LAMOTHE-ABIET
Solutions for winemaking

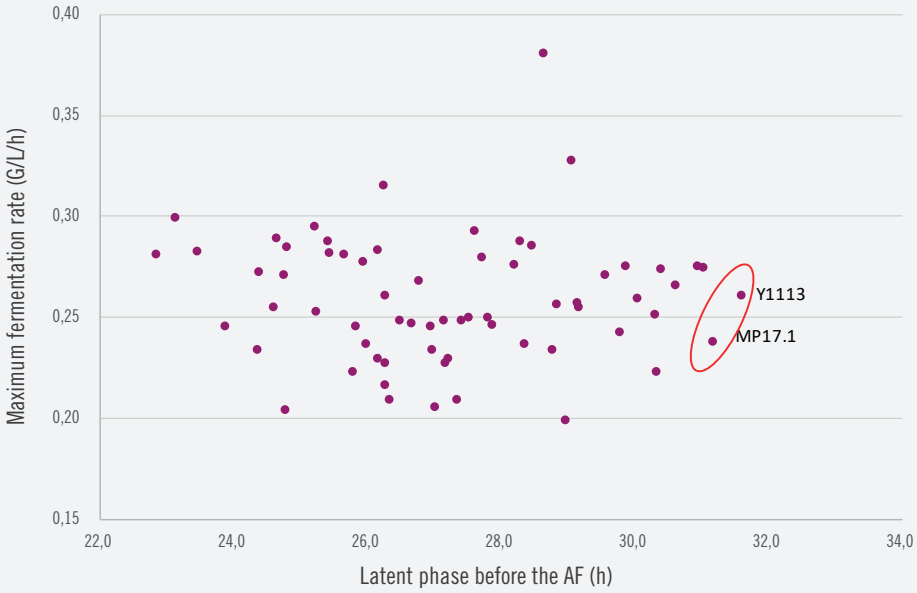
Controlling the microbial flora for winemaking without sulfites

Grape bioprotection using *Metschnikowia pulcherrima*

Reducing sulfites in wines is an important challenge in our industry; a challenge as important as commercialising wines which express their terroir whilst avoiding organoleptic faults due to microbial contamination.

The management of microbiological balances should be planned for starting from the grape harvest, where sulfite addition has, until now, helped to limit the indigenous flora. This study, carried out during the 2017 vintage in different wine regions in France, shows the effect of a biological solution, whose goal is to mitigate the SO_2's antiseptic effect on grapes. It is a strain of non-*saccharomyces* yeast of the *Metschnikowia pulcherrima* species, resulting from a specific selection project aiming to meet this "bioprotection" function.
The study was equally concerned with the microbiological equilibriums at key steps of vinification as with the analytical and organoleptic characteristics of the finished wines.

Graph 1: fermentary capacity of yeast strains, depending on the maximum fermentation rate and the latent phase before the AF



INTRODUCTION

With the evolution of research into wine microbiology, studies carried out at the beginning of the 2000s investigated non-*Saccharomyces* yeasts.
This research noted the technological benefits of certain non-*saccharomyces* yeasts for wine, creating interest in them. Enological use of this type of yeast has been common for about ten years now. Certain species have interesting properties, particularly in regards to their organoleptic effects. To mention just a few, some strains of *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* or *Kluyveromyces thermotolerans* are used for their ability to improve wines' aromas and taste. In most cases, it is necessary to inoculate afterwards with a strain of *Saccharomyces cerevisiae* in order to complete the fermentation. Other species are interesting as an alternative to the use of SO_2 on grapes, a technique which has shown its efficacy for ten years: bioprotection.
Indeed, from a microbiological point of view, sulfite addition destroys all or part of the indigineous flora, thus limiting the development of undesirable microorganisms (Lonvaud-Funel et al, 2010). On the other hand, bioprotection involves adding a selected microorganism which can colonise the ecological niche on the grapes, thus preventing the development of spoilage microorganisms such as *Brettanoymces*, acetic bacteria, or non-*Saccharomyces* yeasts that might be detrimental to the wine's quality (Lonvaud-Funel et al, 2010 ; Bartolini et al, 2010). Instead of eliminating the indigenous flora by sulfite addition, and creating an ecological vacuum that is susceptible to contaminations, bioprotection helps to control microbiological equilibriums (Immelé, 2010). It is well known that the *Saccharomyces cerevisiae* species have a high fermentary capacity (Ribéreau-Gayon, 1998). Therefore, most bioprotection solutions are non-*Saccharomyces* yeasts.
A joint research programme between two universities, including the University of Bordeaux, was launched to study a bank of over 70 non-*Saccharomyces* yeasts. Through their characterization, the goal was to determine whether one or several yeasts had interesting features for enological use.

