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Research Article

Bioprotection on Chardonnay Grape: Limits and Impacts of Settling Parameters

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Background and Aims. If bioprotection has already been proven to limit the development of spoilage flora on musts, its effectiveness against oxidation depends on the winemaking process. To optimize its application, this study analyzed the evolution of the chemical composition of the bioprotected musts and wines, according to different settling routes. Their impacts on the organoleptic characteristics of wines were also studied. *Methods and Results*. A bioprotected must was subjected to 6 different maceration routes before AF (triplicates), varying the duration and temperature parameters. A temperature value ≤12°C was the main factor independently of the duration which allowed a good implantation of the bioprotectant. An increase of the maceration duration at 12°C led to browning of the must, without significant effect on the final color of the wine, which was felt as more "floral," with more length in the mouth. *Conclusions*. The bioprotectant implantation and its effectiveness on the sensory profile of the wine was guaranteed at maceration temperature values lower than 12°C. *Significance of the Study*. This study participates in the improvement of the bioprotection management in white winemaking, with the guarantee of a positive impact of the prefermentation maceration without sulphites on the organoleptic profile of the wines.

1. Introduction

The agrifood system is in a state of flux: changing consumer needs and eating habits, restrictions and bans on certain chemicals, and climate and demographic changes [1]. In addition, many food pathogens are constantly evolving, such as bacteria, viruses, parasites, or fungi that have been found in some foods, creating serious public health problems (e.g., listeriosis and cholera) [2, 3]. To respond to these challenges, innovative approaches have been and are being developed. In this context, the concept of bioprotection or biocontrol, i.e., adding microbial antagonists to biologically inhibit pathogens, has emerged as an obvious alternative method. In the oenological world, bioprotection appeared later with the use of non-*Saccharomyces* yeast strains to reduce the quantity of sulphite, intolerance to this molecule by some people, while controlling the development of undesirable flora in winemaking [4, 5]. Non-*Saccharomyces* yeast species such as *Metschnikowia pulcherrima*, Metschnikowia-*fructicola*, *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Pichia kluyveri* are now marketed as bioprotection strains [6]. The latter yeasts have a strong capacity for implantation in prefermentation conditions [7–12] and potentially inhibitory activities on spoilage microorganisms such as *Brettanomyces bruxellensis* yeasts and acetic acid bacteria. The mechanisms of action depend on the yeast species used: competition for nutrients and oxygen, production of killer toxins, production of inhibitory compounds other than killer toxins, and quorum sensing [13–21]. Many field trials have been carried out, but only a few preliminary

scientific studies have highlighted some effects, antimicrobial and antioxidant, examining the role of grape maturity, the timing of bioprotection yeast addition without testing the impact of winemaking routes on the addition of bioprotection [22–25]. White must bioprotection by early addition before settling of one *T. delbrueckii* strain was previously tested in real white winemaking conditions [22]. The implantation of the strain was successful and had no impact on the fermentation kinetics. This implantation reduced biodiversity during the prefermentation stages compared to the sulphited condition. However, it would prevent, with the same effectiveness as sulphiting, the development of spoilage microorganisms (*Brettanomyces* yeast and acetic acid bacteria). The strain used could also protect the wine from enzymatic and chemical oxidation, but this protection depends on the winemaking conditions (temperature of settling) and the must/wine matrix. To better control its effectiveness, a monitoring of bioprotection was carried out during the 2018 harvest, according to different technical settling routes during white wine winemaking. The impacts of time and temperature on the effects of bioprotection were analyzed. Three temperatures (7°C, 12°C, and 18°C) coupled with two settling durations (36 and 72 hours) were tested in triplicate in the winery Château de l'Eclair (SICAREX Beaujolais—Liergues (Rhône)—France).

2. Materials and Methods

2.1. Winemaking and Experimental Design. The wines were produced with Chardonnay grapes (clone 95) from "Le Saint Vincent" plot. Manual harvest was carried out on September 13, 2018. The health status of the harvest was satisfactory, without trace of cryptogamic diseases. The grapes were placed in a pneumatic press. The bioprotection strain used was *M. pulcherrima* MCR 24 (Primaflora VB-AEB group) reactivated by rehydration according to the manufacturer's instructions. It was added (final concentration of 50 mg/L which corresponds to 5.10^5 CFU/mL) in two stages: the first half in the giraffe leading to the press and the second half in the tray under the press. The grape juices were then transferred into 25L demijohns. Three temperatures were tested: 7°C, considered as a cold temperature compared to those of classical winemaking practices; 12°C, temperature usually fixed for raking; 18°C, a temperature usually reserved for fermentation. For each of these temperatures, two settling times were carried out: 36 hours and 72 hours. The reference was set for a settling duration of 36 hours at 12°C because these parameters are those usually used during raking and correspond to those used in previous experiments [22]. Table 1 summarizes the experiments with encoding for each test. After settling, all tests were placed at 18°C and inoculated with the same strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) Fermol Chardonnay (AEB group) (200 mg/L corresponding to a concentration of 2.10^6 CFU/mL after rehydration and inoculation). At the end of the alcoholic fermentation, the wines were sulphited at 30 mg/L total, and then filtered and stored in the cellar.

For each test, samples were collected on must before bioprotection addition, on clarified must (36 h, 72 h), at midalcoholic fermentation, and at the end of alcoholic fermentation.

2.2. Microbiological Analysis and Monitoring of Fermentation Kinetics

2.2.1. Detection of Different Populations of Microorganisms and Monitoring of Implantation of the S. cerevisiae Strain. The YPD medium was used for the enumeration of total yeasts (20 g/L glucose, 5 g/L yeast extract, 10 g/L peptone, 0.2 g/L chloramphenicol, and 20 g/L agar). The populations of *B. bruxellensis* were determined on a specific medium composed of 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone, 0.1 g/L p-coumaric acid, 0.1 g/L ferulic acid, 0.03 g/L bromocresol green, 0.006% cycloheximide, 0.2 g/L chloramphenicol, and 20 g/L agar-pH 4.8 [26]. Acetic acid bacteria were enumerated on mannitol medium (25 g/L mannitol, 10 g/L yeast extracts, 20 g/L agar, 10 mL/L Delvocid® at 1% (m/v), and 10 mL/L of 0.5% (m/v) penicillin). For the identification of yeast species, 30 colonies per sample were isolated on YPD medium. Each colony was then identified by ITS-RFLP PCR as described by Esteve–Zarzoso et al. [27]. Implantation of the *S. cerevisiae* strain was verified by Inter Delta PCR [28] at midalcoholic fermentation.

