

Bio-Dealcoholization of Wines

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The link between viticulture and climate change has become more evident in recent years. The temperature rise has been a crucial factor in the early ripening of grapes, which has resulted in wines with imbalanced acidity, higher alcohol content, and higher pH values. Consumers are seeking high-quality and healthy products, and this trend has extended to wine consumption.

Keywords: climate change ; post-fermentation ; membrane separation ; heat treatment

1. Climate Change and Wine Quality

Climate change, primarily driven by anthropogenic influence, induces significant transformations in the Earth's system [1][2], including temperature extremes and modifications in precipitation patterns [3][4]. The latest Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) [5] predicts a continued rise in global surface temperature and a decrease in annual precipitation across emission scenarios, with intensifying and more frequent extreme events globally [4][6]. The Mediterranean region is particularly vulnerable to global warming, with projected severe climate events affecting agriculture and leading to economic and food security challenges [7].

As indicated by Lamonaca et al. [8], detrimental outcomes, such as reductions in agricultural production and economic conditions associated with an increase in food insecurity, are anticipated to exacerbate, significantly affecting the agricultural sector [9][10]. This emphasizes the considerable vulnerability of agriculture to climate change, including viticulture.

In 2022, wine exports from these three European countries amounted to around EUR 23 billion, emphasizing the economic importance of viticulture in Europe and globally, highlighting wine grapes as one of the most valuable fruit crops worldwide [2][11][12]. Additionally, global wine production in 2022 reached 258 million hectoliters, slightly lower than the previous year's output of 261 million hectoliters. Nevertheless, the vineyard area has decreased by approximately 1.18% over the past five years (from 2018 to 2022) due to extreme weather changes [13], underscoring that climate factors, particularly temperature, exert a more significant influence on vine development and berry composition relative to other factors such as soil or variety [14][15]. However, it is essential to acknowledge the multifaceted nature of these interactions, and the impact can vary based on specific vineyard locations [15].

This demonstrates that, as stated earlier, due to the strong relationship between viticulture and climate, fluctuations in temperature and frost events (early or late frosts) can directly impact grape production and the quality of the wine produced [3][6][16]. Increasing temperatures lead to premature grape ripening, culminating in an undesirable rise in sugar levels and a decrease in organic acids and phenolic compounds. This results in higher alcohol content, reduced acidity, and modified sensory profiles [16][17][18], ultimately altering wine quality and typicity [2][19][20]. Given the potential compromise of crop yields and viticulture productivity under future climatic conditions, the urgent adoption of cultivation approaches and strategies is crucial to mitigate the effects of climate change on wine production and quality.

Moreover, recent data indicate a growing consumer preference for low-alcohol wines (9% to 13%). This preference is associated with increased social awareness of the harmful effects of alcohol on human health (calorific intake and possibility of alcohol-related diseases) as consumers seek a healthier lifestyle [21][22][23][24]. This has led to an increase in the volume of no and low-alcohol beverages of over 7% across the ten major global markets in 2022. This upward trajectory is projected to outpace the growth observed in the past four years, with a forecasted Compound Annual Growth Rate (CAGR) of over 7% from 2022 to 2026. This forecasted rate represents an increase compared to the 5% CAGR recorded from 2018 to 2022 (**Figure 1**). In this context, wine producers are innovatively lowering alcohol content to meet consumer demands and address challenges posed by high grape sugar concentration during winemaking. Musts with high sugar content usually present difficulty in performing alcoholic fermentation, causing stuck or sluggish fermentation, leading to prolonged and intricate processes or even complete stoppage [22][23][25][26]. These issues arise from the

osmotically stressful environment of high sugar concentrations, hindering water absorption and slowing yeast metabolic activity. The resulting alcohol production can reach toxic levels for the yeast, and the must's nutritional conditions and the potential presence of fermentation inhibitors further contribute to the complications [27]. Furthermore, in some countries, such as the USA, Finland, Sweden, Ireland, and the United Kingdom, exceeding 14.5% (v/v) alcohol content results in higher taxes [22][28][29].

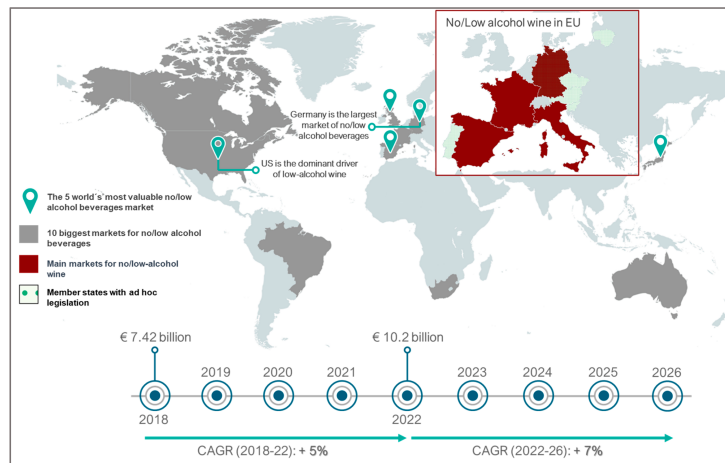


Figure 1. Overview of global market trends for no-alcohol and low-alcohol beverages, including wine.

