BIO-PROTECTION IN OENOLOGY: A REAL ALTERNATIVE TO SULFITES?

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Introduction

Since more than ten years, the use of sulfites in food and wine industries faces challenges regarding consumers acceptance. Sulfites represent a chemical additive that could trigger various reactions in sensitive individuals (Lisanti et al., 2019). The use of sulfur dioxide (SO_2) as wine additive is able to ensure both antioxidant protection and microbiological stability. In spite of these undeniable advantages, in the last two decades the presence of SO_2 in wine has raised concerns about potential adverse clinical effects in sensitive individuals.

The winemaking industry has followed the general trend towards the reduction of SO2 concentrations in food, by expressing at the same time the need for alternative control methods allowing reduction or even elimination of SO_2. In the light of this, research has been strongly oriented toward the study of alternatives to the use of SO_2 in wine. Most of the studies have focused on methods able to replace the antimicrobial activity of SO_2.

This review article gives a comprehensive overview of the current state-of-the-art about the chemical additives and the innovative physical techniques that have been proposed for this purpose. After a focus on the chemistry and properties of SO_2 in wine, as well as on wine spoilage and on the conventional methods used for the microbiological stabilization of wine, recent advances on alternative methods proposed to replace the antimicrobial activity of SO_2 in winemaking are presented and discussed.

Even though many of the alternatives to SO_2 showed good efficacy, nowadays no other physical technique or additive can deliver the efficacy and broad spectrum of action as SO_2 (both antioxidant and antimicrobial), therefore the alternative methods should be considered a complement to SO_2 in low-sulfite winemaking, rather than being seen as its substitutes (Lisanti et al., 2019). Furthermore, different studies have reported that consumers would be willing to pay more for wines with reduced amount or without sulfites (Amato et al., 2017; Deneulin and Dupraz, 2018). For these reasons many efforts have been conducted by the professionals to reduce sulfites content in wine.

Various alternatives can be used to replace either the antioxidant, antioxidasic properties of sulfites or their antimicrobial properties (Lisanti et al., 2019). Among these alternatives, the use of tannins, inactivated dry yeast enriched in glutathione, ascorbic acid as antioxidant (Bahut et al., 2019; Comuzzo et al., 2017; Vignault et al., 2019a; Vignault et al., 2019), and the use of DMDC, lysozyme, sorbic acid as antimicrobial alternatives were reviewed recently by Lisanti et al. (2019). Most of these alternatives present some disadvantages or limitations and constitute complementary tools rather than real alternatives.

The use of non-*Saccharomyces* yeasts (NS yeasts) as a bio-preservative agent in must has been recently proposed by the wine industry as a possible alternative to sulfites. NS yeasts inoculation on grapes or must has been tested considerably in recent years in order to bioprotect the must by directly colonizing the environment and preventing the development of spoilage microorganisms. The industrial objective is to reduce the sulfites dose and to substitute its effect as much as possible. However, up to now no scientific data has supported or demonstrated the bio-protective effect of this practice during the winemaking process. During the last years we have studied the bioprotection strategy in both white and red grapes to assess the efficiency of this sulfite alternative at the beginning of the winemaking process.

Bioprotection strategy for white grapes:

Aligoté grapes were harvested manually. The *T. delbrueckii* strain BBMV 3FA5 (Primaflora VB - AEB group) was added as bioprotective strain during juice extraction at a concentration of 5×10^5 CFU/mL corresponding to 50 mg/L. No sulfite was added in the bioprotection modality. For the control modality (S control modality), SO_2 was added using a 5% sulfite solution at a concentration of 30 mg/L. Exactly the same experiment was conducted with Aligoté in two different wineries using the same process. After pressing, musts were cold racked during 24h, the alcoholic fermentation was performed at 20°C after addition of a commercial strain of *Saccharomyces cerevisiae* ($2X10^6$ CFU/mL corresponding to 200 mg/L). Malolactic fermentation was conducted using a malolactic starter.

Analysis of the different populations levels during winemaking process is presented in Figure 1. Firstly, the high percentage (50%) of Td identification on bioprotected modality strongly suggests its implantation and persistence during racking. The results indicate that the evolution of the microorganisms (acetic acid, lactic acid bacteria, *Brettanomyces*) follow the same trend in both modalities. This result underlines that bioprotection in our condition was as efficient as sulfite to limit the development of spoilage microorganisms during the winemaking process.

