

Review

# The Effect of Non-*Saccharomyces* and *Saccharomyces* Non-*Cerevisiae* Yeasts on Ethanol and Glycerol Levels in Wine

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**Abstract:** Non-*Saccharomyces* and *Saccharomyces* non-*cerevisiae* studies have increased in recent years due to an interest in uninoculated fermentations, consumer preferences, wine technology, and the effect of climate change on the chemical composition of grapes, juice, and wine. The use of these yeasts to reduce alcohol levels in wines has garnered the attention of researchers and winemakers alike. This review critically analyses recent studies concerning the impact of non-*Saccharomyces* and *Saccharomyces* non-*cerevisiae* on two important parameters in wine: ethanol and glycerol. The influence they have in sequential, co-fermentations, and solo fermentations on ethanol and glycerol content is examined. This review highlights the need for further studies concerning inoculum rates, aeration techniques (amount and flow rate), and the length of time before *Saccharomyces cerevisiae* sequential inoculation occurs. Challenges include the application of such sequential inoculations in commercial wineries during harvest time.

**Keywords:** non-*Saccharomyces*; *Saccharomyces* non-*cerevisiae*; yeast; wine; ethanol; glycerol

## 1. Introduction

After carbon dioxide (CO<sub>2</sub>), ethanol and glycerol are the most abundant compounds produced during alcoholic fermentation. The levels of ethanol and glycerol in wine depend upon many factors, such as seasonal events affecting the concentration of grape sugar, and winemaking decisions, including fermentation conditions and fermenting yeasts [1].

At a commercial scale, inoculations with *Saccharomyces cerevisiae* strains are often preferred over those with non-*Saccharomyces* or *S. non-cerevisiae* yeasts, because the latter is considered responsible for incomplete fermentations (and consequently high levels of residual sugar in wine), and they produce high concentrations of acetic acid and ethyl acetate [2,3]. Nevertheless, non-*Saccharomyces* or *S. non-cerevisiae* yeasts are important to winemakers, particularly to those who target wines with unique sensory characters that are popularly recognised as typical of their geographical origin or variety [4–7]. These yeasts are also popular among winemakers who choose to produce less alcoholic wines [8].

Although non-*Saccharomyces* and *S. non-cerevisiae* yeasts are sought after for their specific oenological characteristics, it is a challenge for some of these yeasts to conduct a complete fermentation to a desired level of dryness. This is very important to winemakers, in part because finished wines with higher levels of residual sugars above 0.5 g/L require high doses of sulfur dioxide (SO<sub>2</sub>) to ensure their microbial stability to prevent wine spoilage. Therefore, inoculations of non-*Saccharomyces/S. non-cerevisiae* in mixed cultures with *S. cerevisiae* strains, which have higher fermentation rates, have been studied to ensure complete fermentation.

The strategy of using selected mixed cultures for alcoholic fermentation is believed to be the key to produce wines with desirable characteristics that meet changing market demands with less ethanol but still with flavours comparable to standard wines [9]. This strategy is carried out by two different methods of inoculation: (1) co-inoculation, which involves concurrent inoculations of non-*Saccharomyces/S. non-cerevisiae* yeasts at high cell concentration (e.g., 10<sup>7</sup> cell/mL) with *S. cerevisiae*; and/or (2) sequential inoculation, which involves inoculating non-*Saccharomyces/S. non-cerevisiae* yeasts to start the fermentation and continue for a determined amount of time alone, and inoculating *S. cerevisiae* to take over and complete the fermentation [9,10]. The time period before carrying out the sequential *S. cerevisiae* inoculation and the *Saccharomyces/non-Saccharomyces* or *Saccharomyces non-cerevisiae* inoculum ratio are both important parameters that affect the fermentation kinetics and oenological outcomes, and the former generally varies between 1 and 3 days [11–14].

Many reviews have studied different perspectives of non-*Saccharomyces/S. non-cerevisiae* yeasts for modern winemaking practices, including their influence on different wine quality parameters with an emphasis on traits such as the primary (or varietal) and secondary (or fermentative) aromas of wines, acidity, freshness, as well as specific styles of wines (such as traditional method sparkling wines and red table wines) [3,9,15–21].

The aim of this review is to highlight those studies that have shown a direct link between the use of non-*Saccharomyces* or *S. non-cerevisiae* and the concentration of ethanol and glycerol in wine or synthetic media. The first part of this review provides an overview of ethanol and glycerol as contributors to wine sensory characteristics, and a general overview of non-*S. cerevisiae* or *S. non-cerevisiae* yeasts. The second part of this review provides more specific details of individual non-*S. cerevisiae* or *S. non-cerevisiae* species that are relevant to the wine industry. We conclude this review by suggesting what additional research might help winemakers have greater control over wine quality outcomes.

## 2. Ethanol Reduction

Ethanol is produced by yeast during the alcoholic fermentation and is generally found in the range of 11.5–15% *v/v* in wines. It is an important wine component that directly effects organoleptic properties, aging, and wine stability [22]. The impact of ethanol on the sensory profile of wines and other alcoholic beverages has been recently reviewed [23]. Ethanol influences taste and mouthfeel sensations, alters the sensation of sweetness, increases bitterness, decreases sourness, and contributes to the hotness sensation and body of the wine [24–27]. Ethanol can also decrease the volatility of aroma compounds by increasing their solubility in the wine [28], making small compounds such as fruity-driven ethyl esters and acetates less recognisable by human senses.

According to the International Organisation of Vine and Wine (OIV), the alcohol strength of wines must be a minimum of 8.5% *v/v*, although in cool climate wine regions, this value can be lowered to 7% *v/v* [29]. Over the past two decades, ethanol content in wines has been noticeably increasing in some regions by 0.1–1% per year [30,31]. Apart from hotter climates leading to higher sugar berry levels at harvest and therefore, higher alcohol contents in wine [32], one of the main reasons behind this progressive increase is consumer demand for specific wine styles, which are described as rich, well-structured, with a flavour profile dominated by dark, ripe fruits [33]. This style requires optimal grape maturity and higher sugar content of 240 g/L or more [34].

Nonetheless, an increasing trend for reduced alcohol in beverages (broadly defined as containing 9% to 13% *v/v* ethanol), and low-alcohol (0.5–2% *v/v*) wines by consumers has been recently

observed [35,36]. Increasing health and safety consciousness and global initiatives towards moderating alcohol consumption are reasons for producing lower alcohol wines that appeal to wine drinkers [37]. Wines may also be subjected to higher taxation depending on their alcohol content, which increases the final cost of wines to the consumer [38]. Since ethanol is the main source of caloric content in wine, there is also a risk of a negative impact on wine export to countries where health labeling of foods and beverages served at restaurants is voluntary or mandatory [39].

