Chapter 17

Viticultural and Biotechnological Strategies to Reduce Alcohol Content in Red Wines

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Abstract

Viticultural and biotechnological strategies are two approaches to deal with higher must sugar levels at harvest time. A wide range of factors could significantly affect sugar accumulation in the grape such as choice of vineyard site, soil composition, irrigation strategy, rootstock, and grape cultivar selection as well as grape yield. In this sense, approaches to canopy management are continually evolving in response to changes in other vineyard management practices; some of these could contribute to reduce soluble sugars on grape berries at harvest time. On the other hand, among possible biotechnological strategies, one of the most relevant is the control of the fermentative process by using selected yeast strains. In this chapter, we will show how some viticultural practices have influenced the accumulation of soluble sugars and other enological parameters in grape berries at harvest time. We will also report how a careful yeast selection and the implementation of different fermentation strategies can also contribute to reduce ethanol content in wines.

Keywords: auxins, microfermentation, total soluble solids, veraison, yeast

1. Introduction

The current demand by consumers toward well-structured, full body wines has driven the requirement for late harvests. These practices ensure an optimal phenolic maturity, which entails very mature grapes with high level of sugars [1, 2]. Additionally, the timing of harvest is probably the single most important viticultural decision taken each season. "Critical ripening period" and "physiological maturity" are phrases used by winemakers that appear frequently in conjunction with wine grape harvests, on winery websites, and in wine press reviews of vintages, winegrowing regions, and wines [3]. Thus, the properties of the grapes at harvest set limits on the quality of the wine potentially produced [4]. Grape is a nonclimacteric fruit and does not ripen further after harvest, so harvesting at the proper stage of maturity is essential for optimal grape quality in terms of soluble solids, berry weight, titratable acidity, and overall sensory characteristic. This is a very important period that influences grape composition and determines varietal characteristics [5].

There are several measurable parameters in grapes that relate in some way to quality factors. One of these is some measure of sugar concentration, which usually is accomplished by estimating the amount of dissolved compounds in the juice [6]. The ripening of grape berries is accompanied by a massive accumulation of soluble sugars, and by the synthesis and accumulation of a wide range of phenolic compounds and aroma precursors. All of these processes play major roles in the quality of the berries and wine. Sugars accumulate in the vacuoles of flesh (mesocarp) cells, which account for 65–91% of the fresh weight in a mature berry [7]. Most of those soluble sugars are two hexoses easily metabolized by yeasts and bacteria, glucose, and fructose, which decrease the perception of sourness, bitterness, and astringency, enhancing the "mouthfeel", "body", or "balance" of wines [8]. From veraison, and throughout ripening, the berries accumulate roughly equal amounts of glucose and fructose [7]. However, while glucose and fructose concentration increases in the grape berry during ripening, there are multiple biochemical processes affecting the concentration of grape-derived compounds, which may, positively or negatively, influence wine composition and sensory properties [9]. Thus, determining grape harvest date for commercial winemaking usually involves a delicate balance, minimizing potential negative characters and maximizing positive flavor and phenolic substances, while avoiding excessive sugar concentration [10].

Although ethanol is very important for wine quality (most aroma volatiles are more soluble in ethanol than in water), wine's aroma is declined with increasing ethanol content [11]. Additionally, higher sugar levels at harvest produce not only higher alcohol content on wines, but also alter the content of yeast-derived metabolites [12]. Thus, one of the major issues of higher alcohol content in wines is its effect on the sensory properties of the wine, in such a way that relatively small changes in alcohol content could have a great influence on how the wines are perceived. Another major concern has to do with market trends due to the leading critics around the world, whose ratings have a strong effect on sales. Accordingly, because of the significance of viticulture and the winemaking socioeconomic sector in Europe and other areas of the world, it is important for wineries to consider market demands when adjusting alcohol levels in wines derived from their vineyards.

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On average, wines have gradually increased in alcohol content and pH in recent years and winemakers are concerned about the problem. Moreover, climate change may increase this tendency. Changes in rainfall distribution and average temperatures will probable affect vine and grape physiology, and impact wine composition and quality [13]. Under a future warmer climate, higher temperatures may inhibit the formation of anthocyanin, increasing volatilization of aroma compounds [14] and total soluble solids, suggesting a decrease in wine quality. Hence, high alcohol levels in wines should receive more prominent attention to improve the technologies for reducing alcohol content of wines by conserving organoleptic balance, flavor, and high quality. The strategies to achieve moderate alcohol levels fit mainly into four basic groups as viticultural, prefermentation, fermentation, and postfermentation strategies [15]. Prefermentation and fermentation applications can be include under the name of biotechnological strategies.

Viticultural and biotechnological strategies are two approaches to deal with higher must sugar levels at harvest time. The former involves practices as partial defoliation in vineyards, which has as main objectives increasing sunlight and ventilation for the fruit, aiming to improve color and maturity in red grapes, and helping to reduce fungal diseases, which should result in better wine quality [16]. A wide range of factors could significantly affect sugar accumulation in the grape such as choice of vineyard site, soil composition and vine nutrition, irrigation strategy, rootstock, and grape cultivar selection as well as grape yield [15]. In this sense, approaches to canopy management are continually evolving in response to changes in other vineyard management practices; some of these could contribute to reduce soluble sugars on grape berries at harvest time. On the other hand, a review among putative biotechnological-based strategies has been carried out, mainly related to the use of yeast strains in wine elaboration. Between all approaches one of the most relevant is the amendment of the fermentative process by using selected yeast strains and making changes in the way to proceed. A procedure consisting in the use of mixed yeasts inoculum was development and wines with up to one degree less alcohol strength were obtained. This chapter attempts to show how different viticultural and biotechnological strategies impact on the potential alcohol concentration in wines.

2. Managing the time of grape ripening

There is an increasing interest in using a number of plant growth regulators (PGRs) to manipulate berry composition for the benefit of the wine industries. PGRs that control the coordination of berry ripening and act to coordinate global changes in gene expression during crucial events of plant development could become ideal targets for altering ripening in a global manner [17]. Research on the role of auxins as PGRs in grape berry development to manipulate the timing of the onset of ripening, harvest date, and berry composition [18, 19] has showed lower total soluble solids levels in those grapes treated with auxins at harvest time. Since extending the time before harvest increases sugar concentration, which in turn leads to wines with elevated ethanol concentration [10], it could be advisable the use of auxins to delay grape maturity. The mechanism by which auxins delay ripening is unknown, but auxin treatments

maintain the berry in the preveraison state, as judged by a delay in the physical and biochemical changes normally associated with ripening. These include a delay in the accumulation of sugars and anthocyanins, and also a delayed decrease in acidity and chlorophyll [18].