2.2.2. Monitoring of Fermentation Kinetics. The alcoholic fermentation (AF) was monitored by Fourier transform infrared spectroscopy (FTIR) (OenoFoss®). Classical oenological parameters were analyzed from 200 μL of initial must, fermented must, or finished wine. The concentrations of volatile acidity (VA in g/L of acetic acid) and assimilable nitrogen (YAN in mg/L) were determined on clarified musts and at the end of AF. SO_2 contents (mg/L), volatile acidity (VA) values, ethanol contents (% v/v), and residuals sugars concentrations were also measured in wines by the same method. The kinetics of alcoholic fermentation were monitored by gravity measures with a mustimeter.

2.3. Specific Chemical Analysis. These analyses focused on the evaluation of a potential oxidation state of musts or wines during different winemaking routes.

2.3.1. Color Determination by Tristimular Coordinates (L, a, b). At the end of the settling, musts color measurements were carried out by tristimular coordinates (L, a, b) with a CM-5 Konica Minolta ™ spectrophotometer. The principle of this measure is based on a reflectance scan of the entire visible spectrum, ranging from 380 nm to 700 nm, to obtain CIE colorimetric values ($L \times a \times b$) [29]. The samples of clarified musts were centrifuged (3 min at 20°C, 10,000 g), and then 15 mL of the supernatant were transferred to a recommended spectral cuvette (CM-A98 glass cuvette with parallel faces, 50 × 38 mm, path 10 mm lens). In the CIE color space ($L \times a \times b$), L^* represents clarity, a^* the red axis, and b^* the yellow axis. The color difference between the samples is based on the calculation of the color difference: $\Delta E = ((\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)^{1/2}$. If $\Delta E > 2$ between two samples, the color difference is significantly accepted.

Table 1: Summary and tests coding.

Settling temperature	7°C	12°C	18°C
Duration 36 h (short)	Triplicates ($S+7$) Tests 607-608-609	**Triplicates (S + 12)** **Tests 610-611-612**	Triplicates ($S+18$) Tests 613-614-615
Duration 72 h (long)	Triplicates ($L+7$) Tests 616-617-618	Triplicates ($L+12$) Tests 619-620-621	Triplicates ($L+18$) Tests 622-623-624

Note: The reference mode is in bold.

2.3.2. Determination of the Total Polyphenol Index (TPI). At the end of the settling, must samples were centrifuged (5 min at 20°C, 5000 g). The supernatants were transferred in a quartz cuvette and the absorbance values were measured at 280 nm with a UV/visible spectrophotometer.

2.3.3. Analysis of Phenolic Compounds by UHPLC. The phenolic compounds were analyzed at the end of AF by UHPLC (ultrahigh pressure liquid chromatography). Chromatography was performed in reverse phase. The ACQUITY UPLC H-Class (waters) chromatographic system was equipped with a diode array detector and a Raptor ARC-18 column. The column oven was thermostatically controlled at 35°C and the sampling system at 12°C. A water-methanol-TFA (trifluoroacetic acid) mixture in volume proportion 100 : 5 : 0.28 was used for solvent A and methanol (100%) for solvent B with a variable flow rate ranging from 0.36 to 0.50 mL/min. A gradient elution is presented in Table S1.

The UV-visible spectrum was recorded between 210 and 610 nm with a resolution of 1.2 nm and an acquisition rate of 20 points/sec. The fluorometer recorded two pairs of excitation/emission wavelengths (270 nm/322 nm and 270 nm/420 nm, respectively). Pure standards of gallic acid, protocatechic acid, hydroxybenzoic acid, hydroxytyrosol, tyrosol, catechin, epicatechin, B1 and B2 dimers, caftaric acid, gentisic acid, caffeic acid, coumaric acid, chlorogenic acid, and ferulic acid were injected under the same chromatographic conditions in order to identify and quantify these compounds in the samples. Data acquisition and processing were carried out with Waters Empower software. Gallic acid, hydroxybenzoic acid, hydroxytyrosol, tyrosol, catechin, epicatechin, dimers B1 and B2, grape reaction product (GRP), coutaric acid, caftaric acid, gentisic acid, caffeic acid, coumaric acid, and ferulic acid have been detected and grouped into families of phenolic compounds. Gallic acid, hydroxybenzoic acid, caftaric acid, caffeic acid, coutaric acid, coumaric acid, gentisic acid, and ferulic acid belong to the family of phenol acids. Catechin, epicatechin, dimers B1, and B2 belong to the group of flavan-(3)-ol, hydroxytyrosol, and tyrosol to the family of tyrosols. GRP alone represents its group.

2.3.4. Analysis of Antioxidant Capacity Using the DPPH Method. This method is based on the ability of DPPH (1,1-diphenyl-2-pycrilhydrazyl) which is a radical stable in the solution to fix free radicals and to stop the oxidative chain reaction; it makes it possible to judge the trapping capacity of DPPH, and therefore to evaluate the antioxidant capacity of a matrix. The DPPH test was carried out according to the method of Yamaguchi et al. [30]. First, 600 μL of the test sample or methanol (control) were mixed with 400 μL of Tris-HCl buffer (100 mM, pH 7.4). Then, 1 mL of DPPH (500 μM, diluted in 100% ethanol) was added. The final mixtures were placed in the dark at 20°C for 30 min before a measure of absorbance at 517 nm. The trapping capacity of DPPH was calculated from the following equation:

$$DPPH \text{ trapping activity}(\%) = 1 - \left[\frac{(Abs \text{ sample} - Abs \text{ blank})}{(Abs \text{ control} - Abs \text{ blank})}\right] * 100 \text{ with Abs: value of absorbance at } 514\, nm. \quad (1)$$

2.4. Sensory Analysis. Pivot profile© (PP) was used with a wine professional panel from the Beaujolais Vineyard to highlight the main sensory differences among the modes [31, 32]. The panel was composed of 15 wine professionals (2 women, 13 men, and mean age = 49) including winemakers, vine and wine consultants, vine and wine engineers, and laboratory technicians located around Villefranche-sur-Saône who are very familiar with both the Chardonnay wines and the PP methodology. The wine tasting was carried out in a single session during February, 2020, after one year of bottling.

The PP consists in describing each sample of wine by directly comparing it to a reference, called the "pivot" wine. As samples, only two out of three replicates were chosen within each mode to eliminate the faulty ones: 607 and 608 samples for the $S+7$ mode, 613 and 615 for the $S+18$ mode, 616 and 617 for the $L+7$ mode, and 619 and 620 for the $L+12$ mode (Table 2). The samples of $L+18$ mode were excluded from the sensory analysis due to spontaneous fermentation observed during settling (see Results-section*Sensory analyses of wines using the Pivot profile*©). Each sample corresponded to a blend of two preselected

Table 2: Representation of the blend of selected wine replicates in each mode tested.

Modes	Blend of selected wine replicates
$S+12$ (pivot wine-reference mode)	610; 611
$S+7$	607; 608
$S+18$	613; 615
$L+7$	616; 617
$L+12$	619; 620

replicates within each mode, as presented in Table 3. The choice of preselected replicates is justified in the results part. The pivot wine corresponded to the $S+12$ reference mode, as also a blend of only two replicates (610 and 611 samples).