During winemaking, high sugar and therefore ethanol can cause sluggish and stuck alcoholic fermentations and can be challenging for successful malolactic fermentations [40,41]. As a means to address these issues, methods have been studied that include lowering the final ethanol content of wine using a wide selection of interventions. These can be grouped into (1) pre- (e.g., viticultural, juice dilution, and fermentation of early harvest fruit); (2) concurrent (e.g., non-*Saccharomyces* yeasts, modified yeasts, and arrested fermentation) [42,43]; and (3) post-fermentation (e.g., non-membrane and membrane ethanol removal) [43–45]. The use of microbiological approaches such as inoculation with non-*Saccharomyces* yeasts for producing wines with less ethanol is a promising alternative to the removal of ethanol by membrane based-approaches [11,46–48]. The advantages associated with the use of low-ethanol/high-glycerol yielding yeasts include their relatively easy application and lower costs when compared to more expensive and less eco-friendly approaches, such as membrane contactors, nanofiltration, or the spinning cone column [49,50]. However, it is important to acknowledge that low-ethanol/high-glycerol yielding yeasts are much less effective than the membrane-based processes in terms of ethanol reductions [44], and they are perhaps only suitable when winemakers want to achieve a reduction in ethanol content by up to 3.0% *v/v* [49].

Inoculations with non-*Saccharomyces* yeasts can be used as a strategy to produce lower alcohol wines due to the yeasts' different sugar utilisation pathways, including respiration, alcoholic fermentation, and glycerol–pyruvic metabolisms, and different regulatory mechanisms, in comparison to *S. cerevisiae* [51]. While the theoretical sugar-to-ethanol yield for a complete fermentation by *S. cerevisiae* generally ranges from 90% to 95%, the residual sugar is consumed via alternative metabolic pathways and biomass biosynthesis [52]. On the other hand, ethanol yield and the by-products formed vary immensely amongst non-*Saccharomyces* yeasts [23]. For example, due to the Crabtree effect, *S. cerevisiae* prefers fermentation metabolism rather than respiration when the sugar amount exceeds 10 g/L [40]. In contrast, among non-*Saccharomyces* yeasts, there are strains and species that can consume sugar with aerobic respiration regardless of sugar concentration [53,54] without contributing significantly to the final ethanol level of the wine. Therefore, non-*Saccharomyces* yeasts have been studied under partial and controlled aeration strategies during fermentation to achieve lower ethanol by allowing part of the sugar to be consumed via respiration rather than alcoholic fermentation [47,55]. However, an increase in undesirable volatile compounds, such as acetic acid and ethyl acetate, are the main limiting factors of the aeration strategies that require the application of a proper aeration regime [55–57].

As a response to interest in reduced alcohol levels in wines, researchers studied non-*S. cerevisiae* and *S. non-cerevisiae* yeast species [49,56,58–60] (Table 1). Several non-*Saccharomyces* yeast strains were identified, including *Metschnikowia pulcherrima*, and two species of *Kluyveromyces*, which have the capacity to decrease ethanol yields by respiration [59]. *M. pulcherrima* AWRI 1149 was identified as a potential yeast to produce wine with a reduced ethanol concentration, having been identified following the evaluation of 50 non-*Saccharomyces* isolates under limited aeration conditions, and in sequential inoculations with *S. cerevisiae* [49]. A similar study with 48 non-*Saccharomyces* yeast isolates identified *Torulaspora delbrueckii* AWRI 1152 and *Zygosaccharomyces bailii* AWRI 1578 yeasts as suitable for reducing ethanol [56]. More recently, the respiratory, fermentative, and physiological characteristics of 114 non-*Saccharomyces* yeasts were evaluated [60]. Taking into account their ability to reduce ethanol content *Hanseniaspora uvarum* BHu9 and BHu11, *Hanseniaspora osmophila* BHo51, *Starmerella bacillaris* (synonym. *Candida zemplinina*) BSb55, and *Candida membranaefaciens* BCm71 were selected as candidates for co-fermentations.

### 3. Glycerol

Glycerol is the most abundant yeast metabolism by-product after ethanol and CO<sub>2</sub>. It is produced from dihydroxyacetone phosphate, which is first reduced to glycerol-3-phosphate via glycerol-3-phosphate dehydrogenase (GPDH), and then converted into glycerol by a specific phosphatase. This is a non-volatile 3-hydroxy alcohol, which is a polyol also known as a sugar alcohol. Glycerol is a viscous liquid at room temperature and appears to contribute to mouthfeel and viscosity at, or above 28 g/L and sweetness in the range of 5–12 g/L [42,61–63]. However, Nieuwoudt, et al. [64] did not find any link between wine quality (expressed as the number of medals received at a wine competition) and concentrations of glycerol in wine, and Goold et al. [42] concluded in their review that glycerol had only a minimal influence on the viscosity of wine.

The synthesis of glycerol and acetic acid, in addition to ethanol, are both linked to redox balance [22,42]. The significance of glycerol synthesis to redox balance has been suggested to be due to the inability of mutants (unable to synthesise glycerol) to grow in anaerobic conditions [22,65]. During the stationary phase of yeast during fermentation, glycerol synthesis has been found to be associated with redox balance by removing excess reducing power [22,66].

Many factors can influence the production of glycerol, which is in general more abundant in wines fermented with non-*Saccharomyces* than those fermented with *S. cerevisiae* [67–69], and in red wine (approximately 10.5 g/L) compared to white wines (approximately 7 g/L) [64]. Glycerol is generally more abundant in red wines in part because red juice typically ferments at higher temperatures (20–25 °C) than white wines (<20 °C). Yet, fermenting temperatures positively influence the production of glycerol by yeasts, and non-*Saccharomyces* and *S. non-cerevisiae* are not an exception. For example, increasing the fermentation temperature from 16 to 20 °C increased the glycerol content from 1.69 to 3.04 g/L in co-fermentations of *Candida stellata* and *S. cerevisiae* [52]. A significant increase in the glycerol content of a grape juice was also observed after increasing the temperature from 12 to 25 °C, with fermentations carried out by pure *Saccharomyces paradoxus* reporting an increment of approximately 2.5 g/L, for example [12]. The sugar level of grapes at harvest (and therefore in the juice) also influences the production of glycerol, because this compound is accumulated by yeast to combat dehydration by balancing the intracellular osmolarity with that of the medium [1]. This effect has become even more evident in recent times due to hotter seasonal temperatures compressing the ripening windows of different grape varieties in warm climates. This has meant that winemakers delay the harvest date because of wineries operating at full capacity, thereby causing a part of the crop to overripe in the vineyard [70]. Hranilovic, et al. [71] reported that the glycerol content of a Shiraz wine from early harvest grapes (approximately 265 g/L TSS) was much higher than those from the late harvest (approximately 325 g/L TSS), with a *M. pulcherrima* strain (followed by a *S. cerevisiae* inoculum), producing early and late harvest wines with 10.51 and 12.59 g/L glycerol, respectively. Juice with high sugar concentration also leads to an excess in acetic acid, which can be explained by yeasts trying to maintain redox balances by using surplus NAD(P)<sup>+</sup> accumulated during the synthesis of fermentation metabolites [62,72–74].

The growth of non-*Saccharomyces* yeast species such as *Lachancea thermotolerans*, *T. delbrueckii*, and *M. pulcherrima* strictly depends upon oxygen availability [57,75,76]. When the effect of oxygen availability on glycerol production by non-*Saccharomyces* was evaluated, oxygenation at three dissolved oxygen levels of 0.08, 0.41, and 1.71 mg/L resulted in glycerol reduction [76]. This was evident for a *T. delbrueckii* strain in co-fermentation with *S. cerevisiae*, with the glycerol content decreasing from 6.79 g/L in the *T. delbrueckii* anaerobic treatment up to 1.09 g/L in the co-inoculation treatment with 1.71 mg/L of dissolved oxygen. Different results were reported by Morales et al. [57], who observed increased glycerol yields for *M. pulcherrima*/*S. cerevisiae* mixed cultures under controlled oxygenation conditions (sparged with pure air, nitrogen, or mixtures of both) during the first 48 h of fermentation, and anaerobic conditions thereafter.

