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Non-*Saccharomyces* yeasts as bioprotection in the composition of red wine and in the reduction of sulfur dioxide

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ABSTRACT

Non-*Saccharomyces* yeasts have been used for many years due to their technological potential, particularly as a "booster" of wine fruity aroma in mixed fermentations with *Saccharomyces cerevisiae*. Recently, a new application has emerged, bioprotection, which consists in colonizing the environment in the context of sulfite reduction in wines. The chemical and sensory impact of non-*Saccharomyces* yeast according to different modes of application in a context of fermentation without addition of SO_2 was evaluated through trial with Merlot N. (*Vitis vinifera* L.). An effective niche occupation by non-*Saccharomyces* yeasts was highlighted during the prefermentary stages by Quantitative-PCR and MALDI-TOF MS identification. Chemical analysis (GC-MS and GC MS/MS) of finish wine showed the significant impact of the dose applications, with bioprotection characterized by linear esters and sequential application by acetates of higher alcohol contents. Moreover, a separation according to the species used in bioprotection was revealed. Finally, using a panel trained, the sensory analysis confirmed that the use of non-*Saccharomyces* yeast was a fruity booster in sequential inoculation and, to a less extent, when used as bioprotection. This study shows for the first time that the use of non-*Saccharomyces* yeast as a bioprotection has a significant impact on the aromatic profile of wines.

1. Introduction

In recent years, many investigations have shown a particular interest in non-*Saccharomyces* yeasts due to their interesting biotechnological characteristics (Ciani et al., 2010; Contreras et al., 2014), with a focus on their organoleptic impact (Esteve-Zarzoso et al., 1998; Jolly et al., 2003). Due to their low ethanol tolerance, pure cultures of non-*Saccharomyces* yeast are not considered and are only applied as a mixed fermentation alongside *Saccharomyces cerevisiae,* either in co-inoculation, i.e., added at the same time as *Saccharomyces cerevisiae*, or in sequential inoculation. This latter application consists in inoculating fairly high population levels of non-*Saccharomyces* ($\sim 10^7$ cells. mL^{-1}) so that they can initiate alcoholic fermentation by producing molecules of interest over a given period of time. Then, when the degradation of sugars is initiated (generally after 24–72 h), *Saccharomyces cerevisiae* are subsequently added. The literature highlights a "booster" effect of this practice on the fruitiness of wine (Renault et al., 2015; Rodríguez et al., 2010; Zott et al., 2011). Different compounds can contribute to the fruitiness of red wines, such as esters (Lytra et al., 2017), volatile thiols (Bouchilloux et al., 1998), lactones to a lesser extent through aromas of cooked fruits (Ferreira et al., 2004; Pons et al., 2017), and furanones which play a positive role in the perception of red fruit notes (Ferreira et al., 2016; Kotseridis, 1999).

Recently, to meet current social demand, alternatives to sulfur dioxide have emerged, such as bioprotection through the addition of non-*Saccharomyces* yeasts. Bioprotection is defined as the use of antagonistic cultures that are added to inhibit pathogens and/or to extend the shelf life without negatively changing the sensory properties of products (Lücke, 2000). The control of fungal damage using antifungal microorganisms depends on the interactions between the food itself (including its natural microbiota) and the antifungal microorganisms. Bioprotection through competition for nutrients, by occupying the space of

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the ecosystem, by antifungal component synthesis impacts on the growth, bioactivity of pathogens and thenutrients bio-disponibility (Leyva Salas et al., 2017). Sulfur dioxide is indeed a source of controversy: consumers increasingly want to eat and drink "healthier" products that are designed with fewer and fewer chemical preservatives (Forbes et al., 2009; Pérès et al., 2018; Poveda et al., 2005). Furthermore, studies have shown that sulfur dioxide may have harmful effects on consumers: intolerance of sulfites (Timbo et al., 2004; Warner et al., 2000).

Among the non-*Saccharomyces* yeasts used as bioprotective agents or in a sequential application, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* species have been considered. The former, in association with *Saccharomyces cerevisiae* in sequential inoculation, has been shown to release esters and thus increase the intensity of the fruitiness of red wines (Benito, 2018; Renault et al., 2015). In addition, *Torulaspora delbrueckii* can release volatile thiols through its β-lyase activity (Azzolini et al., 2015; Belda et al., 2017; Renault et al., 2016; Zott et al., 2011). On the other hand, *Metschnikowia pulcherrima* produces higher concentrations of phenylethanol and 2-phenylacetate in association with *Saccharomyces cerevisiae* (Varela et al., 2016). Also, *Metschnikowia pulcherrima* has β-glucosidase activities, leading to the release of varietal aromas from the non-volatile precursors on grapes, such as terpenes or thiols (Fernández et al., 2000; Nguyen & Panon, 1998; Oro et al., 2014).

The sensory impact of non-*Saccharomyces* yeast as a bioprotection in oenology has been poorly studied to date. Simonin et al. (2020) showed noticeable but non-repeatable differences from one winery to another in Chardonnay with *Metschnikowia pulcherrima*. In practice, winemakers frequently report an increase in fruitiness in fermented red wines without sulfites with the use of non-*Saccharomyces* yeasts as a bioprotection. But this empirical observation has not been scientifically demonstrated.

In a context of strong recent industrial development of non-*Saccharomyces* yeast, the objective of this study is to evaluate the chemical and sensory impact of two commercial yeast products of non-*Saccharomyces* yeasts used for bioprotection. Two modes of application of the same product of non-*Saccharomyces* were tested: bioprotection and sequential inoculation, with *Saccharomyces cerevisiae* on harvested grapes as control. The experiment took place in a context of sulfite reduction in red wines, so the wines were not sulfited during the winemaking and wine aging process until bottling.

2. Materials and methods

2.1. Winemaking process and treatments

The trial was carried out with Merlot N (*Vitis vinifera* L.). grapes from vineyards located in the Pessac Léognan Appellation, Bordeaux, France, from the 2019 vintage. Clusters were separated into five batches according to the following treatments (Table 1): *Torulaspora delbrueckii/Metschnikowia pulcherrima* at 50 mg.L^{-1} on harvested grapes (ZE5, Zymaflore®Egide), *Torulaspora delbrueckii/Metschnikowia pulcherrima* at 300 mg.L^{-1} at the vatting stage (ZE30, Zymaflore®Egide), *Torulaspora delbrueckii* at 50 mg.L^{-1} on harvested grapes (Zα5, Zymaflore®Alpha), *Torulaspora delbrueckii* at 300 mg.L^{-1} at the vatting stage (Zα30, Zymaflore®alpha) and *Saccharomyces cerevisiae* on harvested grapes as control (Sc5, Zymaflore®XPure). Strains were used underan Active-Dry-Yeast (ADY) form and rehydration was done according to the manufacturer's protocol. The *Torulaspora delbrueckii* strain is the same in Zymaflore®Egide and Zymaflore®alpha. For the bioprotection treatments (ZE5/Zα5/Sc5), ADY was applied using sprinklers on the crop throughout application. For the sequential application (Zα30/ZE30), non-*Saccaharomyces* ADY were added directly after rehydration into the grape juice at the vatting stage (Fig. 1).

Table 1
Applications and species used according to treatment.

Modalities	Application	Species	ADY Products
ZE5	50 mg.L^{-1} on harvest (bioprotection application)	*Torulaspora delbrueckii/ Metschnikowia pulcherrima* (50/50)	Zymaflore ® Egide
ZE30	300 mg.L^{-1} at vatting stage (sequential application)	*Torulaspora delbrueckii/ Metschnikowia pulcherrima* (50/50)	Zymaflore ® Egide
Zα5	50 mg.L^{-1} on harvest	*Torulaspora delbrueckii*	Zymaflore ® alpha
Zα30	300 mg.L^{-1} at vatting stage (séquential application)	*Torulaspora delbrueckii*	Zymaflore ® alpha
Sc5	50 mg.L^{-1} on harvest (bioprotection application)	*Saccharomyces cerevisiae*	Zymaflore ® XPure

The grapes were crushed according to standard practice and divided in two 9 kgstainless steel tanks (10L) by modality. No sulfur dioxide was added to any of the treatments. Then the vats were moved to a room at 10 °C for prefermentary maceration for 48h. All modalities were inoculated at 200 mg.L^{-1} with the commercial ADY *Saccharomyces cerevisiae* Zymaflore®XPure, after 48h of maceration for the bioprotection modalities (ZE5/Zα5/Sc5) and after a loss of ten points of density according to the manufacturer's protocol for the sequential modalities (ZE30/Zα30). Zymaflore®XPure was selected by breeding process to produce low amount of H_2S, SO_2 and sulfur combining products and is recommended for red wine vinification. Finally, all treatments were sulfited at 40 mg.L^{-1} and filtered before bottling after malolactic fermentation.