CHARACTERISATION OF STRAINS' FERMENTARY POTENTIAL

The goal of the first study was the estimate the fermentary potential of each strain. To do so, their ethanol tolerance was evaluated. The same Sauvignon Blanc must was inoculated at a rate of 5.105 CFU/ml, equivalent to 5 g/hL.
Once the fermentations were finished, it was observed that none of the yeasts were able to ferment beyond 4% abv. This low alcohol resistance makes their use for fermentation impossible. For this reason, it was decided at this stage of the study to focus on other applications of non-*Saccharomyces* yeasts, in particular bioprotection.
This characterisation of the yeasts' ethanol tolerance gave data on their fermentation kinetics. It is agreed that low sugar consumption and a long latent phase for the AF are desirable attributes for a bioprotection yeast. A quick start to the alcoholic fermentation would not allow the clear juice to be separated from the lees for white and rosé wine production. Of the 70 strains investigated, 2 yeasts were particularly interesting for their low fermentary capacity: MP17.1 and Y1113 ***(graph 1)***.
It should be noted that, from the beginning, one of the two strains stood out from the others for its ability to give pleasant aromas during fermentation, which was not the case for the other strain tested which gave reduced, rubbery aromas. It was therefore decided to focus on characterising the first yeast, a *Metschnikowia pulcherrima*, with the code MP17.1. ▸

CHARACTERISATION OF MP17.1 FOR USE IN "BIOPROTECTION"

One identified strain (MP17.1) was particularly interesting for its high implantation capacity and its very low fermentary activity. It remained to be seen whether it could fulfil the other essential criteria for use in bioprotection. As well as having a low fermentary activity, a bioprotection yeast should be resistant to extreme conditions (low temperature, low pH) and easy to use.

Resistance to extreme conditions

The lowest temperature that it resists was 2 °C. In these conditions MP17.1 remains viable and is even able to develop. This is particularly interesting since it allows for pre-fermentation cold soaks or stabilisation, even at very low temperatures. The higher the temperature, the greater the population development ***(graph 2)***. It can also withstand a very low pH, up to 3.

Yeast viability after rehydration

The yeast's viability after rehydration is an essential parameter in bioprotection from a practical point of view. Viability must last long enough. A study was made by Lamothe-Abiet's Research and Development department. The results obtained indicated that the bioprotection yeast could be used up to four hours after rehydration. Beyond this time, there was significant loss of viability and decreased effectiveness ***(graph 3)***.

Application method

Two application methods are possible. The first involves rehydrating the yeast in 10 times its weight in water at 30 °C.
The second method involves directly sprinkling the yeasts onto the harvest or the must. Internal laboratory trials showed no significant different in the yeasts' viability between the two application methods ***(graph 4)***. Nevertheless, rehydration is recommended in order to better distribute the yeasts in the harvest bin, receival vat, tank or press.

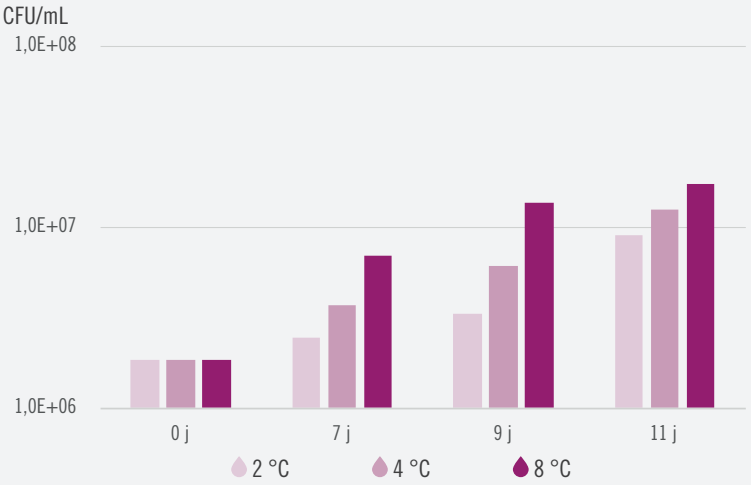
Trial protocols

The use of bioprotection yeasts should be planned for as early as possible in the vinification process. Indeed, the highly fertile environment of grape must is extremely favourable for the multiplication of all kinds of microorganisms.
Therefore, adding a bioprotection yeast beforehand is all the more important. It is nowadays possible to spray certain adapted bioprotection yeasts into the harvest bin thanks to spray jets, however the large majority of users add it directly into the receival bin or in the press. This is the application method which was used in the trials of MP17.1.
Several comparative trials were carried out during the 2017 vintage in different wine regions in France.
The different trial modalities were as follows:

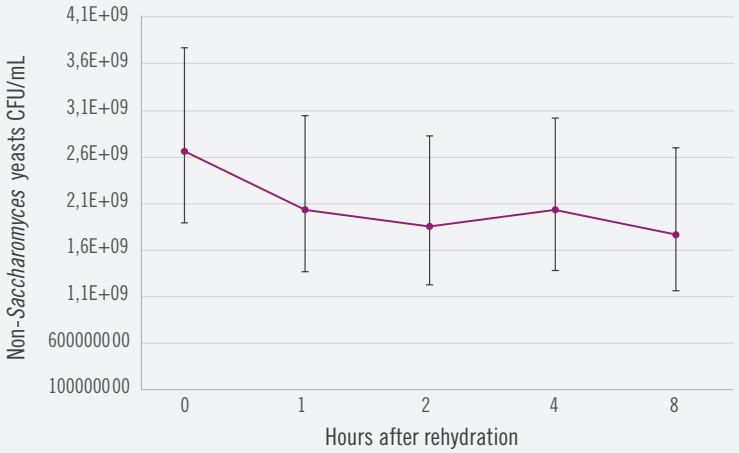
- A classical sulfite addition protocol using 5 g/hL
- A protocol of MP17.1 inoculation on the grapes at 5 g/hL for white wines, and 10 g/hL for red wines.
- A protocol without sulfite addition and without bioprotection (for just one trial).

Obviously, the grapes for each trial came from the same block, enabling meaningful comparisons. Must analyses were systematically made in order to check the homogeneity of the different tanks. The vinification protocols were strictly identical for the different modalities.

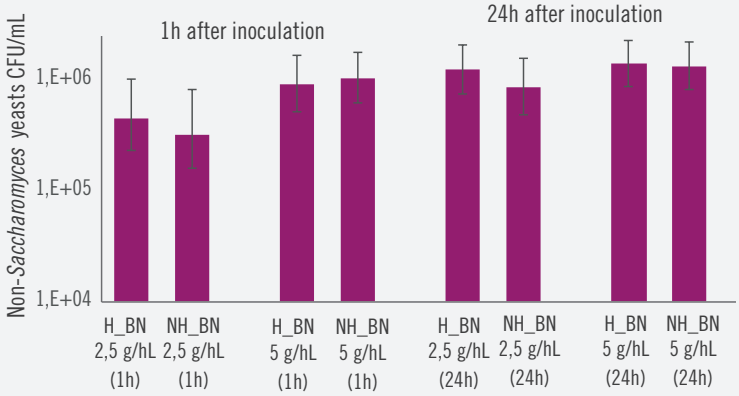
Graph 2: Evolution of MP17.1 populations (inoculation at 5 g/hL)



Graph 3: yeast's viability over time after rehydration



Graph 4: Impact of the non-rehydration of the yeast on the inoculation



These trials had several objectives:
- To ensure that MP17.1 was properly implanted into the must using microbiological analyses done on WLD non-*Saccharomyces* selective mediums. Before sulfite addition or MP17.1 inoculation on the grapes, an analysis of non-*Saccharomyces* yeasts was carried out on the free-run juice to estimate their concentration and composition. The same analysis was carried out after SO_2 or yeast addition for each of the two protocols. Then, in all of the trials, 24 to 48 hour of stabilisation was carried in each winery, after which a new microbiological analysis was made to determine the evolution of the non-*Saccharomyces* microorganisms' populations.
- To observe the impact of bioprotection on the wines' analytical characteristics, in particular on the quantity of SO_2 and SO_2 combining elements.
- To determine whether the chosen protocol has a significant effect on the wines' organoleptic properties. ▸

RESULTS AND DISCUSSION

Microbiological analysis of musts

Almost all of the indigenous populations are composed of *Hanseniaspora uvarum*, highly recognizable by its large green colonies ***(figure 1)***.