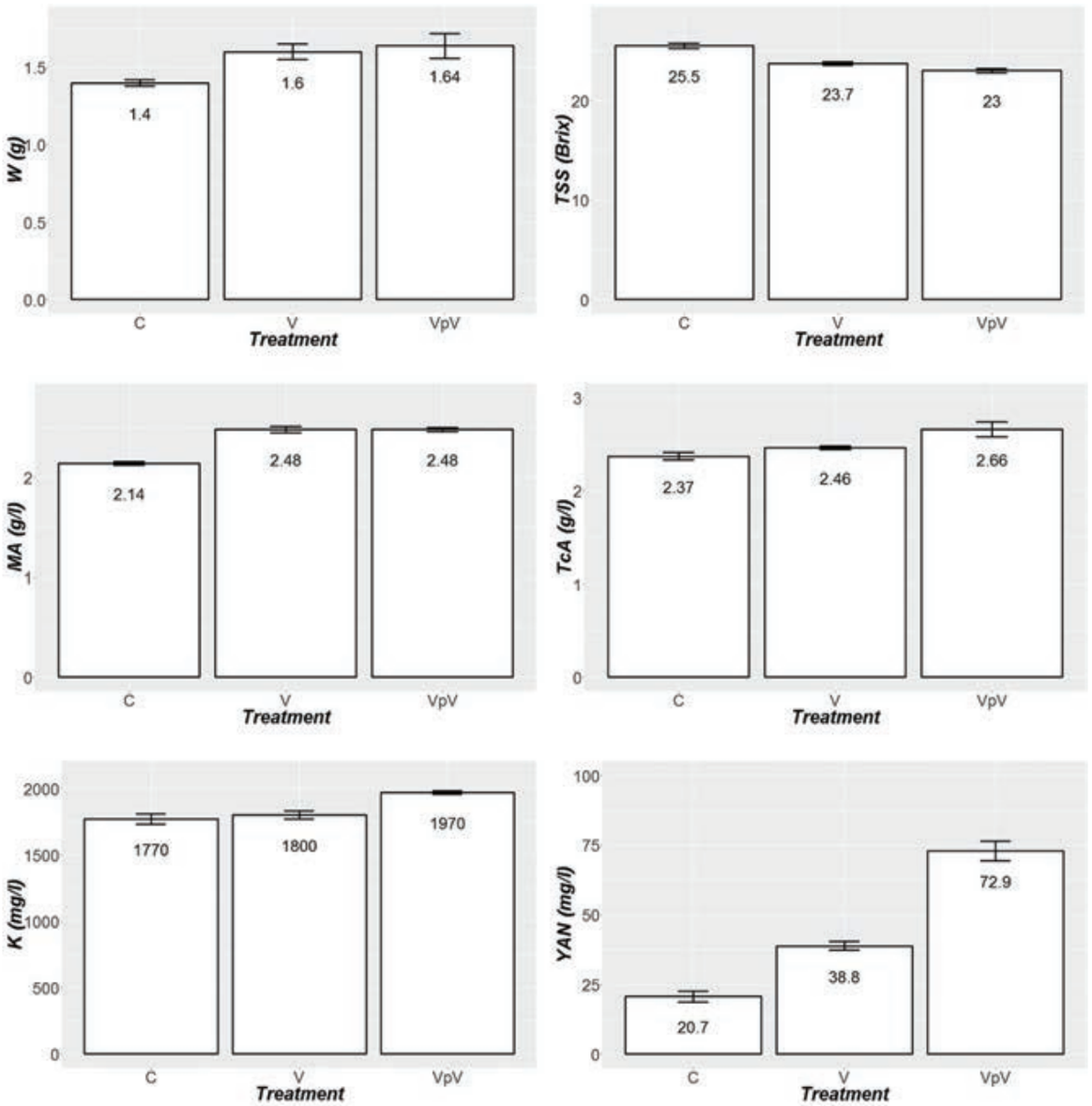

Figure 1. Bar graphs of berry weight (W), total soluble solids (TSS), malic acid (MA), tartaric acid (TcA), potassium in must (K), and yeast assimilable nitrogen (YAN) (2015). Average values are displayed within bar graphs. Standard errors are shown as bars (±1 SE mean). C: control; V: NAA sprayed 5 days preveraison; VpV: NAA sprayed 5 days pre- and postveraison.

Figure 1 reports differences in maturity of *Vitis vinifera* L. Tinta de Toro grapes at harvest time due to the synthetic auxin 1-naphthaleneacetic acid (NAA) treatments. A commercial vineyard representative of vineyard lands in the Protected Designation of Origin (PDO) Toro (Spain) was selected. The trial consisted on a randomized triplicate design with control and NAA treatments randomized over adjacent replicates. Each replicate consisted of 100 treated vines

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in each subplot. Bunches were sprayed 5 days preveraison (V) and 5 days pre- and postveraison (VpV) with 50 mg/l NAA in water. Control fruit (C) was not sprayed. Veraison stage was followed by color development and it was established when approximately 50% of cluster berries begin to color. Three hundred berries were sampled for each of the three replicates at harvest time (September 2015). Several analysis of variance (ANOVA) *F*-tests, performed using R software, were carried out to study the effect of auxins on berry weight (W), total soluble solids (TSS), malic acid (MA), tartaric acid (TcA), potassium in must (K), and yeast assimilable nitrogen (YAN, which comprises both ammonia and alpha-amino acids). When *F*-ratios were statistically significant ($p < 0.05$), *post hoc* tests (Holm corrections) were carried out to determine where the differences between groups lay.