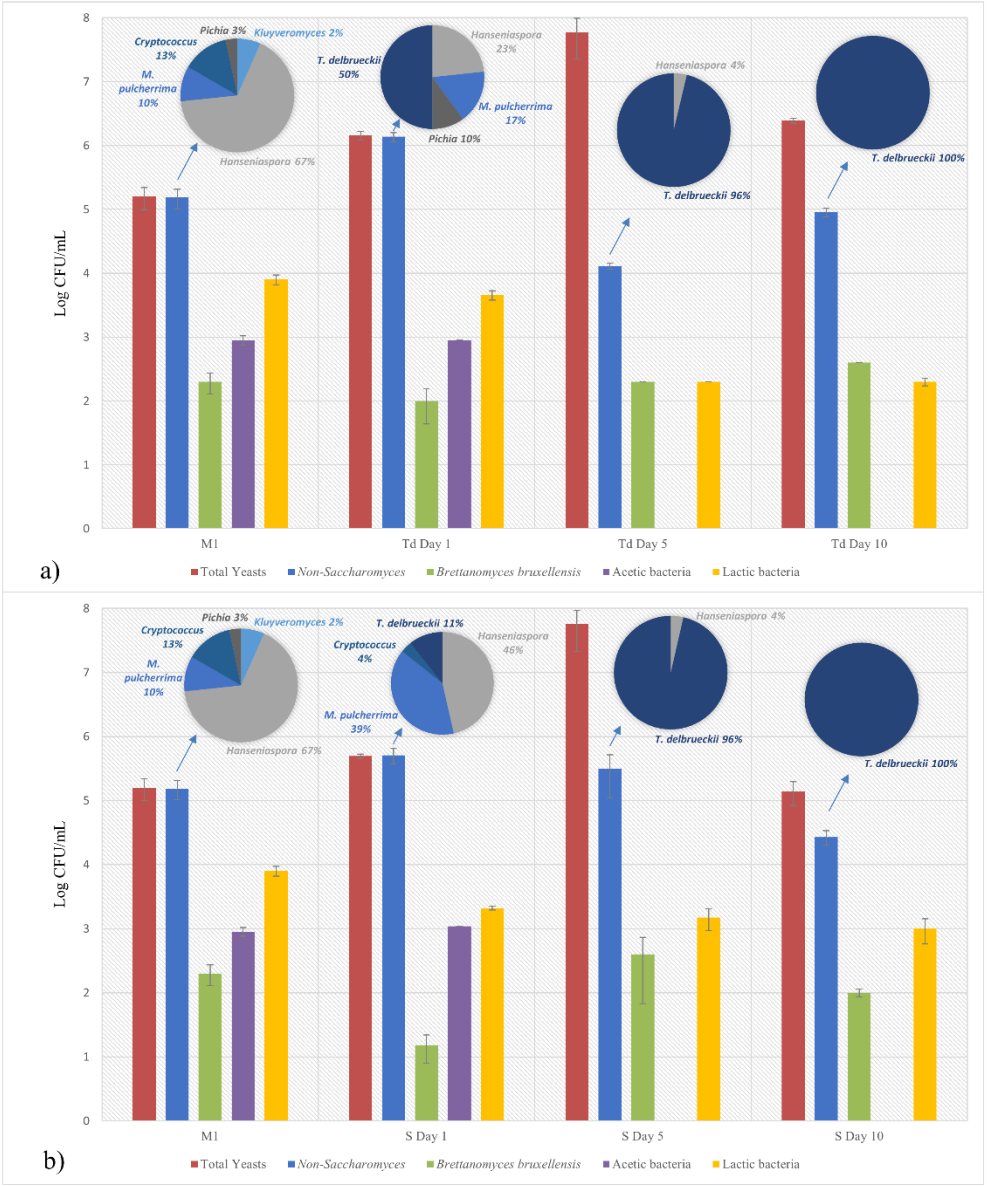

Figure 1 : Numeration of the different populations during winemaking process: total yeasts, non-Saccharomyces yeasts, Brettanomyces bruxellensis, acetic bacteria and lactic acid bacteria on different agar media at different winemaking times in winery 1. a) Td modality; b) S control modality. M1 represents the must before the addition of T. delbrueckii strain or sulfite; Day 1 : End of the pre-fermentative stage ; Day 5 : Mid-alcoholic fermentation ; Day 10 : End of alcoholic fermentation. The percentage of the different NS genera/species are represented by a pie chart. Standard deviation is based on technical replicates of the same population (Simonin et al., 2018).