The non-*Saccharomyces* yeasts, which have the capacity to redirect the sugar consumption for the production of alternative compounds, rather than ethanol, have been studied in wines

with reduced ethanol content. These alternative compounds could be glycerol and pyruvic acid produced via glycerol–pyruvic metabolisms. Alternatively, before sugar is utilised during alcoholic fermentation, sugars can be consumed via respiratory metabolism [40], which is the case with various non-*Saccharomyces* yeasts with lower Crabtree effect.

Other factors can have an impact on the formation of glycerol by non-*Saccharomyces*. These include the concentration of nitrogen and sulfites [8]. Limited nitrogen concentrations in the must (in the form of amino acids and ammonium) can lead to a significant increase of glycerol production. By contrast, higher levels of sulfur dioxide lead to higher levels of glycerol [30]. Increased glycerol production has been found to be linked to increased acetic acid, which is easily detected due to its vinegar smell [3,18,77].

Upon a thorough literature review, we identified five non-*Saccharomyces* yeasts that have been widely studied due to their different oenological traits; *Schizosaccharomyces pombe*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, *Candida stellata*, and *Torulasporea delbrueckii*. These five non-*Saccharomyces* yeasts have different pathways that result in ethanol reduction and glycerol production while influencing different parameters of the chemical composition of wines. The following sections of this review present current knowledge regarding the effect of alternative yeasts that influence ethanol and glycerol concentrations while highlighting gaps in our knowledge that require further research.

#### 4. *Schizosaccharomyces Pombe*

*Schizosaccharomyces pombe* is a widely studied yeast due to its particular ability to moderate wine acidity via malic acid degradation [78]. Among its other promising traits is the ability to enhance the color of red wines and reduce Ochratoxin A, biogenic amines, and ethyl carbamate [79–81]. The most detrimental metabolites produced by spoilage yeasts in pure culture or spoiled juices have been found to decrease in mixed fermentations carried out at the laboratory scale [2,82]. The main characteristics of *S. pombe* and its application in winemaking were reviewed recently [83,84]. *S. pombe* is commercially available as an alternative method to de-acidity wine [19].

Unlike some non-*Saccharomyces* yeast species, *S. pombe* is capable of fermenting wines up to comparable concentrations of *Saccharomyces*, in the range of 10–15% *v/v* ethanol, depending on the strain and presence of aeration [85]. Malo-alcoholic fermentation [86] and the glycerol–pyruvic pathway [87] observed in *S. pombe* inoculations have different impacts on the final ethanol content of the wines. Therefore, while some studies reported an ethanol reduction with inoculations involving *S. pombe* [87], others have reported no difference or even an increase [88–91].

The use of *S. pombe* (strain 938) for white winemaking was investigated by Benito et al. [87], with sole, mixed, and sequential fermentations in conjunction with *S. cerevisiae* (*Cru Blanc*). All the strains in the study were able to ferment the wines to dryness. The sole fermentation of *S. pombe* showed 0.65% *v/v* lower ethanol compared to *S. cerevisiae* alone, values being 13.18% *v/v* and 12.53% *v/v*, respectively. Similar results were obtained with the sequential inoculation, with 0.4% *v/v* lower ethanol value in comparison to the control treatment [87]. Loira et al. [88] studied the effect on Syrah wine sensory quality of *S. pombe* strains (938, V1 and 4.2) in mixed and sequential fermentations with *S. cerevisiae* strain 7VA. *S. pombe* was not used as a sole inoculum in this study. Ethanol reduction did not occur in the mixed or sequential fermentations with *S. pombe* and *S. cerevisiae* compared to treatments with a sole inoculum of *S. cerevisiae*. In this study, slightly higher ethanol values that were not statistically significant were reported for mixed and sequential fermentations with *S. pombe* strains (13.2% to 13.5% *v/v*) compared to *S. cerevisiae* (13.2% *v/v*) [88]. Although the same strains of *S. pombe* (938) were used in these studies, different delay times were applied for the sequential inoculation of *S. cerevisiae* (48 h versus 7 days), and different strains of *S. cerevisiae* were used for the comparison.

Increased ethanol levels have been reported in studies that used *S. pombe* where juices had high malic acid content. *S. pombe* (Y0119) was used in a sequential inoculation with *S. cerevisiae* (NT116) to ferment Kei-apple (*Dovyalis caffra* L.) juice, which contained high malic acid (45 g/L). An increase in



ethanol level (6.08% *v/v*) was reported compared to sole inoculation with *S. cerevisiae* (4.67% *v/v*) [91]. Similarly, slightly higher ethanol values (approximately 0.2% to 0.5% *v/v*) were reported in a study where *S. pombe* was used in sequential inoculation with *S. cerevisiae* for fermenting plum juice (*Prunus domestica* L.), compared to *S. cerevisiae* as a control [90]. Studies that included *S. pombe* that measured glycerol reported that the yeast produced lower levels of glycerol than the other yeasts in the study (Table 2) [20].

## 5. Metschnikowia Pulcherrima

*Metschnikowia pulcherrima* is a non-*Saccharomyces* yeast that is commercially available from many manufacturers [10,19]. Its ability to enhance varietal aroma compounds [50,92] and reduce the ethanol content of wines has raised interest in its commercial use [93,94]. Recently, the impact of *M. pulcherrima* in winemaking has been reviewed [95].

The ability of *M. pulcherrima* for withstanding ethanol concentrations of up to 7% *v/v* has been reported by Combina et al. [96]. Recently, four strains of *M. pulcherrima* were identified as being able to actively grow at a higher ethanol concentration (9% *v/v*) [97]. Sixty-two of the 65 strains isolated from the Douro region of Portugal in this study were able to tolerate a 6% *v/v* ethanol level. On the other hand, its ability to ferment was reported up to levels of 4% *v/v* in micro-fermentations conducted in pasteurised grape must [13]. Consequently, different co-inoculation and sequential inoculation strategies have been studied [14,57,93].