Means and standard errors of all evaluated parameters arranged by treatment are shown in **Figure 1**. According to ANOVAs there were significant effects ($p < 0.05$) of treatment factor on all the measured parameters. Thus, at harvest time Brix levels tended to significantly decrease in the sequence C (25.5 °Brix ± 0.53 95% CI) > V (23.7 °Brix ± 0.33 95% CI) > VpV (23.0 °Brix ± 0.39 95% CI). One of the issues that emerges from the findings showed in **Figure 1** let support the hypothesis that both NAA treatments (V and VpV) predispose to a higher levels in YAN levels compared with control subplots, and specifically VpV more than V. The latter would indicate the ability of the NAA treatments to increase the assimilable nitrogen for yeasts in grapes at harvest time. This finding could have important implications in those musts which are very low in YAN levels by varietal causes. Additionally, since high levels of auxin in development are thought to be involved in cell division and expansion, it is not surprising the higher weight berries in both NAA treatments than Control subplots.

In the same year (2015), another study with NAA was performed in Villafranca de Duero (Spain). The trial established two parcels within two *Vitis vinifera* L. cv.: Cabernet Sauvignon and Syrah. At this time, the trial consisted on a randomized quadrupled design with control and NAA treatments. Each replicate consisted of 10 treated vines (~150 bunches) in each subplot. Treated bunches were sprayed 5 days pre- and postveraison (VpV) with 50 mg/l NAA in water. A total of 100 berries were sampled for each of the four replicates at harvest time (September 2015). Several *t*-tests, separately for each cv., were carried out to study the effect of auxins on W, TSS, MA, TcA, K, and YAN (**Figure 2**). According to the *t*-tests NAA had no significant ($p > 0.05$) effects on any of the parameters evaluated on cv. Cabernet at harvest time. However, in the case of cv. Syrah the levels of K in must significantly ($p < 0.05$) increased as a consequence of NAA application. Thus, must K levels tended to significantly increase in the sequence Control (2310 mg/l ± 196 95% CI) < VpV (2690 mg/l ± 207 95% CI). Because an "oenological excess" of K ions in red wines especially reflects an unfavorable ionic balance and a detrimental high pH value [20], this finding could be of great importance to winemakers. Although there are no significant differences with the control subplots in terms of TSS a decreasing trend in both cultivars could be observed in NAA-treated subplots in the sequence Control (24.3 °Brix ± 0.61 95% CI) > VpV (24.1 °Brix ± 0.53 95% CI) for cv. Cabernet Sauvignon and Control (24.6 °Brix ± 1.86 95% CI) > VpV (23.6 °Brix ± 1.39 95% CI) for cv. Syrah. One of the issues that emerges from these findings is that varietal factor might be of significant importance in the context of NAA effect on grape ripening.

With the data set obtained in both experiments with NAA (cvs. Tempranillo, Cabernet Sauvignon, and Syrah), the relationship between YAN, MA, and TSS levels in order to assess the intercorrelations among these must quality parameters was studied (**Figure 3**). From the data in **Figure 3**, it is apparent a possible linear relation between YAN and MA levels. Furthermore, levels of MA are positively correlated with levels of YAN. On the other hand, the findings do not indicate an apparent pattern in case of TSS with any of the other parameters.

Although the mechanisms that control the ripening of the nonclimacteric grape berry are poorly understood [21], the results of this study indicate the ability of NAA to decrease TSS at harvest time. Although the lower levels of TSS in treated berries may be mainly due to a delay in sugar accumulation, these data suggest that auxin treatments may be useful in controlling high must sugar levels at harvest time.

