAAACCTCTCTTTGGA	
M. pulcherrima	MP2-F	AGACACTTAACTGGGCCAGC	García et al., 2017
	MP2-R	GGGGTGGTGTGGAAGTAAGG	
T. delbrueckii	Tods L2	CAAAGTCATCCAAGCCAGC	Zott et al., 2010
	Tods R2	TTCTCAAACAATCATGTTTGGTAG	
Z. bailii	ZBF1	CATGGTGTTTTGCGCC	Rawsthorne and Phister, 2006
	ZBR1	CGTCCGCCACGAAGTGGTAGA	

The mixture for the amplification

Primer Forward (10 μM)	0.8 μL
Primer Reverse (10 μM)	0.8 μL
ROX Reference Dye (50X)	0.08 μL
TB Green Premix Ex Taq II (2X)	10 μL
DNA	2 μL
Sterilized Milli-Q water	6.32 μL
Total	20 μL

The amplification reaction



9. References

Andorrà, I.; Esteve-Zarzoso, B.; Guillamón, J.M.; Mas, A. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. *Int. J. Food Microbiol.* **2010**, 144, 257–262, doi:10.1016/j.ijfoodmicro.2010.10.003.

Beltran, G.; Novo, M.; Rozès, N.; Mas, A.; Guillamón, J.M. Nitrogen catabolite repression in during wine fermentations. *FEMS Yeast Res.* **2004**, 4, 625–632, doi:10.1016/j.femsyr.2003.12.004.

Cavazza, A.; Grando, M. S.; Zini, C. Rilevazione della flora microbica dimostie vini. Vignevini. **1992**, 9, 17–20.

Esteve-Zarzoso, B.; Belloch, C.; Uruburu, F.; Querol, A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* **1999**, 49, 329–337, doi:10.1099/00207713-49-1-329.

García, M.; Esteve-Zarzoso, B.; Crespo, J.; Cabellos, J.M.; Arroyo, T. Yeast monitoring of wine mixed or sequential fermentations made by native strains from D.O. "Vinos de Madrid" using real-time quantitative PCR. *Front. Microbiol.* **2017**, 8, 2520, doi:10.3389/fmicb.2017.02520.

Hierro, N.; Esteve-Zarzoso, B.;González, A.; Mas, A.; Guillamón J.M. Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* **2006**, 72, 7148–7155, doi:10.1128/AEM.00388-06.

Hierro, N.; Esteve-Zarzoso, B.; Mas, A.; Guillamón, J.M. Monitoring of *Saccharomyces* and *Hanseniaspora* populations during alcoholic fermentation by real-time quantitative PCR. *FEMS Yeast Res.* **2007**, 7, 1340–1349, doi:10.1111/j.1567-1364.2007.00304.x.

Navarro, Y.; Torija, M.J.; Mas, A.; Beltran, G. Viability-PCR allows monitoring yeast population dynamics in mixed fermentations including viable but non-culturable yeasts. *Foods.* **2020**, 9, 1373, doi:10.3390/foods9101373.

Quirós, M.; Gonzalez-Ramos, D.; Tabera, L.; Gonzalez, R. A new methodology to obtain wine yeast strains overproducing mannoproteins. Int. J. *Food Microbiol.* **2010**, 139, 9–14, doi:10.1016/j.ijfoodmicro.2014.04.024.

Rawsthorne, H.; Phister, T.G. A real-time PCR assay for the enumeration and detection of *Zygosaccharomyces bailii* from wine and fruit juices. *Int. J. Food Microbiol.* **2006**, 112, 1–7, doi:10.1016/j.ijfoodmicro.2006.05.003.

Zott, K.; Claisse, O.; Lucas, P.; Coulon, J.; Lonvaud-Funel, A.; Masneuf-Pomarede, I. Characterization of the yeast ecosystem in grape must and wine using real-time PCR. *Food Microbiol.* **2010**, 27, 559–567, doi:10.1016/j.fm.2010. 01.006.

ANNEX II

Publications

microorganisms

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Article

A Rapid Method for Selecting Non-*Saccharomyces* Strains with a Low Ethanol Yield

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Abstract: The alcohol content in wine has increased due to external factors in recent decades. In recent reports, some non-*Saccharomyces* yeast species have been confirmed to reduce ethanol during the alcoholic fermentation process. Thus, an efficient screening of non-*Saccharomyces* yeasts with low ethanol yield is required due to the broad diversity of these yeasts. In this study, we proposed a rapid method for selecting strains with a low ethanol yield from forty-five non-*Saccharomyces* yeasts belonging to eighteen species. Single fermentations were carried out for this rapid selection. Then, sequential fermentations in synthetic and natural must were conducted with the selected strains to confirm their capacity to reduce ethanol compared with that of *Saccharomyces cerevisiae*. The results showed that ten non-*Saccharomyces* strains were able to reduce the ethanol content, namely, *Hanseniaspora uvarum* (2), *Issatchenkia terricola* (1), *Metschnikowia pulcherrima* (2), *Lachancea thermotolerans* (1), *Saccharomycodes ludwigii* (1), *Torulaspora delbrueckii* (2), and *Zygosaccharomyces bailii* (1). Compared with *S. cerevisiae*, the ethanol reduction of the selected strains ranged from 0.29 to 1.39% (v/v). Sequential inoculations of *M. pulcherrima* (Mp51 and Mp FA) and *S. cerevisiae* reduced the highest concentration of ethanol by 1.17 to 1.39% (v/v) in synthetic or natural must. Second, sequential fermentations with *Z. bailii* (Zb43) and *T. delbrueckii* (Td Pt) performed in natural must yielded ethanol reductions of 1.02 and 0.84% (v/v), respectively.