All wine samples were served with 50 mL at 12°C in black ISO glasses identified by a random 3-digit code and covered with plastic Petri dishes. The pivot wine was served as the same way but with twice the volume. The wines were thus presented in pairs, each composed of the pivot wine and the sample, for which the assessors had to freely describe with their own terms the sensory characteristics they perceived "less" and/or "more" intense in each wine sample when comparing it to the pivot wine. They had to mention only descriptive terms in a positive form, without expressing hedonic meaning or making sentences.

2.5. Statistical Analysis. Data has been processed with Xlstat© software (2016.02.27444). A Tukey test (confidence interval: 95%) was applied to the following results: number of microorganisms, oenological parameter values (VA, YAN, TPI, and SO_2), phenolic compounds concentrations, and DPPH values. For the colorimetric analyses, significant differences were indicated by the colorimetric difference (ΔE), as explained previously.

Concerning sensory analysis, PP data was lemmatized and categorized by regrouping the terms with the same meaning together. This categorization task was performed independently by three oenologists and then, each individual categorization was compared to establish a common list of terms. Then, all hapax (i.e., terms used only once) were eliminated. After that, a contingency table was realized by following the procedure of [32]. First, the positive and negative frequencies of each term for each wine sample were computed. Then, for each term, the negative frequency was subtracted from the positive frequency for which this value was reported in a "subtraction" column for each wine. To finish, the minimum frequency in all subtraction columns was added to all values of the subtraction column and reported in a "translation" column to obtain only positive values for each term. Only the translation columns were then compiled in a term ∗ wine contingency table, from which, we eliminated the terms that did not allow a large enough discrimination, that is a similar distribution between the wine modes for the same term. This reduced contingency table was then submitted to correspondence analysis (CA). All statistical analyses for sensory data were performed by using the free software *R*, version 3.6.2 for Windows [33] with the additional *R* packages FactoMineR [34], factoextra [35], and corrplot [36].

Table 3: Tristimular coordinates (L, a, b) of musts after settling.

Modes	L	a	b	Overall color difference (ΔE)	Difference with the reference $S+12$
$S+7$	64.55	9.47	42.83	78.04	0.87
S + 12	**63.72**	**9.57**	**42.48**	**77.17**	**0.00**
$S+18$	63.81	9.07	41.64	76.73	−0.44
$L+7$	65.62	8.87	42.47	78.66	1,49
$L+12$	83.12	2.74	30.24	88.49	11,32
$L+18$	82.02	1.66	24.98	85.75	8.58

Note The color difference was calculated using the following formula: $\Delta E = ((\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)^{1/2}$. The reference mode is in bold.

Finally, statistical analyses were carried out: principal component analysis (PCA) with a Biplot representation; classification with hierarchical cluster analysis (HCA) using the SIMCA-P software (v15, Umetrics©) for a global analysis of the data.

3. Results

3.1. Microbial Populations during the Prefermentation Phase. The initial population on grapes was $1.4.10^5$ CFU/mL with 50% of *M. pulcherrima* species, 10% of *Aureobasidium pullulans*, and 40% of unidentified genera and species (Figure 1). The population levels of *Brettanomyces bruxellensis* and acetic acid bacteria were $1.5.10^2$ and $9.7.10^3$ CFU/mL, respectively. The inoculation with the bioprotection strain *M. pulcherrima* MCR 24 led to an increase in the concentration of total yeasts of about one log in all conditions compared to the initial must. It was counted between $8.0.10^5$ and $1.4.10^6$ CFU/mL of total yeasts at the end of settling in all musts (Figure 1). Even if significant differences in total yeast populations have been observed, particularly between $S+18/S+7$ modes and $S+7/L+18$ modes, these differences remained small. Except for the condition $L+18$, the species *M. pulcherrima* represented at least 97% of the total yeasts, indicating a high probability of implantation of the bioprotective strain. For $L+18$ mode, the species *M. pulcherrima* represented 56% of the total yeast at the end of settling (Figure 1). A temperature of 18°C associated with a long settling time (72 hours) did not allow the implantation of the bioprotective strain. Under these conditions, it is likely that indigenous *S. cerevisiae* yeasts were developed (43%) at the expense of the bioprotective strain. Moreover, early starts in alcoholic fermentation were noticed in triplicates of the mode $L+18$. For *B. bruxellensis* yeasts, significant differences in populations were observed on clarified musts from $S+7$ (4.0.101 CFU/mL) and $L+18$ (5.3.101 CFU/mL) compared to other clarified musts. However, these populations remained very small and these differences appeared minor at this stage of winemaking. Regarding acetic acid bacteria, no significant difference was found between the different musts with relatively low concentrations (<4.2.102 CFU/mL).