Contreras et al. [49] identified a *M. pulcherrima* strain that can reduce the ethanol content of wine through part of a sequential inoculation with *S. cerevisiae*. Lower ethanol levels of 0.9% and 1.6% *v/v* were achieved in Chardonnay and Shiraz wines, respectively, compared to a sole inoculum of *S. cerevisiae*, which found ethanol levels of 15.1% *v/v* in Chardonnay and 13.8% *v/v* in Shiraz wines. A similar result of 0.9% *v/v* ethanol reduction was reported in a study carried out with the sequential inoculation of *M. pulcherrima* with *S. cerevisiae* compared to a sole inoculum of *S. cerevisiae* that produced ethanol levels of 13.2% *v/v* [93]. Furthermore, a mixed inoculum of *M. pulcherrima* and *S. uvarum* with sequential inoculation of *S. cerevisiae* was conducted. Two different *M. pulcherrima*/*S. uvarum* inoculum ratios were applied ( $1 \times 10^6$  cells/mL to  $1 \times 10^5$  cells/mL, and  $1 \times 10^6$  cells/mL to  $1 \times 10^4$  cells/mL). Both resulted in an ethanol reduction of 1.7% *v/v*, along with higher concentrations of succinic acid and glycerol, compared to wine fermented with *S. cerevisiae* [93]. Varela et al. [50] studied *M. pulcherrima* and *S. uvarum*, both of which were found to be able to produce wines with reduced ethanol content. Along with the ethanol reduction, the sensory profile and volatile aromatic composition of Merlot wines were studied. Fermentation was conducted via co-inoculation using *M. pulcherrima* ( $1 \times 10^6$  cells/mL) and *S. cerevisiae* ( $1 \times 10^5$  cells/mL). An ethanol reduction of 1.0% *v/v*, along with higher concentrations of ethyl acetate, total esters, total higher alcohols, and total sulfur compounds were detected in wines fermented with a co-inoculation of *M. pulcherrima* and *S. cerevisiae*, compared to wines fermented with *S. cerevisiae*. Although a higher production of ethyl acetate and total sulfur-containing compounds was detected, the sensory panel did not detect associated negative attributes in the wines [50].

The effect of different aeration regimes and immobilisation on the ethanol reduction with selected strains of *M. pulcherrima* has been studied [98]. An ethanol reduction of 1.38% *v/v* was achieved in first 72 h of fermentation of Verdicchio must with *M. pulcherrima* under an aeration flow of 20 mL/L/min, compared to the control that used *S. cerevisiae* [98]. A blend of Malvasia and Viura (Macabeo) must was fermented in a study by Morales et al. [57] using a mixed culture of *M. pulcherrima* and *S. cerevisiae*, and using different aeration regimes (sparged with air or nitrogen). The lowest ethanol values were reported for the treatments sparged with air and fermented with the mixed culture of *M. pulcherrima* and *S. cerevisiae*, regardless of the inoculum level of *S. cerevisiae* (1% or 10%). In these treatments, 11% *v/v* ethanol was obtained, compared to 14.7% *v/v* and 12.9% *v/v* ethanol in the treatments fermented with *S. cerevisiae* sparged with nitrogen and air, respectively. However, high acetic acid values (higher than 0.65 g/L) obtained under air-sparged treatment produced wines that would have been unacceptable for consumers and do not meet market regulations were reported. In contrast, treatments sparged

with nitrogen and fermented with the mixed culture of *M. pulcherrima* and *S. cerevisiae* had an ethanol reduction of 0.8% *v/v* compared to those fermented with *S. cerevisiae*, with acceptable levels of acetic acid (lower than 0.1 g/L) [57]. The same level of ethanol reduction was achieved (0.8% *v/v*) using a sequential fermentation with Viura/Macabeo and Malvasia varieties using *M. pulcherrima* with *S. cerevisiae* compared to the control (*S. cerevisiae*) under non-aerated conditions [99].

The ability of selected immobilised non-*Saccharomyces* yeasts (*Starmerella bombicola* (formerly named *Candida stellata*), *Metschnikowia pulcherrima*, *Hanseniaspora osmophila*, and *Hanseniaspora uvarum*) to reduce the ethanol content in wine via sequential fermentation has been also studied [14]. In synthetic grape juice, the sequential inoculation of *M. pulcherrima* had 1.14% to 1.35% *v/v*, with 48 and 72 h delays in inoculation of *S. cerevisiae*, respectively. Ethanol concentration was reduced by 1.10% to 1.46% *v/v* in natural grape juice in the same study [14]. Similarly, Röcker et al. [47] studied five non-*Saccharomyces* yeast strains including *M. pulcherrima* for a sequential inoculation with *S. cerevisiae* var. *bayanus* strain under three different aeration conditions (aeration with sterile pressured air during 15 and 5 days, and under regulated oxygen content of 20% dissolved oxygen), to ferment Riesling must. Ethanol concentration was reduced by up to 3.8% *v/v* within 3 days of aeration, although the authors reported an increase in vinegar (associated with acetic acid) and oxidation sensory attributes [47].

Three non-*Saccharomyces* yeast strains (*M. pulcherrima*, *T. delbrueckii*, and *Zygosaccharomyces bailii*) fermented Chardonnay using sequential inoculation with *S. cerevisiae* under three different aeration conditions (no air addition, 5 mL/min aeration (0.025 VVM), 10 mL/min aeration (0.05 VVM)). The authors reported that the sequential inoculation with *M. pulcherrima* reduced alcohol by 1.6% *v/v*, which was the highest ethanol reduction among all the non-*Saccharomyces* yeast strains in the study, compared to *S. cerevisiae* [43]. In this study, applied aeration conditions did not cause an increase in the acetic acid production. However, in the wines produced with a sequential inoculation of *M. pulcherrima* and *S. cerevisiae*, an over-production of ethyl acetate (280 mg/L) was detected under the aeration regime of 0.05 VVM. This indicates an oxygen threshold for the over-production of this compound [43], which causes unpleasant odors such as nail polish remover and vinegar [100].

The ability of *M. pulcherrima* to reduce the final ethanol content via its respiratory characteristic has been shown with various studies (Table 1). Recently, *M. pulcherrima* has been reported to produce lower levels of glycerol under semi-anaerobic conditions than when the air flow into the fermentations was 1 mL/L/min and 20 mL/L/min (Table 2). Therefore, taken together in respect to ethanol and glycerol content, future research with *M. pulcherrima* (solo, sequential, or co-fermentations) should include a range of aeration strategies, a range of inoculum rates, and an investigation into the length of time before inoculation of *S. cerevisiae* yeast, to render its application feasible at a commercial winemaking scale to achieve ethanol reduction without compromising or enhancing sensory characteristics [14,43,47,59,93,97].

## 6. Lachancea Thermotolerans

*Lachancea thermotolerans* (previously *Kluyveromyces thermotolerans*) is available commercially from yeast manufacturers [19,101]. This yeast has specific oenological traits including a positive influence on wines' sensory profile [102] and total acidity [13,103]. The main characteristics of *L. thermotolerans* and its effects on winemaking were recently reviewed [101,104–106].

*L. thermotolerans* in pure culture was able to reach 10.46% *v/v* ethanol levels in micro-fermentations of pasteurised commercial white wine must, conducted at 25 °C, by leaving more than 50 g/L residual sugar. The control wine inoculated with a pure culture of *S. cerevisiae* EC-1118 fermented the must to dryness (less than 6 g/L residual sugar), reaching an ethanol level of 13.04% *v/v* [102]. The study continued at industrial scale, and an ethanol reduction of 0.7% *v/v* was achieved with a Sangiovese must [102]. In the study by Del Fresno et al. [89], a higher ethanol reduction of 1.2% *v/v* was achieved. This was compared to a different *S. cerevisiae* strain (7VA), which was sequentially inoculated later than the previous study (6 days), with a higher inoculum amount (both at 10<sup>8</sup> cell/mL). The laboratory-scale

fermentation using Tempranillo must conducted at 25 °C and 12.66% *v/v* ethanol level used sequential inoculation, while sole inoculation of *S. cerevisiae* 7VA was 13.84% *v/v* [89].