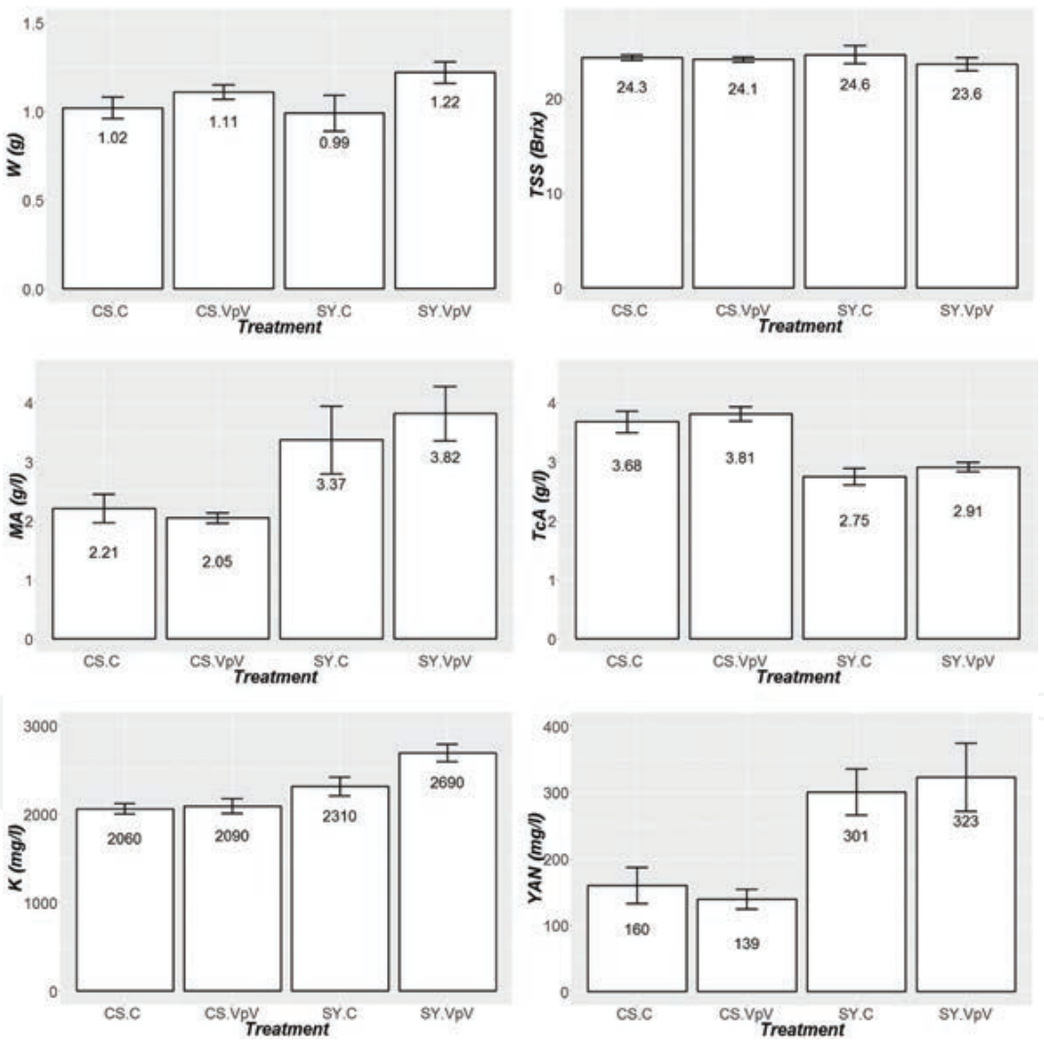

Figure 2. Bar graphs of berry weight (W), total soluble solids (TSS), malic acid (MA), tartaric acid (TcA), potassium in must (K), and yeast assimilable nitrogen (YAN) (2015). Average values are displayed within bar graphs. Standard errors are shown as bars (±1 SE mean). CS.C and SY.C: control in Cabernet sauvignon and Syrah, respectively; CS.VpV and SY.VpV: NAA sprayed 5 days pre- and postveraison in Cabernet sauvignon and Syrah, respectively.

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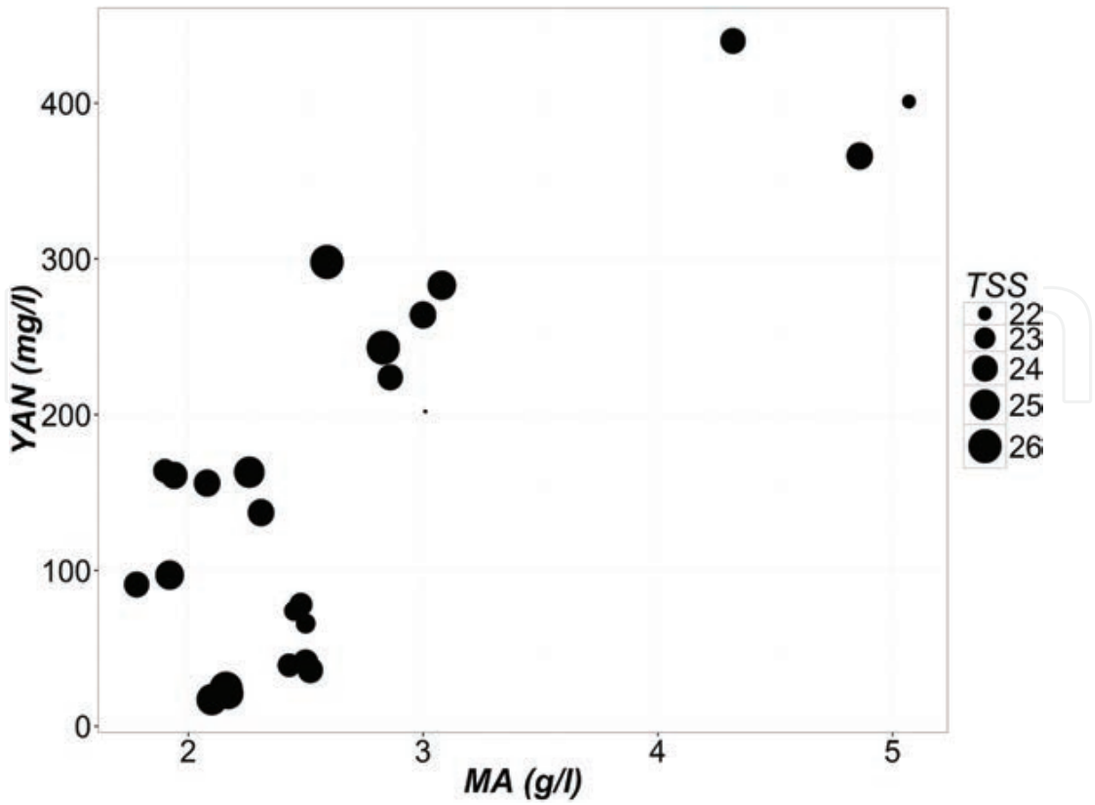

Figure 3. Relationship between malic acid (MA), yeast assimilable nitrogen (YAN), and total soluble solids (TSS) levels in grapes at harvest time. TSS in Brix scale.

3. Effects of Mg^{2+} foliar fertilization on berry sugar content

It must be recognized that grapevine nutrition remains an important part of managing a vineyard since it impacts on berry development and, finally, wine quality is derived to a large degree from berry composition. Some grape growers avoid any fertilizer for fear of overstimulating growth, whereas in other cases vineyard blocks might be fertilized when only specific areas of the block require fertilizer. Therefore, it is important that growers have a sound basis for determining the fertilizer needs of their vines [22]. Elsewhere, since the general relationship between vine nutritional status (in both nutrient macro- and microelements) and grape composition is obscure, further efforts are necessary to acquire greater knowledge in this topic. This is an important knowledge gap because these elements should necessarily influence grape juice quality and, therefore, the vinification process.

It is recognized that plants need K^+ for the formation of sugars and starches, for protein synthesis, and for cell division. Additionally, K^+ also neutralizes organic acids, regulates the activities of other mineral nutrients in plants, activates certain enzymes, and helps adjust water relationships (Hewitt, cited by [20]), but free potassium ions are released when the grape cell membranes are broken during grape processing, and form crystals with tartrate, which drop grape juice and wine acidity [11]. On the basis of the antagonistic interaction between levels

of K^+ and Mg^{2+} reported by several authors at the root-soil interface [23, 24], another study was performed during 2014 vintage in the PDO area of Ribera del Duero, Spain. The impact of Mg^{2+} supply on berry chemistry attributes from this trial is shown below.