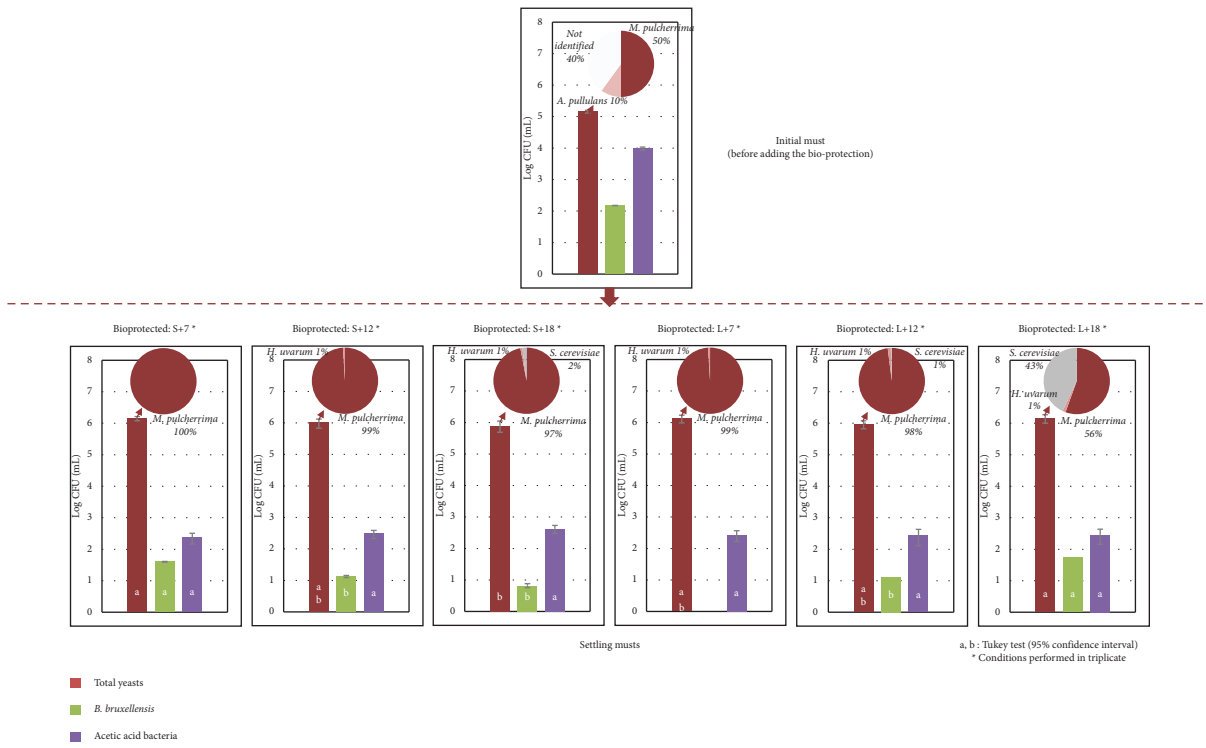

FIGURE 1: Enumeration of total yeasts, *B. bruxellensis* yeasts, and acetic acid bacteria on initial musts and clarified musts. For total yeasts, the different species are represented in a circular diagram (*H. uvarum*: *Hanseniaspora uvarum*). All conditions were performed in triplicate and subjected to the Tukey statistical test (95% confidence interval). Statistical analysis was performed in each microorganism detection method without matching with the other ones.

3.2. Implantation of S. cerevisiae Strain. After settling, must temperatures were brought to 18°C and the strain of *S. cerevisiae* Fermol Chardonnay was inoculated in all tests (initial concentration of $2.0.10^6$ CFU/mL). The control of implantation of the strain was realized at midalcoholic fermentation to verify the success of the inoculation and to validate the relevance of sensory analysis (Figure 2).

Sample 2 of wine in $S+18$ mode showed an Interdelta PCR profile different from that of the reference (Figure 2). Different bands were observed between these two profiles, with in particular a 240 bp band characteristic of the *S. cerevisiae* reference strain, which was missing in sample 2 in $S+18$ mode. The inoculated *S. cerevisiae* strain was therefore not implanted in this test. Concerning samples 2 and 3 of wines from $L+18$ mode, Interdelta PCR profiles were slightly different compared to the reference. All bands of the reference profile were present for both samples, but additional bands at 120 and 140 bp were also noted, not specific to the strain inoculated. These results suggested that the ADY strain are therefore partially developed in these musts with other indigenous *S. cerevisiae* strains. These results confirm early alcoholic fermentation starts in two out of three tests for $L+18$ mode (see part *Fermentation kinetics*). For all other samples, Interdelta PCR profiles were identical to the profile of the reference, indicating that *S. cerevisiae* strain was implanted in these musts. The successful *S. cerevisiae* strain implantation in 20 tests out of 23 underlined that the addition of the bioprotective strain *M. pulcherrima* MCR 24 did not prevent the development of *S. cerevisiae* starter to carry out alcoholic fermentation. In addition, in the wines from bioprotected trials 2 and 3, resulting from $L+18$ mode, the partial implantation of the starter highlighted the limits of bioprotection at high temperatures with a long settling time (72 hours).

3.3. Microbial Populations at the End of Alcoholic Fermentation. No *B. bruxellensis* yeast was detected in all samples, but we cannot exclude a viable but not cultivable (VNC) state of this yeast at this stage of winemaking. For acetic acid bacteria, significant differences were observed at the end of alcoholic fermentation. The concentration of acetic acid bacteria was significantly higher with $3.1.10^5$ CFU/mL in wines from $L+18$ mode, compared to the other samples: $7.8.10^1$ CFU/mL for $S+7$, <30 CFU/mL for $S+12$, $5.0.10^2$ CFU/mL for $S+18$, <30 CFU/mL for $L+7$, and <30 CFU/mL for $L+12$ modes. The lack of implementation of the bioprotection yeast in $L+18$ mode seemed to induce a long-term development of these spoilage bacteria.

3.4. Fermentation kinetics. All fermentations were completed in 8 days, except for mode $L+18$ where the fermentation time was 9 days. The kinetics were similar between the different modes, except for $L+18$ where the density loss was faster than the first 3 days (loss of density 0.55 *versus* 0.47 for other tests), with a sluggish end of AF (Figure S1). The standard deviations were low within a triplicate, reflecting the good repeatability between the tests of the same mode.

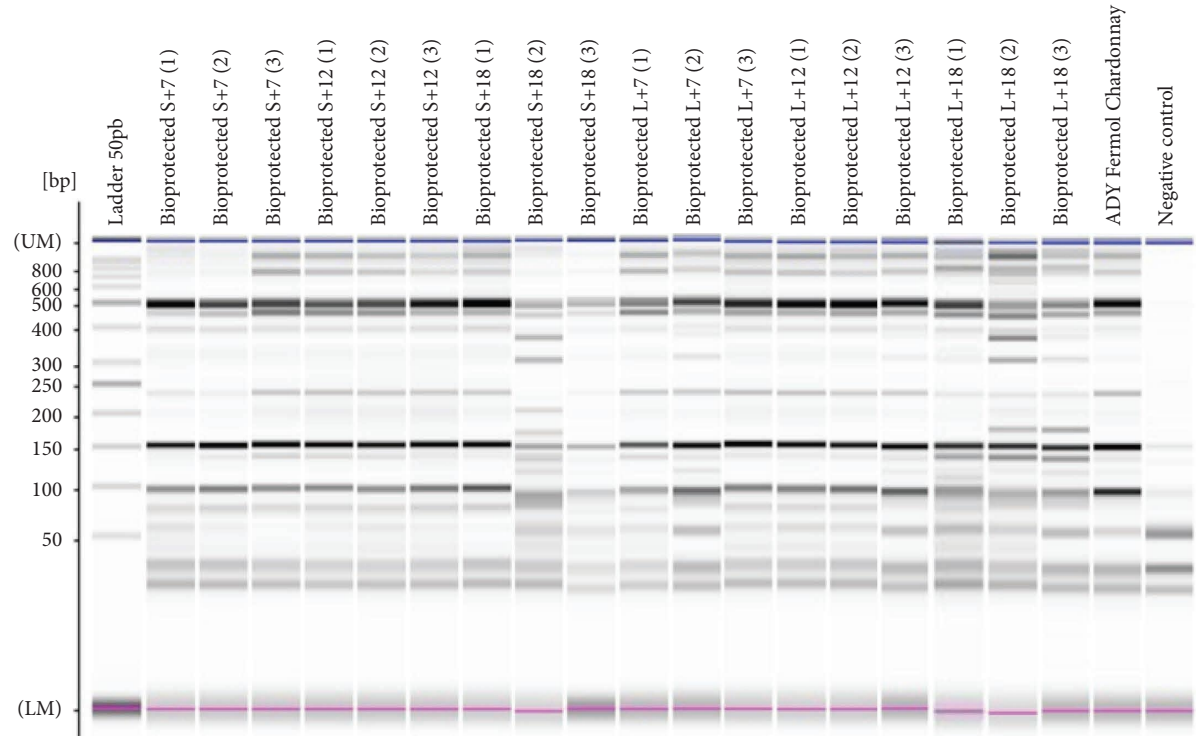

Figure 2: Control of the implantation of the yeast *S. cerevisiae* (Fermol Chardonnay-AEB) at midalcoholic fermentation. Interdelta PCR was used to characterize strains of *S. cerevisiae*. The result of these amplifications was analyzed by the MultiNA MCE-202©.

