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Influence of sulphur dioxide management on microbial populations during wine ageing

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ABSTRACT

The use of food preservatives is controversial these days, and sulphur dioxide (SO_2) is no exception. Microbial communities have been studied particularly during the prefermentative and fermentative stages in the absence of added SO_2. However, the microbial risks associated with the reduction or absence of SO_2, particularly during the wine ageing process, have been little studied. The microbiological control of wine ageing is a key issue for winemakers wishing to produce high-quality wines without added SO_2. The present study aims to evaluate the impact of different wine ageing strategies, depending on whether or not SO_2 is added, on microbial population levels and diversity. Sulphite management had minimal impact on the population levels of yeast and acetic acid bacteria. Conversely, the population of lactic acid bacteria, whether from inoculation or spontaneous malolactic fermentation (MLF), was significantly impacted by SO_2 management. High and stable population levels of around 10^5 CFU/mL were observed in the SO_2-free modalities. Sulphiting after malolactic fermentation has a greater effect on lactic acid bacteria up to four months of ageing than standard SO_2 addition at the time of vatting. *Oenococcus oeni* was the only species detected during ageing using a culturable approach. After five months of ageing, a high genetic diversity of *Oenococcus oeni* was highlighted in all modalities, even in the inoculated one with a commercial strain. The high population level of *Oenococcus oeni* was associated with acetic acid and D-lactic acid production, as well as mousy off-flavours. One sulfite treatment at the end of MLF inhibits the growth of *Oenococcus oeni*, but only temporarily. Winemakers should consider the stage and number of treatments to limit the development of lactic acid bacteria (LAB) during ageing and preserve the quality of red wine.

KEYWORDS: No SO_2, red wine ageing, MALDI-TOF MS, *Oenococcus oeni*, VNTR, microbial populations

INTRODUCTION

Sulfur dioxide has been used in oenology for many years because of its many properties. This additive is an antimicrobial, an antioxidant, and an antioxidasic (Boulton *et al.*, 1999). Winemakers need to adapt their practices and reduce their use for several reasons. Firstly, from a health point of view, exposure to sulphur dioxide is associated with adverse reactions in 'sulphite-sensitive' individuals in the form of intolerance (Papazian, 1996). According to the OMS, the acceptable daily intake (ADI) of sulfite is 0.7 mg/kg bw per day (Joint & Additives, 2009). Professionals must also comply with the regulation. For example, for red wines (less than 2 g/L of sugar), the dose must be less than 150 mg/L of total SO_2. For organic dry wines, the doses allowed in winemaking are reduced by 50 mg/L compared to conventional practices (European regulations (Conventional wines: Regulation (EC) N° 606/2009 Annex1B). In addition, the Commission Delegated Regulation (EU) 2019/934 of 12 March 1999 mentions that "In the light of the results of scientific studies [...] the values could be re-examined at a later date with a view to reducing them". Finally, wine producers need to adapt their winemaking processes to consumer demands. Indeed, wine consumption patterns are increasingly integrating environmental and social objectives and considerations (D'Amico *et al.*, 2016; Delmas & Gergaud, 2021; Vecchio *et al.*, 2023). Consumers' willingness to pay (WTP) for sustainable wines is higher and increased by the "no sulfite added" label (Apaolaza *et al.*, 2017; Giraud-Héraud *et al.*, 2019; Raineau *et al.*, 2023).

To meet this demand, winemakers can reduce the dose or eliminate sulfites at different stages of the winemaking process (Lisanti *et al.*, 2019). During the pre-fermentation stages, bioprotection could be used to replace SO_2; this consists of the addition of microorganisms directly on grapes or at vatting for their antimicrobial and chemical properties, and also to limit oxidation phenomena (Windholtz *et al.*, 2021a; Windholtz *et al.*, 2023; Windholtz *et al.*, 2021b). Regarding the ageing stage, winemakers have several alternatives to SO_2. For example, chitosan has been proposed for its antimicrobial properties, particularly against *Brettanomyces bruxellensis* (Paulin *et al.*, 2020). Filtration and cross-flow filtration can be used during bottling to microbiologically stabilise the wine in the bottle. The cost of these alternatives is not negligible for professionals. Many scientific studies have been carried out on these SO_2 alternatives, but few on the rational use of sulfur dioxide, particularly during the ageing period.

If sulphites are not added, microbial development can occur during wine ageing, which can lead to off-flavours (Pelonnier-Magimel *et al.*, 2020). The best described is related to the production of volatile phenols by *Brettanomyces bruxellensis*, which is associated with barnyard, horse sweat or burnt plastic aromas (Chatonnet *et al.*, 1992; Kheir *et al.*, 2013). An increase in volatile acidity is another microbiological defect that may occur during wine ageing due to lactic acid bacteria (LAB), acetic acid bacteria (AAB) or *Brettanomyces bruxellensis*. Recently, the occurrence of mousy off-flavours in wines has increased, partly due to the significant reduction in SO_2 addition during ageing. Indeed, recent studies make the relationship between the increase in acetic acid and/or mousy off-flavours and high population levels of LAB during wine ageing (Moulis *et al.*, 2023). Mousy off-flavours have been reported to be produced, probably among others, by *Brettanomyces bruxellensis*, *Lentilactobacillus hilgardii* or *Oenococcus oeni* in wine (Snowdon *et al.*, 2006; Moulis *et al.*, 2023) and beer (Martusevice *et al.*, 2024a). Three chemical compounds were identified as responsible for mousy off-flavour in wine: ETHP, 2-acetyl-tetrahydropyridine (ATHP), and 2-acetylpyrroline (APY). ATHP is found in many agri-food products (Martusevice *et al.*, 2024a; Snowdon *et al.*, 2006), and it was described sensorially as "biscuit cracker" (Bartowsky & Henschke, 1995). ATHP detection threshold in water is 1.6 $\mu g.L^{-1}$ (Teranishi *et al.*, 1975), and ATHP concentrations can reach 60 $\mu g.L^{-1}$ in some beers (Martusevice *et al.*, 2024b). ETHP detection threshold in water was determined at 150 $\mu g.L^{-1}$ (Craig & Heresztyn, 1984). Previous studies have reported on the impact of *Oenococcus oeni* strains on the production of the mousy off-flavour (Romano *et al.*, 2008; Moulis *et al.*, 2023). Different authors (El Khoury *et al.*, 2017; Franquès *et al.*, 2018; Lorentzen *et al.*, 2019) have documented the genetic diversity and technological traits of *Oenococcus oeni*, the main species responsible for MLF and its impact on wine sensory properties. However, this documentation is lacking during the wine ageing process.