2.2. Microbial analysis

During the prefermentary maceration, 10 mL of must was sampled in sterile conditions at different times, namely at vatting and after 48h of maceration, and were immediately processed.

2.2.1. Quantitative PCR

10 mL of must was centrifuged at 9000g for 10 min and the resulting cell pellet was rinsed twice with 2 mL 0.05 M EDTA pH8. The cells were lysed using a FastPrep-24 instrument (MP Biomedicals, Illkirch, France) for DNA extraction according to the protocol described by Zott et al. (2010). The DNA samples were conserved at −20 °C. The quantitative PCR (Q-PCR) method was implemented to monitor the population levels of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* communities using specific primers (Table S.1).

Samples for 20 μL reactions were prepared as described by Zott et al. (2010). For each sample, four amplifications were considered: DNA extract and DNA diluted per 10, both in duplicate.

2.2.2. MALDI-TOF MS

For each sample, 24 colonies (at vatting and after 48h of maceration) and 16 colonies (at mid alcoholic fermentation) were randomly isolated on the same dilution on the LT medium (20 g.L^{-1} glucose, 10 g.L^{-1} yeast extract, 10g.L^{-1} peptone, agar 20 g.L^{-1}, 0.1 mg mL^{-1} Chloramphenicol and 0.15 mg mL^{-1} Biphenyl) and then were subcloned on the YPG agar medium. Colonies were identified at species level using Matrix-assisted-Laser-Desorption-Ionization-Time-of-Flight Mass-Spectrometry (MALDI -TOF MS).

The manufacturer's protocol for the extended direct transfer method (eDT) was followed. The mass profiles were compared with the MBT Compass LibraryTM and with an additional database created in the laboratory (94 new strains isolated from must and wine) using the MBT Explorer Module (Bruker Daltonics).

2.3. Chemical analysis

2.3.1. Classical analysis

Grape must parameters at vatting stage and wine analyzed for each

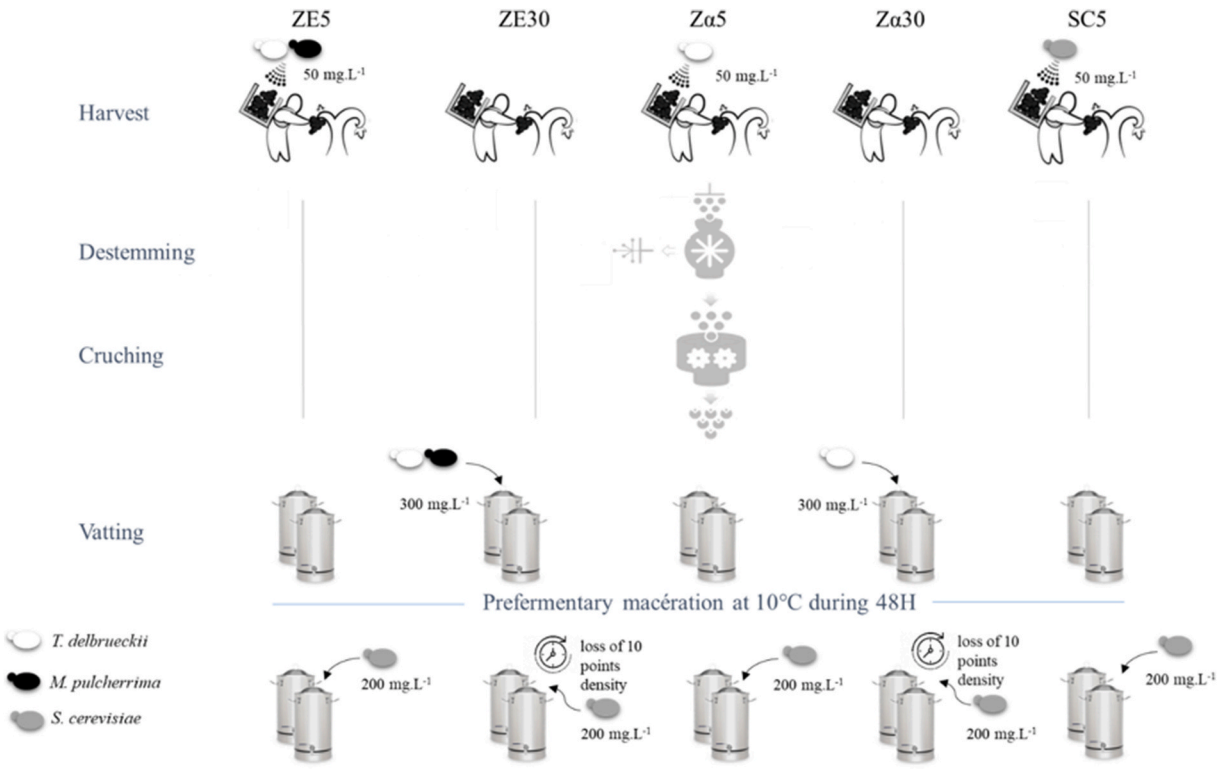

Fig. 1. Experimental design of the study.

modality were obtained with Enology Analyzer Y15 (BioSystems, Spain) and a WineScan™ Flex (Foss, Hillroed, Denmark) coupled to the Foss Integrator 2 software (version 2.0.2). The must analyses are presented in (Table S.2).

2.3.2. Ester analysis

The esters were assayed using solid-phase microextraction (SPME) according to the optimization protocol of Antalick et al. (2010). Briefly, 100 μm of polydimethylsiloxane film (PDMS-100) was used as the stationary phase to cover the fiber (Supelco, Bellefonte, PA). 10 mL of wine was placed in a 20 mL head-space vial supplemented with 3.5 g sodium chloride. 10 μL of a stock solution of internal standards (ethyl-d_5 butanoate, ethyl-d_5 hexanoate and ethyl-d_5 octanoate at about 200 $mg.L^{-1}$ each in absolute ethanol) was added. The vials were tightly sealed with a PTFE-lined cap. The samples were homogenized with a vortex shaker and then loaded on to a Gerstel (Mülheim an der Ruhr, Germany) auto sampling device. The program involved swirling the vial at 500 rpm at 40 °C for 2 min, after which the fiber was inserted into the headspace at 40 °C for 30 min as the solution was swirled again. Gas chromatography analyses were carried out on an HP 5890 GC system coupled to an HP 5972 quadrupole mass spectrometer (Hewlett-Packard), equipped with a Gerstel MPS2 autosampler. Injections were in splitless mode (injector temperature, 250 °C, interface temperature, 280 °C) and a BP21 capillary column (50 m × 0.32 mm, film thickness, 0.25 μm; SGE, Ringwook, Australia). The oven temperature was programmed at 40 °C for 5 min, then raised to 220 °C at 3 °C/min, and held at that temperature for 30 min. The mass spectrometer was operated in electron ionization mode at 70 eV in selected-ion-monitoring (SIM) mode.

2.3.3. Volatile thiols, lactones, furanones and oxidative compounds

The 3-Sulfanylhexanol (3SH), lactones (massoia lactone, γ-nonalactone, γ-decalactone, and δ–decalactone), furanones (Furaneol, Homofuraneol and Norfuraneol) and compounds associated with the characteristic flavors reminiscent of oxidation (Thibon et al., 2015) (methional, phenylacetaldehyde, and o-aminoacetophenone) were quantified in the wines by gas chromatography–tandem mass spectrometry (GC-MS/MS) adapted from Thibon et al. (2015). 50 μL of internal standard mix containing 6-sulfanylhexanol (6SH, 500 $\mu g.L^{-1}$, EtOH), 4-methoxy-2-methyl-2-sulfanylbutan (MMSB, 500 $\mu g.L^{-1}$), ethyl maltol (EM, 1 $mg.L^{-1}$, EtOH), and 3-octanol (1 $mg.L^{-1}$, EtOH) was added in 20 mL of wine sample. The sample was percolated through a conditioned SPE column (HR-X, 500 mg 6 mL, Macherey Nagel, France). Then, the SPE columns were rinsed twice with 2 mL of hydro-alcoholic solution (10%) before eluting with 3 mL of pentane/dichloromethane (50/50; v/v) followed by 3 mL of dichloromethane/methanol (95/5; v/v). The organic phases obtained were blended, dried over anhydrous sodium sulfate, and concentrated to 150 μL under a nitrogen stream.