This study also focused on the effect of bioprotection on the oxidation of must. Colour was measured by Tristimulus coordinates (L*a*b*) on racked must, at the end of AF and at the end of MLF in both wineries (Figure 2). The racked must with sulfites was significantly less brown than the must inoculated with *T. delbrueckii*, whatever the winery. However, at the end of alcoholic and malolactic fermentations, no difference between the two modalities could be observed in winery 1. For winery 2, the bioprotected modality was still browner after alcoholic fermentation, but no significant difference was observed at the end of the malolactic fermentation. These data point out that while the bioprotection was efficient in limiting the development of the indigenous microorganisms, the protection against oxidation was matrix dependent.

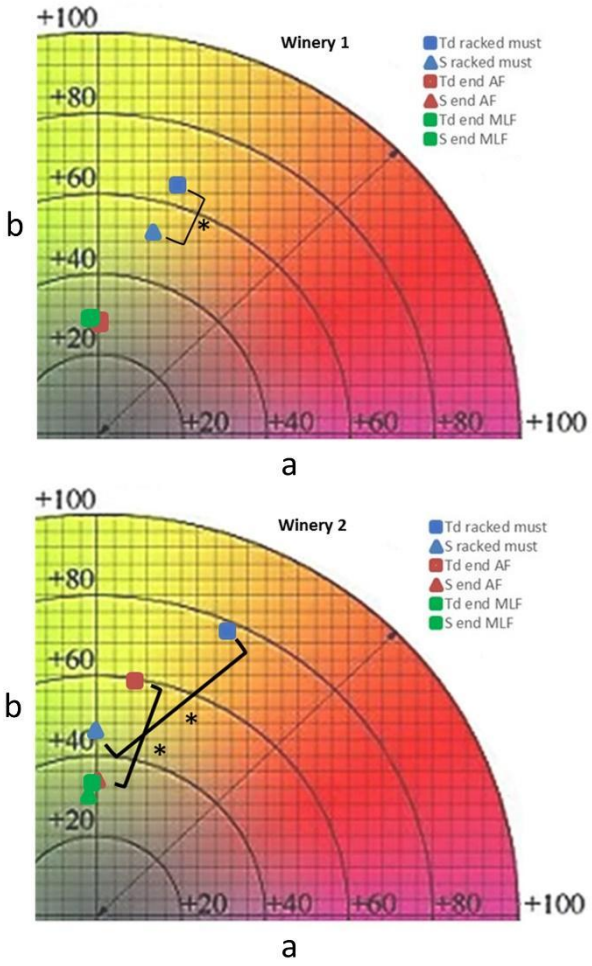

*Figure 2 : Graphic representing the Tristimulus coordinates (L*a*b*) from the samples on racked must (blue), after alcoholic fermentation (red) and malolactic fermentation (green) for both wineries. L corresponds to lightness/darkness, a to red/green chromaticity, b to yellow/blue chromaticity. Td represents the test modality with the addition of the T. delbrueckii strain and S the control modality with the sulfite addition (Simonin et al., 2018).*

Bioprotection strategy for red grapes:

Pinot noir grapes were harvested manually from three different wineries. The strain *M. pulcherrima* MCR 24 3FA5 (Primaflora VR – AEB group) was added as bioprotective strain during vating at the beginning of the cold pre-fermentation maceration at a concentration of 5×10^5 CFU/mL (50 mg/L). No sulfite was added in the bioprotection modality. For the control modality (S control modality), SO_2 was added using a 5% sulfite solution at a concentration of 30 mg/L. After 3 days of cold (12°C) maceration, *S. cerevisiae* strain Levulia PN® was added for fermentations in the three wineries (200 mg/L corresponding to $2x10^6$ CFU/mL). At the end of alcoholic fermentation, the wines were inoculated with a malolactic starter. After malolactic fermentations, all the wines were bottled with 30 mg/L of SO_2 (5% (w/v)). The sensorial analysis was carried out two months after bottling.