The microbial dynamics in terms of population and diversity, as well as the potential risks of reducing or eliminating sulfur dioxide (SO_2), especially during wine ageing, have not been extensively studied. However, controlling the microbiology of wine ageing is essential for winemakers who want to produce high-quality wines without adding or limiting SO_2. This study evaluates the impact of various ageing strategies, including SO_2 addition and MLF management, on microbial population dynamics and diversity. The following research questions were addressed: i) Is it possible to reduce the frequency of sulfiting while preserving wine quality? ii) Does inoculation with selected lactic acid bacteria limit the risk of microbial deviation during wine ageing?

MATERIALS AND METHODS

1. Winemaking process, treatments, and sampling

Merlot N (*Vitis vinifera* L.) grapes from the 2022 vintage were produced in the Cadillac Côtes de Bordeaux appellation (Bordeaux, south-west France) in a winery using European organic farming methods. The grapes were divided into six homogeneous batches according to the treatment shown in Figure 1. The grapes were crushed, and each batch was divided in triplicate into 30 L stainless steel tanks. After a cold soak at 10 °C for three days, vats were inoculated with 200 mg/L, corresponding to 1 to 2 × 10^7 CFU/mL of *Saccharomyces cerevisiae* (Actiflore® F33, Laffort, France)

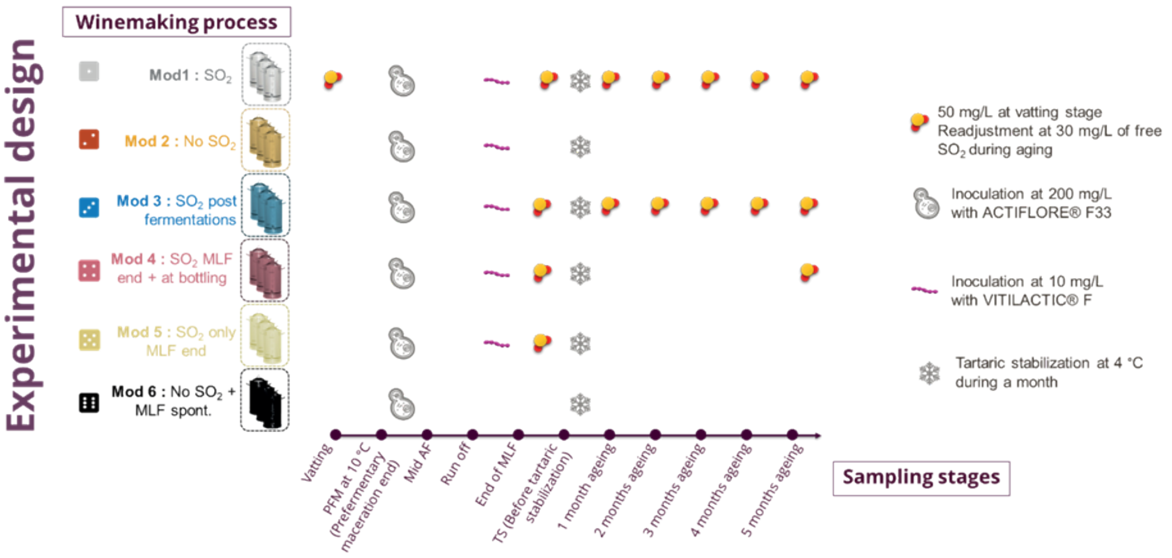

FIGURE 1. Experimental design: SO_2 treatments during the winemaking process, MLF management, and sampling.

Mod1: SO_2: 50 mg/L of total SO_2 at vatting stage, 30 mg/L of free SO_2 adjustments every month after MLF; Mod2: No SO_2: no SO_2 addition during ageing; Mod3: SO_2 post fermentations: adjustment at 30 mg/L of free SO_2 every month after MLF; Mod4: SO_2 at MLF ending + bottling (30 mg/L of free SO_2); Mod5: SO_2 only MLF ending (adjustment at 30 mg/L of free SO_2) Mod6: No SO_2 + MLF spon.: no SO_2 addition and spontaneous MLF.

for alcoholic fermentation (AF). For MLF, the modalities were inoculated with a commercial *O. oeni* (Vitilactic® F, Martin Vialatte, France) at 10 mg/L corresponding to 1 to 2 × 10^6 CFU/mL except for the last modality (Mod 6) where the MLF was spontaneous. After tartaric stabilisation, wines were aged for five months before bottling. The different SO_2 additions and MLF management according to the modalities are presented in Figure 1. During the experiment, 10 ml of must or wine were sampled under sterile conditions at each stage and processed immediately.