2. Techniques to Decrease Alcohol Content in Wines

In recent years, consumer preferences have evolved substantially as they have become increasingly demanding, conscious of the characteristics of the goods they choose, and focused on products that promote a healthier lifestyle. This trend extends to the consumption of wine, where consumers are more attentive and concerned about the health risks associated with alcohol consumption. As a result, there is an increasing demand for wines with reduced alcohol content [22][23][24], thus driving the production and sale of such wines. In this regard, winemakers have been actively seeking technological strategies to decrease or eliminate the alcohol content of wines. Furthermore, given the context of climate change, which has significantly contributed to the production of grapes with higher sugar levels and, consequently, an increase in the alcohol content of wines, the search for innovative methods to meet this demand becomes imperative.

These strategies for winemakers to produce wines with reduced alcohol levels can be categorized in several approaches. Still, more commonly, they are separated depending on when they are applied in the wine production process. Some authors [30][31][32] indicate four different categories, while other authors [33][34] divide these strategies into three, as will be discussed in this document (**Figure 2**).

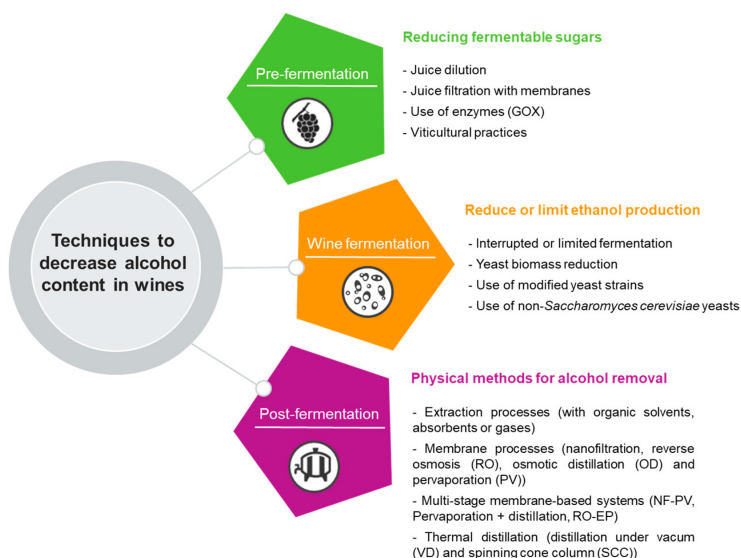


Figure 2. Techniques to decrease alcohol content in wines and fermented beverages.

2.1. Non-Microbial Alcohol Reduction in Wines

2.1.1. Reducing Fermentable Sugars in the Grapes

The first category, used during the pre-fermentation stage, focuses on reducing fermentable sugars. This limits the amount of alcohol produced during fermentation, resulting in wines with lower alcohol content right from the start [34]. It is one of the most common methods for producing wines with lower or reduced alcohol content. Techniques such as juice dilution and juice filtration with membranes or enzymes are employed at this stage. Additionally, viticultural practices such as early harvesting, growth regulators, reducing leaf area to limit photosynthetic rate, and pre-harvest irrigation are cited by Schelezk et al. [35]. Specific viticultural methods can produce a wine with lower alcohol content and higher acidity, which can then be blended with a more mature fermented juice. Although these methods are known to decrease ethanol concentration by 3% (v/v), the resulting wines may have unpleasant, acidic, and unripe flavors [36][37].

Juice Dilution

The addition of water to the must to reduce the concentration of fermentable sugars effectively reduced the characteristics of Cabernet Sauvignon and Shiraz wines. However, it is necessary to find a limit to avoid changes in the sensory profile of wines, as demonstrated in other studies [38].

Juice Filtration with Membranes

Nanofiltration, ultrafiltration, or reverse osmosis membranes retain sugar from the must before fermentation. The filtered juice is then fermented to produce wine with reduced alcohol content. Several studies have investigated this technology's use and reported positive results that do not affect essential compounds such as polyphenols, malic acid, and tartaric acid, with no significant changes in the sensory profile of the wines [39][40][41][42][43].

Use of Enzymes

The use of enzymes, specifically glucose oxidase (GOX), has shown to be an effective technique in reducing the alcohol content of wines by reducing glucose in grape juice before the fermentation process and has been the subject of numerous studies [44][45][46][47][48]. Derived from the fungus *Aspergillus niger*, the GOX enzyme promotes the conversion of β -D-glucose into D-glucono-lactone, releasing H_2O_2 , and subsequently catalyzes the conversion of D-glucono-lactone into gluconic acid, producing gluconic acid [45]. These reactions are responsible for the oxidation of fermentable sugars in the juice, thus preventing ethanol formation during fermentation. Applying the GOX enzyme has been shown in assays to decrease alcohol content by approximately 2% v/v [46] to 4.3% v/v [47]. Petkova et al. [48] found a 28% reduction in glucose after enzyme application, preserving some glucose and fructose for yeast fermentation in a second treatment. Additional studies observed not only reduced alcohol content but also decreases in heptyl acetate concentration, certain alcohols with floral notes, and ketones with floral and fruity notes in the wines [44].

Viticultural Practices

Early grape harvesting is another widely used pre-fermentation method to reduce alcohol in wines, involving either early harvesting or blending mature grapes with early-harvested ones. This approach has shown promise, achieving about a 3% v/v decrease in ethanol content [49][50]. In studies with Pinot Noir and Tannat grape varieties, this technique not only lowered alcohol content but also decreased pH and total acidity without affecting other wine constituents [51].

Therefore, regarding viticultural practices, it is essential to carefully consider early harvest to maximize grape flavor compounds for the final wine style. However, this approach may not be appropriate for all wine styles. Early harvest can be beneficial when there is a rapid onset of berry shrivel. Still, options for color enhancement in red wines should be considered depending on how early the harvest is conducted [52][53].