3.5. Color by Tristimular Coordinates (L, a, b) Determination. The color estimation was carried out at the end of settling by the tristimular coordinates (L, a, b) determination (Table 3). A difference in color is significant when the overall color difference (ΔE) between the two samples is greater than 2.

With the reference fixed to $S+12$, only the musts from $L+12$ and $L+18$ had a less brown color. No difference in color was noted between the modes including a short settling time or low temperature (7°C). The analysis of phenolic compounds at the end of alcoholic fermentation will indicate whether the different parameters applied during settling had an influence on the composition and concentrations of these molecules. Moreover, the color differences were attenuated during fermentation and finally disappeared before the sensory analysis (data not shown).

3.6. Analysis of Oenological Parameters. The means oenological parameters were determined on musts after settling and wines. The values obtained are presented in Table 4. The initial concentrations of assimilable nitrogen and sugars in the must were 237 mg/L and 233 g/L, respectively.

At the end of settling, significant differences were found for available nitrogen and TPI. First, the samples from $L+18$ mode showed a significant decrease of YAN with a loss of more than 50 mg/L (161 mg/L) compared to the other modes (>212 mg/L). Secondly, significant decreases of TPI values were observed for samples from $L+12$ and $L+18$ modes with index values less than 6.5 compared to the other samples where the values were greater than 9.2. These data suggested that a long settling time coupled with temperature values above 12°C would not be favorable for the protection of the phenolic compounds of the must.

In wines, the volatile acidity concentration was significantly higher in wines from $S+7$ mode with 0.28 g/L of acetic acid (Table 4). The total SO_2 concentrations were statistically different for each wine. However, given the precision of the method (±10 mg/L), the differences are difficult to interpret. We can just note a strong combination of total SO_2 under all conditions (>68%) probably with secondary AF products such as acetaldehyde. The different settling modes did not influence ethanol contents and residual sugar concentrations in wines.

3.7. Phenolic Compounds at the End of Alcoholic Fermentation. Contents of phenolic compounds of families such as tyrosol, phenol acids, flavan-3-ol, and GRP (grape reaction product) were quantified at the end of the alcoholic fermentation for all samples (Figure 3). The concentration of "tyrosol" compounds was significantly lower in the wines from $L+18$ mode with 6.68 mg/L, while in all other conditions, the concentrations were greater than 7.67 mg/L.

The concentration of phenol acids was significantly higher in wines from $L+7$ mode with a concentration of 4.82 mg/L compared to the wines from $L+12$, $S+18$, and $L+18$ modes, suggesting a better protection of these compounds at low temperature. GRP concentration of wines was significantly higher when settling was carried out at 18°C, with 4.44 mg/L for wines from $S+18$ mode and 4.57 mg/L

Table 4: Oenological parameters analyzed at the end of settling and at the end of AF with volatile acidity (VA) in g/L of acetic acid, assimilable nitrogen (YAN in mg/L), TPI (total polyphenol index) in mg/L equivalent of gallic acid, combined SO_2 (mg/L), total SO_2 (mg/L), residual sugars (g/L), and ethanol (% v/v) ethanol. These data were subjected to two separated Tukey statistical tests (must and wines) (95% conf. interval). The reference mode is in bold. Standard deviations are present in the supplementary data (Table S3).

	Modes	VA (g/L acetic ac.)	YAN (mg/L)	TPI (mg/L eq. gallic ac.)	Combined SO_2 (mg/L)	Total SO_2 (mg/L)	Residual sugars (g/L)	Ethanol (%v/v)
Settling musts	$S+7$	0^{a}	227^{a}	9.7^{a}	—	—	—	—
	S + 12	**0** ^{a}	**214** ^{a}	**9.9** ^{a}	—	—	—	—
	$S+18$	0^{a}	215^{a}	9.9^{a}	—	—	—	—
	$L+7$	0^{a}	223^{a}	9.3^{b}	—	—	—	—
	$L+12$	0^{a}	213^{a}	6.4^{c}	—	—	—	—
	$L+18$	0^{a}	161^{b}	5.6^{d}	—	—	—	—
Wines	$S+7$	0.28^{a}	—	—	43^{a}	53^{abc}	0.24^{d}	$12,53^{a}$
	S + 12	**0.22** ^{b}	—	—	**41** ^{a}	**50** ^{bc}	**0.25** ^{cd}	**12,53** ^{a}
	$S+18$	0.12^{d}	—	—	33^{b}	44^{d}	0.34^{ab}	$12,47^{a}$
	$L+7$	0.22^{b}	—	—	45^{a}	57^{a}	0.30^{bc}	$12,56^{a}$
	$L+12$	0.15^{c}	—	—	42^{a}	55^{ab}	0.37^{a}	$12,56^{a}$
	$L+18$	0.17^{c}	—	—	33^{b}	48^{cd}	0.37^{a}	$12,62^{a}$

Note (—) not determined.