2. Microbial analysis

2.1. Quantification of population levels

Samples were plated on selective agar media for different microorganisms. For total yeast, LT medium with agar (20 g/L D-glucose, 10 g/L yeast extract, 10 g/L peptone, 0.1 mg/mL chloramphenicol, and 0.15 mg/mL biphenyl) was used, and plates were incubated at 30 °C for five days. For bacteria, pasteurised grape juice diluted to a quarter (final sugar concentration 37.5 g/L) with 5 g/L of yeast extract and 1 mL/L of Tween 80 with 30 g/L of agar was used. Pimaricin (5 g/L) and penicillin (1.25 g/L) were added to select acetic acid bacteria (AAB). The plates were incubated at 25 °C for seven days in aerobiosis. Only pimaricin was added to select LAB, and plates were incubated in anaerobiosis at 25 °C for 12 days.

2.2. Species identification by MALDI-TOF MS

For each replicate, 10 colonies were randomly isolated at the same dilution for each specific medium. Colonies were identified at the species level by MALDI-TOF MS. For the extended direct Transfer (eDT) method, the manufacturer's protocol (Bruker, Karlsruhe, Germany) was followed. Mass profiles were compared with the MBT Compass Library™ completed with the additional database generated in the laboratory (Vallet-Courbin *et al.*, 2022; Windholtz *et al.*, 2021a).

2.3. *Oenococcus oeni* strain typing

Colonies that were identified as *O. oeni* by MALDI-TOF MS were suspended in H_2O for storage at –20 °C. A multiple-locus Variable Number of Tandem Repeats (VNTR) analysis was used to discriminate the strain of *O. oeni* according to the method adapted from Claisse and Lonvaud-Funel (2012). Among the five VNTR loci proposed, only the TR1 and TR2 loci were targeted, as they are reported to be the most discriminative. The PCR mix targeting TR1 or TR2 consisted of: 4 µL of the suspension, 1 µL of 2.5 µM primer mix and 5 µL of Qiagen Multiplex PCR Master Mix 2X (Qiagen, Deutschland). The PCR cycle was as follows: reactions were cycled for 15 min at 95 °C, then for 30 cycles of 94 °C for 30 s, 62 °C for 1 min 30 s, and 72 °C for 1 min 30 s, then followed by a final extension period of 30 min at 60 °C. PCR products were analysed using a microchip electrophoresis system (MultiNA Shimadzu). A homemade R script was used to normalise results with a reference strain for size correction of the amplified DNA fragments. This normalisation makes it possible to compare amplifications obtained between different batches of samples.

3. Mousy off-flavour analysis

After six months in a bottle, wines were analysed for mousy off-flavours by gas chromatography-mass spectrometry coupled with stir bar sorptive extraction according to the protocol of Kiyomichi *et al.* (2023). Three compounds correlated with mousy off-flavours were quantified: 2-acetyl-1-pyrroline (APY), 2-acetyltetrahydropyridine (ATHP) and 2-ethyltetrahydropyridine (ETHP).

4. Classical chemical analysis

Classical chemical analysis (residual sugars, total acidity, malic acid, pH, ethanol *(v/v)*, and acetic acid) was performed with the Enology Analyzer Y15 (BioSystems, Spain). Analysis of D and L lactic acid was performed by HPLC.

5. Statistical analysis

Statistical analysis was performed in RStudio (RStudio Team, 2022.07.1). The Shapiro–Wilk test was used to control the parameters of normality and homogeneity of variances, and Levene's tests were performed using the "car" package (Fox & Weisberg, 2018). The package "agricolae" allowed the analyses of variance by ANOVA (Mendiburu, 2023) and the determination of significant differences. The Tukey HSD test was used to classify significant modalities. Results are presented as scatterplots, polar coordinates or boxplots by using the package "ggplot2" (Wickham *et al.*, 2016).

RESULTS

In this work, the dynamics of culturable yeast, LAB and AAB populations were monitored during the winemaking process, and colonies were identified at the species level using MALDI-TOF MS. During the experiment, 1,890 yeast isolates, 1,343 LAB isolates and 132 AAB isolates were identified by MALDI-TOF MS on the six modalities studied.

1. Population dynamics of microorganisms

The dynamics of culturable yeast populations during the winemaking process are shown in Figure 2A. At vatting, yeast populations were similar and around 10^5 CFU/mL in all modalities. After cold soak, at 10 °C, the population reached 5.10^6 CFU/mL, except for the SO_2 modality, where the population increased slightly to 2.10^5 CFU/mL. From the middle of the fermentation to the end of the MLF, the population decreased to 2.10^3 CFU/mL, before stabilising at 20 CFU/mL after one month of ageing. Then, the population levels did not differ significantly between the modalities, reaching 10^3 to 10^4 CFU/mL after two months of ageing and remained stable until bottling.

Identification of yeast colonies isolated in LT medium at the species level using MALDI-TOF MS is shown in Figure 2B. At least six different yeast species were found in the must, with *Hanseniaspora uvarum* and *Metschnikowia* sp. being the most dominant in all modalities from vatting to the end of prefermentative maceration. However, from AF to bottling, as expected, colonies were identified as mainly belonging to *Saccharomyces cerevisiae*.