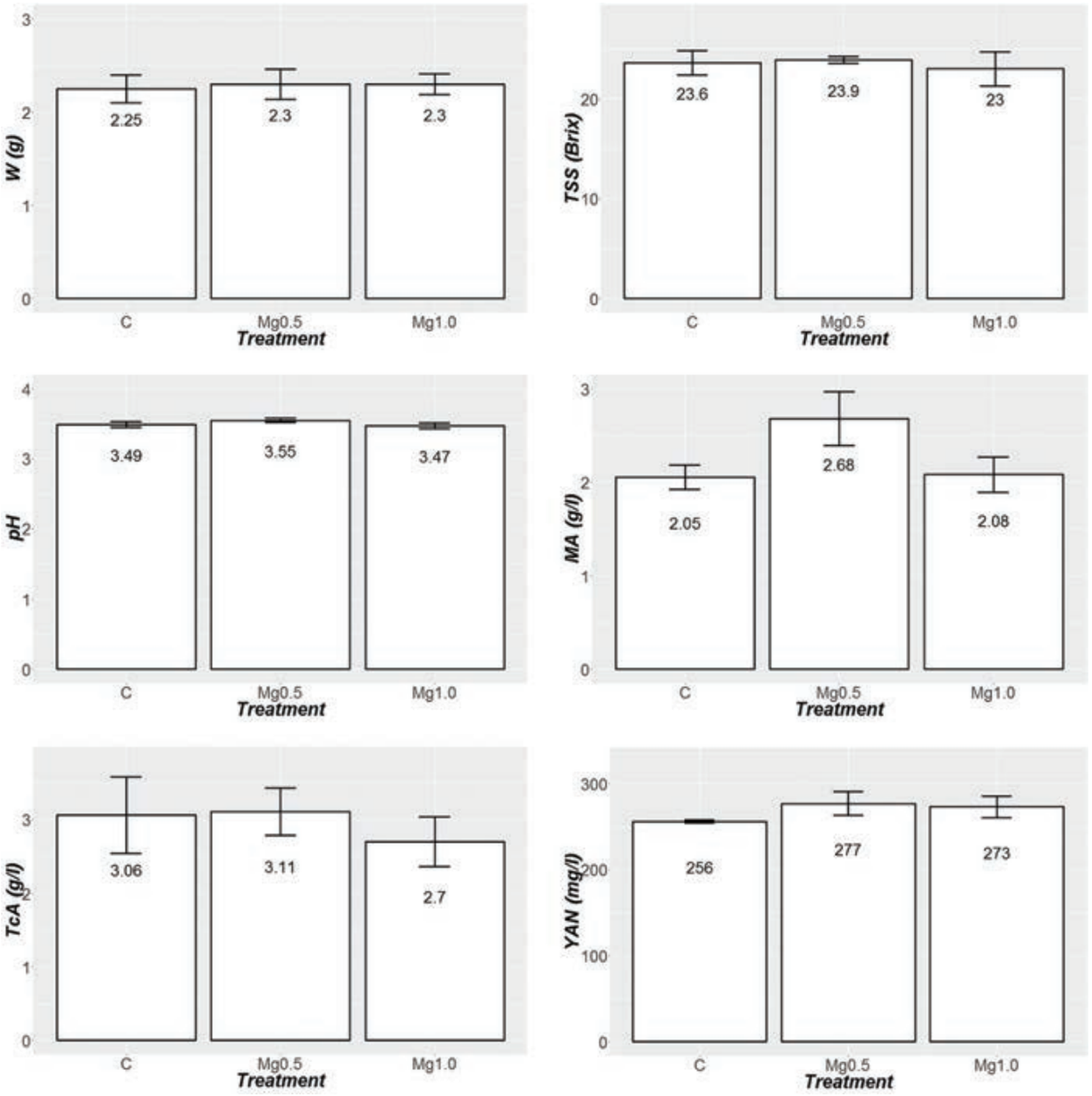

Figure 4. Bar graphs of berry weight (W), total soluble solids (TSS), real acidity (pH), malic acid (MA), tartaric acid (TcA), and yeast assimilable nitrogen (YAN) (2014). Average values are displayed within bar graphs. Standard errors are shown as bars (±1 SE mean). C: control; Mg0.5: foliar Mg at 0.5 kg/ha; Mg1.0: foliar Mg at 1.0 kg/ha.

The cultivar chosen, *Vitis vinifera* L. Tempranillo, is important in Spanish PDOs such as Rioja, Navarra, and Toro, and in many other countries [25]. The trial consisted on a randomized triplicate design with control and a foliar Mg^{2+} spray which was applied to cv. Tempranillo at two doses (0.5 kg/ha (Mg0.5) and 1.0 kg/ha (Mg1.0)) at veraison stage. Each replicate consisted

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of 10 treated vines (~150 bunches) in each replicate. The treatments were evaluated by data from cluster samples (10 clusters per replicate) at harvest time. One hundred berries from the clusters were removed and weighed (W). The berries were then crushed and several parameters (pH, TSS, MA, TcA, and YAN) were determined in the must. Means and standard errors of all evaluated parameters arranged by treatment are shown in **Figure 4**.

Several *F*-tests were carried out to study the effect of foliar Mg on W, TSS, pH, MA, TcA, and YAN. According to the ANOVA, foliar Mg treatments had no significant effects on any of the must quality parameters evaluated. Although none of TSS differences were statistically significant, as can be seen from **Figure 3** only Mg1.0 treatment showed a lower TSS level than control subplots, whereas Mg0.5 showed a greater TSS level than other treatments. Interestingly, although there are no significant differences with the control subplots in terms of grape weight (W) an increasing trend could be observed in Mg treated subplots (Control (2.25 g ± 0.29 95% CI) < Mg0.5 (2.30 g ± 0.31 95% CI) < Mg1.0 (2.31 g ± 0.22 95% CI)). In a similar way, leaf and shoot removal also affected YAN levels in such way that both Mg treatments may be associated with an increase in this key quality parameter. However, with a small sample size ($n = 9$ and 1 year), caution must be applied in both cases (W and YAN), because it is important to bear in mind the possible bias in the response to a foliar Mg treatment. Thus, if we consider our results collectively, they do not allow us to draw clear conclusions about the impact of foliar Mg treatments on harvest parameters, and therefore on TSS.

