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Population Dynamics and Yeast Diversity in Early Winemaking Stages without Sulfites Revealed by Three Complementary Approaches

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Abstract: Nowadays, the use of sulfur dioxide (SO_2) during the winemaking process is a controversial societal issue. In order to reduce its use, various alternatives are emerging, in particular bioprotection by adding yeasts, with different impacts on yeast microbiota in early winemaking stages. In this study, quantitative-PCR and metabarcoding high-throughput sequencing (HTS) were combined with MALDI-TOF-MS to monitor yeast population dynamic and diversity in the early stages of red winemaking process without sulfites and with bioprotection by *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* addition. By using standard procedures for yeast protein extraction and a laboratory-specific database of wine yeasts, identification at species level of 95% of the isolates was successfully achieved by MALDI-TOF-MS, thus confirming that it is a promising method for wine yeast identification. The different approaches confirmed the implantation and the niche occupation of bioprotection leading to the decrease of fungal communities (HTS) and *Hanseniaspora uvarum* cultivable population (MALDI-TOF MS). Yeast and fungi diversity was impacted by stage of maceration and, to a lesser extent, by bioprotection and SO_2, resulting in a modification of the nature and abundance of the operational taxonomic units (OTUs) diversity.

Keywords: MALDI-TOF MS; oenological laboratory specific database; winemaking without SO_2; bioprotection; metabarcoding HTS; quantitative-PCR

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1. Introduction

Yeast microbiota on the grape berry surface is the main source of the fermentative microbial community responsible for alcoholic fermentation and organoleptic quality of wine. Numerous studies have been carried out to characterize the yeast microbiota during the fermentation process. Different environmental factors (vintage, climate) [1] and technical parameters (temperature, carbon dioxide, inoculation with starters) can impact fungal diversity and population dynamics in grape must during the prefermentary stage [2–4] and alcoholic fermentation [5–7]. Previous studies reported the impact of sulfur dioxide addition on wine microbial diversity [1,8–10] and the yeast population dynamic during alcoholic fermentation [11–13]. More recently, the impact of bioprotection, as an alternative to sulfites, on the microbial characteristics of wines has also been considered [14,15].

Different methods based on culture-independent approaches are available to study yeast microbiota from grapes to wine, such as quantitative-PCR (Q-PCR) and high-throughput

sequencing (HTS). The Q-PCR method allows the population dynamics of targeted microorganisms to be analyzed, and was applied to characterize the must and wine microbial community [6,7], and to study the impact of non-*Saccharomyces* on wine quality [16] or the effect of oenological practices on grape must microbial populations [17].

High-throughput sequencing (HTS) methods allow relative abundance and biodiversity indices to be calculated from the sequences obtained. The HTS method has already been developed in oenology [18] using the 454 pyrosequencing method to study the microbial ecology of grape berries or in wine [19,20]. However, this latter technology is no longer used and has now been surpassed by Illumina [21].

Traditionally, sequencing analysis of the internal transcribed spacer and 26S rDNA was used for yeast colonies identification at species level. For many years, matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has replaced phenotypic or genetic sequencing identification techniques in the medical environment to become a routine analysis technique [22,23]. It is a fast, simple, accurate, and cost-efficient tool for the identification of microorganisms [24]. Furthermore, beverage sectors such as the beer industry have implemented this method for the identification of spoilage microorganisms or fermentative yeasts [25–27]. Cells are co-crystallized with the matrix in a way that yields a sufficient number of medium-sized ions in the mass spectra. The identification of microbiological samples by this method relies on the acquisition of mass fingerprints and subsequent comparison with a Biotyper database.

In oenology, several studies have aimed to optimize protein extraction protocols, improve yeast identification by creating specific wine microorganism databases [28], or obtain finer identification by the extraction of high mass range moieties [29]. MALDI-TOF MS Biotyper has been used to differentiate oenological yeast at the genus or species level, such as *Saccharomyces* [30], to identify different groups of *Saccharomyces cerevisiae* [31,32], and to describe the grape berry microbiota [33]. However, it has been applied little until now for describing the yeast diversity of a must or wine-related environment, and some wine yeast species have not yet been identified due to their absence from the Biotyper database [34].

Today, societal demand tends to reduce chemical inputs in the food industry, with no exception in oenology. Indeed, sulfur dioxide (SO_2) is particularly targeted as the most used chemical input for winemaking. Microbiological alternatives have emerged, such as "bioprotection". The term "bioprotection" refers to the use of microorganisms or their metabolites to inhibit or even eliminate unwanted microorganisms in foods in order to guarantee hygienic qualities of the products and thus increase their shelf-life without altering their sensory properties [35,36]. In oenology, bioprotection is particularly used during prefermentary phase and till now, only pure culture of non-*Saccharomyces* species was considered [14,15].

The aim of this study was to evaluate a mix of two non-*Saccharomyces* yeasts as bioprotection (*Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) during the prefermentary stages without sulfur dioxide addition at the industrial scale. The MALDI-TOF MS Biotyper analysis was used to study the yeast community diversity during the prefermentary stages and to monitor the implantation of both non-*Saccharomyces* yeasts used as bioprotection. In a first step, the existing Biotyper database was extended to a laboratory-specific database made with 17 additional new species specific to the wine environment (in total, 43 yeast strains). In parallel to MALDI-TOF MS, two complementary approaches were applied: Q-PCR to monitor population levels of targeted species, and HTS metabarcoding to analyze fungi diversity.

2. Materials and Methods

2.1. Yeast Isolation Procedure

Yeasts growing were assessed using a specific YPG-based medium (10 g/L Yeast extract, 10 g/L Peptone, 20 g/L Glucose, and 25 g/L agar, pH adjusted to 4.8 with orthophosphoric acid) named LT (supplemented with 0.15 g/L biphenyl (Fluka, Paris, France)

and 0.1 g/L chloramphenicol (Sigma Aldrich, Saint-Quentin Fallavier, France)) to inhibit mold development and bacterial growth, respectively. Samples were spread at tenfold serial dilution in triplicate and incubated under aerobic conditions at 26 °C for 5 days. Plates containing between 30 and 300 colonies were counted, and colony-forming units (CFU) per mL were recorded and type of colony enumerated. For samples at prefermentary stages and start of alcoholic fermentation, around 30 colonies were picked according to the proportion of each type of colony and plated onto fresh LT plates.

2.2. MALDI-TOF MS

2.2.1. Validation of Yeast Identification by MALDI-TOF MS Biotyper

Biological material from a freshly-grown single colony was used in parallel for identification at species level (i) by sequencing 26S rDNA using NL1-NL4 primers for amplification [37] and (ii) by MALDI-TOF MS Biotyper. For MALDI-TOF MS analysis, a fresh colony was spotted onto an MSP 96 target polished steel BC (Bruker, Karlsruhe, Germany) and allowed to dry at room temperature. The spot of each colony was overlaid with 1 µL 70% formic acid and dried at room temperature. All the samples were overlaid with α-cyano-4-hydroxycinamic acid (HCCA) (1 µL) matrix (Bruker, Germany) for crystallization. MALDI-TOF MS analysis was performed on a Microflex™ LT/SH MALDI-MS System (Bruker Daltonics, Bremen, Germany) using Flex Control (Version 3.1), MTB Compass (Version 3.1) (Bruker Daltonics, Bremen, Germany), and MALDI-Biotyper™ application (Bruker Daltonics, Bremen, Germany), which allows the similarity of the mass profile of an unknown microorganism to be calculated with the mass profiles in a database. To calibrate the mass spectral data generated by the instrument, the Bruker bacterial test standard (BTS) (Bruker, Germany) was added to each plate as a control. The identification of microbiological samples by this method relies on the acquisition of mass fingerprints and subsequent comparison of the data with the Biotyper database. The spectra were analyzed in an m/z of 2 to 20 kDa [38]. Results of the pattern-matching process were expressed as proposed by the manufacturer, with scores ranging from 0 to 3. Scores >2.3 indicated highly probable species identification, score values between 1.7 and 2.0 generally indicated relationships at genus level, and a score <1.7 indicated that the identification was not reliable [39,40].