From vatting to the end of prefermentative maceration, LAB population dynamics were similar for all modalities considered, but with significantly lower population levels with SO_2 (9.10^4 CFU/mL) compared to the other modalities ($3.6 \pm 6.2 \times 10^6$ CFU/mL) (Figure 3A). During alcoholic fermentation, population levels were $5.4 \pm 3.3 \times 10^2$ CFU/mL, except for the SO_2 modality where populations were < 10 CFU/mL. Populations then increased in all modalities to 5.10^6 CFU/mL from run-off to the end of MLF. Different population dynamics were then observed during ageing. In wines supplemented with SO_2 at the end of the MLF (Mod1, Mod3, Mod4, Mod5), the LAB populations decreased strongly during ageing, but not in the same way. Three treatments (Mod3 "SO_2 post fermentations", Mod4 "SO_2 MLF end + bottling" and Mod5 "SO_2 only MLF end") showed a significant decrease, below the detection threshold after three months, whereas the decrease was slower for the Mod1 treatment. Interestingly, repeated sulfiting during the first three months of ageing Mod3 ("SO_2 post fermentations") had the same effect on these populations as single sulfiting after MLF for Mod4 ("SO_2 MLF end + bottling") and Mod5 ("SO_2 only MLF end"). However, after four months, the LAB population increased again, reaching 10^4 CFU/mL before bottling for Mod4 and Mod5, which were sulfited only after MLF. The two modalities Mod2 ("No SO_2") and Mod6 ("No SO_2 + MLF spon."), where no SO_2 was added, had similar population dynamics, although for the indigenous MLF (Mod6 "No SO_2 + MLF spon."), the population levels are significantly higher up to four months of ageing. Before bottling, the population remained high at $3.43 \pm 1.48 \times 10^5$ CFU/mL.

In terms of species identification, nine species were identified in the must (Figure 3B), with *Tatumella* sp. and *Apilactobacillus kunkeei* being the most dominant species from vatting to the end of prefermentative maceration. Only two species were detected during AF: *Lactiplantibacillus plantarum* (formerly known as *Lactobacillus plantarum*) and *O. oeni*. From MLF to bottling, *O. oeni* was the dominant species in the wine, whether inoculated or not.

Finally, the population levels of cultivable AAB were monitored (Figure S1). In the must, the population was at 10^3 CFU/mL in all treatments. Three species of AAB were identified in the must (data not shown): *Gluconobacter cerinus*, *Gluconacetobacter liquefaciens* and *Kozakia baliensis.* Rare isolates were identified as *Acetobacter pasteurianus* during ageing, and population levels did not increase by more than 10^3 CFU/mL, and only episodically (Mod1 and Mod3).

Isolates of *O. oeni* were discriminated to strain level by VNTR using locus TR1 and TR2 at three stages (Figure 4): MLF end, three- and five-months. Four modalities were considered, Mod2, Mod4, and Mod5, where inoculation was performed with a commercial *O. oeni* (Vitilactic® F, Martin Vialatte, France) at 10 mg/L and Mod6, where MLF was performed with indigenous bacteria. In total, 34 different profiles were identified. The genetic identification of *O. oeni* for Mod2, Mod4, and Mod5 was performed on total cultivable biomass. As expected, only the genetic profile of commercial strain VF (Vitilactic F) was identified in Mod2, Mod4, and Mod5 at the end of MLF, whereas five different genetic profiles were identified in Mod6, with a dominant one, profile 0.

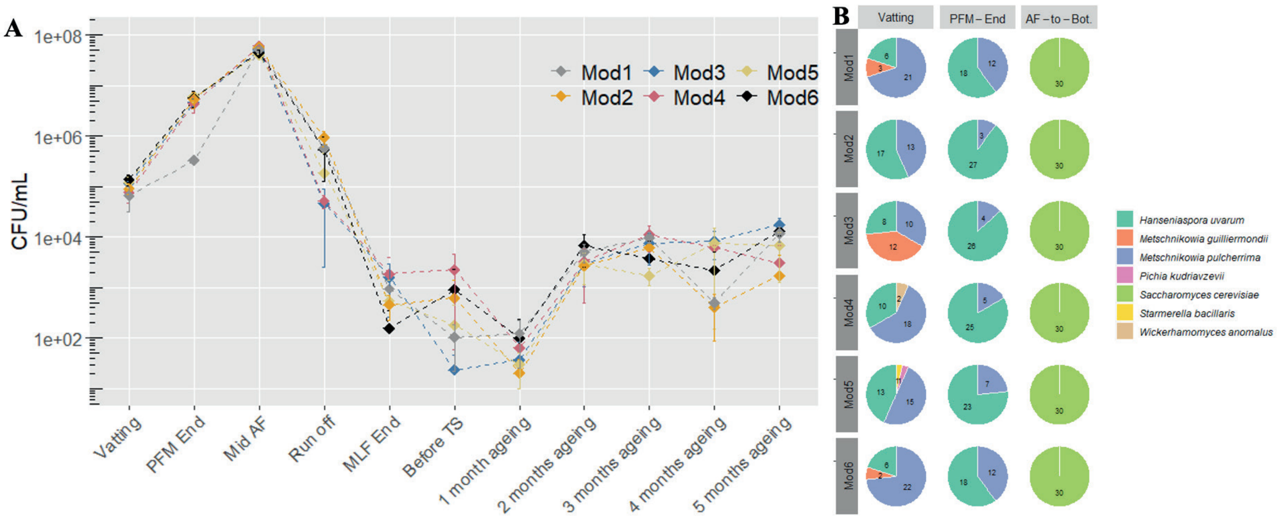

FIGURE 2. Yeast population dynamics during the winemaking process.

(A) Population levels from vatting to five months ageing (before bottling), $n = 3$; PFM: prefermentary maceration; AF: alcoholic fermentation; MLF: malolactic fermentation; TS: tartaric stabilisation. Mod1: SO_2; Mod2: no SO_2; Mod3: SO_2 after fermentations; Mod4: SO_2 only after MLF; Mod5: SO_2 only after MLF + before bottling; Mod6: MLF indigenous and without SO_2.

(B) Yeast identification to species level by MALDI-TOF MS (number in corrplot corresponding to the number of colonies for each modality); AF to bot.: from alcoholic fermentation to bottling.