As well as its ability to decrease the pH of wine, the metabolic pathway of *L. thermotolerans* that can turn sugars into lactic acid is also described as a way to reduce the level of alcohol in wines [18]. A study conducted using micro-vinifications of Airén must conducted at 25 °C [80] with *L. thermotolerans* 617 (100 mL containing  $2.27 \times 10^7$  CFU/mL) followed by the sequential inoculation of *S. cerevisiae* 87 ( $10^7$  CFU/mL of) with 96 h of delay, an ethanol reduction of 0.4% *v/v* was achieved, compared to the sole inoculation of *S. cerevisiae* 87. This sequential inoculation also showed a higher lactic acid production (3.18 g/L) and lower pH (3.52, compared to 3.74 in control wine). The sensory panel perceived this treatment to have better sensorial properties, with higher scores for sweetness, despite similar levels of residual sugar compared to control. This was explained by the possible effect of higher L-lactic acid concentration produced by the effect of *L. thermotolerans*.

Other beneficial compositional effects reported in *L. thermotolerans* treated wines include increases in glycerol at concentrations high enough (>5 g/L) that they could be of sensory relevance. As shown by Kapsopoulou et al. [107] for grape must containing 160 g/L sugar (with 7.4 g/L titratable acidity, at pH 3.5), glycerol formation was significantly higher (5.75 g/L) when *S. cerevisiae* TH941 ( $5 \times 10^5$  cell/mL) was inoculated 3 days after the inoculation of *L. thermotolerans* SCM952 ( $5 \times 10^5$  cell/mL). This was compared to sole *S. cerevisiae* fermentations (4.82 g/L) and other sequential inoculations (after 1 and 2 days). The authors attributed this to the increased survival of the *L. thermotolerans* strain in the co-fermentation treatment in comparison to the other treatments. Likewise, a very high glycerol level (11.22 g/L) was reported in Sangiovese red wines obtained at a commercial scale by the sequential inoculation of *S. cerevisiae* EC1118 ( $10^6$  cell/mL) 2 days after that of *L. thermotolerans* 101 ( $10^7$  cell/mL) [102]. This value was significantly higher compared to the control (9.02 g/L) and the co-inoculated *L. thermotolerans* and *S. cerevisiae* treatments (9.68 g/L). Comitini et al. [13] inoculated non-*Saccharomyces* yeasts in combination with the *S. cerevisiae* EC1118 strain as a starter. All non-*Saccharomyces* strains were inoculated at  $10^7$  cell/mL, while the EC1118 starter strain was inoculated at three different concentrations:  $10^7$ ,  $10^5$ , and  $10^3$  cell/mL. Of all the inoculations tested, those of *L. thermotolerans* with *S. cerevisiae* produced higher levels of glycerol (6.95–7.58 g/L) at all three ratios than when *S. cerevisiae* was inoculated on its own (6.23–6.56 g/L). Contrary to the *C. zemplinina* and *M. pulcherrima* inoculations, no significant differences were found between the three different inoculum ratios (1:1, 100:1, 10,000:1) of *L. thermotolerans* with *S. cerevisiae*. This suggests that glycerol production may not correlate with cell concentration and persistence of the *L. thermotolerans* yeast, at least according to this study. Where the effects of sequential and co-inoculations of *L. thermotolerans* with *S. cerevisiae* yeasts on glycerol concentration were evaluated, the glycerol content in sequentially inoculated fermentations (7.55 g/L) was higher than those observed in the co-inoculated treatments (7.18 g/L) [80].

These findings suggest that sequential fermentation with *L. thermotolerans* and *S. cerevisiae* remains a viable option for winemakers. Further investigations on the sensory quality of the wines could help identify if these variations in glycerol are discernable by consumers and preserve the wines from the formation of off-flavours.

## 7. *Candida Stellata*/*Starmerella Bombicola*

*Candida stellata* is a widely studied yeast due to its positive contributions to wine, including its capacity to produce desirable metabolites such as glycerol [72], and its ability to carry out enzymatic activities that have positive effects on wine sensory attributes. An extensive review of *C. stellata* was carried out by García et al. [108]. Recent studies uncovered the mistake of referring to *C. zemplinina* instead of *C. stellata*, which may explain the disputable characterisations of the oenological traits of these species [109]. For instance, recently, a commonly used strain of *C. stellata*, DBVPG 3827, was reclassified as *Starmerella bombicola* [108].



Traditionally dominating overripe, infected, or botrytised grape berries, *C. stellata* is reported to tolerate at least 9% *v/v* ethanol concentration, while at 15 °C, its growth was recorded at 11% *v/v* ethanol concentration, with decreased tolerance at both 10 °C and 30 °C [22,108,110].

Soden et al. [72] studied the effect of inoculations with *C. stellata* and *S. cerevisiae* on Chardonnay juice. The treatments included sole yeast fermentations of *C. stellata* and *S. cerevisiae*, as well as co-inoculation and sequential inoculation conducted at a room temperature of 18 °C. *C. stellata* in sole fermentation was not able to consume all the sugar, reaching an ethanol concentration of only 5.8% *v/v*. Therefore, *S. cerevisiae* was used for the sequential inoculation, and added after the fermentation activity of *C. stellata* had ceased, with an inoculation density of  $5 \times 10^6$  cells/mL for both yeasts. The resulting wine was dry and had a significantly lower ethanol concentration (11.8% *v/v*) in comparison to the control with a sole inoculation of *S. cerevisiae* (12.5% *v/v*) [72]. The same authors also highlighted in their study the increases in glycerol concentration in the wines fermented with *C. stellata* in comparison to sole *S. cerevisiae* fermentations. The glycerol levels varied from 5.2 g/L in the co-inoculation treatment up to 15.7 g/L in the sequential inoculation trial. Similar results for ethanol reduction were found by Ferraro et al. [111] but using immobilised cells of *C. stellata* and *S. cerevisiae* on Trebbiano Toscano grape must fermented at 20 °C. Inoculum of *S. cerevisiae* at  $5 \times 10^6$  cells/mL was added after 3 days of fermentation. The sequential fermentation of immobilised cells of *C. stellata* and *S. cerevisiae* reached an ethanol level of 10.6% *v/v* compared to the control (11.24% *v/v*), which was inoculated with *S. cerevisiae* only [111]. The ethanol reduction was explained by the significant increase (approximately 70%) in glycerol as a consequence of low fermentation rate and reduced production of ethanol with immobilised cells of *C. stellata* [112].

Immobilised cells of *Starmerella bombicola* (formerly referred to as *Candida stellata*) were used in sequential inoculation with *S. cerevisiae* on Verdicchio grape must. The fermentation was conducted at 25 °C and the inoculation of *S. cerevisiae* ( $1 \times 10^6$  cell/mL) with 72 h of delay. The effect of the removal of the immobilised *S. bombicola* cells was investigated [14]. The 1.07% and 1.64% *v/v* less ethanol achieved with and without the removal of *S. bombicola* beads, respectively, was notable compared to inoculation with *S. cerevisiae*. A significant increase was seen in the concentration of by-products such as glycerol and succinic acid. The ethanol reduction was explained by the production of by-products from glycerol–pyruvic fermentation or other metabolic pathways [14].