2.2.2. MALDI-TOF MS Oenological Laboratory Specific Database (OLS-DB)

The yeast strains chosen to enhance the new laboratory specific database, provided by CRB Oeno (centre de Ressources Biologiques, Unité de Recherche Oenologie, Villenave d'Ornon, France), are listed in Appendix A Table A1. These strains were previously identified by sequencing of 26S rDNA using NL1-NL4 primers [37] (Table A1). An oenological microorganism mass profiles database was created with the MTB Compass Explorer Module (Version 4.1) and Flex Analysis (Version 3.4) (Bruker Daltonics, Bremen, Germany) as follows. A complete extraction was carried out for each strain added to the database. The strains were grown and subcloned on YPG medium. For each isolate, yeast protein extraction was carried out in duplicate in order to generate the reference MALDI-TOF MS spectra for a given strain.

For yeast protein extraction, one fresh colony of each previously-purified isolate was transferred into an Eppendorf tube containing 300 µL of High Performance Liquid Chromatography (HPLC) quality water (VWR Prolabo, Fontenay-sous-bois, France), and a cloudy suspension was obtained after stirring. A total of 900 µL of absolute ethanol (VWR Prolabo, Fontenay-sous-bois, France) was added and then centrifuged at 13,000–15,000 rpm for 2 min. The supernatant was removed, and the resulting pellet was allowed to air dry at room temperature for 5 min. A total of 25 µL of 70% formic acid was added and mixed using a pipette until the pellet was completely dissolved, then 25 µL of 100% acetonitrile (VWR Prolabo, Fontenay-sous-bois, France) was added and all vortexed. Finally, the mixture was centrifuged for 2 min at 13,000–15,000 rpm and 1 µL of the supernatant was deposited onto an MSP 96 target polished steel BC (Bruker, Germany) and allowed to dry at room temperature. All the samples were overlaid with HCCA (1 µL) matrix for crystallization.

For a given strain, four separate deposits from each of the two protein extractions were distributed on the plate. These eight deposits allowed 24 MALDI-TOF MS spectra to be obtained per strain. A baseline check was then performed for each of the 24 spectra, which were compared in pairs: this allowed the homogeneity of the spectra for a given strain to be assessed. The 70 most intense peaks of all spectra of each strain were listed, taking their frequency of occurrence into account.

2.3. Red Wine Vinification Process and Sampling

The trial was carried out in 2018 with Merlot N. (*Vitis vinifera* L.) grapes from vineyards located in the Pomerol region of Bordeaux, France. Grapes were harvested manually from the same plot in small crates, at optimal ripening stage and sanitary status. Clusters were separated into three batches according to the following treatments: bioprotection (BP), SO_2 at 50 mg/L, and without SO_2 (0). SO_2 was added at vatting in the form of potassium metabisulfite (KMS). Bioprotection was composed of a mixture of two species, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (Zymaflore ® Egide–Laffort, Floirac, France), and was applied directly to the grapes at 50 m/L following the manufacturer's protocol and without addition of SO_2. In the winery, the harvest was crushed according to standard practice and distributed between new 225 L French oak barrels. Prefermentary maceration was carried out at 13 °C before inoculation (200 mg/L) with a commercial active dry yeast (ADY) *Saccharomyces cerevisiae* after 48 h. Each treatment was duplicated. During the prefermentary maceration, 10 mL of must were sampled in sterile conditions at different stages for each barrel: vatting, 24 h of maceration, 48 h of maceration, and start of alcoholic fermentation. Samples were transported to the laboratory immediately on ice for processing.

2.4. Yeast and Fungi Community Analysis

2.4.1. DNA Extraction

The cells were collected from samples after centrifuging at 9000 rpm during 10 min and were rinsed twice with EDTA 50 mM before being frozen and conserved at −20 °C until subsequent DNA extraction. For DNA extraction, the protocol was followed according to Zott et al. (2010). DNAs were conserved at −20 °C.

2.4.2. Population Dynamics of Targeted Microorganisms by Quantitative PCR

The Q-PCR method was chosen to monitor the population levels of different species and target communities using specific primers (Table A2): *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, and *Hanseniaspora* sp.

The Q-PCR program was one 5-min cycle at 95 °C, followed by 40 cycles at 95 °C for 10 s, 60–63 °C (differed according to primer pairs) for 30 s, and 72 °C for 30 s, completed by the post-PCR. To obtain the melting temperature, the temperature was increased by 0.3 °C every 10 s from 63 °C to 95 °C for each specific Q-PCR. 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. Standard curves were built for each yeast species in triplicate, using DNA extracted from 10-fold serial dilutions of fresh cultures in pasteurized red must.

2.4.3. Yeast Biodiversity Analysis

- Meta-barcoding and high-throughput sequencing analysis (HTS)

HTS analysis targeting rDNA 18S (fungal) was applied to all samples. DNA libraries for fungi were prepared according to the following protocol: a 350 base (on average) 18S rDNA gene fragment was amplified from each DNA sample with the universal primers FR1 (Amplicon PCR Reverse Primer **overhang adapter** = 5′**GTCTCGTGGGCTCGGAGATGT GTATAAGAGACA**ANCCATTCAATC GGTANT) and FF390 (Amplicon PCR Forward Primer **overhang adapter** = 5′**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCG** ATAACGAACGAGACCT [19]. This first PCR targeting regions with specific primers

including universal sequence primers (amplicon PCR primers) was performed in the laboratory. PCR reactions consisted of 2.5 µL of dilute template (DNAs standardized to 5 ng/µL), 5 µL of each Amplicon PCR Primer 1 µM, 12.5 µL 2X KAPA HiFi HotStart Ready Mix (Roche, Basel, Switzerland). Reactions were cycled for 3 min at 95 °C, then for 35 cycles of 98 °C for 30 s, 52 °C for 30 s, and 72 °C for 60 s, then followed by a final extension period of 8 min at 72 °C.

The second PCR consisted of attaching indices and Illumina sequencing adapters using the Nextera®XT Index Kit (Juno Beach, FL, USA), made by Plateforme Genome-Transcriptome in Bordeaux. Finally, normalized pool libraries for the Illumina paired-end library were prepared, and cluster generation and 2 × 250 bp paired-end sequencing (MiSeq Kit NANO v2) were performed on an Illumina MiSeq instrument.

Data were subsequently imported into the Find Rapidly OTUs with Galaxy Solution (FROGS) Pipeline [41]. The sequences were cleaned as follows. Preprocess: paired-end assembled, with 5′ primer, with 3′ primer, with expected length (<300 and >400 bps), and without N. Then, sequences were dereplicated before being clustered using SWARM [42] with a local clustering threshold with a distance of 3. This single-link method is robust and independent of the sequence at which it is begun. Chimeras were removed with vsearch [43]: chimeras are sequences formed from two or more biological sequences joined together. The resulting sequences were filtered to remove singletons, using Filter phiX (contaminant databank). Taxonomic assignment of operational taxonomic units (OTUs) corresponding to 18S rDNA sequences was performed using silva132 18S [44] as the reference database. Sequences were filtered on BLAST with a percentage of identity (97%) and percentage of coverage (95%). An affiliation postprocess allowed inclusive amplicon ambiguities to be resolved and OTUs aggregated based on alignment metrics. Finally, OTUs corresponding to *Vitis* sp. were removed.

- Yeast diversity analysis by MALDI-TOF MS

Total yeast population was quantified by the plating method on LT medium. The identification of 30 clones per sample was performed at species level following the manufacturer's protocol (Bruker, Germany) as previously described and using the oenological laboratory specific database.