Using growth regulators also appears to be a viable option, as they can reduce the sugar concentration in the berries, resulting in lower wine alcohol content [54][55][56]. Naphthaleneacetic acid, used as an antitranspirant on Syrah grapes during pre-veraison, led to delayed berry ripening. This delay allowed better management of sugar accumulation and resulted in wines with no significant changes in sensory profiles [57].

Grapevine canopy management, a crucial viticultural practice for vineyard balance, significantly influences sugar accumulation in grapes [58]. Reducing leaf area through severe pruning or leaf removal at various growth stages minimizes fermentable sugar accumulation, lowering ethanol content in the resulting wine [33]. Zhang et al. [59] compared basal and apical defoliation, observing lower alcohol content in Shiraz grapes and wines with minimal impact on aromatic properties. Similar outcomes were reported by Poni et al. [60], achieving significantly reduced total soluble solids and alcohol concentration without affecting phenolic composition through leaf removal above the grape cluster zone in Sangiovese after veraison. Other studies have aimed to reduce the alcohol content in wine through leaf area reduction [61][62][63][64]. However, shoot thinning in Carot Noir grapes (a red interspecific hybrid of *Vitis*) increased soluble solids and alcohol content [65], as it did in Cabernet Sauvignon grapes [66]. Demonstration that the effects of shoot thinning practices

are unclear and depend on the grape variety. A previous study on shoot thinning found that shoot-thinned Marechal Foch (a red interspecific hybrid of *Vitis*) vines showed higher total soluble solids (°Brix) and berry anthocyanin concentrations compared to un-thinned vines [67]. However, the increase in berry anthocyanin did not result in higher anthocyanin concentration in the final wine. Furthermore, shoot thinning did not affect the sensory perception of the “fruitiness” of the wines [67]. In contrast, a study focused on Corot noir and the implementation of shoot thinning provided inconsistent results in grape and wine quality across a two-year evaluation (2008–2009). The evaluation was determined by soluble sugars (°Brix), pH, titratable acidity (TA), wine anthocyanin, berry, and wine tannin content. The study found that shoot thinning increased berry °Brix, wine alcohol concentration, and anthocyanin concentration only in the second year [65].

2.1.2. Reduce or Limit Ethanol after Winemaking

The following category relates to post-fermentation procedures. In this phase, physical methods for alcohol removal, including extraction, membrane separation, or distillation, are applied.

Alcohol Removal via Extraction Methods

Extraction can be carried out using gases, organic solvents (not for wine used commercially), or adsorbents.

The unique properties of carbon dioxide (CO₂), including its ability to transform into a supercritical fluid under specific temperature and pressure conditions, make it an effective compound for extracting organic compounds, such as ethanol in wine. In its liquid state within the wine, CO₂ has an affinity for the carbon chain of ethanol, facilitating its dissolution. As it transitions back to a gaseous state, it carries the dissolved ethanol, reducing the ethanol content in the wine [68]. According to Ruiz-Rodríguez et al. [69], extraction carried out with supercritical CO₂ in white, red, and rosé wines has proven to be an advantageous alternative. Besides removing ethanol from beverages, it leaves no residues in the wines and does not alter their aromatic profile or antioxidant activity. Furthermore, it is an economical, safe, and easy-to-handle solvent [34]. Other organic solvents like pentane and hexane are also used to remove ethanol from wines but may remove other aromatic compounds, affecting the final taste of the wines [70]. Hydrophobic adsorbents like zeolites are used in the production of dealcoholized wine. Nikolaou et al. [71] reported a conversion rate of 69.2% of malic acid content and the production of wines with low ethanol content. According to Akyereko et al. [72], this method enables the production of wines with an ethanol content of 0.5% v/v.

Alcohol Removal through Membrane-Based Processes

The separation through the use of membranes refers to physical separation techniques aimed at reducing or eliminating the alcohol content of wine through a semipermeable membrane. In this method, a natural osmotic pressure occurs due to the difference in concentration between two solutions flowing through the semipermeable membrane (tangentially, in parallel, or circularly). As a result, alcohol and water from the wine move from the more concentrated solution to the less concentrated one, reducing or eliminating ethanol from the wine [40][73][74]. The most commonly used membrane separation techniques include nanofiltration, reverse osmosis (RO), osmotic distillation (OD), and pervaporation (PV).

Besides its application in pre-fermentation to lower the sugar content in the must, nanofiltration can also be employed for alcohol removal from finished wines [40][75][76]. The semipermeable membrane has a size ranging from 1 to 10 nm, allowing the rejection of smaller molecules (such as sugars and proteins) at a pressure of about 75 bar, surpassing ultrafiltration membranes [77]. Some studies demonstrate the advantages and effectiveness of this technique compared to the reverse osmosis technique, which, in addition to reducing the alcohol content of wine, preserves its organoleptic characteristics with fewer losses in anthocyanins at lower pressures, making the process more cost-effective [43][78][79][80].

On the other hand, the RO process involves the application of high pressures, in the range of 60 to 80 bar, under which water and ethanol molecules are forced through the semi-permeable membrane, leaving behind a retentate with the remaining compounds. Simultaneously, a permeate flow is generated, containing higher amounts of water and ethanol due to their smaller molecular size [23][81]. However, adding water to the retentate is necessary to achieve efficient dealcoholization, which becomes a disadvantage of this process since it is generally illegal or restricted in many wine-producing countries. Additionally, this technique has other challenges related to dilution due to potential alterations in the sensory properties of wines and the operation at high pressures [79][82][83].