Both growing in similar environmental conditions, including high sugar-containing musts, and possessing similar taxonomic and oenological profile characteristics, *C. zemplinina* (synonym *Starmerella bacillaris* [113]) and *C. stellata* have produced contrasting results in previous literature. Magyar and Tóth [114] evaluated the oenological characteristics of some yeast strains, including four strains of both *C. stellata* and *C. zemplinina*. While both *C. stellata* and *C. zemplinina* showed a strong fructophilic character, *C. stellata* showed higher ethanol and glycerol yield and the same level of volatile acidity compared to *C. zemplinina* [114].

The role of *Candida zemplinina* (synonym *Starmerella bacillaris*) as a tool to produce wines with less ethanol levels but higher glycerol concentrations has been extensively studied and recently reviewed [115]. *Candida* isolates were obtained from Sicilian musts by Di Maio et al. [116] and sequential inoculations with three different *C. zemplinina* strains and *S. cerevisiae* (NDA21) were conducted on Nero d'Avola must. The highest ethanol reduction was 0.3% *v/v* obtained with the sequential inoculation of *C. zemplinina* Cz3 strain, compared to a pure inoculation of *S. cerevisiae* (NDA21), along with higher glycerol content [116]. A similar level of ethanol reduction (0.3% *v/v*) and higher glycerol production was recorded in the study conducted by Rolle et al. [117] on Barbera must. In this study, two different *C. zemplinina* strains were used (FC54 and C.z03). The inoculation of *C. zemplinina* ( $10^6$  cells/mL) was followed by the inoculation of *S. cerevisiae* Uvaferm BC ( $10^6$  cells/mL) after 2 days. Fermentations of natural grape must by sequential inoculations of *C. zemplinina* with *S. cerevisiae* EC1118 increased the level of glycerol (5.45–6.30 g/L in the final wine), and remarkably produced less acetaldehyde and total SO<sub>2</sub> compared to the other yeasts [118].

## 8. *Torulaspota Delbrueckii*

*Torulaspota delbrueckii* was one of the first commercially available non-*Saccharomyces* yeasts and is currently available in dry or frozen form from many yeast manufacturers and suppliers [3,10,19]. Some of the oenological traits of *T. delbrueckii* reported in studies include optimising wine quality parameters i.e., enhancing aroma composition, and positively impacting the foam properties for traditional methods of sparkling wine [119–122]. Studies have been conducted using this yeast on different wine styles, and its effect on the winemaking practices was recently reviewed [123–125].

Significant ethanol reductions due to the utilisation of *T. delbrueckii* in sequential fermentations have been reported when compared to pure inoculations with *S. cerevisiae* (Table 1). Nevertheless, some studies reported slight ethanol reductions (lower than 0.2% *v/v*) [125] or none [88]. Additionally, higher ethanol reduction levels were achieved when aeration was integrated during the fermentation process, which stimulates aerobic metabolism [56]. Contreras et al. [56] carried out a sequential inoculation with *T. delbrueckii* AWRI1152, followed by inoculation of *S. cerevisiae* AWRI1631 when 50% of sugar was consumed. Four different aeration regimes were applied (air or nitrogen), and fermentations were conducted at 22 °C with agitation (200 rpm) in a chemically defined grape juice medium. With the aeration at 5 mL/min (0.025 VVM) for the first 24 h of the fermentation, an ethanol reduction of 1.5% *v/v* was achieved, compared to the control of *S. cerevisiae* under anaerobic conditions. No increase in acetic acid levels occurred; however, the impact on the flavour profile of the wine requires further investigation [56]. To answer this question, Canonico et al. [43] studied three non-*Saccharomyces* yeast strains (*M. pulcherrima*, *T. delbrueckii*, and *Zygosaccharomyces bailii*) and their ability to reduce ethanol under limited aeration conditions. In this study, sequential inoculation with *T. delbrueckii* resulted in 0.9% to 1% *v/v* lower ethanol, depending on the aeration strategy (0.025 VVM and 0.05 VVM) compared to control of *S. cerevisiae* under anaerobic conditions. Furthermore, wines fermented with *T. delbrueckii* under aerobic conditions showed a favorable balance between ethanol reduction and volatile profile [43].

Recent studies showed that the nutrient supplementation has a positive correlation on the ethanol yield of *T. delbrueckii* [126,127]. Additionally, Mecca et al. [127] studied three commercially available *T. delbrueckii* strains and compared some oenological characteristics. Significant differences were reported in ethanol yields, as well as in the volatile aroma compounds [127]. Concerning the glycerol content of wines made using *T. delbrueckii*, a range of 4.1–8.9 g/L has been reported in wines (Table 2) [125,128,129]. It has been suggested that *T. delbrueckii* has a more developed glycerol–pyruvic pathway than other yeasts, although some studies have found no differences in glycerol production [91,124,130].

## 9. Other Non-*Saccharomyces* and *Saccharomyces Non-cerevisiae* Yeasts

In addition to the yeasts mentioned in the sections above, new yeasts are constantly being investigated for their ability to ferment wines and their role in uninoculated fermentations. Contreras et al. [56] studied *Zygosaccharomyces bailii* in sequential inoculation under different aeration conditions in chemically defined grape juice medium. With the aeration at 5 mL/min (0.025 VVM) throughout the fermentation, an ethanol reduction of 2% (*v/v*) was achieved, as well as a significantly lower acetic acid compared to the control of *S. cerevisiae* under anaerobic conditions. Using the same *Z. bailii* strain in sequential inoculation, in Chardonnay must, Canonico et al. [43] reported a significant ethanol reduction (1% *v/v*) under aeration (0.05 VVM). In this study, a lower ethanol reduction (0.8% *v/v*) was achieved with a lower aeration (0.025 VVM); however, the resultant wine had a promising volatile profile including individual esters, higher alcohols, and volatile acidity.

Various studies have reported ethanol reduction with non-*Saccharomyces* yeasts from different genus, including *Pichia* and *Hanseniaspora* (Table 1). Maturano et al. [11] studied two non-*Saccharomyces* yeasts, *H. uvarum* and *C. membranaefaciens*, with the sequential inoculation with *S. cerevisiae* on Malbec must. Taking a step further, three fermentation factors—inoculum size, time prior to inoculation of *Saccharomyces cerevisiae*, and fermentation temperature—were optimised using a Box–Behnken

experimental design [11]. By applying the optimised factors, the highest ethanol reduction with *H. uvarum* was achieved with an inoculum size of  $5 \times 10^6$  cells/mL and a delay of 48 h 37 min before *S. cerevisiae* inoculation with the fermentation temperature of 25 °C. However, for *C. membranaefaciens*, the optimised factors were different: an inoculum size of  $2.72 \times 10^6$  cells/mL, delay of 24 h 15 min before *S. cerevisiae* inoculation, and fermentation temperature of 25 °C was used. The study showed that the time before the inoculation of *S. cerevisiae* affected the ethanol production of the non-*Saccharomyces* yeasts. The high sugar consumption ability of *H. uvarum* via oxidative metabolism was reported to be the reason for this [11].