2.5. Statistical Analysis

The α-diversity was calculated by using R package phyloseq [45] from the OTU matrix generated by FROGS as input [41]. "Observed" concerns the number of OTUs; "Chao1" estimate the number of unobserved species from those observed one or two times. Shannon and Inverse Simpson are quantitative index; it takes into account the abundance of each OTUs.

The data were assumed to be normally distributed (Shapiro–Wilks normality test, $p > 0.05$) and the variance homogeneity was verified (Leven test, $p > 0.05$). 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, $p > 0.05$). All tests were carried out using the R Studio program.

3. Results

3.1. Optimization of Wine Yeast Identification by MALDI-TOF MS

3.1.1. Comparative Analysis of Species Identification by MALDI-TOF MS Biotyper Database and 26S rDNA Sequencing

In a first study, 623 yeast clones isolated from the prefermentary stages and beginning of alcoholic fermentation were first identified using 26S rDNA sequencing. All the yeast isolates were identified successfully by sequencing at species level, resulting in 17 yeast species among traditional oenological ones (Figure 1). A majority of the yeast isolates (66%) were identified correctly at species level (score > 2) by the MALDI-TOF MS with Biotyper database and according to the 26S rDNA sequencing identification, whereas 6% were identified only at genus level (score between 1.8–2). Finally, 28% of yeast isolates were not

identified (176 clones out of 623) using the MALDI-TOF MS Biotyper database, probably because the colony extraction and/or identification scores were not correct. Although the *S. cerevisiae* species is well represented in the Biotyper database (13 strains), only 54% of isolates identified by 26S rDNA sequencing (191/353 clones) were correctly identified using the MALDI-TOF MS Biotyper database (score >2) (Figure 1). For the 162 *S. cerevisiae* isolates not correctly identified by the MALDI-TOF MS Biotyper database, the scores were lower than 1.8 (134 isolates), probably due to the non-wine origin of the *S. cerevisiae* in the Biotyper database. For *H. uvarum*, representing 20% of the isolates by 26S rDNA sequencing, 90% of total *H. uvarum* isolates were identified correctly by the MALDI-TOF MS Biotyper database that contains eight strains.

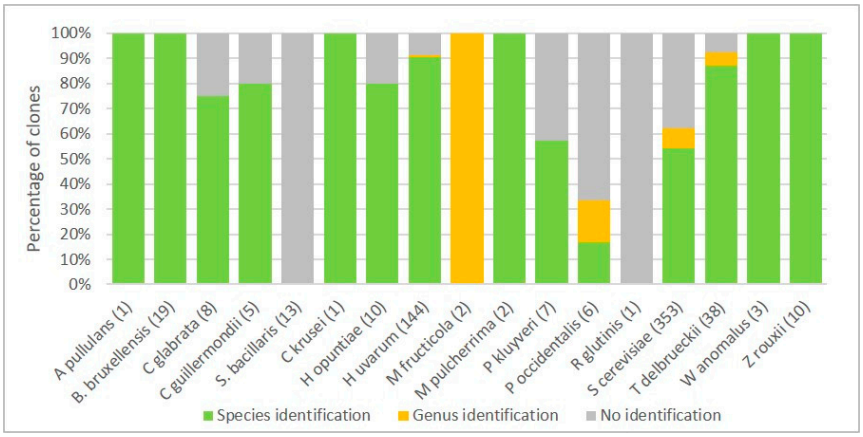


Figure 1. Percentage of clones given identical identification by 26S rDNA sequencing and MALDI-TOF MS (the number of clones considered is given in brackets).

3.1.2. Oenological Laboratory Specific Database of MALDI-TOF MS

The oenological laboratory specific Biotyper database was created with yeast species and strains specific to the wine environment. Yeast strains originating from must and wine were provided by CRB OENO. For all isolates, species identification was validated by 26S rDNA sequencing, and the sequence and match information on BLAST are presented as Table A1. To implement the database, different situations were considered: (i) when strains of a given species were present in the Bruker database and its identification result was <2.0, only one or two strains isolated from the wine environment were approved in the database (as for example for *Hanseniaspora uvarum* or *Lachancea thermotolerans*), (ii) when species were not present in the database (*Shizosaccharomyces japonicus* or *Starmerella bacillaris),* only one or two strains were added, leading to an identification score >2.

For *Saccharomyces cerevisiae,* the scores with wine isolates were not correct (<2.0), and five additional *S. cerevisiae* strains originating from a wine environment were therefore added, and the resulting identification scores were found to be greater than 2 using these two databases. For *Pichia guilliermondii*, 14 strains were already present in the Biotyper database, while the identification results were >2.0 for wine isolates; only one strain of *Pichia guilliermondii* related to the wine environment was added. Considering the genetic and phenotypic diversity of *Brettanomyces bruxellensis* [46], 15 strains representative of the genetic groups of the species were added. Finally, 17 distinct species and 43 different strains were added to the existing database (Table 1).

Table 1. Numbers of strains per species in the Biotyper database (Biotyper DB) and the oenological laboratory specific database (OLS DB).

Genus	Species	Number of Strains in Biotyper DB	Number of Strains in OLS DB
Aureobasidium	*pullulans*	3	2
Brettanomyces	*acidodurans*	0	1
Brettanomyces	*bruxellensis*	5	15
Candida	*cantarelli*	0	2
Starmerella	*bacillaris*	0	2
Hanseniaspora	*uvarum*	8	2
Lanchancea	*thermotolerans*	3	1
Metschnikowia	*pulcherima*	4	2
Pichia (Candida)	*guiliermondii*	14	1
Pichia	*kluyveri*	1	1
Pichia (Candida)	*membranifaciens*	2	1
Saccharomyces	*cerevisiae*	13	5
Shizosaccharomyces	*japonicus*	0	1
Shizosaccharomyces	*octosporus*	0	1
Shizosaccharomyces	*pombe*	4	3
Torulaspora	*delbrueckii*	5	2
Zygosaccharomyces	*bailii*	3	1

3.2. Yeast and Fungi Community during the Early Stages of Winemaking without Sulfites

Merlot N. (*Vitis vinifera* L.) grapes collected in 2018 were separated into three batches according to the following treatments: bioprotection (BP), SO_2 at 50 mg/L, and without SO_2 (0). Grape must was collected at four different stages (vatting, 24 h, 48 h, beginning of alcoholic fermentation), thus resulting in 18 samples for further analysis. Chemical analysis of the Merlot grape must and wine at the end of the alcoholic fermentation are given in Table A3; no significant differences from one modality to another were noticed for grape must enological parameters, except for the total SO_2 that was logically higher for the SO_2 modality comparing with bioprotection and without SO_2 modalities. The wine analysis showed no significant difference concerning the acetic acid content except for the residual sugars for the without sulfites modality (Table A3).

3.2.1. Population Dynamics of *Hanseniaspora* spp. and Non-*Saccharomyces* Yeasts Used as Bioprotection

Three yeast species and genera (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, and *Hanseniaspora* spp.) were targeted to monitor population dynamics using Q-PCR. Results are given in Figure 2 and Table A4. Population levels of *Hanseniaspora* sp. were relatively stable during the prefermentary stages, ranging from 2.8×10^2 to 1.2×10^4 cells/mL, whatever the modality considered, and then increased during the start of the alcoholic fermentation reaching 1.4–1.2×10^6 cells/mL. Levels of indigenous populations of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (with SO_2 and without SO_2 treatment) were low and below the detection limit of Q-PCR (<100 cells/mL), except for duplicates at the vatting stage without SO_2 (*Metschnikowia pulcherrima* at 3.9×10^2 cells/mL and 5.8×10^2 cells/mL, respectively) and the start of alcoholic fermentation. Population levels of both species inoculated as the bioprotection treatment at the vatting stage confirmed the effective implantation of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*, with averages of 5.7×10^4 and 4.4×10^6 cells/mL, respectively. *Torulaspora delbrueckii* population levels were relatively stable during the prefermentary stages, whereas the *Metschnikowia pulcherrima* population decreased to 3.4×10^4 cells/mL on average after 48 h of maceration and then increased to 1.2×10^6 at the start of alcoholic fermentation.