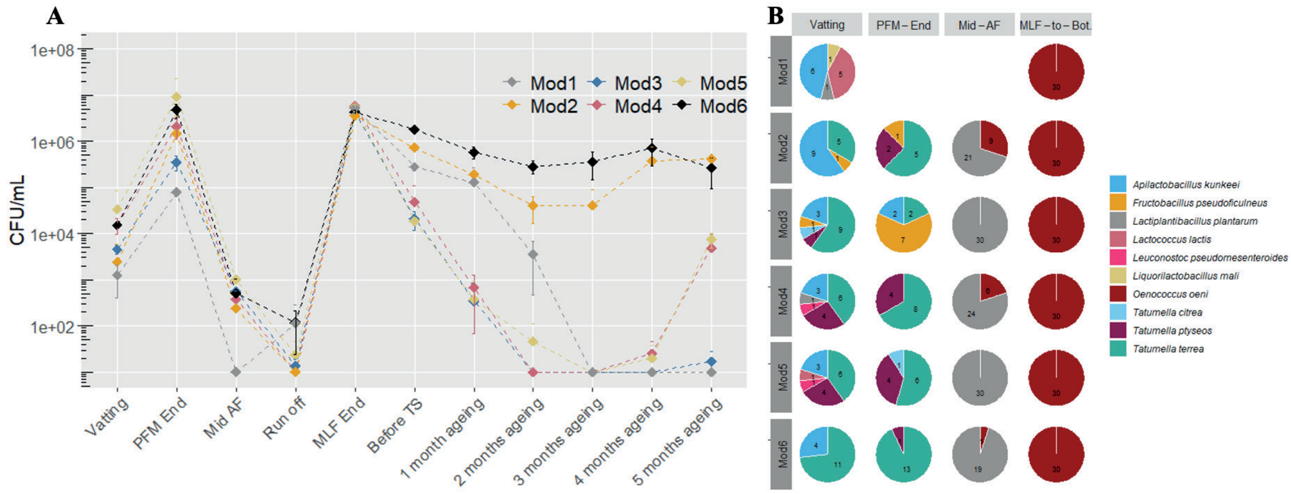

FIGURE 3. LAB populations during winemaking.

(A) Population levels at vatting to five months ageing (before bottling), $n = 3$.

(B) Species identification by MALDI-TOF MS (number in the corrplot corresponds to the number of colonies). MLF to bot. represents the same result: *O. oeni* was found at 100 % from MLF to bottling. *Oenococcus oeni* genetic diversity.

In the middle of ageing, genetic identification was only possible for Mod2 and Mod6, as no colonies were isolated for Mod4 and Mod5 with SO_2 addition. Few isolates were identified as VF in both treatments (inoculated and non-inoculated). Compared to the end of the MLF, the emergence of new dominant profiles (profiles 1, 3 and 4) of *Oenococcus oeni* was highlighted.

New profiles appeared in Mod2, Mod4, and Mod5, with no repeatability in the nature and frequency of certain profiles between replicates at the end of ageing. Surprisingly, after five months of ageing, a genetic profile similar to that of the commercial *O. oeni* was detected in Mod6.

2. Wines chemical analysis

There were no significant differences in terms of ethanol, residual sugar, L-lactic acid and pH for the different modalities in the wines after six months of ageing and bottling (Table S1). Acetic acid was monitored throughout the ageing process (Figure S2). In the “No SO_2” modalities, there was a gradual increase in acetic acid levels (Mod2 and Mod6), reaching 0.38 and 0.45 g/L of acetic acid, respectively. In the SO_2 modalities (Mod1 and Mod3), acetic acid levels remained stable. In the last month, there was a slight significant increase for the SO_2 treatments after MLF (Mod4 and Mod5) compared to the other treatments (Mod1, Mod3, Mod4, and Mod5).