Inoculation of *M. pulcherrima* MCR 24 strain in the 3 wineries was successful as shown by the high level identified *M. pulcherrima* yeast. However, we two main behaviours, with the persitance of *M. pucherrima* during all the process in winery 1, while in winery 2 & 3 *M. pulcherria* level decrease during the cold maceration. Whatever the winery, the bioprotection prevent or limit the development of spoilage microorganism as efficiently as the sulfited modality (Table 1). Indeed, the addition of a low dose of sulfite allows to either eliminate or decrease the concentration of microorganism in the must or limit their growth (Table 1). The different observed effect of sulfite corresponds to the previously reported antimicrobial action of sulfites on different microorganisms, dependent on the starting must and the combination of free sulfite with must compounds (Divol et al., 2012). If we compare the impact of the bioprotection *versus the* sulphite modality at the end of the cold soak maceration, we can observe a slight increase of *B. bruxellensis* species in all the wineries. The evolution of acetic bacteria was dependent on the experimental sites, with an increase in wineries 2 and 3 and a decrease in winery 1 compared to the starting musts. If we compare these results with those of sulfited modalities, few significant differences were found between modalities. These results suggest that bioprotection was as efficient as sulfites in maintaining a low level of potential spoilage microorganisms in must. After alcoholic fermentation, no significant difference could be observed for most of the spoilage microorganisms in the three wineries.

		Winery 1		Winery 2		Winery 3	
Initial must composition	Non-*Saccharomyces* (CFU/mL)	$4.33x10^4$ - **(33% *Mp*)**		$9.67x10^4$ - **(7% *Mp*)**		$9.00x10^4$ - **(10% *Mp*)**	
	B. bruxellensis (CFU/mL)	$< 3.00 x10^1$		$6.67 x10^0$		$2.03 x10^3$	
	Acetic acid bacteria (CFU/mL)	$2,33 x10^2$		$5,00 x10^3$		$4,00 x10^2$	
		Sulfited	Bioprotection	Sulfited	Bioprotection	Sulfited	Bioprotection
After cold maceration	Non-*Saccharomyces* (CFU/mL)	$1.00 x10^{2\ b}$ - **(57% *Mp*)**	$7.00 x10^{5\ a}$ - **(100% *Mp*)**	$2.00 x10^{4\ b}$ - **(0% *Mp*)**	$4.00 x10^{5\ a}$ - **(10% *Mp*)**	$2.87 x10^{3\ b}$ - **(18% *Mp*)**	$1.13 x10^{7\ a}$ - **(21% *Mp*)**
	B. bruxellensis (CFU/mL)	$3.33 x10^{1\ a}$	$< 3.00 x10^{1\ a}$	$7.10 x10^{2\ a}$	$8.00 x10^{1\ b}$	$3.33 x10^{2\ a}$	$4.23 x10^{3\ a}$
	Acetic acid bacteria (CFU/mL)	ND ^{a}	$7.00 x10^{1\ a}$	$3.33 x10^{4\ b}$	$7.00 x10^{5\ a}$	$5.00 x10^{3\ b}$	$1.70 x10^{4\ a}$
After Alcoholic fermentation	Non-*Saccharomyces* (CFU/mL)	ND	ND	$1.03 x10^{3\ b}$ - **(0% *Mp*)**	$2.63 x10^{3\ a}$ - **(0% *Mp*)**	$4.63 x10^{3\ a}$ - **(40% *Mp*)**	$1.07 x10^{3\ b}$ - **(7% *Mp*)**
	B. bruxellensis (CFU/mL)	ND	ND	$< 3.00 x10^{1\ a}$	$8.00 x10^{1\ a}$	$4.67 x10^{2\ a}$	$5.67 x10^{2\ a}$
	Acetic acid bacteria (CFU/mL)	ND	ND	$1.83 x10^{4\ a}$	$6.33 x10^{3\ b}$	$7.62 x10^{2\ a}$	ND ^{b}

The analysis of the phenolic compounds for the three wineries revealed that no significant difference was noticed between both modalities (data not shown). The Figure 3 represents the average scores for each attribute and each sample for the three wineries, after sensory analysis. Very few attributes showed significant effects between treatments and none of the effects were common to the three wineries. Our results show that in our conditions, bioprotection did not change markedly the organoleptic profile of wines.