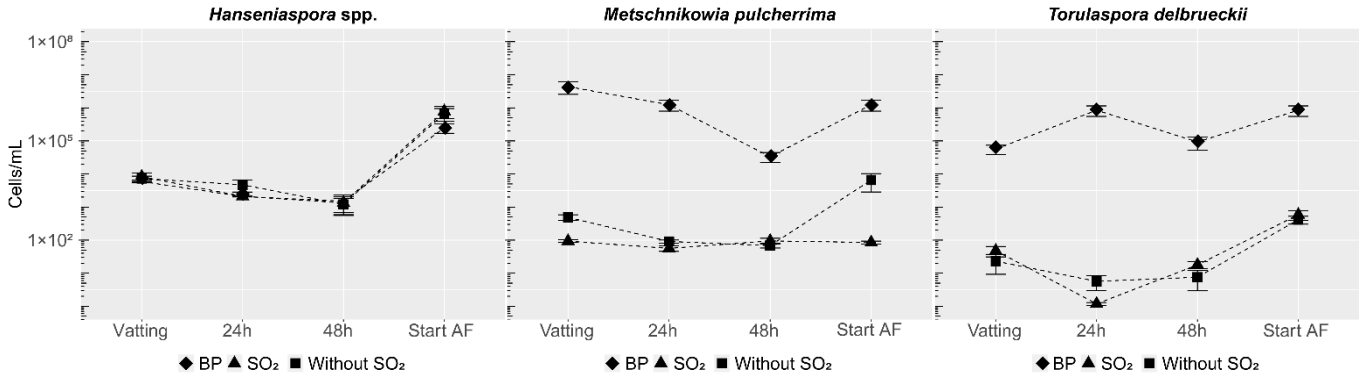


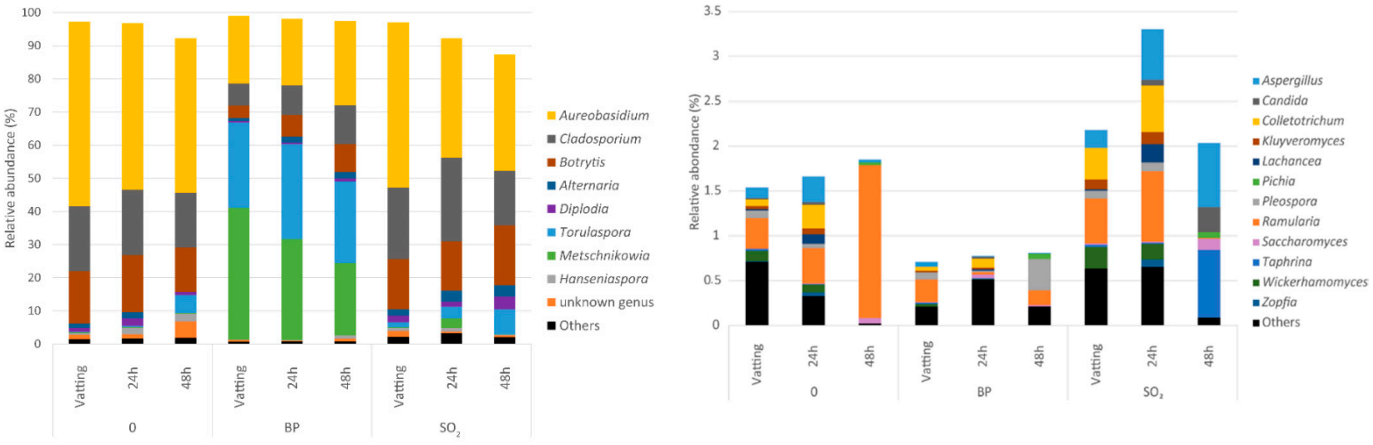
Figure 2. Population dynamics of *Hanseniaspora* spp., *Metschnikowia pulcherrima*, and *Torulaspora delbrueckii*. During prefermentary stages (vatting, 24 h, and 48 h of maceration, start of alcoholic fermentation). Bioprotection (BP), SO_2, and without SO_2 treatments. Values indicated as the mean of four technical replicates ± standard deviation.

3.2.2. Yeasts and Fungal Diversity

HTS of the 18S rDNA gene was used to evaluate yeast and fungi microbial diversity during the prefermentary stages. The start of alcoholic fermentation was not considered for HTS due to the inoculation of the yeast with ADY *Saccharomyces cerevisiae*. A total of 18 samples were sequenced, resulting in 190,016 paired-end reads assembled with 5′ primers, 3′primers, with expected length (between 300 and 400 bps), without ambiguous base calls (N characters) in their sequence or barcode. After filtering of chimeras, singletons, clustering SWARM and affiliation OTUs, 161,667 sequences were assigned to 493 OTUs. After a blast filter step (for identity and coverage), a preprocess step, and a *Vitis* sp. removal step (−17.90%), 123 OTUs with 131,864 sequences were obtained. Finally, 7716 sequences were retained on average for each sample, except for one replicate at 48 h of maceration (without SO_2 treatment) that was deleted because of its low number of sequences (729).

Different phyla were detected among all OTUs, mostly within *Ascomycota* (95.9%), followed by *Basidiomycota* (2.3%), *Cryptomycota* (1.6%), and other phyla, but with an abundance below 1% of all OTUs (data not shown). The *Ascomycota* phylum contained 11 classes, including 57.2% *Dothideomycetes* (represented by three genera: *Aureobasidium* (63%), *Cladosporium* (28%); *Alternaria* (3.4%)), 25.9% *Saccharomycetes* (represented by the *Metschnikowia* (47.3%); *Torulaspora* (46.8%) and *Hanseniaspora* (3.2%) genera) and 12.8% *Leotiomycetes* (represented predominantly by the *Botrytis* genus (99.5%)) (Table A4).

Among the eight major genera within the *Ascomycota* phylum (Figure 3A and Table A4), five belonged to molds previously reported on the grape berry (*Alternaria*, *Aureobasidium*, *Cladosporium*, *Botrytis*, and *Diplodia*) [47,48]. The *Aureobasidium*, *Cladosporium*, and *Botrytis* genera were dominant whether the musts were sulfited or not. In accordance with population dynamic results, the *Torulaspora* and *Metschnikowia* genera were most abundant for the bioprotection treatment. At the vatting stage, *Torulaspora* represented 25% of the total relative abundance and this percentage remained stable during prefermentary maceration. By contrast, *Metschnikowia* represented approximately 40% of the total relative abundance and then decreased during maceration (30%), according to the Q-PCR results. Bioprotection led to a decrease in the relative abundance of *Aureobasidium*, *Botrytis*, and *Cladosporium* in comparison with the other treatments. The use of SO_2 at vatting did not lead to any significant changes in the relative abundances obtained from the eight major genera in the samples. *Hanseniaspora* was poorly represented, ranging from 0.1 to 2.4% for all samples. Among the percentage of "Others" in Figure 3A, twelve additional genera were represented, with six fungi genera (*Aspergillus*, *Ramularia*, *Pleospora*, *Colletotrichum*, *Taphrina*, and *Zopfia*) and five yeast genera commonly associated with the grape berry microbial community (*Kluyveromyces*, *Candida*, *Saccharomyces*, *Lachancea*, and *Pichia* are grape yeasts) (Figure 3B). Surprisingly, the *Starmerella bacillaris* species was not identified among the sequences.



(**A**) (**B**)

Figure 3. (**A**) Relative genus abundances based on the taxonomic assignation of high-quality 18S rDNA reads of *Ascomycota* phylum from must samples at prefermentary stages (treatment: without SO_2 (0), SO_2 (SO_2), bioprotection (BP); stages: vatting, 24 h prefermentary maceration, 48 h prefermentary maceration). (**B**) Relative genus abundances in "Others" category for each sample. (Mean of biological replicates).