The aroma compounds were analyzed using a gas chromatograph (SystèmeTrace GC Ultra (Thermo Electron SAS, Courtaboeuf, France)) equipped with a triple quadrupole mass spectrometer (TSQ Quantum XLS; Thermo Electron SAS, Courtaboeuf, France). Data acquisition and analysis were performed using the upgraded Xcalibur Workstation software (Version 4.1, Thermo Fisher Scientific, Brême, Allemagne) supplied by the manufacturer.

2.4. Sensory analysis

2.4.1. General conditions

The sensory analyses were performed as described by Martin and De Revel (2000). The samples were tasted in individual booths (NF EN ISO 8589:2010), using covered black ISO glasses containing about 20 mL of liquid (AFNOR (1977) Sensory analysis – Appartur - Wine tasting glass – ISO 3591. *Analyse Sensorielle*). All panelists were research laboratory staff at Institute of Vine & Wine Science, University of Bordeaux. The objective of the sensory analysis was to determine the impact of these different treatments on the fruity aroma and the potential appearance of aroma defects in the wine.

2.4.2. Discriminative tasting test

15 panelists (including 10 women) formed the panel for the

discriminative test with ages ranging from 24 to 38 years (28.7 ± 4.8, mean ± SD). Discriminative testing was performed triangle tests (NF ISO 4121: 2007) by direct olfaction only. Sc/Zα5, Sc/Zα30, Sc/ZE5, Sc/ZE30, Zα5/Zα30, Zα5/ZE5, ZE30/Zα30 and ZE5/ZE30 were the combinations used for the triangle tests (Table 4). In total, each judge had to carry out the sixteen triangular tests.

2.4.3. Descriptive test

2.4.3.1. Training. 17 judges (including 12 women) formed panel 2 with ages ranging from 23 to 40 years (28.0 ± 5.4, mean ± SD). The panelists received olfactory training on the descriptors linked to wine defects ("Oxidation", "Reduction", "Vegetal") and to two specific descriptors for the present study: "Fruitiness" and "Fermentary aroma". "Fruitiness" descriptors concerned aromas correlated with red and black-berry fruits while "Fermentary aromas" were characterized by candy and banana aromas. The products and concentrations used are presented in Table S.3. Participants had not been informed about the characteristics of the study. They had all provided informed written consent.

The training was carried out over three sessions based on protocol of Pelonnier-Magimel et al. (2020). The first session consisted in presenting the references of descriptors that the judges had to recognize blindly. During the second session, the judges had to classify the intensity of "Vegetal", "Fruitiness" and "Fermentary aromas" in the hydroalcoholic solution; each set was composed of four samples (one control and three with increasing concentrations). The concentrations were determined internally with expert tasters. The previously described protocol was also used in the last session; the only difference was that the sets consisted of three different wine matrix samples (one control and two with increasing concentrations).

2.4.3.2. Sensory profile. Before beginning the descriptive analysis, each taster had to smell the references of each descriptor: "Oxidation" (Escudero et al., 2000), "Reduction" (Ugliano et al., 2012), "Vegetal" (Roujou de Boubée et al., 2000), "Fermentary aroma" and "Fruitiness" (Lytra et al., 2014). The sensory profile was performed using black ISO glasses coded with three-digit numbers. The wines were tasted according to a semi-monadic presentation. The descriptors were evaluated one by one for all wines at olfactory level only. One tasting box was associated with one descriptor and the wines were presented with a different code with three-digit random numbers in all boxes. The tasting booths and the order of presentation of the samples were randomized in a Latin square arrangement. Sensory profiles were carried out using a continuous line scale (AFNOR, 2003). For each sample, the panelists rated the intensity of five descriptors on a 10 cm scale printed on paper, labeled "no odor perceived" on the left and "very intense" on the right.

3. Results

3.1. Microbiological analysis

During prefermentary maceration, two samplings were carried out at the vatting and end-of-maceration stages to quantify *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* populations by Q-PCR and to identify yeast species by MALDI-TOF-MS.

The population quantification of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* by Q-PCR confirmed the effective implantation of the two species in all modalities when added (Table 2). The indigenous populations of *M. pulcherrima* were on average 3.1×10^4 cells.mL^{-1} (Zα30/Zα5/Sc5), contrary to the indigenous populations of *Torulaspora delbrueckii* which were on average 5.5×10^2 cells.mL^{-1} at vatting (Sc5).

As expected, the initial population of *Torulaspora delbrueckii* was higher for the sequential inoculation than for the bioprotection modality and increased by one log after 48h in cold maceration, from 10^6 to 10^7 cells.mL^{-1} for Zα30 and ZE30, and from 10^5 to 10^6 cells.mL^{-1} for ZE5 and Zα5. The population levels of the indigenous population of *Metschnikowia pulcherrima* remained stable in bioprotection, whereas the population strongly decreased in sequential inoculation (from 10^7 cells.mL^{-1} to 10^4 cells.mL^{-1}).

A total of 480 colonies were analyzed by MALDI-TOF-MS at vatting and after 48h of prefermentary maceration (Fig. 2). Seven species were identified: *Issatchenkia terricola*, *Pichia kluyveri*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Starmerella bacillaris*, *Hanseniaspora uvarum* and *Saccharomyces cerevisiae*. Some colonies (0.83%) did not grow during transplantation ("No growing") and some colonies (1.6%) could not be identified ("No ID").

As expected in Zα5 and Zα30, *Torulaspora delbrueckii* was the main species at vatting (85.4% for both treatments) and at the end of the maceration (77%–95%, respectively).

In the ZE5 and ZE30 samples, *Torulaspora delbrueckii* represented 52% of the total colonies analyzed at the vatting stage and 77% and 95%, respectively, after 48h of maceration. *Metschnikowia pulcherrima* was at 39.5% and 29.2% at the first stage and then decreased to 16.7% and 20.9% after 48h, in the ZE5 and ZE30 samples, respectively.

With regard to the control treatment (Sc5), *Saccharomyces cerevisiae* represented only 39.6% and 25% out of the total amount of colonies analyzed at vatting and after 48h of maceration, respectively. *Hanseniaspora uvarum* was dominant in the Sc5 modality (43.8% then 56.3%) but its proportion did not exceed 17% in the other modalities.

At mid-alcoholic fermentation, a total of 160 colonies were identified by MALDI-TOF-MS in order to determine the implantation of *Saccharomyces cerevisiae* (Fig. S.1). *Saccharomyces cerevisiae* implantation was effective with the *S*c5 and ZE5 modalities (100% of isolates were *Saccharomyces cerevisiae*). *Torulaspora delbrueckii* was found at 37.5%

Table 2
Quantification of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* obtained by Q-PCR methods (cells.mL^{-1}) and total yeast counts obtained by culture-dependent method (UFC.mL^{-1}) at vatting and after 48h of maceration.

Stage	Treatment	*Torulaspora delbrueckii* (cells.mL^{-1})	*Metschnikowia pulcherrima* (cells.mL^{-1})	Total yeast (UFC.ml^{-1})
1.Vatting	Zα5	$3.35 \pm 0.77 \times 10^5$	$1.34 \pm 0.97 \times 10^4$	$1.17 \pm 0.29 \times 10^7$
	Zα30	$6.57 \pm 1.81 \times 10^6$	$2.84 \pm 1.59 \times 10^4$	$2.52 \pm 0.62 \times 10^7$
	ZE5	$1.39 \pm 0.46 \times 10^5$	$1.16 \pm 0.68 \times 10^6$	$7.38 \pm 2.57 \times 10^6$
	ZE30	$7.16 \pm 4.74 \times 10^5$	$3.20 \pm 1.73 \times 10^7$	$1.19 \pm 0.35 \times 10^7$
	Sc5	$5.51 \pm 2.30 \times 10^2$	$3.35 \pm 1.61 \times 10^4$	$4.03 \pm 1.35 \times 10^6$
2. 48h of maceration	Zα5	$1.69 \pm 0.47 \times 10^6$	$3.26 \pm 1.82 \times 10^4$	$2.44 \pm 0.97 \times 10^7$
	Zα30	$3.32 \pm 1.94 \times 10^7$	$4.81 \pm 2.20 \times 10^4$	$4.55 \pm 1.37 \times 10^7$
	ZE5	$5.22 \pm 3.64 \times 10^5$	$1.95 \pm 0.56 \times 10^7$	$1.18 \pm 0.17 \times 10^7$
	ZE30	$1.58 \pm 0.04 \times 10^7$	$4.05 \pm 2.00 \times 10^4$	$6.88 \pm 0.85 \times 10^7$
	Sc5	$1.28 \pm 0.27 \times 10^3$	$2.88 \pm 1.30 \times 10^4$	$3.30 \pm 2.58 \times 10^7$

Treatments: Control (Sc5), *Torulaspora delbrueckii* bioprotection application (Zα5) and sequential application (Zα30); mix of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* bioprotection application (ZE5) and sequential application (ZE30). Values indicated as the mean of four technical replicates and two biological replicates ± standard deviation.