4. Effects of leaf removal and lateral shoot removal on berry sugar content

One of the most important and commonly applied summer canopy management operations in viticulture is the removal of leaves [26] and shoots in the fruit zone. Both practices are performed on grapevines to increase air circulation, light exposure, penetration of fungicide sprays, as well as decrease disease incidence. In general, exposing fruit to the sun will increase fruit temperature along with the enzymatic activities therein. Consequently, when compared to shaded fruit, exposed fruit will normally contain higher soluble solids [27]. Nevertheless, it should be noted that these actions on the vine canopy microclimate, which basically depends on the amount and distribution of leaf area in space and its interaction with above-ground climate [28], will have different effects on harvest quality according to the time and the shoot position when they were carried out. Most canopy microclimate components are of different values than those around the canopy, due to attenuation by the canopy. The degree of shading within grapevine canopies can be altered by three principal means: by varying the shoot number, the vine vigor, and/or the training system employed [28]. At the same time, a number of viticultural practices in wine grape improvement programs have been a topic of discussion in the scientific community in order to improve grape quality at harvest: optimum balance in vine pruning, shoot thinning, leaf and lateral shoot removal, early cluster thinning, late cluster thinning, shoot positioning, and tipping or irrigation scheduling.

On the basis of the above, a research was performed during 2014 vintage in the PDO area of Ribera del Duero, Spain. The cultivar chosen was *Vitis vinifera* L. Tempranillo. The trial

consisted on a randomized triplicate design with control and two leaf removal treatments both at veraison stage. One of the treatments was a leaf and lateral shoots removal between the clusters positioned at bottom and top in the productive shoots (LRbt), whereas the other one was a leaf and lateral shoots removal below the clusters positioned at bottom in the productive shoots (LRbl). Each replicate consisted of 10 treated vines and 100 berries from the clusters were weighed (W) and then crushed to evaluate several must quality parameters at harvest time (pH, TSS, MA, TcA, and YAN). Means and standard errors of must parameters arranged by treatment are shown in **Table 1**.

Must parameter	Control		LRbt		LRbl	
	Mean	SE	Mean	SE	Mean	SE
W (g)	2.25	0.15	2.50	0.25	2.57	0.06
TSS (Brix)	23.6	1.22	23.1	0.66	23.6	0.81
pH	3.49	0.04	3.46	0.05	3.48	0.06
MA (g/l)	2.05	0.13	1.93	0.18	2.29	0.08
TcA (g/l)	3.06	0.52	2.92	0.19	2.98	0.36
YAN (mg/l)	256	1.68	239	8.85	270	22.4

LRbt: leaf and lateral shoots removal between the clusters positioned at bottom and top in the productive shoots; LRbl: leaf and lateral shoots removal below the clusters positioned at bottom in the productive shoots.

Table 1. Means and standard errors (SE) of berry weight (W), total soluble solids (TSS), real acidity (pH), malic acid (MA), tartaric acid (TcA), and yeast assimilable nitrogen (YAN) (2014).

Several *F*-tests were carried out to study the effect of management practices on W, TSS, pH, MA, TcA, and YAN. According to the ANOVA these viticultural practices had no significant effects on any of the must quality parameters evaluated. However, fruit composition has been shown to be affected by microclimate manipulation. While it is true that leaf and shoot removal in both treatments (LRbt and LRbl) had no significant effect on W, both treatments increased consistently this parameter. In an opposite direction both treatments decreased consistently the real acidity (pH). On the other hand, only LRbt treatment showed a lower TSS level than Control (Control (23.6 °Brix ± 2.39 95% CI) > LRbt (23.1 °Brix ± 1.29 95% CI)). In contrast to earlier findings [28, 29] levels of tartaric acid (TcA) are increased by shade (C > LRbl > LRbt). The latter showed that this decrease in the concentrations of tartaric acid in the shaded berries was due to an increase in berry size. Additionally, in contrast to Petrie et al. [30], levels of pH decreased by shade (C > LRbl > LRbt). It is difficult to explain these controversies with previous studies, but it might be related with the time of carrying out both viticultural practices. In this regard, because when veraison begins to occur green growth slows to a stop (while the vine directs energy toward the grape clusters) [31], the choice of another phenological stage for making these viticultural practices might have a different impact on harvest parameters. The lack of scientific evidence and controversies have been reported by several authors. For instance, whereas Bledsoe et al. [32] found that yield and yield components were not signifi-

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cantly affected by the timing of leaf removal in Sauvignon Blanc grapes, Hunter and Visser [33] found that 33% defoliation prior to berries reaching pea size reduced berry size and yield, but had no effect when applied at veraison. Thus, understanding the impact of the timing of leaf and shoot removal on vines is crucial for vineyard managers and winemakers.

5. Biotechnological approaches to reduce the alcohol content in wine

Currently, several different technological strategies are available in order to reduce the ethanol content in final wines. Yeasts are the main microorganisms involved in the ethanol production from grapes and wine production, and accordingly, some of these strategies are based in a different management of wine yeasts, including the isolation of strains with a lower ability to produce ethanol.

Natural screening might be the first attempt to obtain lower ethanol-producing strains. However, this approach is unlikely to succeed because different aspects of the biochemistry, physiology, and genetics of *Saccharomyces cerevisiae*. In fact, this microorganism has evolved by natural selection to boost the ethanol production, even when oxygen is available [1, 34]. On the other hand, an attractive option would be to develop engineered wine yeast with reduced ethanol yield. So far, one of the most promising strategies is to redirect the metabolic flux toward an increased production of glycerol instead ethanol [2, 35]. However, this strategy has shown some unwanted effects like an overproduction of undesirable compounds from an organoleptic point of view [1]. Indeed, glycerol and ethanol produced during alcoholic fermentation are important regulators of the cellular redox balance, and consequently any attempt to redirect carbon flux by gene manipulation would modify the concentration of a range of other metabolites in order to correct the redox imbalance [36]. Acetaldehyde, acetate, succinate, acetoin, diacetyl, and 2,3-butanediol, among others, are some of the compounds to be avoided, since their presence at levels exceeding their sensorial threshold may be detrimental to the final wine quality [1, 36]. Furthermore, the public attitude toward the use of genetically modified organisms (GMOs) in food products and the legal restrictions in their use suggest that novel yeast strains will have to be generated using non-GM approaches.