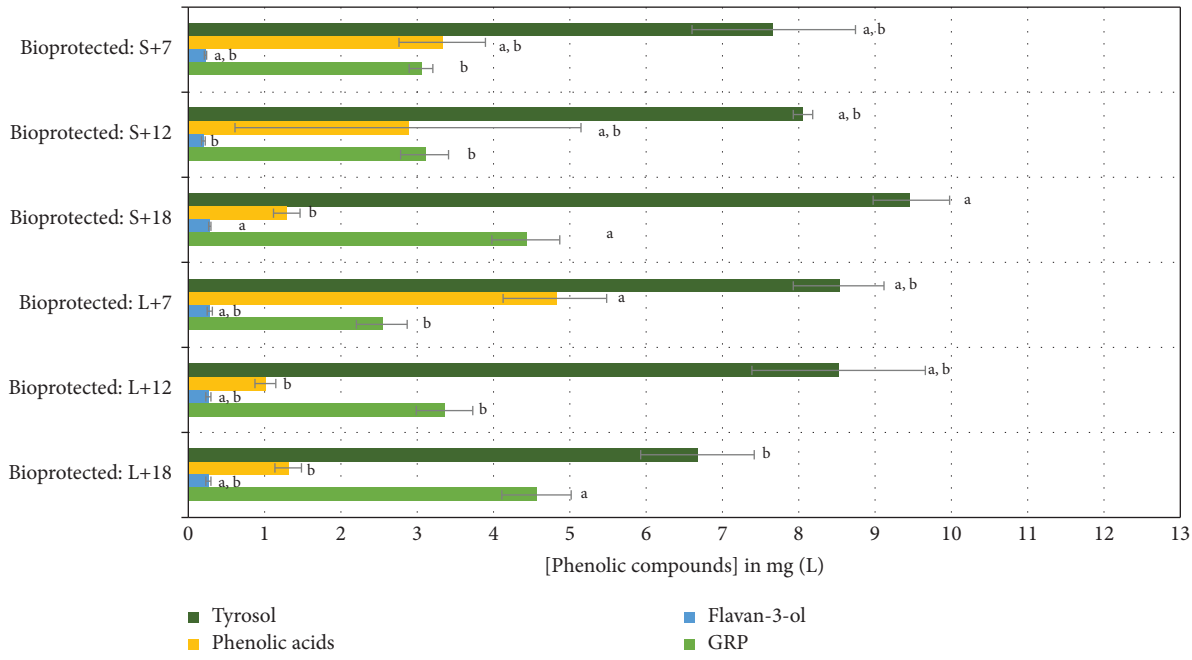

Figure 3: Concentration of families of phenolic compounds (tyrosol, phenolic acids, flavan-3-ol, and GRP) in mg/L at the end of alcoholic fermentation. Data were subjected to the Tukey statistical test (95% conf. interval).

for wines from $L+18$ mode. In fact, the settling time did not really influence the concentrations of phenolic compounds. Only the settling temperatures had a significant impact on the concentration of phenol acids and GRP.

3.8. Antioxidant Capacity Using the DPPH Method. The antioxidant capacity of the wines was evaluated by the DPPH method at the end of alcoholic fermentation (Figure S2). No significant difference in absorbance was noted between the trials: 79.0 for $S+7$, 69.0 for $S+12$, 77.2 for $S+18$, 77.4 for $L+7$, 76.9 for $L+12$, and 73.9 for $L+18$ modes. Temperature and settling time did not influence the antioxidant capacity of the bioprotected wines. The significant differences in phenolic compounds did not affect the antioxidant capacity of these wines.

3.9. Sensory Analyses of Wines Using the Pivot profile©. As samples, only two out of three replicates were chosen within each mode, to eliminate the faulty ones: 607 and 608

samples for the $S+7$ mode, 613 and 615 for the $S+18$ mode, 616 and 617 for the $L+7$ mode, and 619 and 620 for the $L+12$ mode. The samples of $L+18$ mode were excluded from the sensory analysis due to spontaneous fermentation observed during settling. The pivot wine corresponded to the $S+12$ reference mode, as also a blend of only two replicates (610 and 611 samples).

A total of 80 different terms was elicited by the wine professional panel. After lemmatization, categorization, elimination of the hapax, and nondiscriminant terms, a final list of 17 terms was retained. Figure 4 shows the projection of the wine modes and the terms retained on the two first dimensions of the CA. The first and the second dimension explain 91.96% of the variance. The first dimension opposed the acid and lively characteristics to the floral and length ones. The second dimension opposed the buttery, hotness, and floral characteristics to the thiol containing aromatic and fresh ones. Therefore, by comparison with the $S+12$ reference mode (i.e., the pivot wine), Figure 4 shows that the $S+7$ and $L+7$ modes were described in the same way by the wine professionals, that is, these wines were perceived as more acidic, aromatic, and lively and less buttery than the $S+12$ reference mode, with a little more expressiveness for the $S+7$ mode and a little more thiol containing for the $L+7$ mode. At the opposite, the $S+18$ mode was described as more acidic, butter, and lively and less long than the $S+12$ reference mode. The $L+12$ mode was described as more floral, length with a little more richness and toasty notes, less acidic, lively, expressive, and fatter than the $S+12$ reference mode. In addition, whatever the wine mode, the term fruity was often elicited by the wine professionals but seemed to be not enough precise to be discriminating (as it is plotted close to the origin) due to an equivalent frequency of citation as "less" and "more" intense for each wine. Otherwise, any kind of defect seemed to have been perceived in the wine samples.

4. Discussion

The results obtained during the winemaking 2017 [22] have shown that the bioprotection of musts during the pre-fermentation stage of settling in white winemaking has the same effectiveness as sulphiting against the development of potential alteration microorganisms. The early addition of the bioprotection strain can also protect must from enzymatic and chemical oxidation, however, this protection seems to depend on the technical winemaking routes and the must/wine matrix. To deepen the analysis of the impact of the technical parameters during settling on the effects of bioprotection, experiments were carried out during the 2018 vintage, with 6 combinations (time/temperature) applied in triplicate during settling. The mode 12°C for 36 hours ($S+12$) has been set as a reference, these parameters being those usually applied during settling in white winemaking. Although there are currently no molecular tools to confirm the implantation of the *M. pulcherrima* MCR 24 strain, quantitative and qualitative microbiological analyses strongly suggest that the bioprotection strain was well implanted in all modes tested, except in $L+18$ mode where the high temperature and the long settling time (72 hours) were not beneficial for a satisfactory bioprotection strain implantation. In this extreme settling condition, an early alcoholic fermentation started with indigenous *S. cerevisiae* preventing a static settling. A strong reduction in assimilable nitrogen was noted at this stage of the process (approx. 32% of YAN assimilated). Moreover, the strain of *S. cerevisiae* inoculated at the end of settling was partially implanted, illustrated by the coexistence between indigenous *S. cerevisiae* strains and the inoculated strain at midalcoholic fermentation. At midalcoholic fermentation, the speed of fermentation kinetics decreased until the end of AF. One of the probable explanations is an early consumption of essential nutrients, whose amino acids, by indigenous S. cerevisiae and a probable nutritional competition between fermentative strains [37–40]. A significant development of acetic acid bacteria has also been observed in wines in this mode at the end of alcoholic fermentation. However, the little volatile acidity amount measured could be explained by the consumption of a part of acetic acid produced by fermentative yeasts strains [41]. A weak production of acetic acid by some *Acetobacter aceti* strains could be also advanced, relying on the fact that the production of this undesirable volatile compound is strain-dependent [42, 43]. It is interesting to note that this production can also be performed by other bacteria from the genus *Gluconobacter* [44, 45]. The temperature of 18°C associated with a long settling time not only did not allow a good control of the bioprotection and fermentation strains but also indigenous flora (fermentative and undesirable flora). These data highlight the inability of the bioprotective yeast strain to prevent the development of undesirable flora under these "extreme" settling conditions and is not representative of the reality in winemaking [46].