Table 3
Analyses of wines of each modality (duplicate A and B).

	Zα5A	Zα5B	Zα30A	Zα30B	ZE5A	ZE5B	ZE30A	ZE30B	Sc5A	Sc5B
Alcohol By Volume (%vol)	14.6	14.6	14.5	14.6	14.7	14.6	14.5	14.7	14.6	14.7
Residual sugars (g/L)	0.48	0.77	2.50	2.18	0.38	0.42	0.66	1.09	0.39	0.36
Total acidity (tartaric acid g/L)	6.15	6.18	5.85	5.85	6.60	6.49	6.00	5.92	6.52	6.60
Volatile acidity (acetic acid g/L)	0.36	0.37	0.73	0.71	0.33	0.32	0.39	0.40	0.32	0.32
pH	3.26	3.25	3.34	3.32	3.31	3.29	3.29	3.30	3.29	3.30
Total SO_2 ($mg.L^{-1}$)	51	44	45	45	45	46	52	52	46	46
Anthocyanin (mg/L)	613	607	577	576	626	630	555	559	626	629

Treatments: Control (Sc5), *Torulaspora delbrueckii* bioprotection application (Zα5) and sequential application (Zα30); mix of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* bioprotection application (ZE5) and sequential application (ZE30).

Table 4
Olfactory discrimination of different applications of yeast during prefermentary maceration.

	Sc5	Zα5	Zα30	ZE5	ZE30
Sc5		***	***	***	***
Zα5	***		**	=	
Zα30	***	**		=	
ZE5	***	=			=
ZE30	***		=	=	

NB: ***, 0.001 significance level; ** 0.01 significance level; *, 0.05 significance level; = , no significant difference according to binomial distribution. Control (Sc5), *Torulaspora delbrueckii* bioprotection application (Zα5) and sequential application (Zα30); mix of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* bioprotection application (ZE5) and sequential application (ZE30).

(56.3% and 18.8%), 34.4% (31.3% and 37.5%) and, surprisingly, at 90.7% (93.8% and 87.5%) for Zα5, ZE30 and Zα30, respectively. The dominance of *Torulaspora delbrueckii* for Zα30 limited the implantation of *Saccharomyces cerevisiae.*

3.2. Chemical and sensory analysis

The chemical analyses of the grape juice for each modality are given in Table S2. No significant differences were noted in the enological parameters (sugars, total acidity, malic acid, pH, $NH4^+$, amino acids) of the different modalities. Fermentation kinetics were similar for the duplicates within each modality (data not shown). Alcoholic fermentation durations were eleven days for Sc5 and ZE5, twelve days for ZE30 and Zα5 and thirteen days for the ZE30 modality. The chemical analyses of the wines are presented in Table 3. The Zα30 treatment was characterized by a higher concentration of residual sugar (2.34 g/L ± 0.32) and of volatile acidity (0.72 acetic acid g/L ± 0.2). No significant differences were obtained for ABV, pH and total acidity. The dominance of *Torulaspora delbrueckii* at mid-fermentation for the ZE30 modality, to the detriment of *Saccharomyces cerevisiae,* led to sluggish alcoholic fermentation (Fig. S.1).

3.2.1. Volatile compound analysis

Two months after bottling, the volatile compounds of each wine were analyzed (Table S.4). Concerning the ester compounds, the use of *Torulaspora delbrueckii* (bioprotection or sequential inoculation) resulted in significantly higher ethyl propanoate concentrations (298 and 314 μg. L^{-1} for Zα5 and Zα30 respectively) compared to the control (Sc5 242 μg. L^{-1}). The sequential application was characterized by a higher concentration of 2-methylpropyle acetate, isoamyl acetate, phenylethyl acetate and, more particularly for Zα30, of ethyl 2 methylpropanoate (291 $\mu g.L^{-1}$) and hexyl acetate (2 $\mu g.L^{-1}$) compared to the Sc5 control and bioprotection modalities. Lower concentrations of ethyl octanoate and decanoate were also observed for the *Torulaspora delbrueckii* sequential application compared to the bioprotection and Sc5 control. However, the bioprotection application, irrespective of the non-*Saccharomyces* species considered, had significantly higher ethyl butanoate and ethyl hexanoate concentrations than the corresponding sequential inoculation modalities.

Concerning varietal aromas, volatile thiol 3SH was significantly higher in the Sc5 modality compared to bioprotection and the sequential application of non-*Saccharomyces* yeast. On the other hand, the wines from the sequential inoculations (Zα30/ZE30) contained higher concentrations of γ-nonalactone and γ-decalactone. Although significant, these differences were not relevant in terms of enology or contribution to organoleptic component (<10% of the total amount). No significant differences were highlighted for the furanones, except for norfuraneol with a higher concentration for the ZE5 modality. Concerning oxidative compounds, methional and phenylacetaldehyde were higher for ZE5 and Sc5, respectively.

A Principal Component Analysis (PCA) in the form of Biplots was

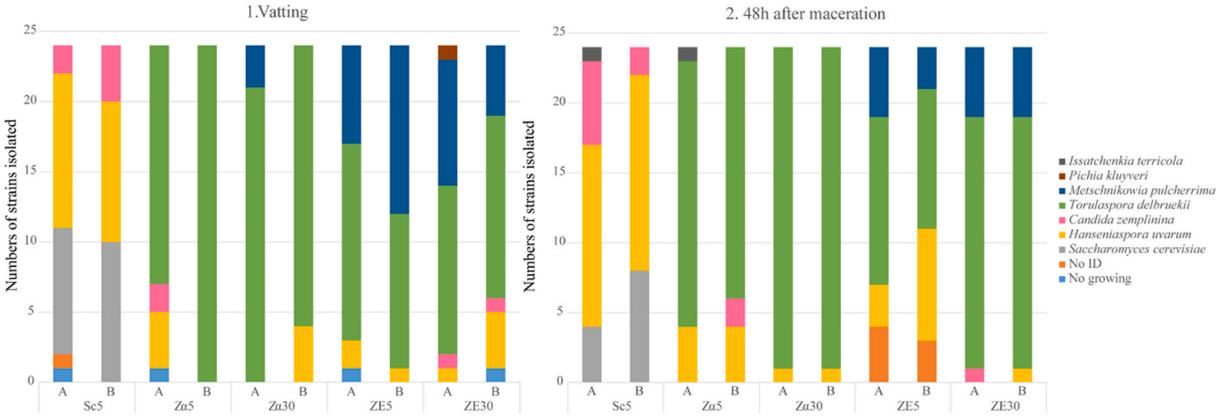

Fig. 2. Yeast identification at species level by MALDI-TOF MS during prefermentary maceration with sequential or bioprotection applications. Sequential (30) and bioprotection (5) applications with *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (ZE), *Torulaspora delbrueckii* (Zα) and *Saccharomyces cerevisiae* (Sc5). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

generated (Fig. 3). Dim 1 separated the application modalities with 63.1% of the variance explained: sequential applications (ZE30/Zα30) to the left of the axis and bioprotection applications (ZE5/Zα5/Sc5) to the right. The second dimension separates the non-*Saccharomyces* samples used as bioprotection (ZE5/Zα5) from the control (Sc5), with a variance explained at 15.8%. The sequential application was correlated to 2-methyl propyle acetate, γ-nonalactone and γ-decalactone content, the bioprotection application to ethyl butanoate, ethyl hexanoate and norfuraneol content, the control to ethyl 3-methylbutanoate, 3SH, ethyl 2-methylbutanoate and ethyl phenylacetate content. Concerning the sequential applications, ZE30 was correlated to propyl acetate and isoamyl acetate and Zα30 to ethyl-2 methylpropanoate and phenylethyl acetate.