Another membrane separation technique for producing low-alcohol wine is osmotic distillation (OD), also referred to as evaporative perstraction (EP) or isothermal membrane distillation [34]. This technique is based on two aqueous phases: the wine containing volatile compounds and the water acting as the stripping liquid. Both circulate in countercurrent on opposite sides of a hydrophobic hollow fiber membrane module. The mechanism of ethanol removal in this technique involves the initial evaporation of ethanol due to increased temperature and the difference in partial pressure between

ethanol in the wine and the space inside the membrane pores. Subsequently, the ethanol vapor diffuses through the membrane pores and, upon emerging from these pores, mixes with the stripping water solution. When the ethanol vapor comes into contact with the stripping water, it condenses into a liquid state [41][74]. This evaporation, diffusion, and condensation process allows ethanol removal from wine, with values ranging from 1.3% to 10.5% v/v, while preserving some essential components [84][85][86]. Reducing alcohol in wine can negatively affect its quality, causing loss of aroma, oxidation, and spoilage due to microorganisms. Therefore, it is crucial to follow the manufacturer's recommendations and perform the alcohol reduction with care [86][87][88].

In contrast to OD, vapor permeation (PV) is a separation technique that employs dense and non-porous membranes to separate components from liquid mixtures based on partial evaporation. In this technique, the liquid mixture comes into direct contact with the selective side of the membrane (chemical affinity). In contrast, the permeate is collected in vapor form on the opposite side of the membrane. Separation occurs due to a driving force, a vacuum, a sweep of inert gas (such as nitrogen), or a temperature difference. This technique has three main steps: adsorption of the target component onto the membrane, diffusion of this component through the membrane, and desorption on the permeate side [89][90]. This technique has been explored as a promising method for reducing the alcohol content in wines due to several advantages applied in this context: it allows for the selective removal of ethanol from wine while preserving its aromatic profile, thus avoiding significant losses in sensory quality; it is more energy-efficient compared to traditional distillation; there is a lower possibility of contamination of the final product; it operates at lower temperatures than other dealcoholization methods; it is a "clean" technique as it produces water and ethanol as byproducts that can be reused [89][91][92]. Nevertheless, this technique faces limitations, such as high investment costs, a limited membrane market, low permeation rates at low temperatures, and the need to optimize operating conditions to achieve desired results [33][74][92].

Alcohol Removal: Thermal Processes in Winemaking

Thermal processes are widely employed to reduce the alcohol content in wines. Vacuum distillation (VD) and spinning cone column (SCC) are methods based on the fundamental principle of heating and evaporation.

Vacuum distillation (VD) is another process for producing low-alcohol wines. It is a thermal process that, under vacuum conditions, concerns evaporation, distillation, and condensation [93]. VD entails heating the wine to relatively low temperatures (typically between 15 °C and 20 °C) compared to traditional distillation. Heating is essential for evaporating volatile compounds, particularly ethanol, from the wine. The alcohol vapor is then separated under vacuum conditions and condensed into liquid form, yielding a distillate containing the extracted alcohol. After alcohol removal, the remaining wine has a significantly lower alcohol content, and retention of the aromas and flavors present in the original wine can occur under certain conditions [33]. Furthermore, it allows for flexible adjustment of wine alcohol content based on producer and consumer preferences. The recovered distillate can be added to the dealcoholized portion [33][89]. Nevertheless, studies indicate that while VD enhances volatile compounds, it may significantly reduce esters, alcohols, and terpenes, impacting the wine's aromatic complexity and sensory profile [34][94].

The SCC technique, widely used in the beverage industry for producing low-alcohol beverages, efficiently preserves aromatic compounds [95]. It involves rotating vertically stacked cones within a column [55]. The alcohol removal occurs in two phases: first, the wine undergoes SCC at a moderate temperature (26–28 °C) and reduced vacuum pressure (0.04 atm) to extract aromatic compounds. In the second phase, at higher pressure and temperature (38 °C), the alcohol content is reduced, resulting in a low-alcohol or non-alcoholic wine, depending on the remaining ethanol. Aromas recovered in the first phase enhance the final wine's aroma [96][97]. Studies highlight the effectiveness of SCC in reducing wine alcohol content and recovering aromatic compounds from red, white, and rosé wines [74][95][96][97][98]. Furthermore, a study conducted by Puglisi et al. [99] suggests SCC, combined with adsorbents, as a profitable strategy for remediating "smoke taint" in wines from grapes affected by wildfires. However, SCC technology is costly and involves expense management [34].

Multi-Stage Membrane-Based Systems

Recently, an innovative approach has emerged for producing low-alcohol beverages, aiming to prevent the loss of desirable aromatic compounds associated with single-membrane dealcoholization methods (such as NF, PV, RO, and OD) and thermal separation processes [23][74][84][85][93]. This approach uses an integrated membrane and distillation system called a multi-stage membrane-based system [74]. This technique combines two or more alcohol removal methods to remove ethanol from wines and beers. Commonly used multi-stage membrane-based systems include nanofiltration and pervaporation (NF–PV) systems [100], pervaporation units combined with distillation [78], and reverse osmosis–evaporative pertraction (RO–EP) systems [101], with the RO–EP system being the most widely used. These combined systems have proven highly efficient for producing low-alcohol wine, as they not only maintain aroma characteristics

similar to the original wine but often achieve improved versions compared to the original product [100][102][103]. Despite some losses of desirable aromatic compounds such as ethyl esters, acetate esters, and monoterpenes [101][104], the ability of these systems to preserve the wine's aroma and volatiles constitutes a promising strategy in the production of low-alcohol or alcohol-free beverages, meeting the growing consumer demand for healthier and high-quality products.