Biodiversity indices were calculated based on high-throughput sequencing data (Figure 4). The number of OTUs at the vatting stage was significantly lower for the bioprotection treatment compared to the other treatments (43 ± 4 OTUs versus 60 ± 3 OTUs). Overall, the number of observed OTUs decreased and α diversity increased ("Shannon" and "invSimpson" index) during prefermentary maceration, whatever the treatment considered. The drop in the number of observed OTUs was particularly marked for the without SO_2 (0) and SO_2 treatments. The "Shannon" index and "InvSimpson" index were significantly higher for the BP and the SO_2 treatment than for without SO_2 for the first two stages (vatting and 24 h of maceration (24 h)).

Table 2 presents the explained variance by "Stage" (vatting, 24 h and 48 h of maceration) and "Treatment" (BP, 0, SO_2) factor and the combination of the two factors for biodiversity indices. The "Stage" factor accounted for the higher percentage of variance (ANOVA $p < 0.05$) for all the biodiversity indices and explained 50% of the variance on average. "Treatment" significantly impacted the number "Observed" and the "InvSimpson" index. The interaction between these two parameters did not show any significant impact whatever the biodiversity index considered.

Table 2. Percentage of variance explained by treatment and stage factors for different biodiversity indices (significance codes for *p* value: ** 0.01 and * 0.05).

	Observed	Chao1	Shannon	InvSimpson
Stage	52.70% **	53.29% **	44.90% *	53.96% **
Treatment	19.13% *	11.25%	13.77%	22.79% *
Treatment*Stage	14.48%	19.67%	4.90%	6.19%
Residuals	13.64%	15.78%	36.33%	17.05%

MALDI-TOF-MS was applied on yeast isolates from samples collected during prefermentary stages and start of alcoholic fermentation.

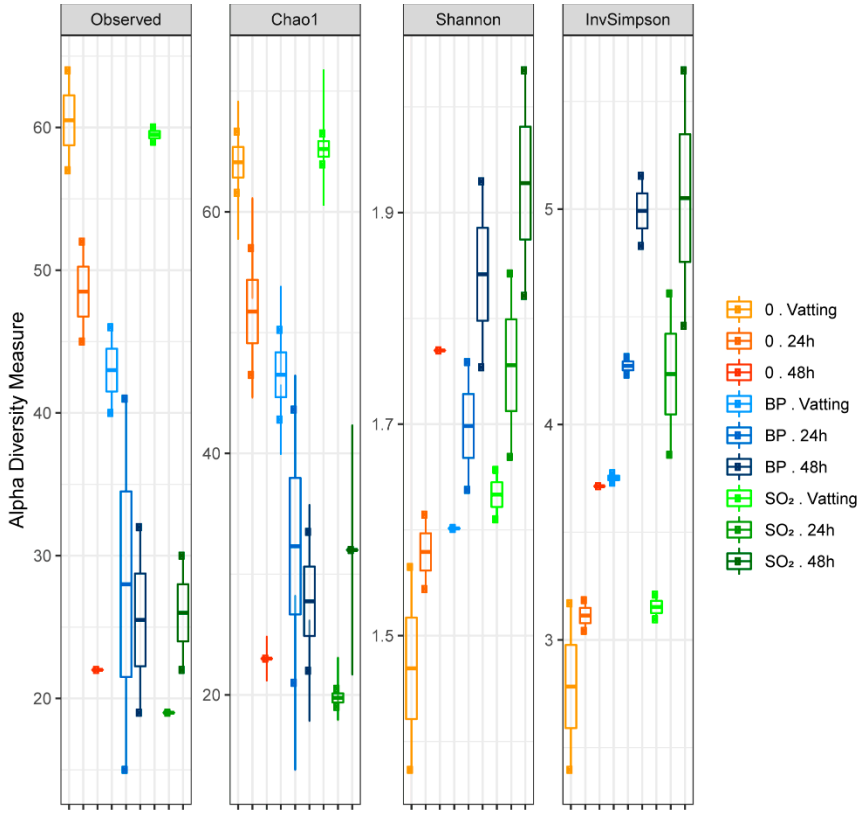


Figure 4. Boxplot of α diversity by four indices based on the taxonomic assignation of high-quality 18S rDNA reads of fungi from must samples at prefermentary stages (treatment: without SO_2 (0), SO_2 (SO_2), bioprotection (BP); stages: vatting, 24 h, and 48 h prefermentary maceration.

Levels of the total yeast population by plating method on LT medium are given in Table A4. Total yeast populations were from 8.3×10^2 to 2.3×10^3 CFU/mL for the vatting stage, except for the bioprotection treatment where the population was logically higher (3.4×10^5 to 4.8×10^5 CU/mL) due to non-*Saccharomyces* addition. Total yeast populations were generally stable during the prefermentary stages and reached 7.7×10^6 to 4×10^7 at the beginning of the alcoholic fermentation. A total of 60 colonies per treatment and 180 colonies per stage (4) were isolated from LT medium and subcloning on YPG medium, resulting in 683 clones to be analyzed by MALDI-TOF MS for species identification (Table A4), instead of the 720 planned. Five isolates did not grow after subcloning and only eleven isolates were isolated from one sample, and this sample was therefore not considered for further analyses. Some isolates (26) were not identified as their profiles did not find a match in the database and, finally, 657 isolates (95%) were identified successfully at species level by MALDI-TOF MS, among which were ten different species (Figure 5). *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, *Aureobasidium pullulans*, and *Hanseniaspora uvarum* were previously reported as OTUs by HTS, whereas *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Whickerhamomyces anomalus*, and *Lachancea thermotolerans* were identified among the "Others". *Candida guillermondii* and *Cryptococcus flavescens* were not identified by HTS. As for the Q-PCR analysis, only *Metschnikowia pulcherrima* was detected with SO_2 or without SO_2 (0) treatment as part of the indigenous population, with a higher percentage without SO_2 at vatting and 24 h of fermentation (24 h).