All analytical data from musts and wines analyses were statistically processed by a principal component analysis (PCA) with a biplot representation coupling observations (in red, blue, and green) and variables (in purple) in two dimensions (Figure 5). This statistical analysis was performed without a priori without any modification of the initial data set. For the realization of this PCA, only the DPPH results were not used due to the absence of a significant difference during a univariate statistical test carried out upstream. The total variance of this statistical model obtained was 61.5%. The $F1$ component explained the data set at 38.3% and the $F2$ component at 23.2%. The main result was that the dataset is divided into three distinct groups (red, green, and blue). This was confirmed using an ascending hierarchical classification (CAH—Figure S3). The first group represented the three tests of $L+18$ mode, in red in Figure 5. The second group represented the three tests of $S+18$ mode (blue). The third group brought all tests together in $S+7$, $L+7$, $S+12$ (reference mode), and $L+12$ modes (light blue). The contribution of the various variables was given in Figure 6.

A strong participation of the variables was observed (>0.75) for the following variables: volatile acidity at the end of FA (AV at the end of FA), YAN in clarified musts, combined SO_2, residual sugars, GRP, and levels of *B. bruxellensis* in clarified musts. These variables should be taken into account in the establishment of parameters and

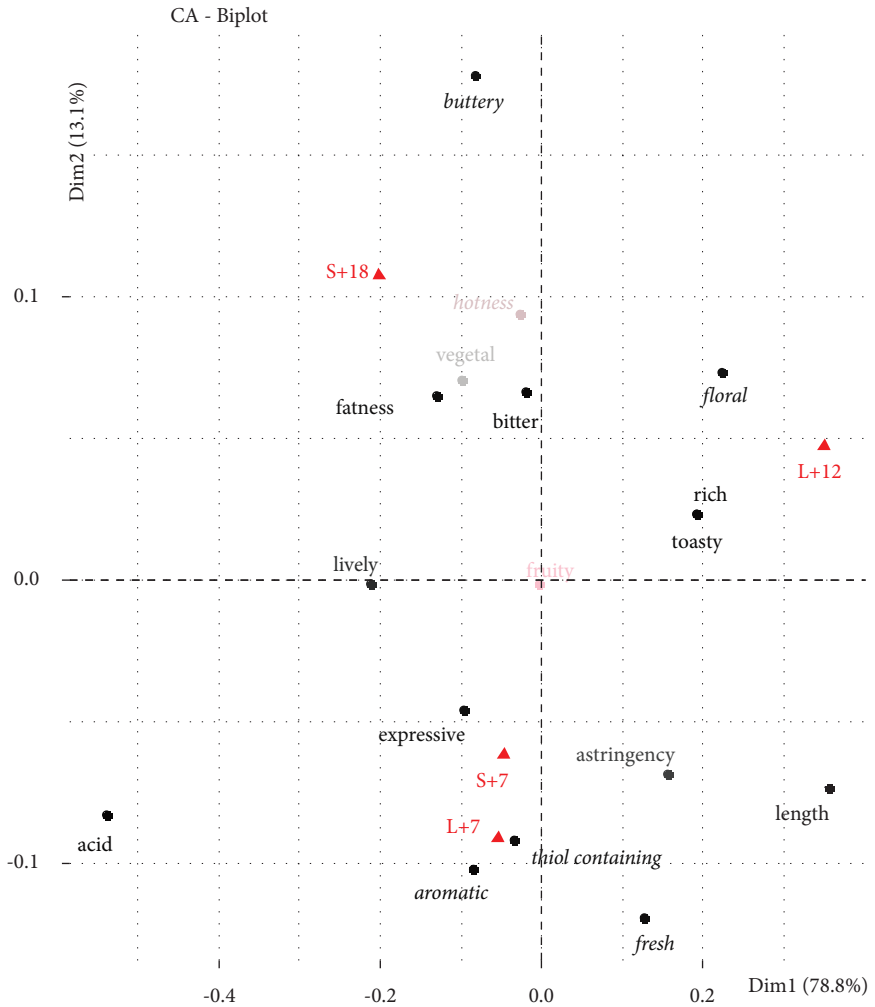

FIGURE 4: Projection of the wine modes ($S+7$, $S+18$, $L+7$, and $L+12$) on the first two dimensions of the correspondence analysis. Terms that are the most contributory variables for each dimension are both represented in **bold** for the first dimension and in *italics* for the second dimension. The quality of representation of the terms in the two dimensions are also represented: terms are colored in black for a $\cos^2$ close to 1, in grey for a $\cos^2$ around 0.5, and in light pink for a $\cos^2$ close to 0.

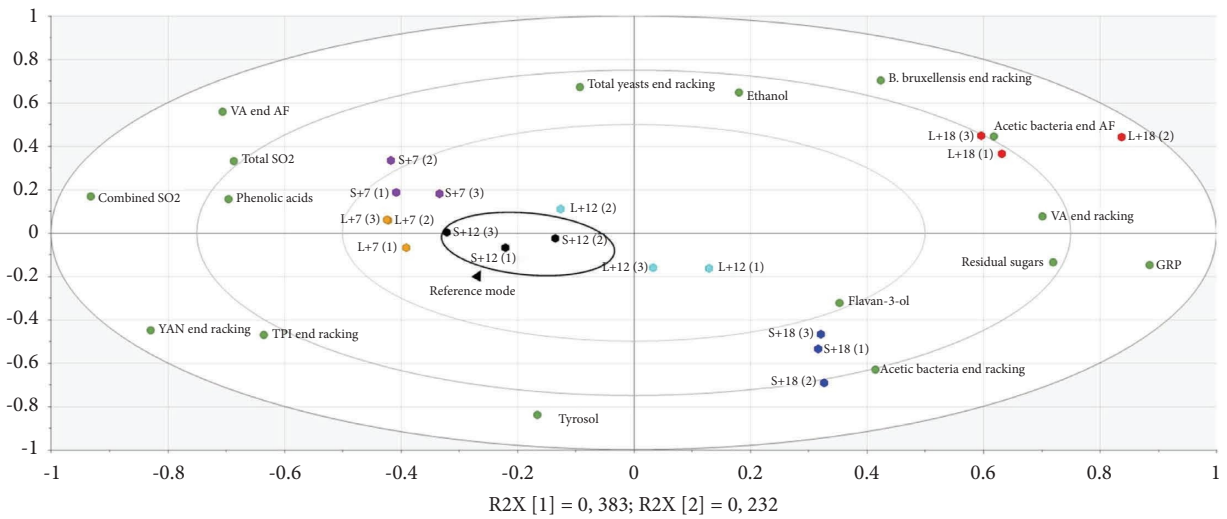

FIGURE 5: Principal component analysis (PCA) with a biplot representation combining the observations "Effect of temperature and settling time," represented in red, blue, and green and the various variables constituting this study are represented in purple.