This apiculate yeast is able to produce large amounts of volatile acidity and ethyl acetate. Sulfite addition on the grapes at 5 g/hL was not enough to completely destroy this indigenous flora. This is also the case for the modality inoculated with low amounts of MP17.1. The small red colonies are characteristic of the *Metschnikowia pulcherrima* strain. Its population is greater than the indigenous population ***(figure 1)***.

If we look at the evolution after one day of stabilisation for the Chenin trial, we can see that MP17.1 dominates the indigenous flora, preventing its development and thus decreasing its presence in the must ***(graph 5)***.

From a microbiological point of view, these trials show the efficacy of bioprotection, since inoculation helped to significantly slow down the development of indigenous microorganisms.

On the other hand, the yeast implantation controls, carried out during the alcoholic fermentations, all verified that no non-*Saccharomyces* yeasts were present during this stage in any modalities, except one. This was the only protocol where no bioprotection or sulfite addition was used. The AF implantation control indicated that two yeasts were present: the selected yeast and another unknown strain.

Therefore, bioprotection does not have any antagonistic effect on the implantation of the yeast selected for fermentation. On the contrary, it enables a better implantation of the fermentation yeast.

Wine analyses

The wines' classical parameters were uniform in each of the trials, thus supporting their significance ***(figure 2)***. One value that should be highlighted is the level of ethyl acetate in the modality that had neither sulfite addition nor bioprotection before the yeast addition (trial in Bordeaux). The concentration of this aroma, which has an odour similar to glue/sticky tape, is greater in this modality compared to those with sulfur or bioprotection. This can be linked to the presence of two strains of yeasts during the fermentation, one of which was unknown. This seems to confirm that the selected yeast was undergoing greater stress, thus affecting the amount of ethyl acetate in the wine.

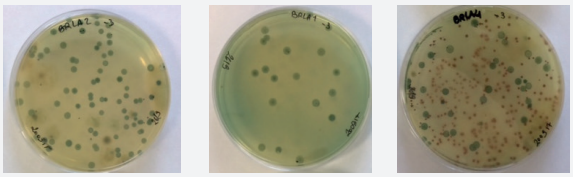
SO_2

The bioprotection modality in the Chenin trial had a total SO_2 content of only 7 mg/L, compared to 26 mg/L for the modality with sulfite addition. The same trend is found in red wine with a total SO_2 of 19 mg/L compared to 3 mg/L for the wine with bioprotection. The reason for this may be because sulfur is toxic for yeasts, they produce ethanal to protect themselves. Ethanal strongly combines SO_2, reacting directly with it to decrease the amount of free SO_2. The amount of sulfite added to the grapes determines the amount of ethanal produced, and the amount of SO_2 that is combined. The same trend was seen in the Pinot Noir trial.

Combining compounds

As well as enabling the user to decrease or totally skip sulfite addition to the harvest, bioprotection also helps to decrease the amount of sulfite added during maturation. ▸

Figure 1: Microbiological analyses of musts on non selective medium non-*Saccharomyces* WLD
Wine Experimental Center, Bordeaux - Graves - Cabernet Sauvignon, 2017



Initial must | 24h after sulfur addition at 5 g/hL | 24h after MP17.1 inoculation

Graph 5: Evolution of non-*Saccharomyces* populations in musts depending on the sulfite addition or the bioprotection
Loire Valley - Touraine, Chenin, 2017