The application of sequential inoculations with long delays prior to *S. cerevisiae* inoculation in winery environments could be challenging. Competitive native or wild *S. cerevisiae* species present in the winery environment can dominate the fermentation before achieving the expected effect from the inoculated non-*Saccharomyces* yeasts [14]. A recent study by Canonico et al. [43] reported the volatile profile of reduced ethanol wines. Finding an acceptable balance between ethanol reduction, volatile aroma profile, and sensory characteristics of the wines is crucial. Therefore, studies conducted on a pilot scale that includes sensory analysis of the reduced alcohol wines made from non-*Saccharomyces* yeasts is lacking. Nitrogen management is an important factor to achieve ethanol reduction, which has been highlighted by authors in previous studies [126,127,131]. The specific nutrient needs of non-*Saccharomyces* yeasts used for ethanol reduction purposes should be further studied.

**Table 1.** Ethanol reduction in wines produced from mixed fermentations with non-*Saccharomyces* and *Saccharomyces non-cerevisiae* with *S. cerevisiae* yeast.

Grape Variety	Wine Style	Ethanol Reduction % (v/v)	Inoculation	Reference
<i>Schizosaccharomyces pombe</i>				
Airén	White still	0.4	Sequential inoculation with <i>S. cerevisiae</i>	[87]
Airén	White still	0.65	Pure inoculation	[87]
<i>Schizosaccharomyces japonicus</i>				
Trebbiano	White still	2.4	Sequential inoculation <i>S. japonicus</i> (immobilised)+ <i>S. cerevisiae</i>	[132]
Trebbiano	White still	1.7	Co-inoculation <i>S. japonicus</i> (immobilised)+ <i>S. cerevisiae</i>	[132]
<i>Metschnikowia pulcherrima</i>				
Malvasia/Viura	White still	0.8	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[99]
Chardonnay	White still	0.9	Sequential inoculation with <i>S. cerevisiae</i>	[49]
Shiraz	Red still	0.9	Sequential inoculation with <i>S. cerevisiae</i>	[93]
Merlot	Red still	1	Co-inoculation with <i>S. cerevisiae</i>	[50]
Synthetic grape juice	–	1.1–1.3	Sequential inoculation <i>M. pulcherrima</i> (immobilised)+ <i>S. cerevisiae</i>	[14]
Verdicchio	White still	1.2–1.6	Sequential inoculation <i>M. pulcherrima</i> (immobilised)+ <i>S. cerevisiae</i>	[14]
Chardonnay	White still	0.7–1.6	Sequential inoculation (aeration)	[43]
Shiraz	Red still	1.6	Sequential inoculation with <i>S. cerevisiae</i>	[49]

Table 1. Cont.

Grape Variety	Wine Style	Ethanol Reduction % (v/v)	Inoculation	Reference
Malvasia/Viura	White still	3.7	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[57]
Riesling	White still	3.8	Sequential inoculation (aeration)	[47]
<i>Lachancea thermotolerans</i>				
Shiraz	Red still	0.4	Sequential inoculation with <i>S. cerevisiae</i>	[71]
Airén	White still	0.4	Sequential inoculation with <i>S. cerevisiae</i>	[80]
Sangiovese	Red still	0.7	Sequential inoculation with <i>S. cerevisiae</i>	[102]
Tempranillo	Red still	1.2	Sequential inoculation with <i>S. cerevisiae</i>	[89]
<i>Candida stellata</i> / <i>Starmerella bombicola</i>				
Trebbiano	White still	0.6	Sequential inoculation <i>C. stellata</i> (immobilised) + <i>S. cerevisiae</i>	[111]
Chardonnay	White still	0.7	Sequential inoculation with <i>S. cerevisiae</i>	[72]
Verdicchio	White still	1.6	Sequential inoculation with <i>S. bombicola</i> (immobilised) + <i>S. cerevisiae</i>	[14]
<i>Candida zemplinina</i> / <i>Starmerella bacillaris</i>				
Nero d'Avola	Rosé still	0.3	Sequential inoculation with <i>S. cerevisiae</i>	[116]
Barbera	Red still	0.3	Sequential inoculation with <i>S. cerevisiae</i>	[117]
Barbera	Red still	0.7	Sequential inoculation with <i>S. cerevisiae</i>	[133]
Riesling	White still	0.8	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[47]
<i>Torulopsis delbrueckii</i>				
Airén	White still	0.3	Sequential inoculation with <i>S. cerevisiae</i>	[134]
Corvina, Rondinella, Corvinone	Red still	0.45	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[135]
Tempranillo	Red still	0.5	Sequential inoculation with <i>S. cerevisiae</i>	[130]
Chardonnay	White still	0.9–1.0	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[43]
Chemically defined grape juice medium	–	1.5	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[56]
Malvasia/Viura	White still	0.5	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[99]
<i>Zygosaccharomyces bailii</i>				
Chardonnay	White still	1.0	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[43]
Chemically defined grape juice medium	–	2.0	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[56]
<i>Pichia kluyveri</i>				
Riesling	White still	0.25	Sequential inoculation with <i>S. cerevisiae</i>	[136]
Riesling	White still	3.0	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[47]
<i>Pichia guilliermodii</i>				
Riesling	White still	2.0	Sequential inoculation with <i>S. cerevisiae</i> (aeration)	[47]
<i>Hanseniaspora uvarum</i>				
Pinotage	Red still	0.8	Sequential inoculation with <i>S. cerevisiae</i>	[137]



Table 1. Cont.

Grape Variety	Wine Style	Ethanol Reduction % (v/v)	Inoculation	Reference
Synthetic grape juice	–	0.8–1.0	Sequential inoculation <i>H. osmophila</i> (immobilised)+ <i>S. cerevisiae</i>	[14]
Verdicchio	White still	1.0–1.2	Sequential inoculation <i>H. osmophila</i> (immobilised)+ <i>S. cerevisiae</i>	[14]
Sauvignon blanc	White still	1.3	Sequential inoculation with <i>S. cerevisiae</i>	[137]
<i>Hanseniaspora opuntiae</i>				
Pinotage	Red still	0.6	Sequential inoculation with <i>S. cerevisiae</i>	[137]
Sauvignon blanc	White still	1.3	Sequential inoculation with <i>S. cerevisiae</i>	[137]
<i>Hanseniaspora osmophila</i>				
Synthetic grape juice	–	0.8–1.3	Sequential inoculation <i>H. osmophila</i> (immobilised)+ <i>S. cerevisiae</i>	[14]
Verdicchio	White still	1	Sequential inoculation <i>H. osmophila</i> (immobilised)+ <i>S. cerevisiae</i>	[14]
<i>Saccharomyces uvarum</i>				
Shiraz	Red still	0.8	Sequential inoculation with <i>S. cerevisiae</i>	[93]
Merlot	Red still	1.7	Pure inoculation	[50]

Recent studies were conducted with the aim of determining the combinations of two non-*Saccharomyces* yeast species (*S. pombe* and *L. thermotolerans*) [138]. The combination of these yeasts was studied at a laboratory scale using micro-vinifications of Tempranillo grape must [79,89,139,140]. Higher levels of glycerol concentrations were reported in mixed fermentations with *L. thermotolerans* (CONCERTO™) and *S. pombe* (inoculated simultaneously at 10<sup>6</sup> cfu/mL), compared to a control with *S. cerevisiae* [79,139,140]. Glycerol increase varied between 0.27 and 0.71 g/L, which may be related to the strain of *S. pombe* used (V2 and 4.5) and control *S. cerevisiae* (88 and CECT 87). A different strain of *S. pombe* (938) was studied in combination with *L. thermotolerans* (CONCERTO™) [89]. Two inoculum ratios of *S. pombe*/*L. thermotolerans* (1:1 and 1:3) were used for the laboratory-scale fermentations with Tempranillo must. The authors reported significantly lower glycerol values in mixed fermentation treatments (5.02 g/L and 6.78 g/L) compared to the control fermentation with *S. cerevisiae* 7VA (7.42 g/L) [89]. Therefore, more studies need to be conducted to understand the effect of the interaction between these two non-*Saccharomyces* yeasts.