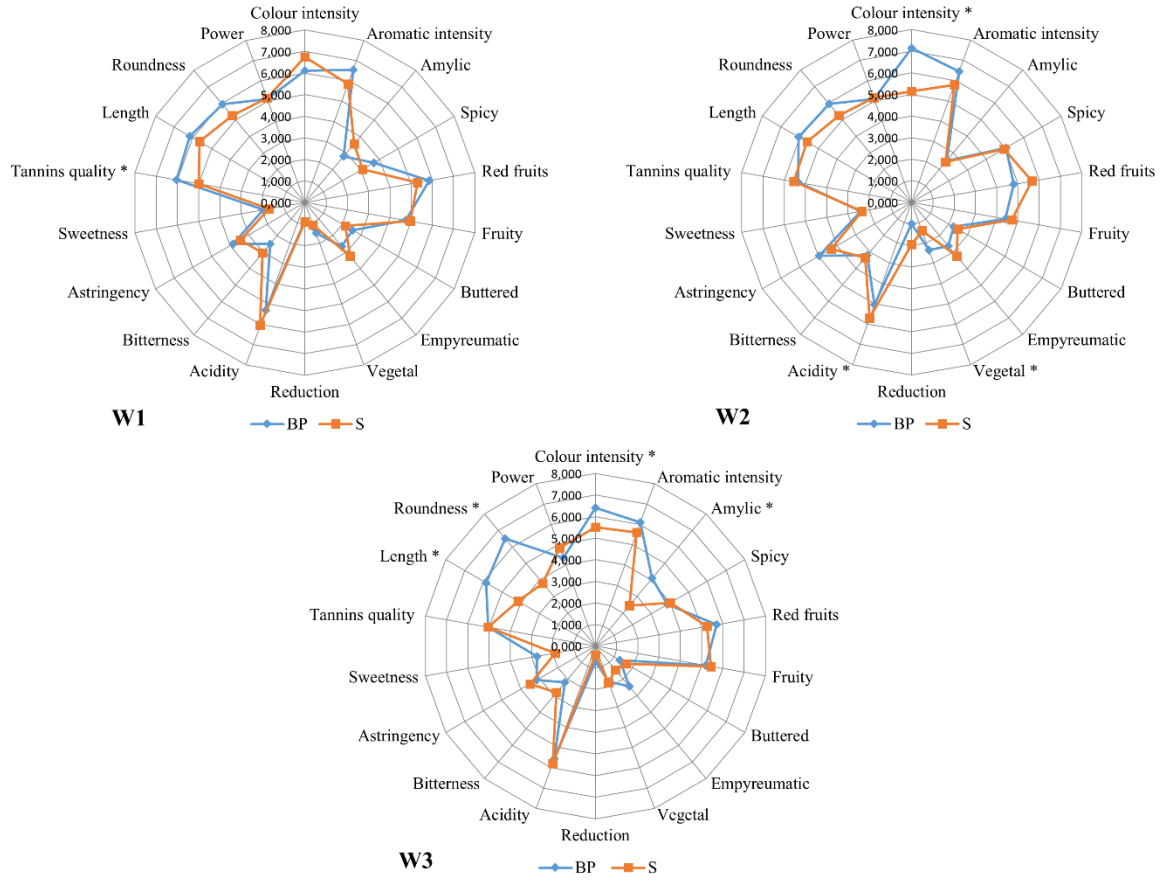

*Figure 3 : Sensory profiles of wine from winery 1 (W1), winery 2 (W2) and winery 3 (W3). Orange lines correspond to S modalities and blue lines correspond to BP modalities. *: significant differences (Tukey test, α= 5%).*

In conclusion, we analyzed for the first time the effects of bio-protection on white and red grape varieties as an alternative to sulphites during the first step of the winemaking process of white and red wines. The comparison of this strategy with sulfite addition showed that, from the microbiological standpoint, the contribution of the bioprotective agents limits the development of natural microbiota, in particular potential spoilage microorganisms *(B. bruxellensis* and acetic acid bacteria) in a manner equivalent to an addition of sulfites. Bioprotection can constitute an interesting biological alternative to sulfiting at the beginning of the winemaking process, which participes to a drastic reduction of the final sulfites concentration in wines.

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