3.2.2. Sensory analysis

A discriminatory olfactory test (triangle test) was used to distinguish

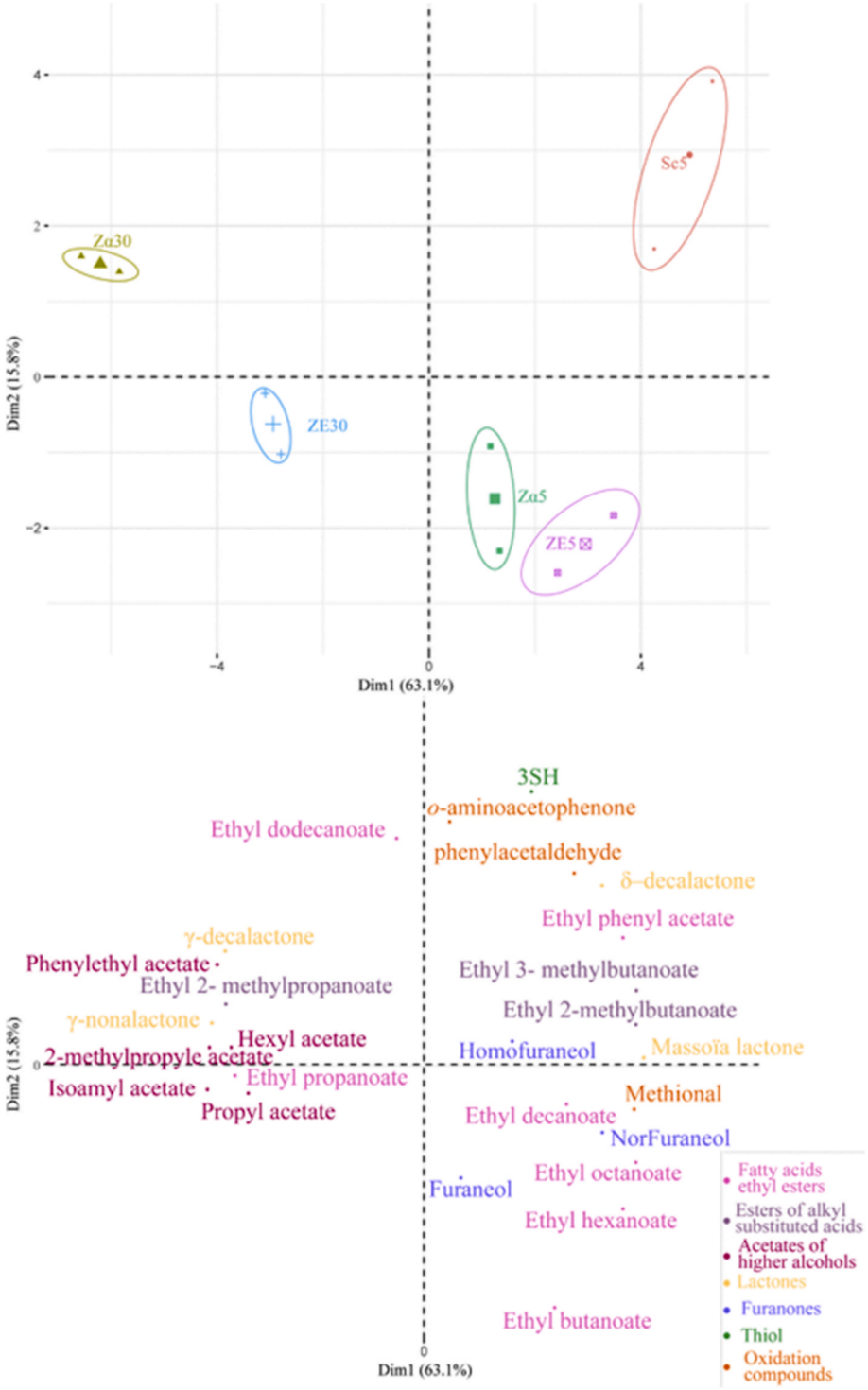

Fig. 3. Principal component analysis (PCA) based on the 26 volatile compound concentrations for sequential (30) and bioprotection (5) applications with *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (ZE), *Torulaspora delbrueckii* (Zα) and *Saccharomyces cerevisiae* (Sc5). Large signs of each treatment correspond to the means of duplicates. PCA were carried out using the Rstudio software (RStudio Team, 2020). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

between the different wines (Table 4). The control wine (Sc5) was significantly different from all other modalities ($p < 0.001$, Table 4). The use of *Torulaspora delbrueckii* either as bioprotection or sequential inoculation (Zα5/Zα30) was discriminated with a $p < 0.01$, contrary to the mix between *T.d* and *Metschnikowia pulcherrima* (ZE5/ZE30). The panel did not perceive any differences between the two applications of non-*Saccharomyces* as a bioprotection (ZE5/Zα5) or as sequential inoculations (ZE30/Zα30).

After a training session, the panel was asked to judge the wines on the basis of five olfactive descriptors: "Fermentary aromas", "Fruitiness", "Oxidation", "Vegetal" and "Reduction". These descriptors were chosen in order to determine the impact of the different applications of non-*Saccharomyces* yeasts on the fruitiness of the wines and on the possible off-flavors that might appear. The wines were not differentiated on the last three descriptors related to wine off-flavors, with scores that did not exceed 3.0 for the descriptors "Oxidation" "Vegetal" and "Reduction" (data not shown). The scores obtained for "Fermentary aromas" and "Fruitiness" are presented in Fig. 4. The control (Sc5) was significantly less intense than the Zα30 treatments for these two descriptors and the ZE30 treatment for "Fermentary aromas".

4. Discussion

Non-*Saccharomyces* yeasts have been used for many years in enology, in mixed fermentations with *S. cerevisiae* to enhance the fruity character and the complexity of wines (Renault et al., 2015; Roudil et al., 2020; Vilela, 2020; Whitener et al., 2017). Sequential inoculation has specifically been proposed; it consists in the early addition of non-*Saccharomyces* at a high population level (10^7 cells.mL^{-1}), followed by the addition of *Saccharomyces cerevisiae* after 24h–72h (at a lower population level (10^6 cells.mL^{-1})) (Renault et al., 2015; Rossouw & Bauer, 2016). Recently, a new application has emerged to meet societal demand for sulfite reduction: bioprotection, via the addition of non-*Saccharomyces* yeasts. This consist in adding yeasts at an early stage to grapes or must, at 50 mg.L^{-1} generally, in order to occupy the ecological niche and limit contaminants (Simonin et al., 2018). The use of non-*Saccharomyces* either as a bioprotection or for sequential inoculation differs greatly in terms of the rate and the time of inoculation. To date, bioprotection has mostly been studied as an alternative to sulfites, but scientific data on its impact on the chemical and sensory composition of wines are scarce. In a first study, Simonin et al. (2020) showed that bioprotection with *Metschnikowia pulcherrima* has no impact on the volatile compounds of red wines, although and specific sensory differences were perceived according to the winery. Rubio-Bretón et al. (2018) tested the use of a mix with two microorganisms, *Lactobacillus plantarum* and *Lachancea thermotolerans,* in bioprotection and showed that black fruits, raspberry and lactic aromas characterized the resulting wines.

The objective of this study was to test whether non-*Saccharomyces* yeast used as a bioprotection could impact the chemical and sensory composition of wine in comparison to sequential inoculation with *S. cerevisiae* in a context of fermentation without sulfites. The final aim was to provide scientific results to shed light on the debate on the different modes of application of non-*Saccharomyces* yeasts in enology.

4.1. *Torulaspora delbrueckii is the most efficient for colonizing the medium*

The Q-PCR and MALDI-TOF MS results confirmed the implantation of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, irrespective of the inoculation rate and time considered in agreement with Windholtz et al. (2021). Furthermore, Simonin et al. (2018) also confirms the implantation of *Torulaspora delbrueckii* in a bioprotection application in aligoté white must and *Metschnikowia pulcherrima* in pinot N. must (Simonin et al., 2020). The *Torulaspora delbrueckii* population increased and was dominant during maceration soaking at 10 °C, unlike *Metschnikowia pulcherrima,* whose population remained stable. Our results confirm those of a recent study by Berbegal et al. (2020), who showed that *Metschnikowia pulcherrima* alone developed from one log of UFC. mL^{-1}, but when associated with other species, its population level remained stable or decreased, unlike that of *Torulaspora delbrueckii*. Non-*Saccharomyces* is considered as bioprotection by occupying the must during the prefermentary stage and limiting undesirable microorganisms. As previously described (Windholtz et al., 2021), in the present study, *Torulaspora delbrueckii* limit the development of *H. uvarum*, that could produced unwanted metabolites such as acetic acid, ethyl acetate, sulfur compounds and biogenic acids (Ciani & Picciotti, 1995; Comi et al., 2001; Romano et al., 2003).