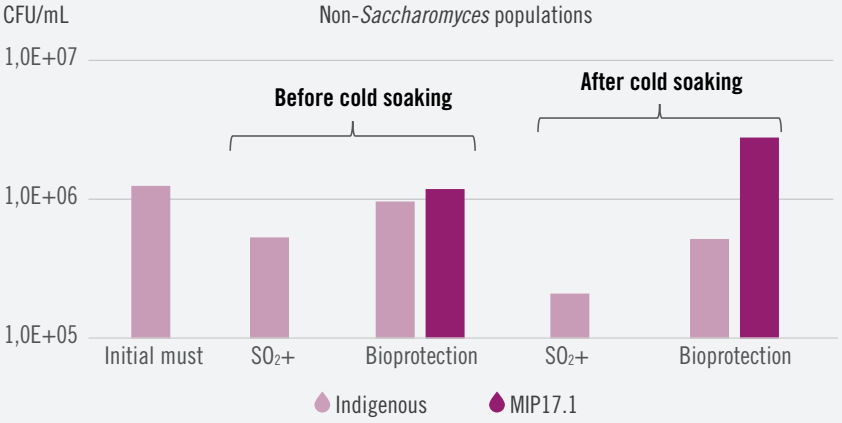


Figure 2: analyses of trial wines

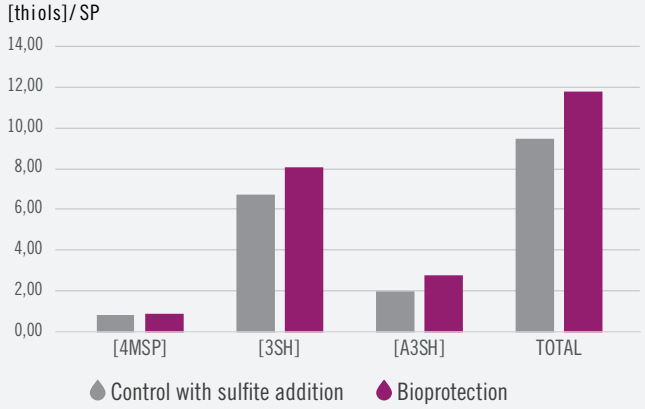
	Chenin (Loire)		Cabernet Sauvignon (Bordeaux) POST MLF			Pinot Noir (Hospices de Beaune) POST AF	
	SO2+	Bioprotection	SO2+	Bioprotection	Ø	SO2+	Bioprotection
ABV(%vol)	12,82	13	12,68	12,65	13,15	13,15	13,11
Total Acidity (g/L H2SO4)	5,86	5,85	3,62	3,76	3,68	5,17	4,96
Volatile Acidity (g/L H2SO4)	0,39	0,43	0,39	0,42	0,47	0,26	0,21
Ethyl acetate (mg/L) Post MLF	/	/	38	33	57	/	/
pH	3,19	3,21	3,72	3,71	3,66	3,27	3,37
Free SO2 (mg/L)	0	0	10	3	0	0	0
Total SO2 (mg/L)	26	7	19	3	0	14	3
Acetaldehyde (mg/L)	24	0	0	0	0	25	0
TL35 estimation (mg/L) Post AF	104	80	85	57	66	89	56
TL35 estimation (mg/L) Post MLF	/	/	57	51	51	/	/

Sulfite addition after the AF on the bioprotection modality will be more effective than on the modality which received a sulfite addition before fermentation. In the Chenin trial, the TL35 of the control modality is 104 mg/L, whereas it is just 80 mg/L for the bioprotection modality (TL35 is the amount of sulfite that must be added to the wine in order to reach 35 mg/L of free SO_2, the higher this value, the more compounds that combine SO_2 are present in the wine). This can be explained by the ethanal content.
Indeed, the control modality has a concentration of 24 mg/L, whereas there is none in the bioprotection modality. In the Bordeaux trial, the same trend was found after the AF, with a TL35 of 85mg/L for the control modality with sulfite addition, 66 mg/L for the modality without sulfite addition or bioprotection, and 57 mg/L for the bioprotection modality. The difference is probably due to the presence of ethanal in the control. This difference is decreased after the malolactic fermentation because the malolactic bacteria are able to consume the wine's ethanal during this second fermentation (Renouf, 2013).
Therefore, after the MLF, the TL35 of the modality with sulfite addition is 57 mg/L, compared to 51 mg/L for the bioprotection modality and the modality with no sulfite addition. The same trend was found for the Pinot Noir trial carried out in Hospices de Beaune in Burgundy.