2.2. Microbial Strategies for Producing Low-Alcohol Wines

The increasing trend of alcohol content in wines, linked to climate change, might result in changes in flavor and complexity and, given the current consumer preferences, might negatively impact commercialization. Therefore, strategies limiting alcohol production or its reduction must be defined. These techniques can be categorized into three fundamental approaches, as mentioned earlier: pre-fermentation, fermentation processes, and post-fermentation techniques [105]. Focusing on microbial strategies, emphasis must be placed on the selection of fermentation microorganisms, their proportion or time of inoculation on grape must, and the conditions during fermentation. The goal is to reduce or restrict ethanol production during the fermentation phase. Specific yeast strains (genetically modified or non-*Saccharomyces* yeasts) can also be used to reduce yeast biomass (keeping the fermentation rate of fermentable sugars as low as possible) [34], or techniques such as interrupted fermentation are employed for this purpose.

2.2.1. GMO Microorganisms

Recently, genetic modifications or adaptive evolution and selection have developed modified yeast strains capable of reducing wine alcohol content during fermentation [69][78][106][107]. However, their use may pose challenges, such as producing undesired secondary metabolites, like acetaldehyde and acetoin, that can affect wine characteristics [108][109]. Another challenge in using such yeast strains is consumer acceptance of genetically modified organisms in food and beverages.

Given the relative simplicity of the yeast genome, its modification can be achieved. To obtain low-ethanol wine, the logical step would be to limit the expression of the enzyme alcohol dehydrogenase (ADH), **Figure 3**, which catalyzes the final step in ethanol production during alcoholic fermentation. However, this approach was deemed unpractical because strains with ADH deletion could not grow under anaerobic conditions and the production of higher levels of acetic acid and acetaldehyde [110][111]. Hence, novel targets had to be found to redirect the metabolism from ethanol production to other end-products [32]. However, these changes in the metabolic pathways must be carefully monitored, as other products can impact the overall quality of the wine [112].

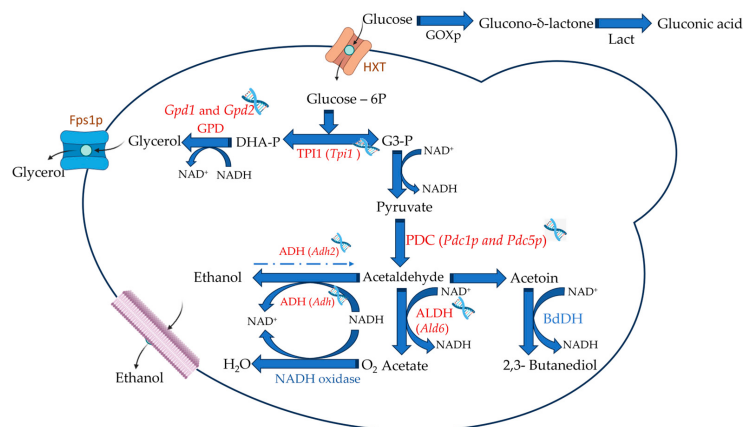


Figure 3. Main target specific enzymes and genes in yeast cells that may lead to lower ethanol yields. HXT—hexose transporter; Fps1p—aquaglyceroporin; GPD—glycerol-3-phosphate dehydrogenase; TPI1—triosephosphate isomerase; PDC—pyruvate decarboxylase; ADH—alcohol dehydrogenase and ALDH—Aldehyde dehydrogenase. GOXp—Glucose-oxidase—and Lact—lactonase—are expressions, in yeasts, of non-yeast genes.

One of the modifications that can be used to reduce ethanol production is linked to the overexpression of glycerol-3-phosphate dehydrogenase isozymes, namely through *Gpd1* and *Gpd2* genes [113], **Figure 3**. Glycerol production usually uses about 4% of grape juice carbon during fermentation by *S. cerevisiae*, generally in the initial stages of biomass formation [114]. Glycerol has two essential functions: to combat osmotic stress and to maintain the oxidation-reduction balance. The reaction behind glycerol formation is linked to the correction of redox balance within cells [115]. The overexpression of these genes increases glycerol synthesis while decreasing ethanol synthesis [116]. This increase in glycerol production can reach as much as 548%, while the reduction in ethanol can be of great significance, as shown in early works [117]. Other results indicate an increase in glycerol production ranging from 109 to 275% and a reduction in ethanol from 3 to 24%, depending on the experimental medium (yeast extract, peptone, dextrose medium, yeast nitrogen

base medium, synthetic medium, grape juice or synthetic Leu-free) or the overexpressed gene (*Gpd1* or *Gpd2*) [112]. However, other metabolites are also more produced and can cause changes in the quality of wine. Of those, succinate, acetate, acetaldehyde, acetoin, and 2,3-butanediol [118][119], **Figure 3**, must be referred to, as their presence above the sensory threshold may be detrimental to perceived wine quality [112][120]. Mutant yeasts with modifications of GPD need further genetic modifications to avoid excessive production of these metabolites. One modification is the deletion of aldehyde dehydrogenases, namely the *Ald6* gene, that contribute to the formation of acetic acid [121]. This modification decreased the formation of acetic acid, with increased glycerol production and lower ethanol yield [119]. However, a subsequent problem arose, as the deletion of *Ald6* increased acetoin production, negatively affecting wine aroma. Ehsani and collaborators [122] obtained a strain that produced lower ethanol levels (3%, v/v less) and higher glycerol production with a reduced impact on sensory parameters in the final wine.