In this study, the use of *Saccharomyces cerevisiae* as a bioprotection did not demonstrate an effective implantation. Therefore, *Hanseniaspora uvarum* represented almost 50% of total colonies at vatting and after 48h of maceration. *Saccharomyces cerevisiae* has been reported to have negative interactions with *Hanseniaspora uvarum* (Wang et al., 2015). The temperature also impacts the growth of microorganisms, especially for *Hanseniaspora uvarum,* which grows better at low temperatures, generally around 15 °C (Albertin et al., 2014; Maturano et al., 2015, 2016). Other studies have also shown that it dominates the must in fermentation at 10 °C (Heard & Fleet, 1988) and in sequential culture

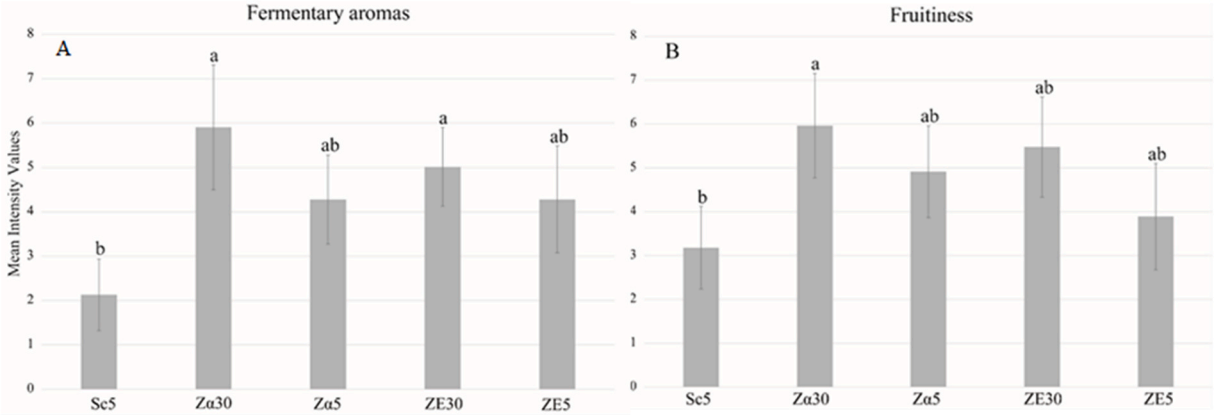

Fig. 4. Descriptive sensory analysis of red wines after blending of duplicates. Grades ranked from 0 (poorly intense) to 8 (very intense) for "Fermentary aromas" (Fig. 4A) and "Fruitiness" (Fig. 4B) descriptors. Sequential (30) and bioprotection (5) applications with *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (ZE), *Torulaspora delbrueckii* (Zα) and *Saccharomyces cerevisiae* (Sc5).
N.B.: The data were then analyzed by single-factor variance analysis (ANOVA, $p<0.05$) and the normal distribution of the residual data was verified (Shapiro-Wilks normality test, $p>0.05$). A Tukey HSD test was applied to classify the different modalities ($p<0.05$). a,b represent significantly different statistical groups.

with *Saccharomyces cerevisiae*; its maximum biomass has been obtained at 15 °C compared to 25 °C for *Saccharomyces cerevisiae* (Mendoza et al., 2019). Sulfur dioxide addition at vatting has been reported to secure the development of *Saccharomyces cerevisiae*. In our experimental conditions, the combination of no sulfites and low temperature could explain the low implantation of *Saccharomyces cerevisiae* during the prefermentary stages.

Another hypothesis that could explain the non-occupation of the niche by *Saccharomyces cerevisiae* is based upon the fact that as this species is applied to grapes, it does not survive on the grape berry surface, unlike other species. Consequently, the lower viable population in the must could explain the non-occupation of the niche by *Saccharomyces cerevisiae*.

4.2. Non-Saccharomyces yeasts, applied either as bioprotection or as sequential inoculation, affect wine sensorial and chemical composition

The comparative study of the impact of non-*Saccharomyces* species as a bioprotection or as sequential inoculation on the chemical and sensory composition of wine was carried out in a context of no added sulfites at vatting. In our experimental conditions, it was possible to obtain wines without off-flavors that could be analyzed for their volatile compound contents and judged on their fruity character.

In view of the chemical results, the wines could be differentiated according to the two applications of non-*Saccharomyces* as well as to the nature of the species used as a bioprotection (both species or *Torulaspora delbrueckii* or *Saccharomyces cerevisiae* only). The wines produced with non-*Saccharomyces* yeasts as a bioprotection were characterized by linear fatty acid ethyl esters predominantly, while with the sequential applications they were characterized by higher alcohol acetates. In the case of bioprotection, non-*Saccharomyces* yeasts could be supplanted by *Saccharomyces cerevisiae* at the end of maceration, thereby leading to the cell death of non-*Saccharomyces* and to the release of lipids in the grape juice. Thus, a pool of lipids may be available for *S. cerevisiae,* which could be used during alcoholic fermentation to produce the ethyl esters of fatty acids. The production of higher alcohol acetates in sequential applications could be explained by a synergistic effect between non-*Saccharomyces* and *Saccharomyces cerevisiae,* inducing an increased production of these compounds as has been previously shown in the literature (Andorrà et al., 2010; Ciani & Comitini, 2015; Ciani et al., 2010; Gobert et al., 2017; Lambrechts & Pretorius, 2000; Sadoudi et al., 2012; Whitener et al., 2017).

Contrary to *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* persists during alcoholic fermentation and probably strongly impacts the wine composition. The results of this study confirm previous works which have highlighted higher concentrations of ethyl 2-methylpropanoate and ethyl propanoate with the use of *Torulaspora delbrueckii* in sequential applications and a higher perception of "Fruitiness" and "Fermentary aromas" compared to the control (Chasseriaud, 2015; Loira et al., 2015; Renault et al., 2015). Taken alone, ethyl 2-methylpropanoate is characterized by a fruity smell of strawberry and kiwi. Pineau (2007) mentioned its contribution to the black and jammy fruit notes perceived in the aromas of red wines, as confirmed by the work of Lytra et al. (2014) who revealed an enhancing effect of this compound on the perception of fruitiness, highlighting its contribution to the black-berry-fruit descriptor. Ethyl propanoate is characterized by aromas of strawberry and blackberry with nuances of solvent, but contributes to the notes of blackberry and jammy-fruit through perceptive interactions (Pineau et al., 2009). Sequential applications are also correlated with higher concentrations of higher alcohol acetates (propyl acetate, isoamyl acetate and phenylethyl acetate). Even though they are characterized by heavier odors of solvent (which can mask the varietal aroma of wine), isoamyl acetates presents candy and banana notes whereas phenyl ethyl acetate shows floral ones (Pineau, 2007; Ribéreau-Gayon et al., 2017). These higher concentrations are in agreement with the present sensory analysis results, where the "Fermentary aromas" descriptor of ZE30 and Zα30 was significantly higher than the control. These compounds could also have an impact on the "Fruitiness" of these wines.

The 3SH content was significantly lower in sequential inoculation with non-*Saccharomyces* than in the control. This result does not agree with previous studies on white wines, which report a higher 3SH concentration in sequential inoculations than with pure cultures of *Saccharomyces cerevisiae* (Belda et al., 2017; Renault et al., 2016). Recently, Seguinot et al. (2020) showed a positive impact on the production of central carbon metabolites and aromas of *Metschnikowia pulcherrima* in sequential applications: the expression of varietal thiols increased strongly as a consequence of positive interactions with *Saccharomyces cerevisiae* and a greater incidence of lipids on the production of fatty acids and their ethyl ester derivatives. Moreover, Renault et al. (2016) highlighted that *Torulaspora delbrueckii* only assimilates the glutathionylated precursor to produce the cysteinylated forms that are then metabolized by *Saccharomyces cerevisiae*. Thus, in sequential inoculation, more cysteinylated precursors should be available and converted to 3SH by *Saccharomyces cerevisiae*. However, it is highly probable that this phenomenon depends on the strain, on the yeast implantation conditions or on the must composition, and more particularly on the cysteinylated/glutathionylated precursor ratio. Indeed, if the ratio is in favor of the cysteinylated precursor (not assimilated by *Torulaspora delbrueckii*), the effect of sequential inoculation should be lower.