Microorganisms, and particularly yeast, have a huge ability to adapt rapidly to different environmental conditions. This property has been used in recent years to modify the natural properties of yeasts by conducting adaptive laboratory evolution (ALE) experiments [2]. This approach mimics the natural evolution, by environmental or metabolic constraints, with the main purpose of obtaining improve yeast strains for several biotechnological applications and, of course, in winemaking processes [37–40]. A recent ALE study, by using KCl as osmotic and salt stress agent during 450 generations, achieved a wine with 0.6% (v/v) less ethanol in pilot scale fermentation when it was compared to the previous ancient strain. Besides that, the use of intrastrain hybrids by breeding techniques (a non-GMO technique) has proven the reduction of the alcoholic strength to 1.3% (v/v) [2].

An alternative approach to modify the final alcohol content of wines is related to the performance of modified fermentation procedures. Although *S. cerevisiae* is the main yeast species

responsible for conducting the alcoholic fermentation, the contribution of a nonnegligible number of other yeast species associated to the initial stages of the fermentation and their contribution to final sensorial properties are well established [41–43]. These strains are naturally present in sound grapes and might be easily isolated from the grape must at initial fermentation stages.

There are significant differences in sugar metabolism between some of these species and *S. cerevisiae*. The non-*Saccharomyces* strains actually allow an increased breakdown of sugars via respiratory pathways than through fermentation. An enhancement of this respiratory catabolism has been suggested by several authors in order to reduce the amount of sugar conversion into ethanol [44, 45]. For this reason, mixed cultures between a non-*Saccharomyces* strain and a *Saccharomyces cerevisiae* strain might be a good alternative to the previously mentioned approaches. Further, an additional advantage of this strategy would be to use autochthonous yeast strains in order to maintain the typicality of wines elaborated in this way [46].

	Muestra/microvinification					
	Young vineyard		**Middle-age vineyard**		**Old vineyard**	
Fermentation phase	**Yeast analyzed**	**Identification**	**Yeast analyzed**	**Identification**	**Yeast analyzed**	**Identification**
Initial	10	**2** *Hanseniaspora uvarum* **1** *Lachancea thermotolerans* **4** *Metschnikowia aff. fructicola* **3** *Metschnikowia pulcherrima*	10	**2** *Lachancea thermotolerans* **4** *Metschnikowia aff. fructicola* **1** *Metschnikowia chrysoperlae* **3** *Metschnikowia pulcherrima*	10	**1** *Debaryomyces hansenii* **1** *Hanseniaspora uvarum* **3** *Lachancea thermotolerans* **2** *Metschnikowia aff. fructicola* **2** *Metschnikowia pulcherrima* **1** *Kluyveromyces dobzhanskii*
Medium	10	**5** *Hanseniaspora uvarum* **1** *Lachancea thermotolerans* **4** *Saccharomyces cerevisiae*	10	**2** *Hanseniaspora uvarum* **5** *Lachancea thermotolerans* **3** *Saccharomyces cerevisiae*	10	**5** *Hanseniaspora uvarum* **1** *Lachancea thermotolerans* **1** *Saccharomyces cerevisiae* **3** *Saccharomyces bayanus*
Final	10	**10** *Saccharomyces cerevisiae*	10	**8** *Saccharomyces cerevisiae* **2** *Saccharomyces paradoxus*	10	**10** *Saccharomyces cerevisiae*

Table 2. Yeast species isolated at different stages of the fermentation process and identified from spontaneous fermentations of natural "Tinta de Toro" grape juice obtained from vineyards of different ages.

Although high levels of ethanol content in final wines is a worldwide issue, as mentioned earlier, in Spain this problem is still more pronounce in the Denomination of Origin (DO) Toro (Toro, Zamora, Spain), whose wines easily reach and exceed 15–16° alcohol content. For this reason a biotechnological-based approach was developed with the final aim to reduce their

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ethanol levels. During 2013 vintage, the population of indigenous strains associated to a winery belonged to DO Toro was characterized. Spontaneous fermentations were carried out on natural grape juice ("*Tinta de Toro*" grape variety), obtained from grapes of three different vineyards. Microfermentations were conducted at fixed temperature (21°C) and were daily monitored by measuring their weight loss until completion (constant weight). Yeast isolation was made from three different phases of the fermentation process: initial, medium, and final stage (final wine). Yeast strains were randomly selected for genetic typing. Yeast identification was carried out by RFLP analysis of the 5.8S-ITS-rRNA region amplified by using ITS1 and ITS4 primers [47], and confirmed by sequencing the D1-D2 regions of 26S rDNA using the NL-1 and NL-4 primers [48]. The results are shown in **Table 2**.

S. cerevisiae strain typing was performed by RFLP-mtDNA analysis with *Alu*I restriction enzyme [49]. The RFLP profiles were compared using the InfoQuest FP software package (Bio-Rad, Hercules, CA, USA). Nine different *S. cerevisiae* strains were identified (**Figure 5**), resulting no matches with the commercial strains routinely used in the winery.

The predominant *S. cerevisiae* strain (**Sc-1**, 44% of *S. cerevisiae* total population) and two non-*Saccharomyces* strains, *Lachancea thermotolerans* and *Kluyveromyces dobzhanskii*, were selected to conduct experimental fermentation with mixed cultures. The final aim was to the decrease the ethanol content by increasing the respiratory catabolism of sugars by non-*Saccharomyces* strains in the initial stages of the alcoholic fermentation. Two different methodologies were assayed for both non-*Saccharomyces* strain: coinoculation with the *S. cerevisiae* strain; and sequential-inoculation, by adding first the non-*Saccharomyces* strain and then (15 days later) the *S. cerevisiae* strain. Microvinifications were conducted in triplicate by inoculation with the selected strains. Microfermentations were carried out at 25°C and monitored by measuring the loss of weight, as described above. Once fermentations were completed, yeast cells were removed by centrifugation and analysis. Samples for quantitative analysis were stored at −20°C until analyses were performed.