Other changes in glycerol metabolism can be achieved by changing the expression of genes linked to Fps1p, an aquaglyceroporin channel that controls the intracellular glycerol concentration [123][124][125]. The production of glycerol is controlled by a regulatory domain located in the N-terminal extension of Fps1p. This domain regulates glycerol transport; when removed, the channel becomes hyperactive. As a result, glycerol continuously leaks out of the cell, and the cell compensates for the loss by producing more glycerol [124][126]. Varela et al. [109] observed that the increased glycerol production, due to deleting the regulatory domain of Fps1p, lowered ethanol formation considerably (**Figure 3**).

Another gene modification to reduce ethanol production can be deleting Pyruvate decarboxylase (PDC) genes. This enzyme catalyzes the decarboxylation of pyruvate to acetaldehyde and CO₂, being three genes known in *S. cerevisiae* (*Pdc1*, *Pdc5*, and *Pdc6*) up-regulated by the transcription factor *Pdc2p*; however, only *Pdc1p* and *Pdc5p* are known to be active in yeast during fermentation [127], **Figure 3**. Modifying the PDC genes resulted in diverse outcomes. Deleting *Pdc1* resulted in lower activity and increased pyruvate levels, which are undesirable, considering microbial stability and balance of sulfur dioxide. Still, no reduction in ethanol, while deleting *Pdc2*, led to a considerable decrease in ethanol levels and increased glycerol production while maintaining sufficient pyruvate decarboxylase activity to support glucose growth [117]. Further changes in this set of genes were performed by Cuello et al. [128], which resulted in a strain with reduced ethanol production without effect on other fermentation kinetics.

Another gene editing that can be used to reduce the production of ethanol is modifying the expression of triose-phosphate isomerase, encoded by the *Tpi1*, which is the enzyme catalyzing the interconversion between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, the two products following the breakdown of fructose 1,6-bisphosphate [111], **Figure 3**. The lack of this gene resulted in high amounts of glycerol, with a reduced ethanol yield [129]. However, the deletion of the *Tpi1* gene caused the inability of this strain to grow in a glucose medium, probably due to the reduced content of NADH, which is produced during the conversion of glyceraldehyde 3-phosphate into pyruvate [130][131]. A complete loss of activity of the TPI1 enzyme seems like it would be more feasible, as a reduction in yeast growth with attendant fermentation problems is likely to occur. Still, a partial reduction in its activity could provide an opportunity for low alcohol-producing yeast. This partial reduction can be achieved by changing regulatory genes, like *Gcr1* and *Gcr2* (transcription factor for glycolytic genes), that can reduce the expression of *Tpi1* [132], with mutations in other genes, like *Reb1* (an essential gene that maps on chromosome), *Rap1* (Repressor Activator Protein), and *Grc1* (component of the minus-end located γ -tubulin ring complex) also able to reduce TPI1 activity [133]. Avoiding glucose repression of respiration has also been a target for producing low-alcohol wines. This approach relied on the use of a chimeric glucose transporter, comprised of the amino-terminal part of HXT and the carboxy-terminal region of *Hxt7* (HXT1, 2, 3, 4, 6, and 7 are intramembrane transporter proteins known to be involved in the transport of glucose) [134]. Using a yeast strain with deleted *Hxt1*, 2, 3, 4, 6, and 7 resulted in a respiratory phenotype with low ethanol production [135], even though results were achieved using low sugar concentrations medium (5%, w/v) compared to grape juice. This shifts the metabolism from the Pasteur effect (under low O₂ concentrations, yeasts conduct alcoholic fermentation forming ethanol and CO₂, or, under high O₂ concentrations, aerobic fermentation occurs with lower glucose consumption) to the Crabtree effect, where, in the presence of high sugar content (about 200 g/L), regardless of O₂ presence, fermentation can occur [136].

A different approach is the expression, in yeasts, of non-yeast genes. Reducing the content of sugars in grape juice before fermentation can be achieved by glucose oxidase (GOXp), an oxygen-dependent dehydrogenase that catalyzes the first step of a two-step process associated with converting glucose to gluconic acid [112]. Besides treating grape juice with GOX to reduce the sugar content, a strategy that has several limitations, even though it can reduce up to 40% of ethanol production [137], the introduction of the gene encoding this enzyme has already been performed in *S. cerevisiae* [138]. Microvinifications of Chardonnay juice samples resulted in wines containing 1.8–2.0% less alcohol, possibly due to the use of glucose to produce D-glucono- δ -lactone and gluconic acid by GOX (**Figure 3**). Other authors overexpressed the gene *noxE* from *Lactococcus lactis*, encoding H₂O-forming NADH oxidase, *NoxEp*, that uses NADH, oxidizing it when oxygen is available [139]. The introduction of this NADH oxidase can decrease the available intracellular NADH pool,

affecting alcohol dehydrogenase (ADH), hence resulting in reduced ethanol formation [32]. This resulted in using only half the available sugar but with paralleled changes in other metabolic pathways, increasing acetaldehyde production, impairing growth, and fermentation performance. All these genetic manipulation approaches to low ethanol-producing yeast, as well as others that might arise, will be dependent on the acceptability of both the industry and consumers of using such yeast, pushing current research on the search for non-GMO alternatives [31].