Moreover, the phenyl acetaldehyde contents detected in the Sc5 control modalities suggest a higher oxidation during the vinification stage. This should be contradictory to a stabilization of thiols in wine (Culleré et al., 2007) (Nikolantonaki et al., 2014), but, in the PCA, the concentration of 3SH and phenyl acetaldehyde was correlated. Thus, one can assume that the oxidation would have taken place during the pre-fermentation stages due to the absence of sulfites. Unlike volatile thiols, the S-conjugated precursors contained in the must are not degraded during oxidation. According to (Roland et al., 2010), the presence of oxygen in musts could lead to a synthesis of certain conjugated forms of 3SH, which might explain the high 3SH content in the control modality.

Furthermore, yeasts are able to produce lactones by β-oxidation of fatty acids (Endrizzi et al., 1996; Lange & Garbe, 2000; Tosi et al., 2012). This release of lactones is dependent in part on the yeast species (Sadoudi et al., 2012), notably the impact of the *Torulaspora delbrueckii* species on γ-nonalactone and δ-decalactone concentrations (Azzolini et al., 2012). This may explain the differences observed in wines, here for γ-nonalactone and δ-decalactone, where the concentration was greater in sequential applications of non-*Saccharomyces*.

Bioprotection treatment was characterized by high levels of fatty acid ethyl esters (ethyl butanoate, ethyl hexanoate and ethyl octanoate). Through perceptive interactions, these esters are involved in the perception of red-berry-fruit and fresh-fruit aroma notes (Pineau et al., 2009) and thus might explain the perception of fruitiness in wines with bioprotection compared to the control, even though these differentiations were not significantly perceived in sensory analysis with a trained panel.

5. Conclusion

In a context of sulfite reduction in winemaking, the use of yeasts with bioprotective activity is presented as an alternative for winemakers with the aim of occupying the niche, limiting the development of spoilage microorganisms and preserving the sensory quality of products. In our experimental conditions, non-*Saccharomyces* yeasts, especially *Torulaspora delbrueckii* have been proven to be good candidates for colonizing the must and limiting wine defects. This study highlights for the first time that the time of inoculation and the dosage of non-*Saccharomyces* impact upon the chemical and sensory perception of red wines without sulfites. In comparison with the use of non-*Saccharomyces* in

sequential applications, which exacerbates the fruitiness of wine, their use in bioprotection has a significant impact on the aromatic profile of wines, with a tendency to be perceived as fruitier in sensory analysis. In a context of winemaking without sulfites, our results confirm the potential of non-*Saccharomyces* yeast used as a bioprotection to preserve and enhance the sensory quality of wines. In order to complete these results, it would have been interesting to evaluate the impact of *Metschnikowia pulcherrima* in red wine chemical and sensory analysis.

CRediT authorship contribution statement

Sara Windholtz: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. **Pascaline Redon:** Resources, Investigation. **Soizic Lacampagne:** Resources, Writing – review & editing. **Laura Farris:** Investigation. **Georgia Lytra:** Conceptualization, Methodology, Writing – review & editing. **Margaux Cameleyre:** Conceptualization, Methodology, Writing – review & editing. **Jean-Christophe Barbe:** Conceptualization, Writing – review & editing. **Cécile Thibon:** Supervision, Writing – review & editing. **Isabelle Masneuf-Pomarède:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111781.

Supplementary data captions

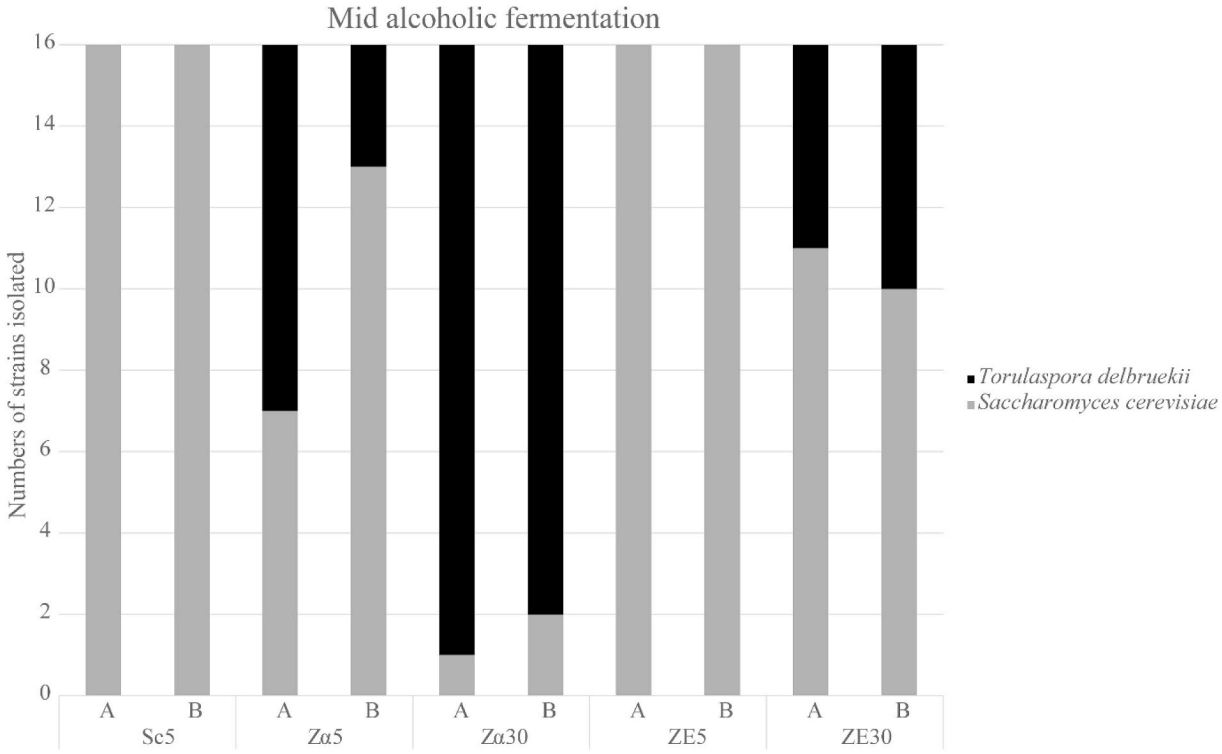

Figure S.1. Yeast identification at species level by MALDI-TOF MS at mid-alcoholic fermentation with sequential or bioprotection applications.

Table S.1
Primers used to quantify population levels of microorganisms by Q-PCR

Species	Primers	References
Metschnikowia pulcherrima	MP2-F AGACACTTAACTGGGCCAGC MP2-R GGGGTGGTGTGGAAGTAAGG	(García et al., 2017)
Torulaspora delbrueckii	TD-F CAAAGTCATCCAAGCCAGC TD-R TTCTCAAACAATCATGTTTGGTAG	Zott et al. (2010)
Hanseniaspora spp.	Hauf 2L — CCCTTTGCCTAAGGTACG Hauf 2R — CGCTGTTCTCGCTGTGATG	Zott et al. (2010)

Table S.2
Chemical analysis of the must for each modality (duplicates A and B).

	Zα5A	Zα5B	Zα30A	Zα30B	ZE5A	ZE5B	ZE30A	ZE30B	Sc5A	Sc5B
Sugars (g/L)	221	212	211	216	218	215	216	219	220	223
Total acidity (tartaric acid g/L)	4.57	4.34	4.54	4.56	4.42	4.36	4.38	4.29	4.56	4.41
Malic acid (g/L)	0.89	0.86	1.03	1.07	0.82	0.80	1.02	1.03	0.85	0.88
pH	3.28	3.28	3.31	3.31	3.26	3.27	3.33	3.37	3.29	3.3
NH_4^+ (mg/L)	117	113	106	108	115	110	108	106	112	109
Amino acids (mg/L)	103	100	106	109	94	91	108	109	98	99

Treatments: Control (Sc5), *Torulaspora delbrueckii* bioprotection application (Zα5) and sequential application (Zα30); mix of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* bioprotection application (ZE5) and sequential application (ZE30)

Table S.3
Attributes and aroma reference standard employed to sensory training.