The analysis of final wines were performed by HPLC using an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) chromatograph equipped with a HyperREZ XP Carbohydrate H^+ column (8 μm particle size, 300 × 7.7 mm) and a HyperREZ XP carbohydrate H^+ Guard pre-column (Thermo Scientific, Waltham, MA, USA), maintained at 50°C. Samples were filtered using 0.45 μm cellulose acetate filters (Costar, Washington, DC, USA) prior to analysis. A refraction index detector (RID) (positive polarity) at a flow rate of 0.8 ml/min with 4 mmol/l H_2SO_4 as mobile phase (injection volume 25 μl) was used to detect glycerol and ethanol. One-way analysis of variance was carried out to determine the influence of the "yeast used" factor on ethanol and glycerol content. The results are shown in **Table 3**. Coinoculation methodology decreased the alcohol level in final wines from 0.55 to 0.62% (v/v) when a *L. thermotolerans* or *K. dobzhanskii* strains had been used, respectively. A two-step (sequential) inoculation strategy achieved a higher reduction in ethanol values: up to 0.79% (v/v) reduction was obtained by using *K. dobzhanskii* strain, whereas a 0.82% (v/v) decrease was obtained by using the *L. thermotolerans* strain. Although some differences in the glycerol content were detected in the final wines, sensory analyses by a panel of expert tasters did not find significant differences in the wines thus elaborated.

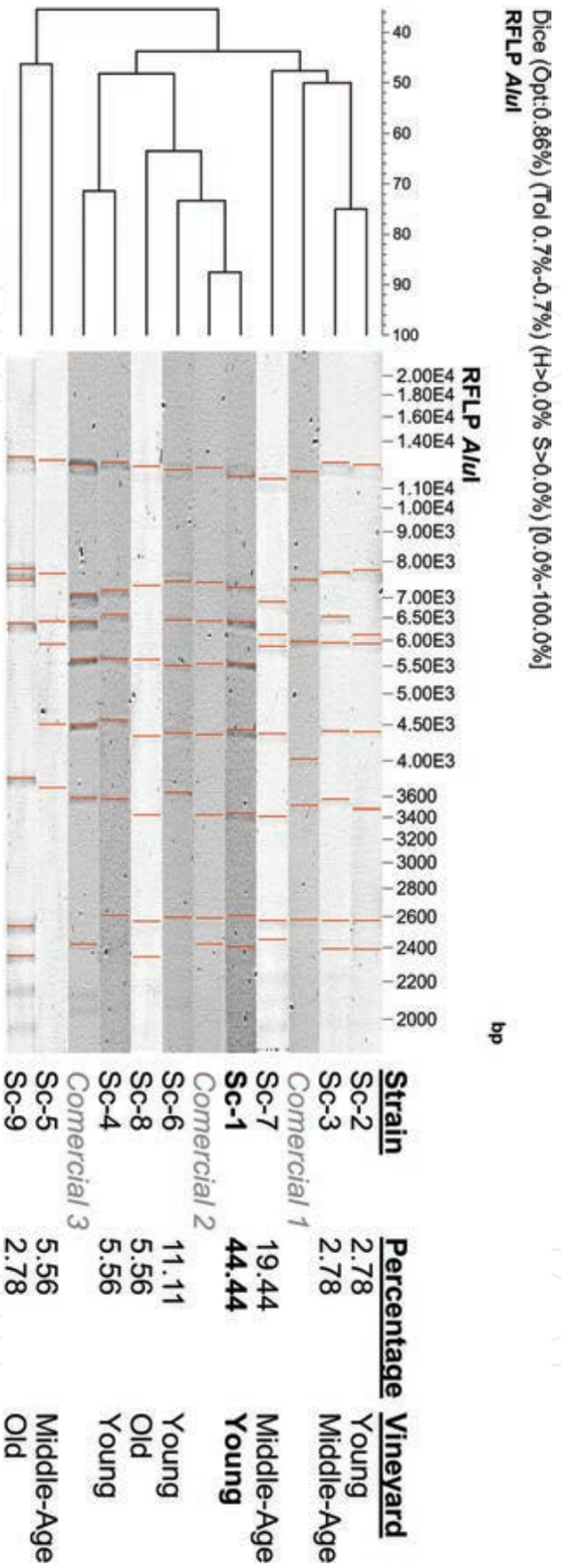

Figure 5. Dendrogram (top) based on RFLP-mtDNA analyses with the restriction endonuclease AluI (center) of all the *S. cerevisiae* strains (bottom) isolated from the three vineyards belonging to the winery (DO Toro, Zamora, Spain). The strain selected for the further oenological approach is highlighted in bold. Commercial strains have not been detected in neither case.

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Compound	Control (Sc-1)	Sc-1 + *K. dobzhanskii*		Sc-1 + *L. thermotolerans*	
		Coinoculation	*Two-step inoculation*	*Coinoculation*	*Two-step inoculation*
Ethanol (%, v/v)	12.70 (0.01)^{a}	12.08 (0.06)^{b,c}	11.90 (0.03)^{c}	12.15 (0.09)^{b}	11.87 (0.15)^{c}
Glycerol (g/l)	4.04 (0.01)^{a}	3.93 (0.15)^{a}	5.57 (0.29)^{b,c}	4.41 (0.16)^{a,b}	6.23 (1.26)^{c}

Values in parentheses correspond to the standard deviation (SD) in each case. Means with different letters are significantly different according to ANOVA results ($p < 0.05$).

Table 3. Ethanol and glycerol content of the final wines analyzed by HPLC.

Recently, a novel study addressed the same issue with a similar experimental design. In fact, Morales et al. [45] used a *Metschnikowia pulcherrima*/*S. cerevisiae* mixed cultures. The authors used an oxygen flux during the first stages of the fermentation. An alcohol reduction of 2.2% (v/v) was achieved with correct levels of volatile acidity. These data suggest that a higher reduction in the ethanol level could be obtained by increasing aeration at the beginning of the alcoholic fermentation, in order to increase the sugar consumption rate by non-*Saccharomyces* strains (respiration).

Therefore, the implementation at the industrial level of strategies to lower the ethanol content of wine, owing to breakdown of sugars by non-*Saccharomyces* yeasts, appears to be an interesting challenge. However, a further optimization is required in several aspects as yeast strains selection, inoculation protocols, aeration conditions, and other requirements.

6. Conclusion

Taken together the findings showed in this chapter, it has become evident that there are several potential efficient practices to overcome high must sugar levels at harvest time. Most favorable results were obtained by using plant growth regulators (auxins) and yeast selection. The generalizability of these results could be subject to certain limitations. Thus, in the case of auxins the cultivar behaves as an important factor to be taken into consideration, whereas in the case of yeast selection, more research is required to determine the efficacy of implementation at the industrial level.

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