2.2.2. Yeast Selection for Low Alcohol Production

One alternative to avoid GMO yeasts, designed for low-ethanol production, is to isolate those that naturally present that trait. However, this effort for *S. cerevisiae* can be complex, as biochemical and physiological characteristics and the underlying genetics of this yeast have been pushed by natural selection to favor the yield of ethanol [31][140]. This has resulted in a slight variation in this phenotype [141], with currently available *S. cerevisiae* wine yeasts resulting in similar ethanol production when fermenting the same must [142]. Hence, the option would be selecting non-*Saccharomyces* (NS) yeasts that preferentially consume sugars by respiration rather than fermentation [136]. Therefore, evaluation of ethanol production variation among NS yeasts has been addressed. Using non-*Saccharomyces* yeasts has garnered significant interest from the scientific community and winemakers, as the available data state (e.g., [143][144][145]). These yeasts can divert carbon or sugar metabolism into other pathways, thus avoiding ethanol production during fermentation [143][144][145][146][147]. Several studies have shown significantly reduced wine ethanol levels when using these yeasts. For instance, Magyar and Toth [148] identified *Saccharomyces uvarum*, *Candida stellata*, and *C. zemplinina* strains with exciting properties. These strains produced, in laboratory fermentations, similar residual concentrations of sugars but with considerable changes in alcohol production, namely for *C. zemplinina*, with approximately half the alcohol content that was recorded for *S. cerevisiae*.

Another exciting work was performed by Gobbi et al. [149], using *Zygosaccharomyces bailii*, *Z. sapae*, *Z. bisporus*, *C. zemplinina*, *C. stellata*, *Hanseniaspora uvarum*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis*, and *S. cerevisiae*, for fermentation tests with grape juices. Results showed significantly low ethanol production in *Z. bailii*, *Z. sapae*, *Z. bisporus*, and *C. zemplinina*, but more prominently when using *H. uvarum*, confirming data observed by other authors [150][151]. Low ethanol production has also been reported for strains of *Metschnikowia pulcherrima*, *Schizosaccharomyces malidevorans* and *C. stellata* [146], *Torulaspora delbrueckii* [150], *Pichia kudriavzevii* and *Z. bailii* [152]. *Schizosaccharomyces pombe* reduced 0.65% of ethanol in the fermentation of white Airén grapes [153], and some *Saccharomyces* species can also provide low ethanol-producing strains. A reduction of 0.7% was achieved with the use of *S. uvarum* [154], with *S. kudriavzevii* also presenting interesting results [155]. However, some adverse effects have also been linked to the use of these alternative strains if partial aeration strategies during fermentation are applied to allow the use of sugar to be consumed via respiration rather than alcoholic fermentation, namely the formation of undesirable volatile compounds, including acetic acid [152][156][157], even though positive effects on sensory characteristics also occur [32].

Additionally, non-*Saccharomyces* yeasts play a multifaceted role in wine fermentation, potentially enhancing its sensory profile and aromas and contributing to wine stability and complexity [143][144][145][146][147][149][157][158][159][160][161][162]. Some non-*Saccharomyces* possess antimicrobial properties, including the production of Killer factors (mycocins), which inhibit the growth of undesirable yeasts (*Zygosaccharomyces* genus, *Brettanomyces bruxellensis*, among others), providing an additional advantage [163]. These “Killer factors”, such as CpKT1 and CpKT2 produced by *Candida pyralidae*, have demonstrated effectiveness in controlling the population of undesired yeast strains, such as *B. bruxellensis*, in winemaking conditions, without adversely affecting the fermentation processes of *S. cerevisiae* or the tested lactic acid bacteria [164]. Other “Killer factors”, such as KTCf20 and Pikt (produced by *Wickerhamomyces anomalus*), Kwkt (produced by *Kluyveromyces wickerhamii*), PMKT and PMKT2 (produced by *Pichia membranifaciens*), have also demonstrated potential in controlling unwanted yeast strains in vinification environments [165][166][167][168].

2.2.3. Co-Inoculations and Sequential Inoculations (Non-*Saccharomyces* and *S. cerevisiae*)

Considering some of the previously referred advantages and drawbacks of using different yeasts, two approaches to reducing the ethanol content in wine are co-inoculation of those yeasts or their sequential introduction in fermentation. The first approach (co-inoculation) involves concurrent inoculations of non-*Saccharomyces* or other *Saccharomyces* non-*cerevisiae* yeasts at high cell concentration with *S. cerevisiae*, and the second approach (sequential inoculation) consists of the start of fermentation with non-*Saccharomyces* or other *Saccharomyces* non-*cerevisiae* yeasts, occurring for a given duration and inoculating *S. cerevisiae* to take over and complete the fermentation [169].

The critical factors affecting fermentation and oenological outcomes of this approach are the time leading to the inoculation of *S. cerevisiae* (in sequential fermentations) and the ratio of *S. cerevisiae* and other yeast [160]. Besides ethanol changes, non-*Saccharomyces* or other *Saccharomyces* non-*cerevisiae* yeasts are essential due to their

contribution to wine aroma and flavor, with several yeasts already described as contributors to that profile. Padilla et al. [160] and Ivit et al. [144] point out several yeasts as having great oenological interest and used in co- or sequential inoculations, which will be briefly reviewed here.