Combinations of non-*Saccharomyces* and *Saccharomyces non-cerevisiae* yeasts for wine fermentations have been investigated, including *M. pulcherrima* and *S. uvarum* [93,141]. *M. pulcherrima* and *S. uvarum* were used in mixed inoculum in the study [93]. Glycerol levels of 12.30 and 12.48 g/L were reported from two different inoculum ratios of *M. pulcherrima*/*S. uvarum* (1 × 10<sup>6</sup> cells/mL to 1 × 10<sup>5</sup> cells/mL, and 1 × 10<sup>6</sup> cells/mL to 1 × 10<sup>4</sup> cells/mL, respectively) in laboratory-scale fermentations of Shiraz must. These values were significantly higher compared to the control wine fermented with *S. cerevisiae*, where 7.91 g/L glycerol concentration was reported. Although the highest level of glycerol (14.55 g/L) was reported in sequential inoculation with *S. uvarum* and *S. cerevisiae*, considering the values of ethanol reduction, a mixed inoculum of *M. pulcherrima* and *S. uvarum* could be a promising combination [93]. The same treatments were applied to Chardonnay must in the study by Varela et al. [141]. Similarly, significantly higher glycerol levels were reported in both inoculum ratios of mixed fermentation of *M. pulcherrima*/*S. uvarum* (11.90 g/L and 12.63 g/L) compared to control wine fermented with *S. cerevisiae* (8.20 g/L). Unlike the study of Contreras et al. [93], the reported values in the mixed fermentations were not significantly different from the sequential inoculation of *S. uvarum* and *S. cerevisiae*, in which a

glycerol level of 11.19 g/L was reported [141]. Considering the ethanol decrease and glycerol levels together, the combined inoculation of *M. pulcherrima* and *S. uvarum* shows promising results.

Applying different non-*Saccharomyces* and *Saccharomyces non-cerevisiae* yeasts, a wide range of glycerol concentrations has been reported in studies (Table 2) of still and sparkling wines, as well as white and red wines (3.5 g/L to 15.9 g/L). Glycerol levels above 5.2 g/L may directly affect the style of the wine by influencing sensory characteristics including the sweetness, body, and structure of the wines [56]. Therefore, the selection criteria for the use of these yeasts should always consider the sensory characteristics of the final wines. In the case of sparkling wines, glycerol levels have an impact on the viscosity, volatile aroma compounds, and foaming [142]. High levels of glycerol produced by some *Saccharomyces non-cerevisiae* yeasts, such as *Saccharomyces kudriavzevii* [143] or *Schizosaccharomyces japonicus* [132] during base wine fermentation, may not make them suitable candidates for achieving a complete second alcoholic fermentation in sparkling winemaking [8]. Additionally, studies have reported an increase of acetic acid when glycerol is produced by some yeasts [8,73].

**Table 2.** Glycerol concentrations in wines from non-*Saccharomyces* and *Saccharomyces non-cerevisiae* yeast studies.

Grape Variety	Wine Style	Glycerol Concentration (g/L)	Method of Detection	Reference
<i>Torulaspora delbrueckii</i>				
Tempranillo	Red still	* 8.6–8.9	Enzymatically with MIURA One oenological analyser	[129]
Chemically defined grape juice medium	N/A	9.3	HPLC	[56]
Tempranillo	Red still	6.7	Y15 enzymatic analyser	[125]
Viura/Macabeo	White still	4.1	Enzymatically with MIURA One oenological analyser	[128]
<i>Hanseniaspora uvarum</i>				
Negromaro	Red still	5.5	HPLC	[144]
Chemically defined grape juice medium	N/A	3.5	HPLC	[56]
<i>Metschnikowia pulcherrima</i>				
Tempranillo	Red still	* 8.2–8.6	Enzymatically with MIURA One oenological analyser	[129]
Viura/Macabeo	White still	4.8	Enzymatically with MIURA One oenological analyser	[128]
Chardonnay	White still	5.5–7.8	HPLC	[43]
<i>Schizosaccharomyces pombe</i> †				
Airén	Sparkling wine	4.7	Y15 enzymatic analyser	[145]
Tempranillo	Red sparkling wine	5.0	Y15 enzymatic analyser	[145]
<i>Schizosaccharomyces japonicus</i>				
Trebbiano	White still	15.9	HPLC	[132]
<i>Saccharomyces ludwigii</i> †				
Airén	Sparkling wine	5.0	Y15 enzymatic analyser	[145]
Tempranillo	Red sparkling wine	5.1	Y15 enzymatic analyser	[145]
<i>Saccharomyces uvarum</i> †				
Synthetic grape must	N/A	5.2	HPLC	[12]
Cabernet franc	Red wine	10–12	Enzymatically using Megazyme International assay kit	[146]
<i>Lachancea thermotolerans</i>				
Tempranillo	Red still	* 8.2–8.3	Enzymatically with MIURA One oenological analyser	[129]
Viura/Macabeo	White still	4.7	Enzymatically with MIURA One oenological analyser	[128]

Table 2. Cont.

Grape Variety	Wine Style	Glycerol Concentration (g/L)	Method of Detection	Reference
<i>Starmerella bacillaris</i>				
Synthetic grape must	N/A	* 7.7–8.2	HPLC	[128]
<i>Williopsis pratensis</i>				
Tempranillo	Red still	8.0	Enzymatically with MIURA One oenological analyser	[129]
<i>Zygosaccharomyces bailii</i>				
Tempranillo	Red still	7.8	Enzymatically with MIURA One oenological analyser	[129]
Viura/Macabeo	White still	5.6	Enzymatically with MIURA One oenological analyser	[128]
<i>Candida vini</i>				
Tempranillo	Red still	7.9	Enzymatically with MIURA One oenological analyser	[129]

\* Two or more products of the same yeast strain used in the study. ‡ *Saccharomyces non-cerevisiae* yeast.

## 10. Conclusions

This review focused on studies concerning non-*Saccharomyces* and *Saccharomyces non-cerevisiae* yeasts, and their effect in solo, sequential, and co-fermentation with *S. cerevisiae* or other non-*Saccharomyces* yeasts, on the ethanol and glycerol content of wines. The application of large-scale sequential inoculations of yeast in a commercial scale winery could be challenging during a busy harvest period, especially if the length of time varies between yeast additions. There is a distinct lack of studies concerning the long-term effect of these yeasts on wine from aging on lees and in bottles, as well as the effect of high glycerol levels in sparkling wines on sensory characteristics. Further research should include the inoculum rate of the yeasts, aging ability of the wines on lees, aeration techniques including flow rate, and time before the sequential yeast is added.

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