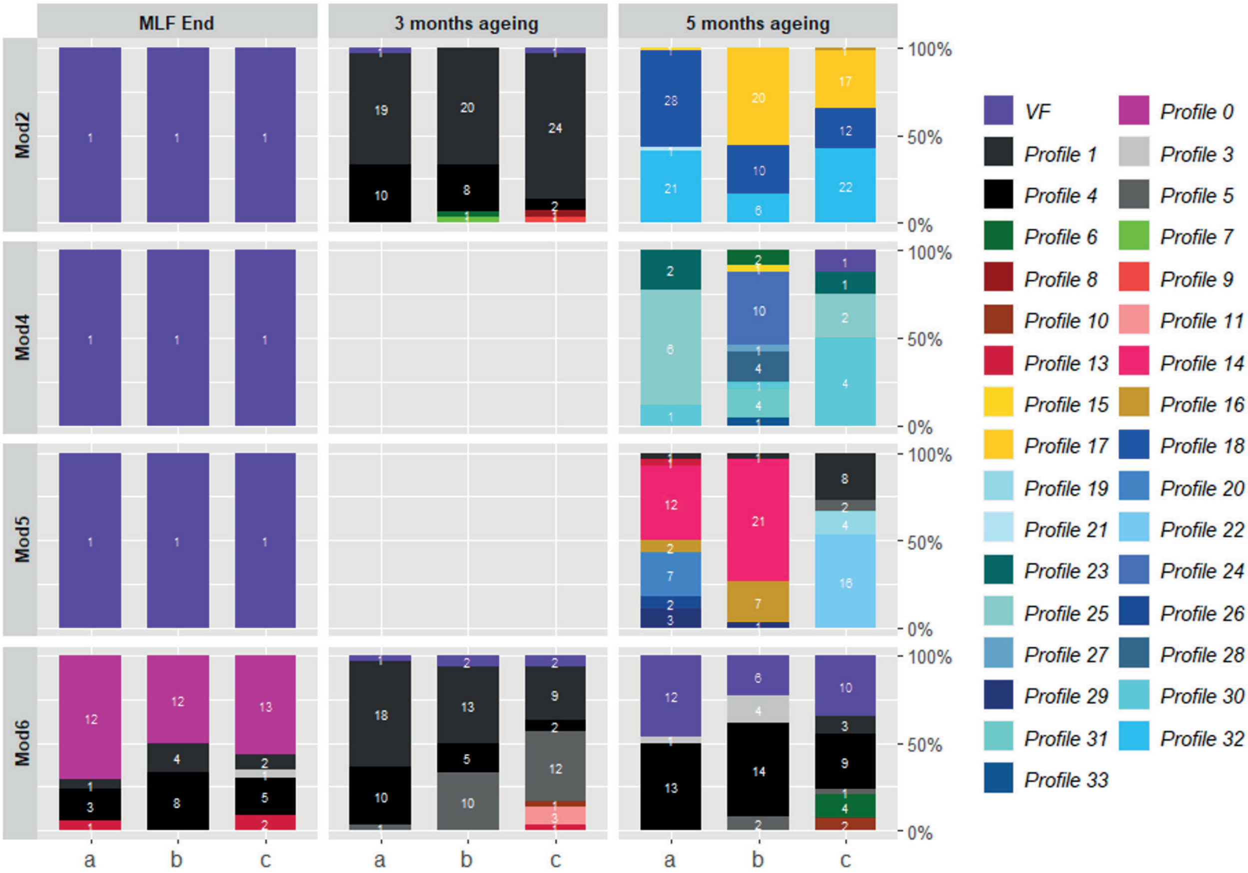

FIGURE 4. *Oenococcus oeni* genetic profile during winemaking and wine ageing process.
a, *b*, *c* are biological replicates. Numbers in barplot represent the number of colonies analysed.
For MLF end stage in Mod2, Mod4, and Mod5 profiles were obtained from total culturable biomass.

Six months after bottling, D-lactic acid, 2-acetyl-1-pyrroline (APY), 2-acetyltetrahydropyridine (ATHP), and 2-ethyltetrahydropyridine (ETHP) were quantified. The compounds responsible for mousy off-flavours were quantified because a suspicious mousy off-flavour was detected by tasting. It was not possible to quantify APY and ETHP in the wines of the different modalities. ATHP was not detected in wines with SO_2 added during ageing (Mod1 and Mod3), whereas it was detected in modalities 2, 4, 5, and 6, the latter with the highest concentration of 21.83 ± 7.1 µg.L^{-1}.

For the D-lactic analysis (Figure 5A), the wines of the Mod1 treatment had significantly lower values than the others, except Mod3. Conversely, in the treatment without SO_2 treatments (Mod2 and Mod6) where D-lactic acid increased throughout the wine ageing process and reached its highest concentration six months after bottling. Citric acid was also quantified (Table S1), but no correlation with D-lactic acid production was observed.

DISCUSSION

The reduction of SO_2 during the winemaking process is a real concern for the wine industry. To date, research has focused on the absence of SO_2 addition during the prefermentative stage or at the time of fermentation, where the absence of SO_2 is easy to manage if the grapes are healthy and ripe. However, there is a lack of information on the influence of SO_2 reduction, particularly during the ageing of red wine. Without protection, red wine aged in barrels or tanks is susceptible to microbial spoilage. Therefore, winemakers are reluctant to change their oenological practices and limit SO_2 during ageing. Previous studies have investigated the effect of SO_2 on the composition of red wine (Arapitsas *et al.*, 2018; Pelonnier-Magimel *et al.*, 2023). However, the effect of SO_2 on the microbial community of both bacteria and yeasts has not been considered recently. The present study aimed to update the knowledge of microbial dynamics, diversity and their impact on wine composition, to support decision-making for red wine management during ageing in the context of no SO_2 addition.

Grape must is a matrix characterised by a high microbiological biodiversity, which tends to decrease over time during the winemaking process. Several factors, such as an increase in ethanol concentration, an increase in temperature, low pH, SO_2 addition, and nutrient depletion, explain this evolution of biodiversity. In our study, the yeast population decreased from mid-fermentation to the end of the MLF, and diversity decreased from vatting to the end of AF. In the grape must, *Hanseniaspora uvarum* and *Metschnikowia* sp. were dominant, in agreement with many previous scientific studies (Beltran *et al.*, 2002; Constantí *et al.*, 1997; Jolly *et al.*, 2014; Windholtz *et al.*, 2021a; Zott *et al.*, 2008; Zott *et al.*, 2010).