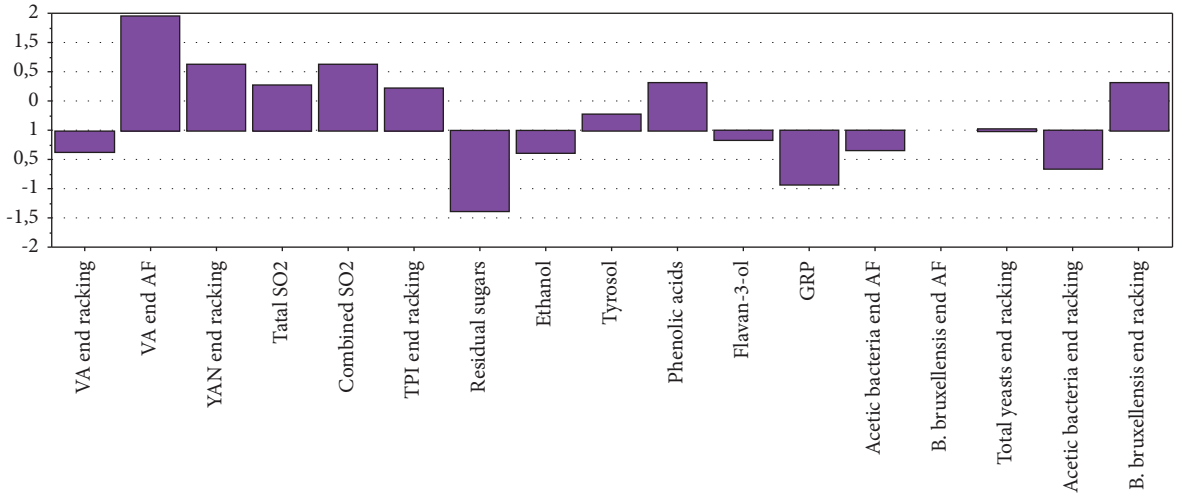

Figure 6: Contribution of the variables.

monitoring of the bioprotection effect. In terms of distance between variables and observations, the variable "the levels of acetic acid bacteria in wines" was very close to the observations gathering the test from $L+18$ mode (Figure 5), which confirms the data described previously. The choice of 18°C did not allow the control of spoilage agents acetic acid bacteria [42, 47]. The variables tyrosol, flavan-3-ol, and phenolic acids had no weight in the construction of the statistical model (Figure 6), suggesting a low impact of temperature and settling time on the phenolic compounds.

If we set as a reference the classic settling mode (12°C/36 hours), extending the settling time at the same temperature or lowering the temperature to 7°C were without consequence on musts and wines. In the context of bioprotection, it would then be preferable to set the temperature values below or equal to 12°C. In this case, the extension of the settling time does not affect the effectiveness of bioprotection. Mechanisms involved in bioprotection by *M. pulcherrima* strains remain unknown today. Species *M. pulcherrima* has never been mentioned in "killer" phenotype. It has only been cited for a potential inhibition of *B. bruxellensis* by capturing the iron present in the medium [17]. The strain of *S. cerevisiae* used in this study has a neutral "killer" phenotype: it did not produce toxins and it was not sensitive to a "killer" toxin. One of the hypotheses that can be made is that the biomass effect from bioprotection applied a selective pressure on the matrix, inhibiting the different spoilage flora [48–51]. A decrease in the overall ratio of yeast and bacterial communities was observed, which could explain the biomass effect of the bioprotection [22, 52, 53]. Another hypothesis is the production of signal molecules inhibiting the same flora [54, 55].

Concerning the sensory analysis part, only five terms are allowed to discriminate the different wine modes, which is not surprisingly considering the relative sensory proximity between those samples (i.e, derived from the same harvest with only settling duration and temperature parameters as variables). Other sensory tests could have been carried out to discriminate between wines such as glasses twice as large [56]. However, links can be made between the physicochemical data and the sensory analysis of wines. Bioprotected wine from a settling at 18°C was mainly characterized by a higher volatile acidity value, with an acidity more particularly felt by the panellists. It is interesting to note that, despite the absence of significant differences in the physicochemical analysis of the bioprotected wines that were macerated at temperatures lower than or equal to 12°C, the longer maceration time at 12°C resulted in more "floral" wines, with more length in the mouth.

The Pivot Profile© constitutes a good first approach to highlight whether there are main sensory differences between wines derived from the same matrix with only a few different winemaking parameters by their direct comparison to a reference. This reference is not always easy to determine. For this study, we chose the $S+12$ reference mode to maintain consistency in the conduct of the full study, as this mode was considered as the most classical winemaking practice. In spite of that, we could have chosen another pivot wine by determining a central blend of all wine modes, which would be composed of an equivolume of each sample, as suggested by Lelièvre-Desmas et al. (2017) and Pearson et al.'s studies [31]. It could represent another good strategy of sensory comparison between all these homogeneous wine modes, especially when the purpose of a study is to compare all samples that are sensorially close to each other, without a predetermined or a priori reference.

5. Conclusion

The temperature of 18°C associated with a long settling time was not favorable to the bioprotection since it appears impossible to control the fermentative microorganisms and/or alterations during winemaking. The other settling conditions did not have a strong impact on the different analyzes. It would then be advisable to use the bioprotection

strain *M. pulcherrima* MCR 24 in a short or long settling at temperatures equal or below 12°C. The few perceived differences had very little influence on the musts and final wines, chemically, microbiologically, or sensorially. It would be interesting to test other bioprotection strains of the same genus and species, available today, in order to compare their effectiveness and also to focus on low fermenting species such as *M. pulcherrima* and *M. fructicola*. To refine these first sensorial results, we could pursue this sensory analysis with a trained panel on these specific sensory characteristics given by wine professionals such as acidity, liveliness, length, floral, and butter aromas. This future work would allow to both precise and quantify the intensity of these attributes that the wine professionals perceived between the samples: What kind of flower notes? How intense the acidity is for the $S+7$ and $L+7$ wine modes in comparison with the $S+12$ reference mode. This next step could be precise if these main sensory characteristics are very subtle or not between the samples, and thus nuance our conclusions of the impact of settling parameters on the sensory attributes of Chardonnay wine. This study presents a more precise action spectrum of bioprotection during settling of white musts and can be a useful tool for better advice to winemakers.

Data Availability

The data in this article are part of a thesis entitled "Study of bioprotection in oenology" carried out between 2016 and 2019 at the University of Burgundy (France). This is confidential but the first author can share the scientific data of this article if needed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: density monitoring as a function of time (days). For a better visibility, the standard deviations have not been added to the graph but are presented in Supplementary data Table S2. Figure S2: percentage of DPPH trapping activity. The wines were analyzed at the end of alcoholic fermentation. Data were subjected to the Tukey statistical test (95% conf. interval). Figure S3: hierarchical ascending classification (HAC) of the different modes for all the variables analyzed in this study, except for the DPPH analysis. Table S1: gradient of solvents A and B for the detection of phenolic compounds in wine. Table S2: density standard deviations as a function of time for all conditions. Table S3: standard deviations for the oenological parameters at the end of settling and at the end of AF. (*Supplementary Materials*)

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