One of the most important genera is *Hanseniaspora*, which comprises at least ten species, *H. uvarum* and *H. guilliermondii* being the most common. Several *Hanseniaspora* species have been tested in sequential or co-inoculated fermentation with *S. cerevisiae*, with a recorded reduction in the ethanol content of wines. Reductions of around 1% were achieved using sequential inoculation of *H. uvarum* and *S. cerevisiae* in synthetic grape juice [158], and, in white (Sauvignon blanc) and red (Pinotage) musts, reductions in ethanol were also achieved of around 1.3% and 0.8%, for white and red musts, respectively [170]. Furthermore, three strains of *H. uvarum*, in sequential or co-inoculated fermentations with *S. cerevisiae*, resulted in lower ethanol concentration when compared to fermentations with the latter only [171]. Also, in synthetic grape juice, a reduction in alcohol was recorded with sequential inoculation of *H. osmophyla* and *S. cerevisiae* [158] and white (Sauvignon blanc) and red (Pinotage) musts; the use of *H. opuntiae* also resulted in less production of ethanol [170]. However, some studies point out the increase in acetic acid when fermentations are performed using *Hanseniaspora* yeast strains [171].

Another important yeast already known to have essential winemaking traits is *Schizosaccharomyces pombe*. Besides being able to moderate wine acidity by metabolizing malic acid, this strain enhances the color of red wine and reduces Ochratoxin A, biogenic amines, and ethyl carbamate [145]. In addition, some *S. pombe* strains used in sequential fermentation resulted in lower ethanol content [153], even though a lack of reduction or even increase in alcohol has been reported when using *S. pombe*, or even the presence of unsuitable aroma produced by the fermentative metabolism of *S. pombe* [172].

An alternative non-*Saccharomyces* yeast of great importance is *Metschnikowia pulcherrima*. This non-*Saccharomyces* yeast is commercially available from many suppliers and is known to improve several organoleptic characteristics of wines [173]. Furthermore, the production of wines with lower ethanol in sequential fermentations with *S. cerevisiae* has been reported in several works, either with grape juice [174], synthetic grape must [175], Chardonnay and Shiraz musts [146], and in white grape must (mixture of Malvasia and Viura varieties) [156]. A reduction of up to 1.6% in ethanol was recorded in Shiraz wines [146], with a drop of alcohol further confirmed in later works [176]. The use of *M. pulcherrima* and *S. uvarum* mixed inoculum, sequentially used with *S. cerevisiae*, reduced 1.7% v/v of ethanol compared to wine fermented with *S. cerevisiae* [176]. When immobilized, the sequential inoculation of *M. pulcherrima* could also reduce ethanol content in synthetic or natural grape juice [158]. However, results are linked to several conditions, namely aeration regimes, that must be carefully monitored [156][177].

Lachancea thermotolerans (previously *Kluyveromyces thermotolerans*) is a commercially available yeast that positively influences wine's sensory profile and total acidity [161]. Besides organoleptic advantages, reduction in ethanol content has been achieved in sequential or co-inoculation. A fermentation started with *L. thermotolerans* and a sequential inoculation, after two days, with *S. cerevisiae*, led to a reduction in ethanol up to 0.7% v/v [159][178][179]. Mixed or sequential fermentation with *L. thermotolerans* and *S. cerevisiae* also reduced alcohol production [180]. Further works prove that sequential fermentations with *L. thermotolerans* and *S. cerevisiae* can reduce the ethanol content in must of Tempranillo grapes. Mixed fermentations of *S. pombe* and *L. thermotolerans* can lower the ethanol content in wine but may also increase the acetaldehyde content [143]. The sensory threshold for acetaldehyde ranges from 100–125 mg/L. Typically, table wines have acetaldehyde levels below 75 mg/L immediately after fermentation. However, if the levels exceed 125 mg/L, it can result in unpleasant odors such as 'over-ripe bruised apples', 'stuck ferment' character, or 'sherry' and 'nut-like' characters.

Torulaspora delbrueckii was one of the first commercially accessible non-*Saccharomyces* yeasts, as they had similar fermentation patterns as *S. cerevisiae* and were able to enhance aroma composition and positively impacting properties for traditional methods of sparkling wine [181]. Several studies have proven that its use in mixed or sequential fermentation reduced ethanol. Most of these studies refer to reductions of 0.5% or below [177][182][183][184][185][186]. Higher reductions of ethanol were recorded in other works, like 1% less alcohol using Chardonnay [162] or less than 1.5% using chemically defined grape juice [176]. However, to achieve higher levels of alcohol reduction, *T. delbrueckii* must be used in regular fermentations with high aeration processes [187].

Another yeast commonly studied due to positive contributions during fermentations is *Starmerella bombicola* (formerly known as *Candida stellata*). Early works by Soden et al. [188] with mixed and sequential fermentations with *C. stellata* and *S. cerevisiae* resulted in less alcohol than the mono-inoculated *S. cerevisiae* control. Further works with sequential fermentations using *Starmerella bombicola* and *S. cerevisiae* in Chardonnay juice yielded lower ethanol concentrations

when compared to *S. cerevisiae* fermentations ^[188]. Immobilizing *Starmerella bombicola* is a practical approach to reducing the final ethanol content in grape must. Studies have shown that using this yeast species in the Trebbiano Toscano grape-must and the Verdicchio grape-must significantly reduce the final ethanol content ^{[158][189]}.

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