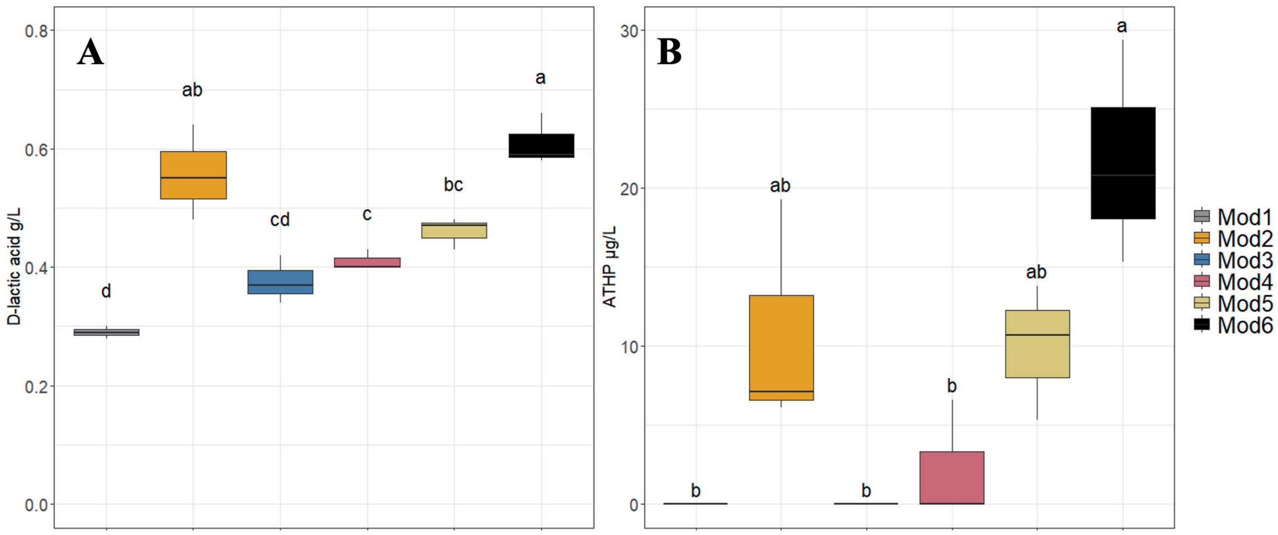

FIGURE 5. Quantification of D-lactic (A) and ATHP (B) after six months of ageing and bottling.

Mod1: with SO_2 during winemaking; Mod2: without SO_2 adding during winemaking; Mod3: SO_2 after fermentations; Mod4: SO_2 only after MLF; Mod5: SO_2 only after MLF + before bottling; Mod6: MLF indigenous and without SO_2. ANOVA (*p*-value < 0.05), HSD *post hoc*, different letters indicate means that are significantly different.

The number of cultivable yeasts decreased with time up to one month of ageing, with a slight increase and then a stable population after five months of ageing. *Saccharomyces cerevisiae* was the dominant species during wine ageing. Previous studies have reported the frequent presence of *Saccharomyces*, *Hanseniaspora*, and *Torulaspora* in wine using either culture or molecular techniques (Saez *et al.*, 2011; Bokulich *et al.*, 2016; Padilla *et al.*, 2016; Pinto *et al.*, 2015). Although *Brettanomyces* was not detected through cultivation with LT medium, it is important to monitor this species in practice, especially during ageing. Molecular methods such as quantitative PCR or digital PCR could provide a more reliable analysis to confirm whether *Brettanomyces* was present during wine ageing in our experimental conditions.

The population and diversity of LAB were also monitored. The population dynamics were similar for all the modalities considered, from racking to the end of the MLF, except for the SO_2 modality (Mod1), where the populations were lower until mid-AF. The negative effect of SO_2 on LAB growth during the winemaking process has already been reported by several authors (Andorrà *et al.*, 2008; Bokulich *et al.*, 2015). As for the yeast community, a higher diversity in grape juice and during PFM was highlighted for LAB, with *Liquorilactobacillus mali* and *Lactococcus lactis* found only at the vatting stage. The former, formerly known as *Lactobacillus mali,* has already been detected in must and during AF (González-Arenzana *et al.*, 2017). According to the scientific literature, *L. lactis* can be isolated from fresh, fermenting plant material and identified in must (Bokulich *et al.*, 2012; Rademaker *et al.*, 2007). Fructophilic species are also present (*Apilactobacillus kunkeei* and *Lactobacillus pseudo ficulneus*) (Filannino *et al.*, 2019). These species can be identified anecdotally in oenology (Bokulich *et al.*, 2012; Haag *et al.*, 1998), but are mostly described in fructose-rich media such as honey, exotic fruits (bananas, figs, cocoa beans), or fermented cocoa beans (Endo *et al.*, 2018). Our data showed the presence of species belonging to the genus *Tatumella* (*terrea*, *citrea*, *ptyseos*) in the must. This genus has been reported from grape must and wine (Maragkoudakis *et al.*, 2013; Pinto *et al.*, 2015). In this study, this genus was observed in must without SO_2, a result that is very interesting as it has already been observed under the same conditions (Morgan *et al.*, 2019; Takahashi *et al.*, 2014).

From mid AF, *Lactiplantibacillus plantarum* dominates, but is then replaced by *Oenococcus oeni* during MLF, as previously reported by Pan *et al.* (1982). Both species are known to have resistance mechanisms that allow them to survive and proliferate in wine (Bravo-Ferrada *et al.*, 2013; Lonvaud-Funel, 2001). Furthermore, *Oenococcus oeni* is the only species found in all the treatments until the end of vinification and bottling. This result is consistent with the study by Bokulich *et al.* (2016), who used high-throughput marker gene sequencing on wine samples after several months of barrel ageing and detected more than 95 % of bacterial sequences belonging to *Leuconostoc* (same family as *Oenococcus*).