Descriptors	References	Concentration (50 mL hydroalcoholic solution)			Concentration (50 mL Côte de Blaye wine)		
Oxidation	Oxidized wine				Control wine	Oxidized wine	
Reduction	Sodium hydrosulfide hydrate (Fisher Scientific, Hampton, USA)				0 µg. L^{-1}	2 µg.L^{-1}	
Vegetal	2-methoxy-3-isobutylpyrazine (Sigma-Aldrich, Australia)	5 ng.L^{-1}	15 ng. L^{-1}	30 ng. L^{-1}	0 ng. L^{-1}	5 ng.L^{-1}	15 ng. L^{-1}
Fruitiness	Hydroalcoholic (12 v/v) macerat of red and black fruits (2/3) and strawberry (1/3) (Picard)	20 mg. L^{-1}	40 mg. L^{-1}	60 mg. L^{-1}	0 mg. L^{-1}	40 mg.L^{-1}	60 mg. L^{-1}
Fermentary aromas	Ethyl propanoate 200 µg.L-1	50 µg.L^{-1}	100 µg. L^{-1}	200 µg. L^{-1}	0 µg. L^{-1}	100 µg.L^{-1}	200 µg. L^{-1}
	Ethyl butanoate 300 µg.L-1	75 µg.L^{-1}	150 µg. L^{-1}	300 µg. L^{-1}	0 µg. L^{-1}	150 µg.L^{-1}	300 µg. L^{-1}
	Ethyl hexanoate 300 µg.L-1	75 µg.L^{-1}	150 µg. L^{-1}	300 µg. L^{-1}	0 µg. L^{-1}	150 µg.L^{-1}	300 µg. L^{-1}
	Ethyl octanoate 300 µg.L-1	75 µg.L^{-1}	150 µg. L^{-1}	300 µg. L^{-1}	0 µg. L^{-1}	150 µg.L^{-1}	300 µg. L^{-1}
	2-methylpropyl acetate 70 µg.L-1	17.5 µg. L^{-1}	35 µg. L^{-1}	70 µg. L^{-1}	0 µg. L^{-1}	35 µg.L^{-1}	70 µg. L^{-1}
	Isoamyl acetate 300 µg.L-1	75 µg.L^{-1}	150 µg. L^{-1}	300 µg. L^{-1}	0 µg. L^{-1}	150 µg.L^{-1}	300 µg. L^{-1}
	Ethyl butanoate 300 µg.L-1	75 µg.L^{-1}	150 µg. L^{-1}	300 µg. L^{-1}	0 µg. L^{-1}	150 µg.L^{-1}	300 µg. L^{-1}

The empty boxes correspond to no addition of product

Table S.4
Concentration of volatile compounds in control (Sc), *Torulaspora delbrueckii* bioprotection application (Zα5) and sequential application (Zα30); mix of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* bioprotection application (ZE5) and sequential application (ZE30).

Compounds	Sc5	Zα5	Zα30	ZE5	ZE30
Fatty acids ethyl esters (µg.L-1)					
Ethyl propanoate	242 ± 12^{b}	298 ± 4^{a}	314 ± 6^{a}	248 ± 1^{b}	293 ± 8^{ab}
Ethyl butanoate	171 ± 9^{bc}	198 ± 11^{a}	147 ± 0.3^{c}	197 ± 8^{a}	179 ± 1^{b}
Ethyl hexanoate	543 ± 17^{ab}	557 ± 34^{a}	337 ± 3^{c}	597 ± 28^{a}	473 ± 5^{bc}
Ethyl octanoate	255 ± 2^{b}	250 ± 1^{b}	125 ± 14^{c}	278 ± 5^{a}	172 ± 22^{c}
Ethyl decanoate	69 ± 9^{a}	65 ± 8^{a}	23 ± 5^{b}	70 ± 12^{a}	31 ± 3^{ab}
Ethyl dodecanoate	12 ± 3^{a}	12 ± 3^{a}	10 ± 0.1^{a}	11 ± 2^{a}	9 ± 0.6^{a}
Ethyl phenylacetate	7 ± 0.3^{a}	4 ± 0.1^{c}	3 ± 0.1^{e}	5 ± 0^{b}	4 ± 0.3^{d}
esters of alkyl substituted acids (µg.L^{-1})					
Ethyl 2- methylpropanoate	124 ± 7^{c}	170 ± 6^{b}	291 ± 4^{a}	142 ± 7^{c}	170 ± 4^{b}
Ethyl 2-methylbutanoate	14 ± 0.8^{a}	13 ± 0.1^{b}	9 ± 0^{e}	12 ± 0.6^{c}	11 ± 0^{d}
Ethyl 3- methylbutanoate	28 ± 0.8^{a}	20 ± 0.7^{bc}	11 ± 0.3^{d}	20 ± 0.8^{ab}	13 ± 0.5^{cd}
Higher alcohols acetate (µg.L^{-1})					
Propyl acetate	9 ± 12^{c}	12 ± 16^{bc}	35 ± 1^{a}	21 ± 1^{bc}	29 ± 0.5^{ab}
2-methylpropyl acetate	27 ± 0.9^{d}	34 ± 0.2^{c}	55 ± 2^{a}	34 ± 0.3^{c}	41 ± 0.9^{b}
Isoamyl acetate	183 ± 13^{d}	223 ± 5^{b}	268 ± 13^{a}	198 ± 7^{c}	250 ± 10^{a}
Hexyl acetate	0.8 ± 0.1^{d}	1 ± 0.2^{ab}	2 ± 0.2^{a}	1 ± 0^{cd}	1 ± 0.1^{bc}
Phenylethyl acetate	26 ± 0.7^{cd}	26 ± 0.8^{bc}	40 ± 1^{a}	24 ± 1^{d}	35 ± 3^{ab}
Thiols (ng.L^{-1})					
3SH	1897 ± 450^{a}	898 ± 201^{b}	1048 ± 18^{ab}	743 ± 22^{bc}	612 ± 29^{c}
Lactones (µg.L^{-1})					
Massoïa lactone	25 ± 0.5^{a}	16.5 ± 0.7^{b}	5.58 ± 0.3^{b}	22.18 ± 1.09^{ab}	8.09 ± 0.19^{b}
γ-nonalactone	22.13 ± 0.08^{c}	22.54 ± 0.28^{bc}	24.19 ± 0.12^{a}	22.06 ± 0.52^{c}	23.93 ± 0.05^{ab}
γ-decalactone	2.29 ± 0.01^{b}	2.25 ± 0.28^{b}	5.82 ± 0.29^{a}	2.01 ± 0.09^{c}	3.37 ± 0.21^{a}
δ–decalactone	2.78 ± 0.24^{a}	1.72 ± 0.28^{ab}	1.45 ± 0.12^{b}	1.88 ± 0.35^{ab}	1.54 ± 0.07^{b}
Furanones (µg.L^{-1})					
Furaneol	4.4 ± 0.9^{a}	5.15 ± 0.7^{a}	3.9 ± 1.2^{a}	4.4 ± 0.2^{a}	4.72 ± 0.2^{a}

(*continued on next page*)

Table S.4 (*continued*)

Compounds	Sc5	Zα5	Zα30	ZE5	ZE30
Homofuraneol	25.1 ± 2.4[a]	27.5 ± 3.8[a]	21.3 ± 2.34[a]	22.5 ± 5.9[a]	18.8 ± 0.09[a]
Norfuraneol	15.07 ± 1.13[b]	15.48 ± 0.1[b]	11.59 ± 0.8[c]	17.26 ± 1.0[a]	9.74 ± 0.24[c]
Oxidative compounds ($\mu g.L^{-1}$)					
methional	2.96 ± 1.53[ab]	2.11 ± 0.06[ab]	1.80 ± 0.13[bc]	4.74 ± 0.43[a]	1.16 ± 0.38[c]
phenylacetaldehyde	33.07 ± 4.36[a]	17.44 ± 1.54[bc]	15.49 ± 0.66[c]	20.38 ± 3.42[b]	22.26 ± 1.08[ab]
o-aminoacetophenone	0.39 ± 0.01[a]	0.31 ± 0.04[a]	0.37 ± 0.05[a]	0.31 ± 0.078[a]	0.29 ± 0.002[a]

Data are average values of two biological replicates ± standard deviation [a,b,c,d,e] represent significantly different statistical groups (Kruskal-Wallis, $p < 0.05$)

References

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