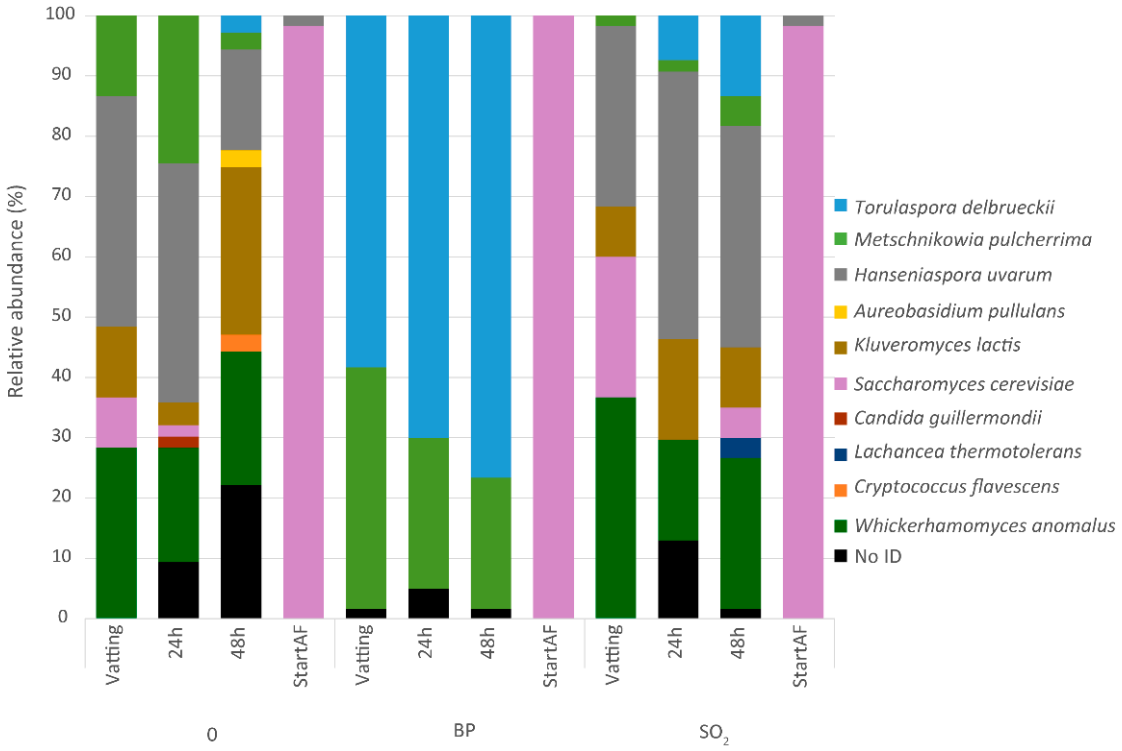


Figure 5. Relative yeast species abundance based on identification by MALDI-TOF MS according to the stages (vatting, 24 h, 48 h of maceration and start of alcoholic fermentation, StartAF) and the modality bioprotection (BP), SO_2, and without SO_2 (0) treatments.

Concerning bioprotection, 90% to 100% of the isolates belonged to the two species *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* as expected, whatever the stage considered before the beginning of the alcoholic fermentation. At vatting, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* represented 58% and 40% of the total clones, respectively. According to the Q-PCR results, *Metschnikowia pulcherrima* decreased during prefermentary maceration, unlike *Torulaspora delbrueckii* for which the percentage of total clones increased (50% to 90% after 24 and 48 h). Except for bioprotection, *Hanseniaspora uvarum*, followed by *Whickerhamomyces anomalus*, were the dominant species during the prefermentary stage. Sulfiting did not result in significant differences in the presence or abundance of other yeast species, except for *Metschnikowia pulcherrima*, for which the percentage was lower with SO_2 addition during the first stage of maceration. As expected, over 98% of the clones analyzed at the start of AF were identified as *S. cerevisiae*.

The "Shannon" index was calculated from data obtained by MALDI-TOF MS (Table 3). Bioprotection treatment logically had a significantly lower Shannon index than the others, whatever the stage considered.

Table 3. Shannon index evaluated by MALDI-TOF MS.

Stage	Treatment	Shannon
Vatting	0	1.34
	SO_2	1.21
	BP	0.68
24 h of maceration	0	1.24
	SO_2	1.16
	BP	0.52
48 h of maceration	0	1.24
	SO_2	1.58
	BP	0.5

0: without any treatment; SO_2: 50 mg/L applied in must after crushed; BP: 50 mg/L of bioprotection applied on grapes and without SO_2.

4. Discussion

Until now, yeast diversity analysis by culture-dependent techniques has been performed using 5.8S-ITS-RFLP analysis and/or 26S rDNA D1/D2 domain sequencing [4,6,15,49–51]. This approach based on PCR analysis and DNA sequencing is time-consuming. Recently, MALDI-TOF MS has been demonstrated to be a rapid and cost-effective tool for the identification of wine yeast at the species level [26,29,33]. In this study, species identification by MALDI-TOF MS was validated for 66% of wine yeast isolated at the prefermentary or beginning of alcoholic fermentation stages, in comparison with 26S rDNA sequencing. These first results revealed that it was necessary to enrich the MALDI-TOF MS Biotyper database not only with missing wine yeast species, such as *Starmerella bacillaris*, but also to add wine strains for some species, such as *S. cerevisiae* or *H. uvarum*, to improve identification. Different authors [28,52] have already highlighted the importance of enriching the yeast database from standard spectra of isolates originated from the oenological environment. In the present study, the Biotyper database was extended by an oenological laboratory-specific database (43 new additional strains corresponding to 17 different species specific to the wine environment). Gutièrrez et al. (2017) [28] reported the successful identification of 95.4% of yeast isolates after optimization of the preanalytical steps and the development of an in-house MS database. By using standard procedures for colony extraction (without optimization of the preanalytical steps) and an oenological laboratory specific extended data base, we were able to obtain the same identification rate with 95% of the isolates successfully identified. Our results confirmed that MALDI-TOF MS is a promising and robust method for wine yeast identification at the species level. However, this method does not currently allow differentiation at strain level in the oenological context, especially for *Saccharomyces cerevisiae* [28,32].

The second aim of the study was to consider a mix of two non-*Saccharomyces* yeasts as bioprotection (*Torulaspora delbrueckii* and *Metschnikowia pulcherrima*) during the prefermentary stages without sulfur dioxide addition at the industrial scale. The MALDI-TOF MS method was used to assess the yeast diversity of the grape juice compared with different commonly-used methods and to monitor the implantation of both bioprotective species.

First, the population dynamics of *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, and *Hanseniaspora* sp. were analyzed using Q-PCR. By targeting known species in the must ecosystem, this technique has the major advantage of establishing their population dynamics with a low detection level. Its major drawback lies in an overestimation that may be caused by the lack of discrimination between live and dying microorganisms. In the present work, this method allowed us to quantify population levels and to confirm the effective implantation of *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* in the bioprotection treatment during the prefermentary stages.

DNA metabarcoding is a method that is increasingly being used to characterize and quantify biodiversity in environmental samples. Illumina metabarcoding generates shorter reads but achieves deeper sequencing than 454 metabarcoding approaches [53]. This

method also allows quantitative information to be obtained on relative abundances of a genus in particular [54], and biodiversity indices from OTU tables. Numerous studies targeting yeasts and fungi have previously been published [53,55–57]. However, various biases have to be taken into account to interpret the data: (i) as for Q-PCR analysis, grape must may contain many PCR inhibitors [58,59], (ii) taxa with low proportions in a community are underrepresented or have a low amplification reproducibility due to primer mismatches or PCR biases [54,60], (iii) amplified DNA does not provide information as to whether yeast are physiologically active or dead, or may be active within the community. In our study, the most abundant OTUs were assigned to grape fungi, mainly *Aureobasidium*, *Botrytis*, and *Cladosporium*. These OTUs represented more than 75% of the total abundance, in line with previous results obtained with samples collected in grape must before the start of alcoholic fermentation [9,10]. Grape must yeast diversity in the present study, as reported previously [19,61,62], was quite low compared to other matrices, such as sugar cane or the soil [53,63,64], but nonetheless richer than in traditional sourdoughs [65]. To gain further insight, it would be interesting to use the metagenomic approach to provide a more in-depth understanding, since it offers a non-targeted taxonomic study [66].

Illumina metabarcoding and MALDI-TOF MS allowed concordant yeast identification both at genus and species level, e.g., *Aureobasidium*, *Hanseniaspora*, *Metschnikowia*, and *Torulaspora*, but with different abundances; *Aureobasidium* was the most abundant OTU for Illumina metabarcoding, whereas it was detected only in one sample through cultivation, probably due to the use of biphenyl in the cultivation medium (LT). Inverse results were obtained for *Hanseniaspora. Saccharomyces* was identified among isolates, but with a very low abundance in Illumina data, which is consistent with other studies based on high-throughput sequence analysis, reporting a near-absence of this genus [1,6,19]. Microbial culturomics, using multiple culture conditions and MALDI-TOF MS, was successfully applied recently to study human gut microbiota [67] and the plant prokaryotic microbiome [68]. In this study, only one medium was used to target yeasts. Higher combinations of various growth media (for example, specific fungi media) and higher number of isolates analyzed per sample dilution would offer a more in-depth estimation of microbial diversity [69]. Moreover, compared to Illumina metabarcoding, culturomics approaches make it possible to collect colonies related to microbial diversity, thus allowing collection enrichment and further phenotypic analysis.