Keywords: alcoholic fermentation; wine yeast; sequential inoculation; ethanol reduction; *Metschnikowia pulcherrima*; *Torulaspora delbrueckii*; *Zygosaccharomyces bailii*

1. Introduction

Global climate change has caused an increase in the alcohol content of wines in recent decades [1–3]. Specifically, global warming has accelerated maturation, increased the total soluble solids content and pH, and unbalanced the maturation of phenolic compounds and the increase in sugar concentration [2]. If grapes are harvested when phenolic compounds are mature, the grape must will have high concentration of sugars and low acidity, which produces wines with a high ethanol content. Otherwise, if the harvest occurs before that point, when sugar accumulation and pH are appropriate, wines will present a reduction in several characteristics (aroma, taste, and astringency) due to insufficient phenolic maturation. Regarding alcoholic fermentation, a high concentration of ethanol may lead to sluggish and stuck fermentations [4–6]. In addition, it can break the balance among acids, sugars, and tannins and develop unpleasant characteristics due to the enhancement of bitterness and burning sensation during tasting [7]. There are other reasons to achieve a lower ethanol content in wines, from their reduction in aromatic profile to the tax increase that will impact the final price of wines.

Previous studies about the reduction of alcohol in wines have focused on viticulture management, prefermentation, and postfermentation treatments and microbiological strategies during

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Article

Effect of a Multistarter Yeast Inoculum on Ethanol Reduction and Population Dynamics in Wine Fermentation

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Abstract: Microbiological strategies are currently being considered as methods for reducing the ethanol content of wine. Fermentations started with a multistarter of three non-*Saccharomyces* yeasts (*Metschnikowia pulcherrima* (Mp), *Torulaspora delbrueckii* (Td) and *Zygosaccharomyces bailii* (Zb)) at different inoculum concentrations. *S. cerevisiae* (Sc) was inoculated into fermentations at 0 h (coinoculation), 48 h or 72 h (sequential fermentations). The microbial populations were analyzed by a culture-dependent approach (Wallerstein Laboratory Nutrient (WLN) culture medium) and a culture-independent method (PMA-qPCR). The results showed that among these three non-*Saccharomyces* yeasts, Td became the dominant non-*Saccharomyces* yeast in all fermentations, and Mp was the minority yeast. Sc was able to grow in all fermentations where it was involved, being the dominant yeast at the end of fermentation. We obtained a significant ethanol reduction of 0.48 to 0.77% (*v*/*v*) in sequential fermentations, with increased concentrations of lactic and acetic acids. The highest reduction was achieved when the inoculum concentration of non-*Saccharomyces* yeast was 10 times higher (10^7 cells/mL) than that of *S. cerevisiae*. However, this reduction was lower than that obtained when these strains were used as single non-*Saccharomyces* species in the starter, indicating that interactions between them affected their performance. Therefore, more combinations of yeast species should be tested to achieve greater ethanol reductions.

Keywords: wine; PMA-qPCR; *Metschnikowia pulcherrima*; *Torulaspora delbrueckii*; *Zygosaccharomyces bailii*; mixed fermentation; coinoculation; sequential fermentation

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1. Introduction

In recent years, the average ethanol concentration in wine has increased, mainly due to climate change and consumer preference for wine styles [1–3]. Different strategies have been applied to reduce ethanol production in wine, such as decreasing the leaf area to lower the sugar content in grape berries, reducing the maturity of grapes, removing sugar from the grape must, developing or screening low-alcohol wine yeasts and removing alcohol from wine (reviewed by [4]). Compared with microbiological strategies, other strategies might have negative effects on wine, such as delaying maturity, reducing the yield of berries, causing a significant reduction in anthocyanins, soluble solids and volatile compounds, and decreasing the wine color [5–8]. Therefore, microbiological strategies were considered to be effective and accompanied by smaller impacts on the wine sensory profile and quality. In particular, the use of non-*Saccharomyces* yeast strains for reducing the alcohol content of wines has been proven to improve the wine aroma complexity and has become a consistent proposal [9–11].

Non-*Saccharomyces* yeasts have been applied in fermentations to reduce ethanol using different inoculation strategies. For example, several studies have shown that single-culture fermentations with *Hanseniaspora uvarum*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Starmerella bombicola*, *Starmerella bacillaris*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces sapae* species are able to reduce the ethanol

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