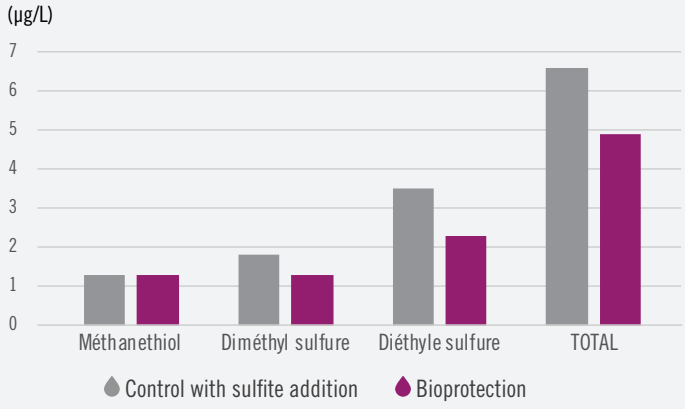
Organoleptic impact

An impact on the wine's aromatic profile could be identified thanks to analyses and tastings.
- Analyses of aromas
In the Chenin trial, within the uncertainty of measurement, volatile thiols analyses were hardly significant, but nevertheless showed that bioprotection does not cause a decrease in volatile thiols in this trial ***(graph 6)***. The analyses also show the relative decrease in undesirable sulfide compounds in the bioprotection modality, indicating that the fermentation yeasts were under lower stress ***(graph 7)***.
- Tastings
For the white wine trial, a panel composed of 16 informed consumers showed a significant difference between the two wines. In a triangular test, 14 tasters out of 16 identified the wine as different, with a significance of 99% according to the Roessler tables. Blind tastings by the same panel also highlighted the difference in the organoleptic profiles of the two modalities. The bioprotection modality was judged to have more pleasant aromas, fewer unpleasant aromas, and a better mouthfeel and overall score ***(graph 8)***. The same trends were found in the trial carried out in Bordeaux ***(graph 9)***.

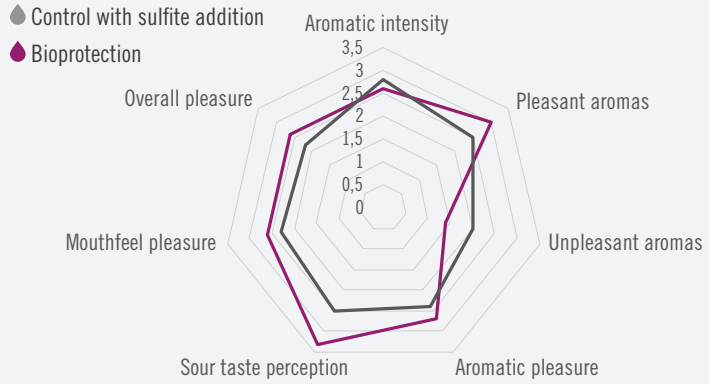
Graph 6: Analyses of volatile thiols
Loire Valley, Chenin, 2017



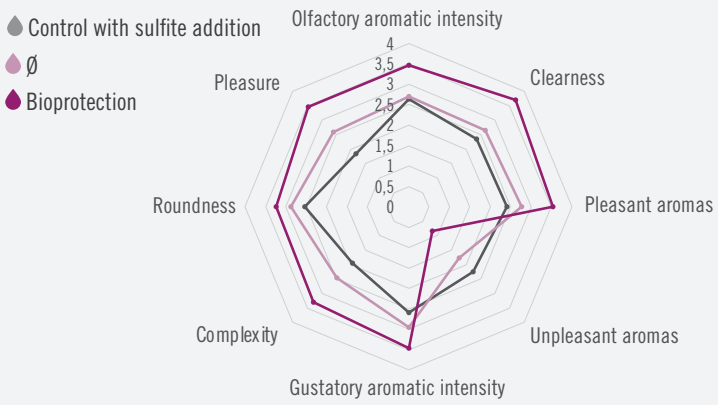
Graph 7: Analyses of undesirable sulfide compounds
Loire Valley, Chenin, 2017



Graph 8: Organoleptic profile of the two modalities using various descriptors. Comparative tasting at the end of AF Loire Valley, Chenin, 2017



Graph 9: Organoleptic profile of the two modalities using various descriptors. Comparative tasting at the end of AF Bordeaux, Cabernet Sauvignon, 2017



CONCLUSIONS AND PERSPECTIVES

The value of bioprotection for controlling the microbial flora in musts during the pre-fermentation phase has been demonstrated in the different trials outlined above. Removing the sulfite addition on the grapes has the benefit of decreasing the amount of S02 and its combining compounds at the end of the fermentation, and to have wines which are more open and clean on the nose. Nevertheless, it is still necessary that the musts' microbiological equilibrium is well controlled. This is made possible using a yeast that is specially selected for this, such as MP17.1. For the production of red wines or polyphenol-rich white wines, musts oxidation does not spoil the future wine. However, this could be problematic for white varieties that are sensitive to oxidation, such as Sauvignon blanc. It would be interesting to study the effects of bioprotection on this type of product. Furthermore, for the production of S02 free wines, solutions are required for the maturation phase. Tannins with antioxidant properties or yeast derivatives rich in reducing molecules could be a solution for winemakers. ■