In oenology, non-*Saccharomyces* yeast preparations are now proposed as bioprotection agents during the prefermentary stages. However, their impact on the microbial community and antiseptic effectiveness have so far received only a few scientific demonstrations [14,15]. Till now, only pure culture of non-*Saccharomyces* yeast was studied. In the present study, the application of a mix of two species was considered. The ability of both bioprotective species *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* to colonize the grape must during prefermentary stages was confirmed by the three methods. However, *Metschnikowia pulcherrima* decreased during the prefermentary stages, whereas *Torulaspora delbrueckii* remained stable. The addition of bioprotection led to a decrease in fungal communities, especially *Aureobasidium* and *Botrytis*, the latter being considered a common grape pathogen. *Hanseniaspora uvarum* is a major species in the grape must microbial community, which can have a negative effect on *Saccharomyces cerevisiae* growth and even lead to delayed alcoholic fermentation [2]. It also produces unwanted metabolites, such as acetic acid, ethyl acetate, sulfur compounds, acetoin, and biogenic amines [70–72]. In this experiment, population levels of *Hanseniaspora* spp. did not differ between treatments, whatever the pre-fermentation stage considered, according to the Q-PCR data. No impact of SO_2 addition on its relative abundance was shown by either the Illumina or MALDI-TOF MS method, contrary to previous studies that showed lower population levels of *Hanseniaspora* spp. in the presence of SO_2 for white wine vinification [2,17]. However, the species was not identified among clones analyzed from the bioprotection samples by MALDI-TOF MS, suggesting a negative impact of the non-*Saccharomyces* species on *Hanseniaspora uvarum*. These results confirmed previous observations by Simonin et al. (2018) [14] who showed

that the use of *Torulaspora delbrueckii* as a bioprotection agent on white must (Aligoté) had a negative impact on the development of *Hanseniaspora uvarum*. Indigenous populations of *Metschnikowia pulcherrima* were negatively impacted by SO_2, according to previous results showing that the growth of this species was affected by the addition of sulfites [15,55]. *Metschnikowia pulcherrima* populations in the bioprotection modality were also shown to decline, irrespective of the analytical method used. Since no SO_2 was added in the bioprotection modality, other factors could explain the population decrease of this species, such as low temperature (13 °C) or negative interaction with *Torulaspora delbrueckii*.

During maceration, the yeast community was affected more by the stage at which the must was analyzed than by the addition of SO_2 or bioprotection. Concerning SO_2 addition, a significant impact on the richness in OTUs from the vatting stage to 24 h of the prefermentary maceration was highlighted, leading to a reduction in the number of OTUs in comparison to the control without SO_2. Similar results were also reported on chardonnay, with a significant decrease in the α diversity in the presence of 40 mg/L SO_2 from pressing through to the end of alcoholic fermentation [10]. The diversity indices of Shannon and invSimpson, which take into account the diversity of OTUs and their abundances, were both impacted significantly by the stage, and, to a lesser extent, by the way the different musts were treated. However, additional experiments on different grape musts are needed to confirm our preliminary results.

5. Conclusions

In conclusion, the use of the MALDI-TOF MS technique allows yeast biodiversity and the implantation control of both bioprotective non-*Saccharomyces* yeasts to be assessed quickly and cheaply, thus confirming that it is a robust method for wine yeast identification at species level, despite the high costs of acquiring and maintaining the equipment. In the future, this technique, combined with the use of different selective media allowing cultivation of a large number of clones, should be considered as an interesting alternative to metabarcoding HTS to analyze yeast diversity from grape, must, and wine. The use of SO_2 significantly impacts the OTUs diversity, affecting their nature and their abundance. Compared with SO_2 modality, bioprotection occupied the niche, leading to a decrease of fungal communities and *Hanseniaspora uvarum* cultivable population. Additional modalities (with pure culture of *Metschnikowia pulcherrima* and *Torulaspora delbrueckii*) are needed to confirm if the use of mix culture of non-*Saccharomyces* yeast is more efficient than the use of a pure culture one.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. List of the strains added to the oenological laboratory specific database with GenBank accession number(s).

Genus	Species	Name CRB	GenBank Accession Number(s)	Species Literature References
Brettanomyces	*acidodurans*	NCAIM Y 2178		[73]
Zygosaccharomyces	*bailii*	L0536	MT950295	[74]
Brettanomyces	*bruxellensis*	CRBO_L0308	MT950279	[46]
Brettanomyces	*bruxellensis*	CRBO_L0417	MT950285	
Brettanomyces	*bruxellensis*	CRBO_L0422	MT950286	
Brettanomyces	*bruxellensis*	CRBO_L0424	MT950287	
Brettanomyces	*bruxellensis*	CRBO_L0463	MT950293	
Brettanomyces	*bruxellensis*	CRBO_L0512	MT950294	
Brettanomyces	*bruxellensis*	CRBO_L0542	MT950296	
Brettanomyces	*bruxellensis*	CRBO_L0611	MT950299	
Brettanomyces	*bruxellensis*	CRBO_L14156	MT950310	
Brettanomyces	*bruxellensis*	CRBO_L14169	MT950311	
Brettanomyces	*bruxellensis*	CRBO_L14173	MT950312	
Brettanomyces	*bruxellensis*	CRBO_L14195	MT950313	
Brettanomyces	*bruxellensis*	CRBO_L1735		
Brettanomyces	*bruxellensis*	CRBO_L1750		
Brettanomyces	*bruxellensis*	CRBO_L1774		
Candida	*cantarelli*	CRBO_L0404	MT950283	
Candida	*cantarelli*	CRBO_L0412	MT950284	
Saccharomyces	*cerevisiae*	522D		[75]
Saccharomyces	*cerevisiae*	CRBO_L0431	MT950288	
Saccharomyces	*cerevisiae*	CRBO_L0439	MT950289	
Saccharomyces	*cerevisiae*	CRBO_L0545	MT950298	
Saccharomyces	*cerevisiae*	CRBO_L1117	MT950308	
Torulaspora	*delbrueckii*	CRBO_L0544	MT950297	[76]
Torulaspora	*delbrueckii*	CRBO_L0630	MT950300	
Pichia	*guiliermondii*	CRBO_L0652	MT950302	[77]
Shizosaccharomyces	*japonicus*	Y13611		
Pichia	*kluyveri*	CRBO_L0677	MT950304	
Pichia	*membranifaciens*	CRBO_L0709	MT950305	
Schizosaccharomyces	*octosporus*	Y-8551		
Schizosaccharomyces	*pombe*	CRBO_L0442	MT950290	[78]
Schizosaccharomyces	*pombe*	CRBO_L0443	MT950291	
Schizosaccharomyces	*pombe*	Y12791		

Table A1. *Cont.*

Genus	Species	Name CRB	GenBank Accession Number(s)	Species Literature References
Metschnikowia	*pulcherima*	CRBO_L0313	MT950282	[79]
Metschnikowia	*pulcherima*	CRBO_L0640	MT950301	
Aureobasidium	*pullulans*	CRBO_L0448	MT950292	[80]
Aureobasidium	*pullulans*	CRBO_L11178	MT950309	
Lanchancea	*thermotolerans*	CRBO_L0672	MT950303	[81]
Hanseniaspora	*uvarum*	CRBO_L0312	MT950281	[82]
Hanseniaspora	*uvarum*	CRBO_L0715	MT950306	
Starmerella	*bacillaris*	CRBO_L0311	MT950280	[83]
Starmerella	*bacillaris*	CRBO_L0740	MT950307	

Table A2. Primers used to quantify population levels of microorganisms.