Contrary to the yeast population dynamics, SO_2 management strongly influenced the population dynamics of cultivable LAB. In his thesis, Millet (2001) studied the effect of different sulfiting doses (0, 30, 50 mg/L of SO_2) after malolactic fermentation (MLF) on three Bordeaux grape varieties in oak barrels. He monitored the population of LAB over three months. In the absence of SO_2, the population level was maintained at around 10^6 CFU/mL, leading to an increase in volatile acidity, while a dose of 50 mg/L of SO_2 inhibited these microorganisms even at high pH (3.75 to 3.95 at the end of MLF). A dose of 30 mg/L of SO_2 was not sufficient to limit these microorganisms. The results referred to wines sulfited at harvest, and only one application of SO_2 was evaluated during ageing. In our study, the effect of SO_2 was not the same whether sulphiting was applied at vatting (Mod1) or not (Mod3). Surprisingly, the absence of SO_2 at vatting allows a more efficient inhibitory effect of SO_2 on LAB after MLF with a population level below the detection threshold after two months of ageing, compared to three months in the SO_2 Mod1 modality. The same influence was then observed whether there was one sulphiting (Mod4 and 5, after MLF) or three sulphiting during the first three months. In our experiment, one sulphiting at the end of the MLF was sufficient to neutralise the LAB during the first four months of ageing. However, after five months of ageing, there was a significant growth of LAB for Mod4 and 5. In a previous study using cultivable counts in the context of wine ageing with SO_2, LAB population was high at the end of the MLF (10^5 to 10^6 CFU/mL), and then decreased to 10^3 to 10^4 CFU/mL after three months of ageing, and was not detectable until the end of the wine ageing process (12 months) (Kioroglou *et al.*, 2020). In the absence of SO_2, whether the MLF was spontaneous or inoculated, the population of LAB was high (around 10^5 CFU/mL) from the end of the MLF until five months of ageing. *Oenococcus oeni* was the only species detected during ageing using a culturable approach, with a high diversity of profiles that evolved throughout the process and according to the different modalities. In the modality without SO_2 Mod2, the profile of Vitilactic F was not detected after three months of ageing, nor at the end. In the case of Mod6, where MLF is spontaneous, four different profiles were detected, but the profile of the commercial strain was detected after three months and at the end of ageing. The resident population in the winery (airborne, biofilms on equipment, or tanks) could be at the origin of a contamination of the three biological replicates, but another hypothesis is that the Vitilactic F strain was already present in the wine, at much lower levels than the indigenous strains, and therefore undetectable according to our experimental design. It is also possible that the genetic profile detected in Mod6 belongs to a strain that is different from Vitilactic F. To confirm this, a more in-depth genetic characterisation would be required.

Previous studies showed that the use of SO_2 affected the diversity of the strains (Reguant *et al.*, 2005), as did Renouf *et al.* (2009). After five months of ageing, the genetic diversity of *Oenococcus oeni* was high in all modalities, with the appearance of new genetic profiles. As mentioned before, different hypotheses could explain the presence of new profiles at this stage, which could originate from the resident population in the cellar or could be initially present at the end of MLF but not detected due to our cultivation approach.

Finally, AAB were at low cultivable population levels in all modalities. However, it has previously been shown to be maintained at constant population levels (10^5 to 10^6 cells/mL) using Q-PCR analysis (Andorrà *et al.*, 2010; Kioroglou *et al.*, 2020). In the study by Kioroglou *et al.* (2020), no cells were detected on AAB medium; the presence of cells at the VBNC stage could explain the difference in population levels between the cultivation and Q-PCR approaches.

The absence of SO_2 during vinification and ageing was associated with higher levels of acetic acid and ATHP. The presence of LAB, especially *Oenococcus oeni,* at high population levels can cause changes in wine quality and an increase in acetic acid content. This is related to the consumption of residual sugars by LAB, as indicated by the high production of D-lactic acid. In addition, the production of mousy-associated compounds, especially ATHP, is noticeable after six months of bottling. Only ATHP was found in wines of the present study at a widely detectable tasting level.

The production of mousy off-flavours by *Oenococcus oeni* in synthetic medium was previously reported by Moulis *et al.* (2023). In the present study, there is strong evidence that LAB and particularly *Oenococcus oeni* species could be at the origin of the mousy off-flavours in red wine. It would have been interesting to test the ability of strains with different genetic profiles highlighted after five months of ageing to produce mousy off-flavours, even if the production of ATPH seemed more related to the absence of SO_2 than to the nature of the genetic profile of *Oenococcus oeni*. Interestingly, Mod4 and 5 differed by only one sulphite addition (at bottling), and ATHP seemed to increase significantly in Mod5 but not in Mod4 after six months. One explanation could be that the formation of this compound by microorganisms could take place in the bottle.

CONCLUSION

The reduction of sulphites during winemaking is an area of current interest, and there is a need to provide winemakers with scientific knowledge. Using a culture-based approach, we demonstrate that the use of SO_2 and the timing of its addition do not affect yeast population diversity. Regarding lactic acid bacteria, however, the genus *Tatumella* was only detected in must without SO_2. We confirmed that *Saccharomyces cerevisiae* and *Oenococcus oeni* were the most prevalent species during wine ageing. After five months, *Oenococcus oeni* exhibited high genetic diversity, with random and unrelated genetic profiles present, independent of the profile observed after malolactic fermentation (MLF), the use of SO_2, or industrial lactic acid bacteria. High population levels of *Oenococcus oeni* were highlighted in the absence of SO_2, whether MLF was inoculated or not. In our study, this high population level of *Oenococcus oeni* was associated with the production of acetic acid, which correlated with the production of D-lactic acid, as well as undesirable aromatic compounds such as mousy off-flavours. For *Saccharomyces cerevisiae*, unlike LAB, the level of SO_2 had no significant effect on cultivable population levels. In our experimental conditions, microbial spoilage associated with the absence of SO_2 was linked to a high and stable population of *Oenococcus oeni* during the wine ageing process, whether through spontaneous or inoculated MLF. Adding SO_2 at the end of MLF inhibits the growth of *Oenococcus oeni*, but only temporarily. Therefore, winemakers could consider reducing the number of sulphite treatments, without eliminating them, to restrict the growth of LAB during ageing and preserve the quality of red wine.

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CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

SW: Conceptualisation, investigation, formal analysis, validation, visualisation, writing—original draft; JM, AVC, ML, CMS: Investigation; EPM: Conceptualisation; VD: Resources; SB: Resources, funding acquisition; EV: Resources, funding acquisition; PL: Conceptualisation; IMP: Conceptualisation, funding acquisition, project administration, supervision, writing review, and editing.

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