Species	Primers	References
Metschnikowia pulcherrima	MP2-F AGACACTTAACTGGGCCAGC MP2-R GGGGTGGTGTGGAAGTAAGG	[16]
Torulaspora delbrueckii	TD-F CAAAGTCATCCAAGCCAGC TD-R TTCTCAAACAATCATGTTTGGTAG	[7]
Hanseniaspora spp.	Hauf 2L—CCCTTTGCCTAAGGTACG Hauf 2R—CGCTGTTCTCGCTGTGATG	[7]

Table A3. Merlot grape must parameters at vatting and wine after alcoholic fermentation analyzed for each modality: SO_2 (added 50 mg/L), without SO_2 (0) and with bioprotection at 50 mg/L (BP). The analyses were performed according to the official methods described by the European Commission. Values correspond to the average of biological replicates.

Chemical Composition in Must				In Wine		
Parameters	**SO_2**	**0**	**BP**	**SO_2**	**0**	**BP**
Reducing sugars (g/L)	245	242	243	1.0	3.0	1.4
Total acidity (g/L)	1.63	1.71	1.67			
Malic acid (g/L)	1.1	1.0	1.1			
pH	3.84	3.84	3.83			
Yeats assimilable nitrogen (mg/L)	116	124	111			
Total SO_2 (mg/L)	127	<10	<10			
Volatil acidity (acetic acid g/L)				0.28	0.30	0.28

Table A4. The yeast and fungi diversity of three treatments (SO_2, 0 and BP) at three stages evaluated by HTS, Q-PCR and MALDI-TOF MS.

	Stage	**1. Vatting**						**2. 24 h of Maceration**						**3. 48 h of Maceration**						**4. Start of AF**					
	Treatments	**0**		**SO_2**		**BP**		**0**		**SO_2**		**BP**		**0**		**SO_2**		**BP**		**0**		**SO_2**		**BP**	
	Duplicats	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
HTS metabarcoding (Relative abundance by Genus in %)	*Aureobasidium*	63.2	48.3	49.7	50.1	20.3	20.3	49.7	50.7	41.2	30.7	13.9	26.3	46.5	-	31.7	38.4	21.9	29.0	-	-	-	-	-	-
	Cladosporium	18.7	20.5	20.2	22.9	7.0	6.5	18.7	20.8	24.2	26.5	10.9	6.9	16.6	-	17.0	15.9	7.1	16.3	-	-	-	-	-	-
	Botrytis	11.1	20.4	15.8	14.5	3.6	3.9	17.7	16.8	15.1	14.5	8.7	4.3	13.4	-	16.4	19.7	9.2	7.8	-	-	-	-	-	-
	Alternaria	0.6	1.9	1.6	2.3	0.8	1.0	1.5	2.1	5.2	1.6	1.5	2.2	-	-	4.3	2.6	2.6	1.3	-	-	-	-	-	-
	Diplodia	0.9	1.6	2.0	2.0	0.6	0.4	2.0	2.7	0.1	2.9	0.1	0.9	0.9	-	1.4	6.2	1.1	0.7	-	-	-	-	-	-
	Torulaspora	0.3	0.2	2.7	0.4	24.8	26.6	0.5	0.2	4.0	3.0	33.4	24.0	5.5	-	7.5	7.7	24.1	25.2	-	-	-	-	-	-
	Metschnikowia	0.1	0.2	0.1	0.1	40.5	39.0	0.3	0.2	5.7	0.1	29.4	31.7	0.1	-	0.0	0.1	27.3	16.2	-	-	-	-	-	-
	Hanseniaspora	0.4	0.5	1.2	0.7	0.1	0.1	3.8	0.1	0.2	2.0	0.1	0.1	2.4	-	0.2	0.2	0.2	1.9	-	-	-	-	-	-
	unknown genus	1.1	1.4	1.9	1.7	0.5	0.5	1.1	1.4	1.0	0.0	0.0	0.5	4.9	-	1.3	0.0	1.6	0.1	-	-	-	-	-	-
	others	1.7	1.4	1.9	2.5	0.8	0.6	1.7	1.7	0.8	5.8	0.2	1.4	1.9	-	3.1	1.0	0.8	0.8	-	-	-	-	-	-
Q-PCR (Cells/mL)	*Torulaspora delbrueckii*	<100	<100	<100	<100	4.5×10^4	6.8×10^4	<100	<100	<100	<100	1.2×106	4.8×105	<100	<100	<100	<100	7.7×10^4	1.1×10^5	3.7×10^2	4.6×10^2	8.8×10^2	2.9×10^2	1.2×10^4	4.8×10^5
	Metschnikowia pulcherrima	3.9×10^2	5.8×10^2	<100	<100	4.5×10^6	4.3×10^6	<100	<100	<100	<100	1.6×106	8.4×105	<100	<100	1.1×10^2	<100	3.8×10^4	2.9×10^4	2.0×10^2	1.3×10^4	<100	<100	1.6×10^6	8.4×10^5
	Hanseniaspora sp.	9.2×10^3	5.4×10^3	1.2×10^4	4.6×10^3	5.2×10^3	6.7×10^3	7.4×10^3	2.1×10^3	2.3×10^3	2.0×10^3	2.1×10^3	2.0×10^3	2.9×10^2	2.1×10^3	2.8×10^2	2.4×10^3	3.4×10^2	2.7×10^3	2.8×10^5	1.1×10^6	1.2×10^6	3.4×10^5	3.6×10^5	1.4×10^5
Culture	Total Yeasts (UFC/mL)	1.2×10^3	2.3×10^3	8.3×10^2	1.3×10^3	4.8×10^5	3.4×10^5	2.4×10^3	2.5×10^3	2.0×10^3	1.6×10^3	5.9×10^5	4.0×10^3	1.2×10^3	4.0×10^3	1.4×10^3	9.8×10^2	5.5×10^5	3.4×10^6	2.0×10^7	2.1×10^7	7.7×10^6	1.8×10^7	2.6×10^7	4.0×10^7

Table A4. *Cont.*

	Stage	1. Vatting						2. 24 h of Maceration						3. 48 h of Maceration						4. Start of AF			
	Treatments	0		SO_2		BP		0		SO_2		BP		0		SO_2		BP		0		SO_2		BP	
	Duplicats	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
MALDI-TOF MS (%)	TOTAL number of colonies analyzed	30	30	30	30	30	30	23	30	24	30	30	30	30	6	30	30	30	30	30	30	30	30	30	30
	Torulaspora delbruekii	-	-	-	-	56.7	60.0	-	-	4.2	10.0	90.0	50.0	3.3	-	16.7	10.0	86.7	66.7	-	-	-	-	-	-
	Metschnikowia pulcherrima	-	26.7	-	3.3	40.0	40.0	13.0	33.3	-	3.3	10.0	40.0	3.3	16.7	3.3	6.7	10.0	33.3	-	-	-	-	-	-
	Hanseniaspora uvarum	40.0	36.7	13.3	46.7	-	-	34.8	43.3	58.3	33.3	-	-	16.7	-	43.3	30.0	-	-	3.0	-	3.0	-	-	-
	Aureobasidium pullulans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Kluveromyces lactis	13.3	10.0	3.3	13.3	-	-	8.7	-	12.5	20.0	-	-	33.3	-	10.0	10.0	-	-	-	-	-	-	-	-
	Saccharomyces cerevisiae	13.3	3.3	36.7	10.0	-	-	4.3	-	-	-	-	-	-	-	-	10.0	-	-	97	100	97	100	100	100
	Candida guillermondii	-	-	-	-	-	-	4.3	-	-	-	-	-	-	16.7	-	-	-	-	-	-	-	-	-	-
	Lachancea thermotolerans	-	-	-	-	-	-	-	-	-	-	-	-	-	16.7	3.3	3.3	-	-	-	-	-	-	-	-
	Cryptococcus flavescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Whickerhamomyces anomalus	33.3	23.3	46.7	26.7	-	-	21.7	16.7	12.5	20.0	-	-	26.7	-	23.3	26.7	-	-	-	-	-	-	-	-
	No ID	-	-	-	-	3.3	-	13.0	6.7	12.5	13.3	-	10.0	16.7	50.0	-	3.3	3.3	-	-	-	-	-	-	-

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